Rapid Measurement of NH_3 and Weak Acid Permeation Through Liposomes and Renal Proximal Tubule Membranes

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=ABSTRACT=

Using the methods of stopped-flow and epifluorescence microscopy with entrapped fluorophore, membrane permeability of NH_3 and weak acids in liposomes, renal brush border (BBMV) and basolateral membrane vesicles (BLMV), and primary culture cells from renal proximal tubule was measured. Permeability coefficient (cm/sec) of NH_3 was 2.9×10^{-2} in phosphatidylcholine liposome (25°C), 5.9×10^{-2} in renal proximal tubule cell (37°C), 4.0×10^{-2} and 2.4×10^{-2} in BBMV and BLMV (25°C), respectively. Formic acid has the highest permeability coefficient among the weak acids tested, which was 4.9×10^{-3} in liposome, 5.0×10^{-3} in renal proximal tubule cell, 9.1×10^{-3} in BBMV and 3.8×10^{-3} in BLMV. There was a linear relationship between external concentration of nonionized formic acid and initial rate of flux of formic acid in liposome, and the slope coincided with the value of permeability coefficient of formic acid measured in pH 7.0.

These results show that techniques of stopped-flow and epifluorescence microscopy with entrapped fluorophore provide the precise method of measurement of very rapid transport of nonelectrolytes through membranes with the advantages of instantaneous mixing effect, good resolution time and easy manipulation.

Key Words: Membrane permeability, Weak acid, NH_3, Stopped-flow technique Fluorescence

INTRODUCTION

The mechanism of passive permeation of non-electrolytes through the biological membranes has been studied for long period of time. In 1902, the significance of membrane was presented by Bernstein (1966) in terms of cell structure and function. It was proposed by Brooks and Brooks (1962) in 1941, and also subsequently by Danielli and Davson (1943), that the membrane maintains the integrity of internal environment. In their membrane model physical characteristics were expressed as a fundamental equation in terms of osmotic permeability. Classical study of Overton (1899 & 1902) indicated that the rate of penetration of substance into a cell was largely determined by it's lipid solubility or partition coefficient. This concept of partition coefficient has been laid foundation in the study of mechanisms of membrane transport. The transport phenomenon of NH_3 and formic acid occupies an important position of acid-base balance and volume regulation by kidney. Especially in proximal renal tubule which is considered to be a major site of ammonia production and secretion, the transfer of synthesized ammonia to final urine depends on "diffusion trapping",

i.e., on the tendency of ammonia to diffuse as NH_3 through apical membrane, and to be protonated and trapped as NH_4^+ in urine (Good & Knepper, 1985: Knepper et al, 1989: Knepper, 1991). In the model of Cl-formate exchange and formic acid recycling in renal proximal tubule, net Na and Cl reabsorption occurs coupled to formic acid recycling (Karniski & Aronson, 1985; Schild et al, 1987). In this pattern of Na and water reabsorption, nonionic diffusion of formic acid through membrane is the rate limiting step.

Since the permeability is fast transport phenomenon, kinetic technique of determination of flux has not been fully developed. According to Overton's rule (1899; 1902), the membrane permeabilities of nonionic forms of many weak acids and ammonia are expected to be high. Therefore the permeabilities of these substances are not readily determined with high accuracy. Since the permeability of unstirred layer is a major component of total permeability, that of the true membrane is extremely difficult to be determined by an assumption of the simple flux system (Gutknecht et al, 1977: Walter et al, 1982). In addition the relatively impermeant ionic forms are known to facilitate the diffusion of nonionic forms through the unstirred layers (Andreoli et al, 1987). Thus, the patterns of permeabilities of weak acids and ammonia through the biological membranes are extremely variable (Klocke et al, 1972; Wolosin & Ginsburg, 1975; Walter & Gntnecht, 1984 & 1986; Golchini & Kurtz, 1988). In the light of the variability and unusual patterns of permeabilities of nonelectrolytes through the biological membranes, it is emphasized that a novel system must be developed to measure the permeability coefficients and the permeability patterns of weak acids and bases.

In the present study, an effort was made to determine the permeability coefficients of NH_3 and weak acids in liposome, renal brush border (BBMV) and basolateral membrane vesicles (BLMV), and primary cultured renal proximal tubule cells. A new method was developed to

improve the resolution time of reaction using stopped-flow technique and epifluorescence microscopy. Changes in fluorescence resulting from diffusion of NH_3 or weak acids were measured and the permeability coefficient was calculated from the initial slope of the tracings.

