Membrane Water and Solute Permeability Determined Quantitatively by Self-Quenching of an Entrapped Fluorophore†

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ABSTRACT: Quantitative determination of rapid water and solute transport and solute reflection coefficients by light-scattering methods is complicated by dependence of vesicle or cell light scattering on nonvolume factors including solution refractive index, cell motion, and membrane aggregation. To overcome these difficulties, a fluorescence technique has been developed to measure accurately (1) osmotic water permeability (P_f), (2) solute permeability (P_s), and (3) solute reflection coefficient (σ). The time course of vesicle volume is determined by the self-quenching of entrapped fluorescein sulfonate (FS), the best of a series of dyes screened for self-quenching, brightness, and vesicle loading/trapping. To validate the method, rabbit renal brush border vesicles (BBV) were loaded with 1–10 mM FS for 12 h at 4 °C and washed to remove extravesicular FS. FS leakage occurred over >6 h at 4 °C and >30 min at 23 °C. FS fluorescence vs vesicle volume was calibrated from the time course of fluorescence decrease (excitation 470 nm, emission >515 nm) in response to a series of inward osmotic gradients in a stopped-flow apparatus. At 23 °C P_f was 0.005 ± 0.001 cm/s, independent of osmotic gradient size, and inhibited 67% by 0.5 mM $HgCl_2$. Urea P_s was 2×10^{-6} cm/s with σ 0.95–1.00 on the basis of the fluorescence time course analysis and the extravesicular [urea] required to obtain zero initial volume flow (null method) when vesicles were loaded with sucrose. P_f and urea P_s determined by light scattering in the presence and absence of FS were the same as those determined by FS fluorescence kinetics. The FS self-quenching method has general applicability for measurement of rapid water and solute transport in small cells and vesicles and in membrane compartments labeled with impermeant fluorescent markers that undergo self-quenching.

The measurement of osmotic water and solute transport in isolated membrane vesicles provides a direct means of examining the permeability characteristics of a functionally homogenous membrane. Interpretation of transport data is uncomplicated by membrane heterogeneity resulting from cell polarity and by unstirred layer effects resulting from complex cell surface geometry and cytoplasmic contents.

Stopped-flow light scattering has been used to measure rapid osmotic water and solute transport in a variety of small cells and membrane vesicles (Verkman et al., 1985; Terwilliger & Solomon, 1981; Illsley & Verkman, 1986; Meyer & Verkman, 1987; Worman et al., 1986; Worman & Field, 1985). The intensity of light scattered from the vesicles provides an instantaneous measure of vesicle volume. The time course of scattered light intensity following exposure of vesicles to a gradient of a permeant solute provides information about the permeability coefficients for osmotic water and solute transport and the solute reflection coefficient. However, vesicle volume is not the sole determinant of scattered light intensity; effects of solute-related refractive index changes (Levitt & Mlekoday, 1983; Chen & Verkman, 1987), artifacts of centrifugal vesicle or cell motion (Mlekoday et al., 1983), solute cavitation, and membrane aggregation limit the quantitative application of light scattering for measurement of rapid solute transport and for determination of solute reflection coefficients.

We report here a new method for measurement of rapid osmotic water and solute transport in vesicles based on the self-quenching of an entrapped fluorophore. Measurement of vesicle fluorescence provides an instantaneous measure of vesicle volume insensitive to changes in solution refractive index and vesicle motion or aggregation artifacts. The method is applied to measurement of rapid osmotic water and nonelectrolyte transport and urea reflection coefficient in brush border membrane vesicles isolated from renal proximal tubule. The method has wide application to studies of water and solute transport in small cells and vesicles and to transport studies in other closed membrane structures, such as endosomes, that can be labeled selectively by an impermeant fluorescent marker.

Materials and Methods

Fluorescein sulfonate (FS) was obtained from Molecular Probes Inc. (Junction City, OR). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Solutions of $HgCl_2$ were prepared immediately prior to use and protected from light exposure.

Membrane Isolation. Brush border vesicles (BBV) from rabbit renal cortex were prepared as described previously (Verkman & Ives, 1986a). The renal cortex from 2–3-kg New Zealand White rabbits was dissected, homogenized, and centrifuged at low speed to remove tissue fragments. To remove nonbrush border membranes, the supernatant was purified by magnesium aggregation and differential centri-

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fugation. Enrichment of specific activity of the brush border enzyme maltase was >15-fold over that in the crude homogenate. BBV were washed twice with 50 mM sucrose containing 5 mM 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid/tris(hydroxymethyl)aminomethane (HEPES/Tris), pH 7.5, maintained at 4 °C, and used within 48 h of preparation.