METHODS

Preparation of liposome

Liposomes were prepared by extrusion procedure (Verkman et al, 1989). Phosphatidylcholine was mixed in chloroform and dried under N_2 stream. The dried lipid was suspended in the loading buffer (100 mM mannitol, 100 mM KCl, 50 mM hepes/tris pH 7.0 or 50 mM mes/tris pH 6.0) containing 1 mM 6-carboxyfluorescein (6-CF) at a concentration of 10 mg/mL. Lipid was subjected to 5 freeze-thaw cycles and 10 passages through a 200-nm Nucleopore filter (Pleasanton, CA) at 200~400 psi using commercial extruder (Lipex Biomembranes, Inc., Vancouver, British Columbia). External 6-CF was removed by passage of liposomes down a G-25M exclusion column; the first 10 drops of cloudy eluate were pooled for subsqunt experiments. The diameter of liposomes extruded through 200-nm filter was reported to be 515 nm as determined by freeze-fraction electron microscopy (Mayer, 1986).

Preparation of membrane vesicles

BBMV and BLMV were prepared as the same method of Chen and Verkman (1989). Briefly, the renal cortex was dissected from the kidneys of 2-3 kg New Zealand white rabbits and homogenized at 4°C in 250 mM sucrose, 10 mM Hepes-Tris, and 5 mM EGTA, pH 7.0. Crude membrane was isolated by differential centrifugation and mixed with 11.3% percoll solution. After 45 min centrifugation at 48,000 g, fractions were collected and assayed for alkaline phosphatase as a brush border

Table 1. Comparison of marker enzyme activities in homogenate, renal brush border and basolateral membrane vesicles

	Homogenate	BBMV	BLMV
Alkaline phosphatase activity (μ mole p-nitrophenol/mg protein/hr)	0.487 $\pm$ 0.051	5.025 $\pm$ 0.823	0.146 $\pm$ 0.009
Na-K-ATPase activity (μ mole Pi/mg protein/hr)	7.52 $\pm$ 0.89	108.61 $\pm$ 9.05	2.13 $\pm$ 0.37

* n=4

membrane marker and Na-K-ATPase activities as a basolateral membrane maker (table 1). BBMV were enriched in alkaline phosphatase activity more than 10-fold and ouabain-sensitive Na-K-ATPase activity less than 0.3-fold over activities in the crude homogenate. BLMV were enriched in Na-K-ATPase specific activity > 15-fold and in alkaline phosphatase activity < 0.3-fold over in the crude homogenate. Vesicles were loaded with 6-CF by incubation of vesicles with the same loading solution containing 1 mM 6-CF at 4°C overnight. Extravesicular 6-CF was removed by three washes in more than 25 volumes of buffer not containing 6-CF. Vesicles were suspended by ten passages through a 23-gauge needle and two passages through 26-gauge needle after each centrifugation (100,000 g, 10min, 4°C). Vesicles were resuspended at a concentration of 10 mg/ml and stored at 4°C until the time of the experiment.

Stopped-flow experiments

A stopped-flow apparatus has a special system for rapid mixing, the stopped-flow block. A diagrammatic representation of the stopped-flow block is shown in figure 1. The two syringes contain the reactant solutions. Flow is initiated by movement of the drive plate. The solutions from the two syringes then flow into a mixing chamber. Incident light passes into one surface of the observation cell and emitted light is monitored at 90°C. The solution from the observation cell then flows into the back

syringe. which acts as a stopping device by hitting a metal block.

The permeability of ammonia and weak acid in liposome and membrane vesicles were measured by a stopped-flow technique (Chen et al, 1988). Aliquots of a microsome suspension labeled with 6-CF were subjected to inward ammonia or weak acid gradients using a Hi-Tech SF-51 stopped flow apparatus (Wiltshire, England). Dead time of the instrument was under 2 ms. Temperature was controlled by a circulating water bath and monitored by an indwelling thermistor. Fluorescence was excited by a stabilized tungsten-halogen lamp and a single grating monochromator (480$\pm$5nm) in series with a six-cavity broad hand (420-490 nm) interference filter. Fluorescence was monitored through two OG515 cut-on filters in series (Schott Glass, PA). Data were recorded at a maximum rate of 10 points/ms and stored on a MINC/23 computer for analysis. 512 time
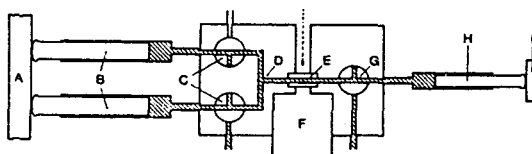

Fig. 1. Diagrammatic representation of a stopped-flow instrument. A, drive; B, reactant syringes; C, filling valves; D, mixer; E, observation cell; F, photomultiplier; G, empting valve; H, back syring; I, stopping bar.

points were repeated 10 times for signal averaging.

The time course of 6-CF fluorescence was fitted to a single exponential function and used to calculate permeability coefficients of NH_3 and weak acids. Calibration of intravesicular 6-CF was performed by monitoring flourescence at the known pH. pHi was set approximately equal to external pH by exposing the vesicles to solution containing 5 μM nigericin.

Preparation of proximal tubule cell

Primary cultures of rabbit kidney proximal tubule cells were prepared by the modification of technique of Chung et al (1982). In brief, two or three New Zealand white rabbits weighing 2-3 kg were sacrificed by decapitation and the renal arteries were cannulated and perfused with modified Hank's media until blanching of the kidney occurred. An additional 2 ml of 0.5% solution of iron oxide was infused until the kidney became a homogeneous gray. The kidneys were removed and the grey cortex was dissected in long strips away from the pale medulla and placed at 4°C. The strips were homogenized in a 7-ml Wheaton Dounce B homogenizer (5 strokes). The homogenate was sequentially filtered through 230 and 74 μm stainless steel screens, retaining proximal tubules and glomeruli on the top of 74 μm screen. Glomeruli containing iron oxide were removed from the suspension with a magnetic stirring bar. The resulting suspension of purified proximal tubules was incubated in DME/F 12 containing 0.125 μg/ml collagenase and 0.0025% soybean trypsin inhibitor for 3 min at 23 °C, and then centrifuged at 150 g for 5 min. The proximal tubules were resuspended in DME/F 12 supplemented with insulin (5 μg/ml), trasferrin (5 μg/ml), and hydrocortisone (5 × 10^{-8} M). This suspension of tubules was then inoculated into 35-mm culture dishes. Cultures were maintained in a humidified 5% CO_2/95% air environment at 37°C. Medium was changed the first day after plating and regularly every 3

days thereafter.

Confluent monolayers of primary proximal tubule cells (7-10-day-old cultures) grown on the coverslip were loaded with 3', 7'-bis (2-carboxyethyl)-5(6)-carboxyfluorescein (BCE-CF by incubation of cells with 10 μM of BCE-CF acetoxymethyl ester (BCECF/AM) at 37°C for 10 min. Impermeant BCECF was formed by the action of esterase and trapped inside the cells. Extracellular dye was removed by washing three times with dye-free solution. After loading, the coverslips were transferred onto a specially devised perfusion chamber to measure the fluorescence.

Fluorescent measurement

BCECF fluorescence of proximal tubule cells was measured using by a Nikon epifluorescent microscope (Diaphot, Japan). Fluorescence was excited at 480±5 nM by 100-W tungsten-halogen lamp powered by a stabilized DC. Emitted light was filtered 510 nm cut-on filter and detected by a model R928S photomultiplier (Hamamatsu Corp., NJ) powered by a high voltage supply/amplifier (Ealing Corp., MA). The amplified signal was digitalized by an analog-to-digital converter (Interactive Microware, PA) at rate of 30 points/s and averaged over 1-s intervals by a IBM PC/AT computer.

Measurement of initial flux and permeability

Figure 2 shows how to measure the permeability of formic acid and NH_3 using fluorophore entrapped in vesicles or cells. In the solution containing the specified solute gradient, ionic and nonionic forms coexist with their concentrations determined by the difference between pK_a or pK_b of the solute and pH of the solution. In this situation even though the concentration gradient of ionic form is much higher than that of nonionic form, the permeation of the ionic form is ignored because of thousand-fold smaller permeability than nonionized form (McLaughlin & Dilger, 1980; Roos & Boron, 1981). Thus, the permeation through the
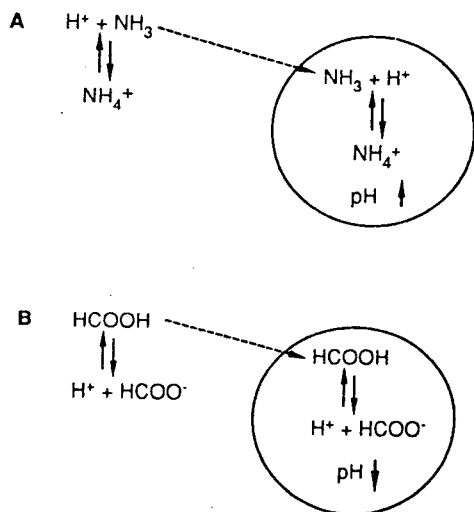
Fig. 2. The diagrams illustrating how the pH and fluorescence inside the vesicle change in the presence of an inwardly directed gradient of ammonia (A) and formic acid (B).

membranes of vesicles or cells occurred mainly in the form of NH_3 in the presence of inwardly directed NH_4Cl gradient (Fig. 2A). As NH_3 permeates into the vesicle or cell, intravesicular pH increases as a result of binding of NH_3 with intravesicular H^+ released from the deprotonated buffers inside the vesicle or cell, and then the fluorescence of 6-CF loaded in vesicle or cell increases. Conversely, as weak acids permeate the vesicles predominantly in the nonionized form, intravesicular pH decreases with decreasing 6-CF fluorescence because inside the vesicle or cell, nonionic forms of formic acid (HCOOH) in this example, dissociate into formate ($HCOO^-$) and hydrogen ion (Fig. 2B).