Transport Measurements. BBV were loaded with FS by incubation of BBV (10 mg of protein/mL) with 10 mM FS for 18 h at 4 °C. Extravesicular FS was removed by three washes in >50 volumes of buffer not containing FS. BBV were homogenized through 23- and 26-gauge needles after each centrifugation (400000*g*, 10 min). BBV were suspended at a concentration of 10 mg of protein/mL and maintained in 50 mM sucrose containing 5 mM HEPES/Tris, pH 7.5, at 4 °C until the time of the experiment (<2 h). Under these conditions, there was less than 4% leakage of FS per hour as judged from measurements of the FS fluorescence response to pH gradients as described previously (Verkman & Ives, 1986b).

Stopped-flow measurements were performed with a Hi-Tech stopped-flow apparatus (Wiltshire, England) interfaced to a MINC/23 computer (Digital Equipment Corp., Maynard, MA). The instrument dead time was <2 ms with a maximum rate of data acquisition of 25 kHz. A 0.075-mL aliquot of BBV (0.1–0.2 mg of protein/mL) was mixed with an equal volume of buffer to give specified solute gradients. Fluorescence was excited at 470 nm with a single-grating monochromator in series with a 450–490-nm interference filter (Omega Optical Co., Brattleboro, VT) and measured through two OG515 cut-on filters (Schott Glass Co., Duryea, PA). Under these conditions, <1% of the measured fluorescence signal was due to scattered light as judged from parallel experiments performed with BBV loaded and not loaded with FS. In some experiments the time course of 90° scattered light intensity at 470 nm was recorded in parallel to the fluorescence measurement. P_f was determined from the time course of FS fluorescence by comparing exponential time constants of experimental data with exponential time constants of theoretical curves calculated from the Kedem–Katchalsky equations in which P_f is varied (Illsley & Verkman, 1986). Single-exponential fits did not deviate systemically from the experimental data. The slightly nonexponential curve shape predicted from the the Kedem–Katchalsky equations becomes almost exponential when a distribution in vesicle surface-to-volume ratios is present (Meyer & Verkman, 1986). P_f was determined from the time course of scattered light intensity in a similar manner as described previously (Verkman & Ives, 1986a).

Steady-state fluorescence spectra and lifetimes were determined on a SLM 48000 fluorometer (SLM Instruments, Urbana, IL). To eliminate inner filter effects, measurements of FS self-quenching in free solution were performed in 1-uL capillary tubes with a Nikon inverted epifluorescence microscope with photometry interfaced to an IBM PC/AT computer.

Results

A series of fluorophores reported to undergo fluorescence self-quenching were screened for applicability as an entrapped indicator of cell and vesicle volume. The indicator requirements for use in measurement of rapid volume changes were (1) rapid loading and slow leakage characteristics, (2) high sensitivity to cell/vesicle volume changes, (3) rapid response to volume changes (<1 ms), (4) bright fluorescence (high quantum yield and molar absorbance) without interference by autofluorescence, (5) no membrane toxicity, and (6) minimal dependence of fluorescence signal on pH and other

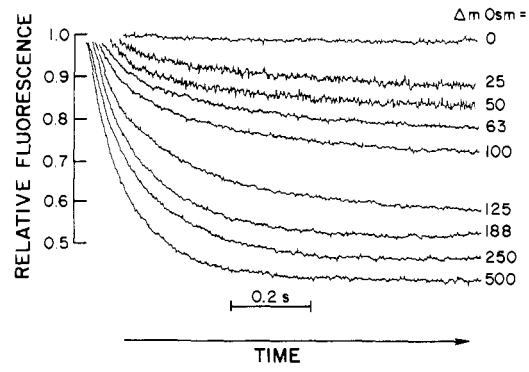

FIGURE 1: Time course of BBV osmotic water transport. BBV in buffer A were mixed with 5 mM HEPES/Tris, pH 7.5, plus varying [sucrose] to give the specified inward sucrose gradients. Curves are offset in the *x* direction for clarity. All measurements were performed at 23 °C.

nonvolume parameters. Fluorophores tested included calcein, fluoresceins, and rhodamines. The best compound tested was the fluorescein derivative fluorescein sulfonate (FS).

In steady-state fluorescence measurements of 0.1 mM FS in 50 mM sucrose containing 5 mM HEPES/Tris, pH 7.5 (buffer A), FS has peak excitation and emission wavelengths of 495 and 525 nm, respectively. In its completely ionized form (>pH 8.0), the FS quantum yield is >0.95 with molar absorbance of 93 000 M^{-1} cm^{-1}. FS fluorescence increased with pH with an apparent pK of 6.3; fluorescence is insensitive to pH (less than 5% change) above pH 7.0. In buffer A, fluorescence vs [FS] is linear in the range 0–5 mM, peaks at 8 mM FS, and then decreases, reaching 50% maximum fluorescence at 14 mM FS. The self-quenching curve is unaffected by neutral solutes including urea, glucose, and sucrose. The fluorescence lifetime of FS is 4.0–4.4 ns below 5 mM FS and decreases to <1 ns at 20 mM FS, consistent with a collisional mechanism for FS self-quenching. Mixture of equal volumes of 20 mM FS in buffer A with buffer A in the stopped-flow apparatus gives a time-independent signal, indicating that the FS fluorescence increase occurs within the instrument dead time (<2 ms), consistent with diffusion-limited FS self-quenching kinetics.

Loading of FS into isolated brush border vesicles (BBV) was accomplished by an 18-h incubation with 10 mM FS in buffer A at 4 °C. At pH 7.5, the half-time for FS leakage from vesicles was >8 h at 4 °C and 45 min at 23 °C, similar to results reported for 6-carboxyfluorescein leakage from BBV (Verkman & Ives, 1986b). At pH 8.5 leakage of FS is very slow (>48 h at 4 °C). Rapid loading of FS into vesicles was possible at lower pH (e.g., 15 min, pH 5, 37 °C) or when using the permeant diacetate derivative of fluorescein sulfonate, which is cleaved by intravesicular esterases into FS.

Osmotic Water Permeability. Figure 1 shows the time course of FS fluorescence following rapid mixture of BBV containing 10 mM FS with hyperosmotic solutions to give specified inward sucrose gradients. There is a monophasic fluorescence decrease due to osmotic water efflux, BBV shrinkage, and FS self-quenching. The amplitude of the signal increased with increasing gradient size due to the smaller final BBV volume. No time-dependent signal was observed in the absence of an osmotic gradient, showing absence of mixing artifacts. At constant inward osmotic gradient, the signal was unaffected by the nature of the impermeant solute used; for 125 mM inward gradients of sucrose, raffinose, and mannitol, exponential time constants and relative preexponential factors were 0.21 ± 0.01 s and 1.00, 0.20 ± 0.02 s and 1.02 ± 0.03, and 0.21 ± 0.01 s and 0.99 ± 0.01, respectively (mean ± SD,

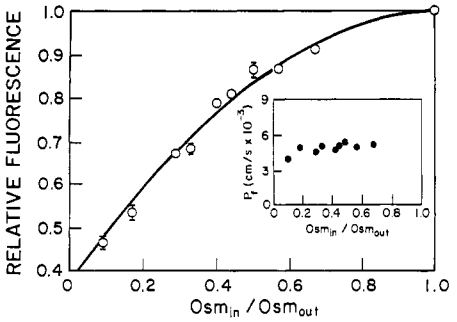

FIGURE 2: Dependence of fluorescence on BBV volume. Curves from Figure 1 were fitted to single-exponential functions. Initial fluorescence intensity was normalized to unity. Relative fluorescence (mean ± SD, $n = 4$), defined as the difference between the initial fluorescence intensity (before volume movement occurs) and that after osmotic equilibration (at infinite time), was determined from the preexponential factors. Data were fitted to the quadratic relation, fluorescence= $AV^2 + BV + C$, where V is relative vesicle volume (Osm_{in}/Osm_{out}), with fitted $A/B = -0.49$. Inset: Dependence of P_f on osmotic gradient size. P_f (mean ± SD) was calculated as described under Materials and Methods with BBV surface-to-volume ratio equal to 2×10^5 cm^{-1} (Verkman et al., 1985).