The flux of NH_3 or weak acids across the membranes was calculated from the time course of change in intravesicular pH on exposure of the vesicles to a specified gradient of NH_3 or weak acids. This calculation was based on the assumption that the rate of H^+ dissipation or accumulation is the same as that of permeation

of NH_3 or weak acids through membrane.

Initial influx of weak acid (HA) at $t=0$ (J_{HA} $t=0$) was calculated by fitting the time course to a single exponential

$$dF/dt = (Ae^{-t}/\tau + Ar)/dt = -A/\tau$$

where A is amplitude, τ is the exponential time constant, and Ar is offset of a single exponential,

$$dpH/dt = dF/dt \times dpH/dF$$

where dpH/dF was obtained from two point calibration using the following equation

$$f_{CF}(pH) = 0.0018(pH)^3 - 0.00104(pH)^2 \\ + 0.00018(pH) - 9.5 \times 10^{-6}$$

Using the values obtained above, J_{HA} $t=0$ was calculated from the following equation,

$$J_{HA}\ t=0\ (mmole/cm^2 \cdot sec) \\ = dpH/dt \times \Delta[HA]/\Delta pH \times V/S$$

where $\Delta[HA]/\Delta pH$ means buffer capacity (β), and S/V is surface to volume ratio of vesicles or cells. The values for S/V of liposome, BBMV, BLMV, and proximal tubule cell used in this equation are 1.3×10^5, 2×10^5, 1.2×10^5, and 4.1×10^4 cm^{-1}, respectively, which were taken from morphometric analyses of other papers (Mary et al, 1987; Verkman & Wong, 1987; Chen et al, 1988). Finally, permeability was calculated from the equation below,

$$P_{HA} = J_{HA}t = 0/\Delta[HA]t = 0$$

where $\Delta[HA] = [TA] \times 10^{pk-pH}$ where the total acid (TA) equals to the sum of ionized form (A^-) and nonionized form (HA).

RESULTS

Measurement of buffer capacity

Intravesicular and intracellular buffer capacity was determined by weak base method using

NH₄Cl pulse technique. It was calculated as the amount of acid load divided by the observed change of cell pH produced by this load. The acid load was estimated as the intracellular NH_4^+, assuming that all NH_4^+ exits the cell as NH_3, giving up H^+ in the process. NH_4^+ was calculated from the last pHi value in the presence of NH₄Cl, assuming a pK of 9.0 and complete equilibration of intra- and extracellular NH_3 at extracellular pH. The change of pH (ΔpH) was the difference between the initial pHi and the last pHi after NH₄Cl withdrawal. Buffering power (β) was calculated according to the following equation.

$$\beta = \Delta[NH_4^+]i/\Delta pHi$$

The values of buffering power of liposome, vesicle and cells obtained withe this method were summarized in table 2.

Permeabilities of NH₃ and weak acids in liposome

Liposome loaded with pH sensitive dye, 6-CF was exposed to the solution containing 100 mM KCl, 100 mannitol, 30 mM NH₄Cl or weak acids and 50 mM hepes-tris at pH 7.0 or mes-tris at pH 6.0. In stopped-flow technique, liposomes were mixed instantaneously with the solution containing weak acids or NH₃ and the rapid change of intravesicular fluorescence was recorded. Initial pH of solution was adjusted as the one that has appropriate steepness of the slope of traces for fitting ; the initial pH of solutions containing NH₄Cl and oxalic acid were 6.0 and those of the formic acid and lactic acid were 7.0. For the calibration the same experiments with the solution of known pH containing nigericin were done in the end of every experiment.

The typical tracing of time course of the changes in fluorescence of liposome was shown in figure 3 and 4. In figure 3, an imposition of an inwardly directed 30 mM NH₄Cl gradient (Δ [NH₃] =0.028 mM) increased the fluorescence intensity very rapidly presumably by increase in

Table 2. Buffer capacity of various experimental systems measured by weak base method

	Buffer capacity mmole/pH unit
50 mM mes/tris pH 6.0	
Liposome	3.71 ± 0.65
BBMV	26.62 ± 3.14
BLMV	28.21 ± 3.96
50 mM hepes/tris pH 7.0	
Liposome	19.84 ± 1.55
BBMV	168.01 ± 24.30
BLMV	164.29 ± 20.71
proximal tubule cell	24.35 ± 3.98

intravesicular pH which results from the influx of NH₃ and formation of NH_4^+ at expense of H^+ inside the liposomes. The average time constant (τ) the time taken for e^{-1} of the total reaction to complete, was 0.004 sec. In figure 4, liposome was exposed to 30 mM gradients of various weak acids where concentration of nonionic form of formic acid, lactic acid and oxalic acid was 0.0169, 0.0131 and 0.00057 mM, respectively. The average time constants were 0. 3, 2.5 and 5 sec, respectively. The permeability coefficients calculated according to the equation above from these tracings were summarized in table 3.

In measuring the permeabilities of highly permeant solutes, transport can be restricted by nonmembraneous barrier, extravesicular or intravesicular unstirred layer. To examine whether the unstirred layer affected the measurement of permeability with this system, liposomes were exposed to various concentrations of nonionized formic acid produced by changing the pH of the external solution(Fig. 5A). Initial rate of influx of formic and acid through the liposome at t=0 (J_{HA} t=0) was calculated and plotted as the function of external concentration of nonionized formic acid, $[HF]_0$. The initial rate of influx of formic acid varied linearly with the external formic acid concentration (Fig. 5B). The slope of this
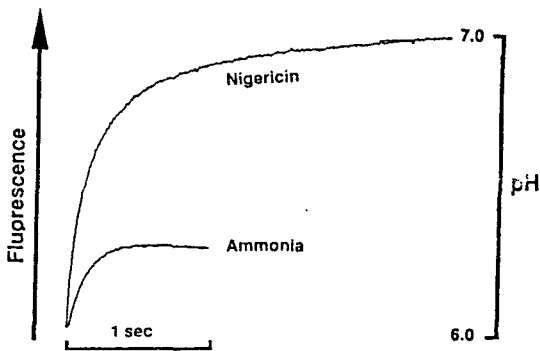
Fig. 3. The time course of the change in intravesicular pH of liposome. Liposomes loaded with 6-CF were exposed to the same buffer solution of pH 6.0 containing 30 mM of NH₄Cl or 5 μm nigericin at 25 ℃. The loading solution was composed of 100 mM mannitol, 100 mM KCl, and 50 mM mes/tris pH 6.0.
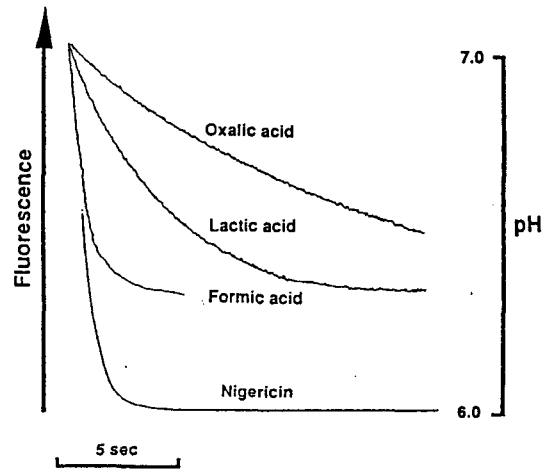

Fig. 4. The time course of the change in intravesicular pH of liposomes. Liposomes loaded with 6-CF were exposed to the same buffer solution of pH 7.0 containing 30 mM of formic acid and lactic acid, pH 6.0 containing 30 mM oxalic acid, and 5 μM nigericin of both pH at 25 ℃.

Table 3. Permeability coefficients of NH₃ and weak acids in various membranes at 25 ℃ and 37 ℃