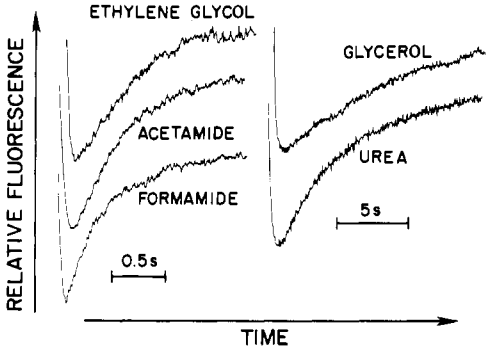

FIGURE 3: Time course of BBV nonelectrolyte transport. BBV in buffer A were subjected to 125 mM inward gradients of the indicated nonelectrolyte solutes. Curves are offset and scaled arbitrarily in the y direction. Fitted exponential time constants for the phase of solute entry (mean ± SD, $n = 4$): 0.58 ● 0.02 s (ethylene glycol), 0.50 ● 0.03 (acetamide), 0.38 ± 0.02 (formamide), 10.5 ± 0.4 (glycerol), and 5.4 ± 0.3 (urea).

$n = 4$).

Calculation of absolute P_f from the time course of fluorescence decrease requires knowledge of the FS fluorescence vs BBV volume relation. Figure 2 shows the relative FS fluorescence, determined from single-exponential regressions to the time course data, as a function of vesicle volume, determined from the ratio of intravesicular/solution osmolarities before osmotic water movement has occurred. The data were fitted empirically to a quadratic relation, which was required to convert fluorescence to volume for determination of absolute permeability coefficients. Calculated P_f was independent of osmotic gradient size (Figure 2, inset), indicating the presence of a single rate-limiting barrier to BBV water transport (plasma membrane) without submembrane resistances (Illsley & Verkman, 1986). Addition of 0.5 mM $HgCl_2$ resulted in a 67 ± 4% (SD, $n = 4$) decrease in P_f for a 125 mM sucrose gradient, similar to results reported by the light-scattering method (Meyer & Verkman, 1987), supporting the existence of protein-bounded water channels in the proximal tubule brush border membrane.

To demonstrate that P_f determined by FS fluorescence was accurate and that FS did not itself alter water transport, parallel measurements of FS fluorescence and light scattering were performed. For inward sucrose gradients of 125 and 250 mM, P_f determined by FS fluorescence and light-scattering methods did not differ significantly (mean ± SD in cm/s × 10^{-3}: 125 mM, 4.5 ± 0.5 fluorescence vs 4.8 ± 0.6 scattering; 250 mM, 4.2 ± 0.3 fluorescence vs 4.3 ± 0.2 scattering). For a 125 mM inward sucrose gradient, P_f determined by light scattering in the presence of 10 mM FS (0.0044 ± 0.0003 cm/s) did not differ from P_f measured in the absence of FS (0.0047 ± 0.0004 cm/s). Interestingly, although FS does not undergo self-quenching in free solution at a 1 mM concentration, 1 mM FS entrapped in BBV did undergo self-quenching. In response to a 125 mM sucrose gradient there was a fluorescence decrease with an exponential time constant of 0.20 ± 0.01 s (corresponding to a P_f of 0.0049 ± 0.0002 cm/s, assuming a fluorescence vs volume calibration similar to that in Figure 2, top) with a signal-to-noise ratio 3–5-fold less than that measured with 10 mM entrapped FS (see Discussion).

Solute Permeabilities and Reflection Coefficients. In response to an inward gradient of a permeant solute, there is a biphasic time course of vesicle volume and FS fluorescence. Initial water efflux results in vesicle shrinkage and increased FS self-quenching, followed by solute and water influx, return of vesicle volume to its initial value, and increase in FS fluorescence (Figure 3). The detailed time course depends on the rates of osmotic water and solute transport and on the solute reflection coefficient. In contrast to data obtained by light-scattering and transmittance methods (Verkman et al., 1985; Pratz et al., 1986), the FS fluorescence data show nearly equal initial and final fluorescence intensities. The reason for different initial and final scattering or transmitted intensities is that the intravesicular refractive index changes with vesicle volume change and solute influx; FS fluorescence is independent of refractive index over the range of values encountered in these studies. In control experiments as above, 10 mM FS does not alter the time course of volume change, as measured by light scattering, in response to 125 mM gradients of urea, glycerol, ethylene glycol, formamide, and acetamide (not shown).

Three approaches have been used for determination of solute reflection coefficients in cells and vesicles based on volume movements induced by solute gradients: (1) null method, (2) analysis of initial water flow, and (3) analysis of the detailed volume vs time curve. In the null method, vesicles are loaded with an impermeant solute. The concentration of an external test solute is varied until no initial volume flow occurs. The ratio of the concentrations of the impermeant to the test solute at the null point gives the reflection coefficient (Goldstein & Solomon, 1960). In the second method, the reflection coefficient is determined from the ratio of the initial volume flows induced by equal osmotic gradients of a test and an impermeant solute. Both methods require accurate volume vs time data at early times. Membrane motion artifacts, refractive index changes, and very rapid solute transport have made difficult the determination of reflection coefficients by these approaches. In the third method, the complete time course or selected parts of the time course (minimum volume and time at minimum volume; Levitt & Mlekoday, 1983; Chasan & Solomon, 1985) are analyzed. Here refractive index effects can lead to a serious misinterpretation of data. In addition, it is probably not correct to assume that the nonequilibrium thermodynamic equations of Kedem and Katchalsky (1958) provide an exact description of the time course of vesicle volume, because (a) most experiments require large perturbations from equilibrium, (b) solute transport is often saturable, and (c) there is ambiguity in the mathematical definition of the mean solute concentration required for solvent drag calculation.

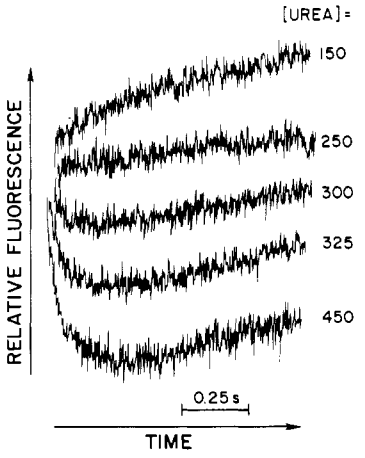

FIGURE 4: Null-point determination of the urea reflection coefficient. BBV in 250 mM sucrose containing 5 mM HEPES/Tris, pH 7.5, were mixed with an equal volume of buffer containing 5 mM HEPES/Tris, pH 7.4, and the indicated concentrations of urea. The decreased signal-to-noise ratio in these curves compared with those in Figure 3 was due to the smaller relative volume change.

Each of the three methods above has been applied to the determination of urea reflection coefficient in BBV using the self-quenching technique to measure vesicle volume change. Figure 4 shows application of the null method. BBV loaded with 250 mM sucrose were mixed with solutions containing varying urea concentrations. For [urea] = 300, 325, and 450 mM, there is an initial decrease in fluorescence due to water efflux. For [urea] = 150 mM, fluorescence increases initially due to water influx. At 250 mM urea there is little initial change in signal, placing the urea reflection coefficient near unity.

Figure 5, top, shows the 10-s time course of FS fluorescence following 75 mM inward gradients of sucrose and urea. Data were obtained with the same BBV suspension and instrument settings so that absolute signal amplitudes can be compared. Initial rates of fluorescence decrease were determined from the initial slopes of single exponentials fitted through the first 250 ms of the fluorescence time course (method 2 for reflection coefficient determination). For the 75 mM gradients, initial slopes were (in arbitrary fluorescence units/time) 1.49 ± 0.13 (SD, n = 4; sucrose) and 1.53 ± 0.15 (urea), giving a urea reflection coefficient of 1.02 ± 0.1. Similar measurements performed with 50 mM paired sucrose and urea gradients gave a urea reflection coefficient of 1.07 ± 0.1.

Figure 5, bottom, shows the theoretical dependence of the fluorescence vs time curve for different values of the urea reflection coefficient. Curves were calculated by using the Kedem–Katchalsky equations with parameters given in the figure legend. P_s (assumed to be nonsaturable) was determined to be 2×10^{-6} cm/s from a fit of the fluorescence time course at 3–10 s, similar to the value of 1.6×10^{-6} cm/s reported for tracer urea uptake in BBV (Chen & Verkman, 1987). For reflection coefficients of 0.5, 0.75, 0.9, and 1.0, it is predicted that the minimum fluorescence for the urea curves would occur at fluorescence levels 38.5, 62.9, 69.7, and 73.5%, respectively, of that for the sucrose curve. Experimentally, the urea curve reached a value 73 ± 2% of that for the sucrose curve. For the same series of reflection coefficients, it is predicted that the minimum fluorescence would occur at 1.11, 0.98, 0.87, and 0.76 s, respectively. Experimentally, the minimum fluorescence occurred at 0.65 ± 0.1 s. Thus, results from all three methods indicate that the BBV urea reflection coefficient is near unity. Results obtained by the third method must be interpreted with caution because of the uncertainties in generating the theoretical curve shape described above.