| | Permeability coefficient(cm/sec) | |
	25 ℃	37 ℃
Liposome		
NH₃	$(2.9 \pm 0.7) \times 10^{-2}$	$(6.8 \pm 1.1) \times 10^{-2}$
Formic acid	$(4.9 \pm 0.2) \times 10^{-3}$	$(8.7 \pm 1.3) \times 10^{-2}$
Lactic acid	$(2.7 \pm 0.6) \times 10^{-4}$	
Oxalic acid	$(1.5 \pm 0.4) \times 10^{-5}$	
BBMV		
NH₃	$(4.0 \pm 0.8) \times 10^{-2}$	$(7.8 \pm 1.9) \times 10^{-2}$
Formic acid	$(9.1 \pm 1.1) \times 10^{-3}$	$(1.7 \pm 0.2) \times 10^{-2}$
Lactic acid	$(2.9 \pm 0.8) \times 10^{-3}$	
Oxalic acid	$(1.3 \pm 0.2) \times 10^{-3}$	
BLMV		
NH₃	$(2.4 \pm 0.1) \times 10^{-2}$	
Formic acid	$(3.8 \pm 0.2) \times 10^{-3}$	
Lactic acid	$(2.8 \pm 0.5) \times 10^{-3}$	
Oxalic acid	$(7.0 \pm 0.8) \times 10^{-4}$	
Proximal tubule cell		
NH₃		$(5.9 \pm 1.0) \times 10^{-2}$
Formic acid		$(5.0 \pm 0.9) \times 10^{-3}$
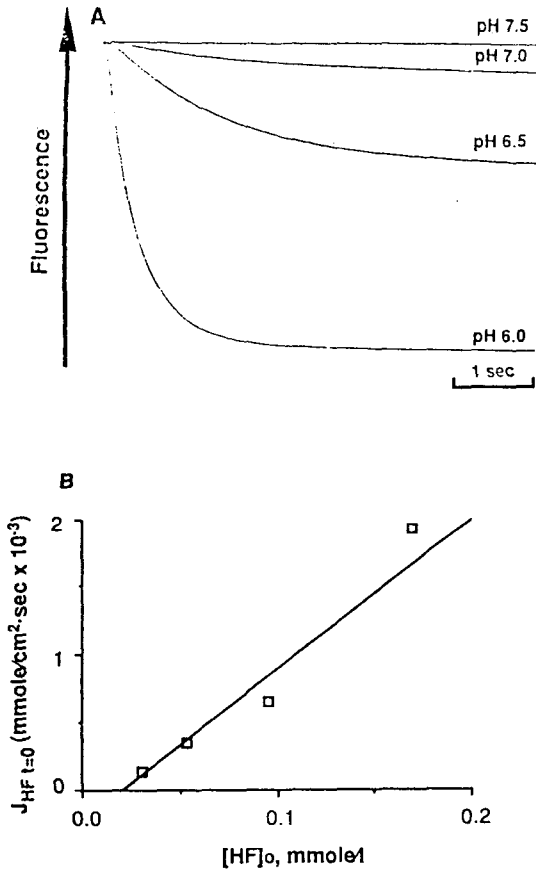
Fig. 5. *A: The time course of decrease in intravesicular pH of liposome by exposure of 30 mM formic acid at various pH_0. B: Initial rate of formic acid influx at various concentrations of nonionic formic acid outside, $[HF]_0$, generated by changes in pH_0.*
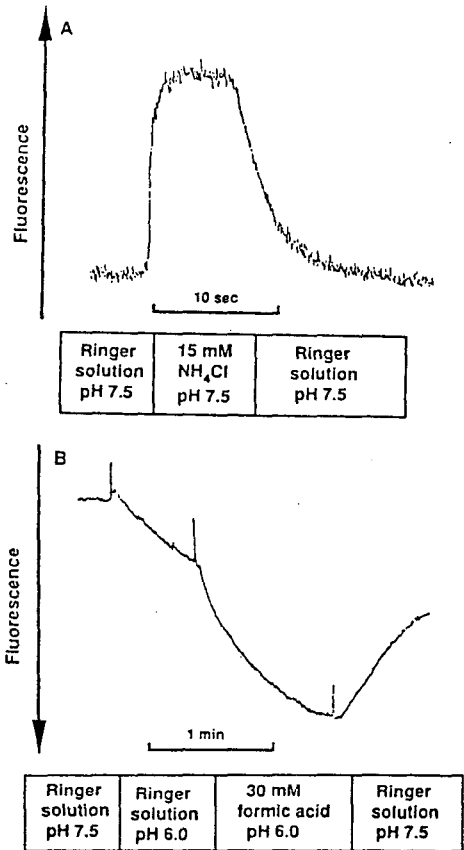

Fig. 6. *The time course of the change in intracellular pH of the proximal tubule cells. A:Cells loaded with BCECF were perfused with the sequence of Ringer solution of pH 7.5 followed by solution containing 15 mM NH_4Cl. B: Cells equilibrated with Ringer solution of pH 6.0 were exposured to 30 mM formic acid. At the end of experiments, cells were perfused again with Ringer solution of pH 7.5.*

relationship, 5.1×10^{-3} cm/sec, was equal to the permeability of formic acid in the liposome calculated from the tracing in Fig. 4. This result indicates that there was no significant unstirred layer effect in stopped-flow measurements with entrapped fluorophore.

Permeabilities of NH_3 and weak acids in renal BBMV and BLMV

The time courses of change in the intravesicular pH of renal BBMV and BLMV

showed the same tracing as those of liposomes except showing slower time constants (data not shown) and higher buffer capacity. The permeability coefficients calculated from these data are summarized in table 3.