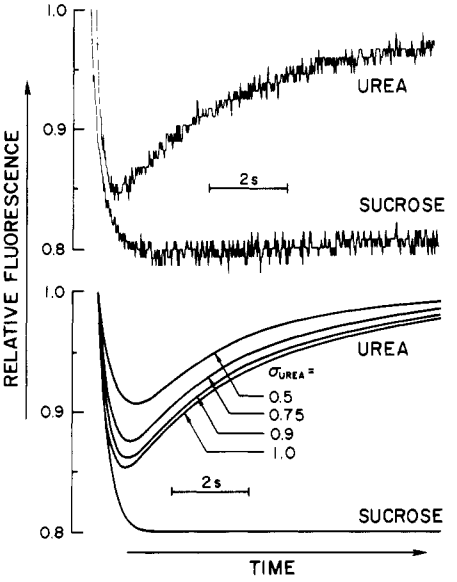

FIGURE 5: Determination of urea reflection coefficient by curve shape analysis. Top: BBV in buffer A were subject to 75 mM inward sucrose and urea gradients. Each curve is the average of quadruplicate stopped-flow experiments. The urea curve is displaced slightly in the x direction for clarity. Bottom: Theoretical curves were calculated with the equations of Kedem and Katchalsky (1958) expressing the mean solute concentration as the arithmetic mean. Volume vs time was converted to fluorescence vs time from the calibration relation given in Figure 2, top. Initial conditions were the same as those used experimentally. P_f and P_{urea} were determined from a fit of the sucrose time course and the late phase (3–10 s) of the urea time course, respectively. Parameters: P_f = 0.006 cm/s, $P_{urea} = 2 \times 10^{-6}$ cm/s with urea reflection coefficients as shown.

Discussion

Measurement of rapid osmotic water and solute permeabilities in small cells and isolated membrane vesicles requires methodology to impose rapidly solute gradients and to measure the instantaneous time course of cell or vesicle volume during dissipation of osmotic and solute gradients. The stopped-flow kinetic method has the required mixing efficiency and dead time (<2 ms) to examine volume transients over ~5 ms to >30 s. Light scattering or transmittance (0° scattering) has been used to measure cell or vesicle volume transients in a wide variety of biological systems including intact red cells (Terwilliger & Solomon, 1983; Mlekoday et al., 1983) and platelets (Meyer & Verkman, 1986) and isolated epithelial vesicles from kidney (Verkman et al., 1985; Meyer & Verkman, 1987), trachea (Worman et al., 1986), intestine (Worman & Field, 1985; van Heeswijk & van Os, 1986), and placenta (Illsley & Verkman, 1986).

Although light-scattering and transmittance studies have provided accurate values for osmotic water permeability and approximate values for solute permeabilities, the determination of solute reflection coefficients and accurate permeabilities for highly permeant solutes has been complicated by several difficulties. (1) Because the intensity of scattered light depends upon both vesicle volume and the refractive indexes of intra- and extravesicular solutions, it is difficult to transform scattering vs time data into volume vs time data, particularly during solute entry, when large changes in intravesicular refractive index occur (Levitt & Mlekoday, 1983). Attempts have been made to account for this effect by assuming that scattered light intensity is a simple sum of volume and refractive index terms and that the refractive index term can be estimated from measurements using dextran (Levitt &