Permeabilities of NH_3 and weak acids in primary cultured proximal tubule cells

Monolayer of proximal tubule cells grown on

Table 4. Comparison of permeability coefficients(cm/sec) measured by different methods

	Present study stopped-flow		Walter flux	Wolosin flux	Klocke continuous flow	Golchini fluorimeter	Knepper micropuncture
	25 ℃	37 ℃	25 ℃	25 ℃	37 ℃	37 ℃	37 ℃
Lipid membrane							
NH₃	2.9×10^{-2}	6.8×10^{-2}	1.3×10^{-1}				
Formic acid	4.9×10^{-3}	8.7×10^{-2}	7.3×10^{-3}	2.3×10^{-4}			
Lactic acid	2.7×10^{-4}		5.0×10^{-5}	0.5×10^{-4}			
Natural membrane							
NH₃	4.0×10^{-2}	7.8×10^{-2}				1.0×10^{-2}	
Formic acid	9.1×10^{-3}	1.7×10^{-2}				1.5×10^{-3}	
Lactic acid	2.9×10^{-3}					1.1×10^{-4}	
Intact renal cell							
NH₃		5.9×10^{-2}				7.1×10^{-3}	6.0×10^{-2}

the coverslip was held in perfusion chamber and the change in fluorescence was monitored with inverted epifluorescence microscopy (Roos & Boron, 1981). Figure 6 shows the typical tracing of the change in intracellular pH of proximal tubule cells when perfused with buffers of several pH containing NH₃ or weak acids. In Fig. 6A BCECF-loaded cells were perfused with the Ringer solution of pH 7.5 and after reaching an equilibrium, reaction was started by perfusion of solution containing 15 mM NH₄Cl. At the end of experiments the cells were perfused with Ringer solution of pH 7.5 again to calculate the leakage of BCECF. The initial portion of the slope after exposure of the 15 mM NH₄Cl was used for calculation of permeability coefficient. In Fig. 5B the cells were perfused with Ringer solution of pH 7.5 first, then changed into the same solution of pH 6.0, which resulted considerable proton leak as manifested by the decrease in BCECF fluorescence before exposure to formic acid gradient. The reaction was started by perfusion of Ringer solution of pH 6.0 containing 30 mM formic acid and was terminated by changing the perfusion solution into Ringer solution of pH 7.5. The initial portion of the slope after changing the solution containing 30 mM formic acid was used for calculation of

permeability coefficient with the correction of proton leak when perfused with solution of pH 6.0 not containing formic acid.

Table 3 summarized permeability coefficients of NH₃ of weak acids in liposome, BBMV, BLMV and proximal tubule cell. NH₃ has the similar permeability coefficient values throughout the various membranes from artificial membrane to cell, which means that NH₃ permeates the plasma membrane via a lipid pathway not other possible pathways such as channels. Among the weak acids tested, formic acid has the highest permeability coefficient value in liposome though all the weak acids have similar partition coefficients, which indicated that small molecules are easier to permeate the lipid bilayer than larger molecules of the same partition coefficient presumably because of structural fitting into tiny holes between acyl chains of lipid molecules.

In table 4, data measured by the stopped-flow technique and epifluorescence microscopy with fluorescence dye were compared with other data measured by different techniques in similar membrane systems. The data of present study correlated well with those of continuous flow method and micropuncture study but not with that of flux study.

DISCUSSION

Knowledge of membrane properties has come largely from studies on model membrane systems. Because biological membranes contain a lipid bilayer, models of membrane system involve as a starting point, the generation of an experimental model of lipid bilayer, that is either planar bilayer of unilammellar bilayer vesicles, i.e., liposomes. Although lipid membrane model system has been useful for physical and chemical understanding of transport, there are some properties of cell membrane that are not mimicked by these systems and some problems of artifact contaminated in the course of preparation. More physiologic information of membrane can be obtained from the membrane vesicles containing membrane proteins. Isolation of membrane derived from a defined pole of the epithelial cell often constitutes an essential step in the investigation of membrane component, membrane properties and their regulation. More biological information can be obtained using the cell system and epithelial cells are the model of choice for transport studies given their polar characteristics and ease of manipulation.

Transport mechanisms in biological system can be studied by a variety of physical or chemical techniques. The most widely used approach for tracing fluxes is rapid filtration method using radioisotope. But this method is limited by it's specific activity, total radioactivity, the efficiency and speed of separation, and the capacity of the biological system to retain it's structural integrity during separation. Since the amount of information obtained from the rapid filtration method is often not precise enough for a thorough kinetic evaluation due to sampling and counting error. Thus this method is inadequate to study transport processes with half-times under 2 s unless special filtration equipment is used. Fluorescence has been considered to be a suitable alternative to the classi-

cal radiotracer method with advantages of high sensitivity and temporal resolution, ease of manipulation, and smaller sample requirement. And also it permits continuous monitoring of transport by fluorescence without requiring periodic sampling.