Mlekoday, 1983; Holmberg et al., 1987). If no corrections are made, P_s is relatively independent of refractive index when $P_f \gg P_s$ and when P_s is determined from analysis of the light-scattering time course at times long compared to the initial phase of osmotic water flux (Chen & Verkman, 1987). (2) A light-scattering artifact is present when cells are studied in a stopped-flow apparatus, probably because of cell alignment along centrifugal flow lines before flow within the observation cell ceases. In red cells or red cell ghosts mixed with isosmotic buffers, there is a marked light-scattering transient within the first 100 ms, which has been taken into account approximately by subtraction procedures (Terwilliger & Solomon, 1981) or by preswelling cells in hypotonic media to convert biconcave disks into spheres (Mlekoday et al., 1983). (3) The intensity of scattered light depends also upon the state of cell or vesicle aggregation. Signal transients may arise from an alteration in the equilibrium state of cell or vesicle aggregation caused by the stopped-flow mixing procedure. In addition, the intensity of scattered light is very sensitive to solution cavitation caused by pressure gradients during the mixing procedure. These effects may be marked at high temperatures or when solutions are degassed inadequately.

Because of these difficulties, it has not been possible to measure confidently the reflection coefficients of common solutes. In the human red cell, a long-standing controversy has existed over the possibility that water and urea share a common pathway. However, despite a variety of stopped-flow light-scattering approaches, red cell urea reflection coefficients have been reported throughout the range 0.55–0.95 (Goldstein & Solomon, 1960; Levitt & Mlekoday, 1983; Chasan & Solomon, 1985).

The use of entrapped fluorophores that undergo fluorescence self-quenching has been reported for studies of vesicle fusion (Kendall & MacDonald, 1982; Blumenthal et al., 1982) and membrane integrity (Chen, 1977; Weinstein et al., 1979). Fluorescein derivatives have been used most often as fluorophores that undergo self-quenching; however, rhodamines and other fluorophores having overlapping excitation and emission spectra often self-quench by nonradiative energy transfer. For transport studies, we selected the fluorescein derivative fluorescein sulfonate after screening a series of compounds for self-quenching, brightness, and vesicle loading/trapping. FS can be loaded into membrane vesicles by prolonged incubation (>24 h at 4 °C), by brief incubation at low pH, or with the diacetate form of FS, which is cleaved by intravesicular esterases into FS. FS leaks out from vesicles on a time scale slow compared to the time required for transport measurements. FS does not itself alter water or urea transport, and P_f and urea P_s values determined by FS fluorescence match those obtained by light scattering. Because self-quenching of FS is a diffusion-limited process, FS fluorescence responds in <1 ms to changes in FS concentration.

There are distinct advantages to measurement of rapid solute transport by fluorescence compared to scattering or absorbance. Fluorescence intensities are insensitive to changes in solution refractive index, cell motion, membrane aggregation, and solution cavitation. This allows for accurate analysis of the early time course of fluorescence following an osmotic gradient and for the direct interpretation of fluorescence intensities in terms of vesicle volume. The transformed volume vs time data can then be fitted to specified models for coupled water and solute movement as shown here for the determination of the urea reflection coefficient in renal brush border vesicles.

The application of self-quenching fluorophores to the measurement of cell or vesicle volume changes requires several control experiments for each system studied. Fluorophore loading and leakage characteristics must be measured. Possible direct toxic effects of the fluorophore on membrane integrity and function should be considered. Quantitative analysis of kinetic data in terms of permeability coefficients requires knowledge of the fluorescence vs volume relation, obtainable from measurements of the fluorescence signal in response to a series of gradients of an impermeant solute. It is interesting that the fluorescence vs [FS] relation is quite different in vesicles from that in free solution; significant self-quenching occurs at <0.1 mM FS in vesicles, but at >10 mM FS in free solution. We have been able to study self-quenching of ~0.1 mM fluorescein–dextran (4000 Da) entrapped in vesicles. The amplified self-quenching response of fluoresceins in vesicles may be due to fluorescein binding to charged proteins at the inner membrane surface or to modification of the ionic environment in the vesicle by membrane-bound proteins. Last, direct effects of the test solute on the fluorescence vs volume relation must be excluded or taken into account in the analysis. While nonelectrolytes such as sucrose, urea, and glucose do not alter the fluorescence self-quenching relation, ionic salts cause a concentration-dependent decrease in self-quenching, probably from an ionic strength dependence for fluorescein–fluorescein interactions.

In addition to the determination of water and solute permeabilities and reflection coefficients, the self-quenching method has application to measurement of water and solute transport in subpopulations of vesicles labeled with a fluorophore that undergoes self-quenching. We recently used 6-carboxyfluorescein and fluorescein–dextran to label endosomal vesicles in intact kidneys (Verkman et al., 1988). Osmotic water transport was measured in the endosomal vesicles by using a crude microsomal preparation, of which <10% of the vesicles contained fluorescein. It was possible to demonstrate the existence of a vasopressin-inducible population of endosomes from papillary collecting duct that contained functional water channels.