The permeability calculation contains the implicit assumption that both intracellular and extracellular fluid spaces are well-stirred compartments without any diffusion gradients. This condition can be accomplished by stopped-flow and continuous-flow method. In continuous-flow method, flow of both solutions through mixer is constant not turbulent, so a lot of samples are required for one measurement. But in the stopped-flow method which has the required mixing efficiency and dead time (2 ms), the flow of two solutions suddenly stop after instantaneous mixing. So, well-stirred condition can be satisfied with smaller requirement of samples.

This study was aimed to improve the methodology of rapid nonelectrolyte transport measurement and to measure the precise permeability in a variety of membrane modes. The permeability of NH_3 and weak acids in phosphtidylcholine liposome, renal brush border and basolateral membrane, and primary cultured renal proximal tubule cells was measured by monitoring the change in intravesicular or intracellular pH on exposure to gradients of NH_4Cl and weak acids. The measured permeability of NH_3 was 2.9×10^{-2} cm/sec at 25 °C in liposome and 5.9×10^{-2} cm/sec at 37 °C in renal proximal tubule cells. This value agrees reasonably well with that Knepper (Knepper, 1991) reported $(6.0 \times 10^{-2}$ cm/sec) in intact tubule at 37 °C.

NH_3 permeabilities of intact cells have been measured in several cell systems using the system of epifluorescence microscopy. Compared the permeability coefficients measured in Swiss 3T3 fibroblasts and MDCK cells, that of renal tubule cell has ten-fold higher value (data not shown). According to Knepper and his col-

leagues who measured NH_3 permeability in several renal tubule segments (Star et al, 1987; Knepper, 1991), proximal tubule has the highest value. These differences among the segments or different cell systems are thought to be due largely to differences in the surface area of each plasma membrane although the differences in the intrinsic NH_3 permeability of each membrane cannot be ruled out.

Generally, the permeation of molecules across cell membrane follows Overton's rule (1899), that is, there is a correlation between the permeability coefficient and the lipid/water partition coefficient of a substance. As has recently been observed in lipid bilayers, Overton's rule appears to underestimate the permeability of smaller molecules (MW < 50) by 2- to 15-fold. In this preparation, the permeability of smaller molecules appears also to be inversely correlated with their molar volume. Formic acid permeates lipid bilayer membrane faster than predicted by Overton's rule (1899 & 1902), i.e., faster than predicted by it's hydrophobicity or by the behavior of other members of it's homologous alkyl series. Possible explanations for this behavior include the "soft polymer" hypothesis, which proposes that very small molecules fit into holes which are abundantly available in the acyl chain region of the bilayer. Another possible explanation is that "transient aqueous pores" exist in lipid bilayers. In the present study, formic acid has the highest permeability among weak acids we tested in liposome.

We used 50 mM hepes/tris or 50 mM mes/tris buffer in this experiments. In poorly buffered solutions, as NH_3 permeates the membrane the pH in the extracellular unstirred layer will decrease and pH in the intracellular unstirred layer will increase. This will result in the $[NH_3]/[NH_4^+]$ in the extracellular unstirred layer being less than the bulk extracellular solution and an increase in the $[NH_3]/[NH_4^+]$ in the intracellular unstirred layer relative to the bulk intracellular solution. The net rate of NH_3 permeation will then decrease. Increasing the buffer capacity of the bulk solution prevents pH gradients from forming in the unstirred layers. So, the buffering system used in this study are sufficiently high to prevent pH gradient from forming in the extracellular unstirred layer, if present.

When the initial rate of formic acid fluxes at several extravesicular pH's was measured and plotted as the function of the concentrations of nonionized form of formic acid, linear relationship was obtained. This implies that the value of permeability measured by the stopped-flow technique is not underestimated by unstirred layer effects.

In summary, the permeabilities of NH_3 and weak acids in liposome, renal BBMV and BLMV, and renal tubule cells were measured using stopped-flow technique and epifluorescence microscope using fluorescent indicator. The values of permeabilities measured are correlated with, but somewhat higher than the data reported previously, which suggests that these systems overcome common opposing difficulties in measuring the rapid transport systems, such as unstirred layer effect, very rapid reaction time, and requiring highly sophisticated skill or large amount of sample in some measuring system. Thus, it can be concluded that stopped-flow technique with fluorescence dye can be established as one of the precise and easy methods in measuring rapid transport of nonelectrolytes.

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