The general approach of tagging vesicles with a volume-sensitive fluorescent marker can be tailored for specialized applications. Increased sensitivity of the fluorescence vs volume relation is possible by measuring sensitized emission with a pair of impermeant, fluid-phase fluorophores that undergo energy transfer. Alternately, volume-sensitive fluorescence can be obtained with an impermeant fluorophore and quencher pair or a fluorophore that undergoes eximer formation with a spectral red shift. With imaging fluorescence microscopy, measurement of water movement and volume changes in intracellular membrane compartments is feasible using the fluorescence methodology reported here.

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Fluorescence Resonance Energy Transfer within the Complex Formed by Actin and Myosin Subfragment 1. Comparison between Weakly and Strongly Attached States†

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ABSTRACT: Fluorescence resonance energy transfer measurements have been made between Cys-374 on actin and Cys-177 on the alkali light chain of myosin subfragment 1 (S1) using several pairs of donor–acceptor chromophores. The labeled light chain was exchanged into subfragment 1 and the resulting fluorescently labeled subfragment 1 isolated by ion-exchange chromatography on SP-Trisacryl. The efficiency of energy transfer was measured by steady-state fluorescence in a strong binding complex of acto–S1 and found to represent a spatial separation between the two probes of 5.6–6.3 nm. The same measurements were then made with weak binding acto–S1 complexes generated in two ways. First, actin was complexed with *p*-phenylenedimaleimide–S1, a stable analogue of S1–adenosine 5′-triphosphate (ATP), obtained by cross-linking the SH_1 and SH_2 heavy-chain thiols of subfragment 1 [Greene, L. E., Chalovich, J. M., & Eisenberg, E. (1986) *Biochemistry 25*, 704–709]. Large increases in transfer efficiency indicated that the two probes had moved closer together by some 3 nm. Second, weak binding complexes were formed between subfragment 1 and actin in the presence of the regulatory proteins troponin and tropomyosin, the absence of calcium, and the presence of ATP [Chalovich, J. M., & Eisenberg, E. (1982) *J. Biol. Chem. 257*, 2432–2437]. The measured efficiency of energy transfer again indicated that the distance between the two labeled sites had moved closer by about 3 nm. These data support the idea that there is a considerable difference in the structure of the acto–S1 complex between the weakly and strongly bound states.

It is now generally accepted that muscle contraction and the motility of many nonmuscle cells result from the relative sliding motion of myosin and actin filaments. These processes are directly linked to adenosine 5′-triphosphate (ATP)[1] hydrolysis and involve the head of the myosin molecule (cross bridge) interacting in cycles of detachment and reattachment with the actin filaments. Currently held theories as to the nature of the force-generating step suggest that during each cycle of ATP hydrolysis the cross bridges alternate between at least two main conformations (Eisenberg & Greene, 1980). The binding of ATP to the myosin induces a cross-bridge structure which binds weakly to actin, the free and actin-bound cross bridges being in rapid equilibrium. Following hydrolysis of ATP, the release of inorganic phosphate transforms the cross bridge to the strong binding conformation. This strong binding or "rigor" complex is easily studied because it is stable and long-lived. In contrast, however, the weakly bound states are

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; 1,5-BrAEDANS, 5-[[2-[(bromoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; 5-IAF, 5-(iodoacetamido)fluorescein; IANBD, 4-[(iodoacetoxy)-ethylmethylamino]-7-nitro-2,1,3-benzoxadiazole; 5-IAS, 5-(iodoacetamido)salicyclic acid; MOPS, 4-morphinepropanesulfonic acid; pPDM, *p*-phenylenedimaleimide; PMSF, phenylmethanesulfonyl fluoride; S1, myosin subfragment 1; S1(A1) and S1(A2), subfragment 1 containing alkali 1 (A1) or alkali 2 (A2) light chains, respectively; TEA, triethanolamine; Tn–TM, troponin–tropomyosin complex; FRET, fluorescence resonance energy transfer; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; HPLC, high-performance liquid chromatography; kDa, kilodalton(s), AS, acetamidosalicylic acid; AF, acetamidofluorescein.

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