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Simultaneous Optical Measurement of Osmotic and Diffusional Water Permeability in Cells and Liposomes†

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ABSTRACT: A quantitative description of transmembrane water transport requires specification of osmotic (P_f) and diffusional (P_d) water permeability coefficients. Methodology has been developed to measure P_f and P_d simultaneously on the basis of the sensitivity and rapid response of the fluorophore aminonaphthalenetrisulfonic acid (ANTS) to solution H_2O/D_2O content. Cells loaded with ANTS in an H_2O buffer were subjected to an inward osmotic gradient with a D_2O buffer in a stopped-flow apparatus. The time courses of cell volume (giving P_f) and H_2O/D_2O content (giving P_d) were recorded with dual photomultiplier detection of scattered light intensity and ANTS fluorescence, respectively. The method was validated by using sealed red cell ghosts and artificial liposomes reconstituted with the pore-forming agent gramicidin D. At 25 °C, red cell ghost P_f was 0.021 cm/s with P_d 0.005 cm/s (H_2O/D_2O exchange time 7.9 ms). P_f and P_d were inhibited by 90% and 45% upon addition of 0.5 mM $HgCl_2$. The activation energy for P_d increased from 5.1 kcal/mol to 10 kcal/mol with addition of $HgCl_2$ (18-35 °C). In 90% phosphatidylcholine (PC)/10% cholesterol liposomes prepared by bath sonication and exclusion chromatography, P_f and P_d were 5.1×10^{-4} and 6.3×10^{-4} cm/s, respectively (23 °C). Addition of gramicidin D (0.1 μg/mg of PC) resulted in a further increment in P_f and P_d of 7×10^{-4} and 3×10^{-4} cm/s, respectively. These results validate the new methodology and demonstrate its utility for rapid determination of P_f/P_d in biological membranes and in liposomes reconstituted with water channels.

While the mechanism of many biologically important membrane transporters is understood at the biochemical and molecular levels, the description of water movement across biological membranes remains empirical. The working definition of a water channel has been derived from data obtained in human red cells (Macey, 1984; Mlekoday et al., 1983; Solomon et al., 1984), the kidney proximal tubule (Verkman & Wong, 1987; Meyer & Verkman, 1987), and vasopressin-sensitive tight epithelia (Harmanci et al., 1978; Levine et al., 1984; Verkman et al., 1988). Compared with pure phospholipid membranes, membranes containing water channels generally have a higher osmotic water permeability coefficient ($P_f > 0.01$ cm/s), an osmotic to diffusional permeability ratio (P_f/P_d) > 1, a low activation energy ($E_a < 5$ kcal/mol), and partial inhibition of P_f by mercurials (Fettiplace & Haydon, 1980; Finkelstein, 1987).

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Quantitative assessment of water permeability in biological membranes and in artificial liposomes reconstituted with water channels requires measurement of P_f, P_f/P_d, and E_a. P_f is measurable accurately from the kinetics of cell or vesicle volume change in response to an imposed transmembrane osmotic gradient; instantaneous volume is inferred from the time course of scattered or transmitted light intensity (Terwilliger & Solomon, 1981; Mlekoday et al., 1983; Verkman et al., 1985) or from the fluorescence of an entrapped fluorophore that undergoes concentration-dependent self-quenching (Chen et al., 1988). P_d has been more difficult to measure because of the rapid water exchange times (<10 ms) in cells and small liposomes or membrane vesicles. Nuclear magnetic resonance methods have been applied for measurement of P_d in blood and kidney cells (Fabry & Eisenstadt, 1978; Wong & Verkman, 1987; Verkman & Wong, 1987); however, very large sample volumes are necessary, relatively high concentrations (generally >20 mM) of an extracellular paramagnetic quencher are required, and there is no approach for simultaneous measurement of P_f. Lawaczek (1984) has reported the use of stopped-flow light scattering and indole fluorescence for measurement of P_d by H_2O/D_2O exchange; however, the very low signal amplitudes, the rapid exchange rates, and the poor fluorescence characteristics of indoles make this approach not feasible to apply in practice (see Discussion).

We report here a direct method for simultaneous measurement of P_f and P_d in closed membranes including cells and liposomes. P_f is measured by stopped-flow light scattering or transmittance. P_d is measured in parallel from the fluorescence time course of entrapped aminonaphthalenetrisulfonic acid (ANTS), a fluorophore that has 10 times the sensitivity of indoles to solution H_2O/D_2O composition, with a visible fluorescence spectrum (Kuwahara & Verkman, 1988). The methodology is validated by using sealed red cell ghost membranes and artificial liposomes containing gramicidin. These results establish a quantitative approach for determination of P_d and P_f/P_d in native and reconstituted membranes, particularly important for the functional analysis of reconstituted water channels.

Materials and Methods

Materials. ANTS and 6-carboxyfluorescein (6CF) were purchased from Molecular Probes, Inc. (Junction City, OR). Egg phosphatidylcholine (PC), cholesterol, D_2O (99.9%), and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Membrane Preparation. Sealed red cell ghost membranes were prepared by a modification of the method of Dodge et al. (1963). Recently outdated (<24 h) packed red cells were washed twice in 150 mM NaCl and 5 mM sodium phosphate, pH 7.4 (PBS), and lysed in >30 volumes of 5 mM sodium phosphate, pH 8.0 (4 °C). Unsealed ghosts were pelleted (20000*g*, 30 min) and washed three times in the lysis buffer. Ghosts were sealed by incubation in >30 volumes of 50 mM NaCl and 5 mM sodium phosphate, pH 8.0, for 45 min at 37 °C, pelleted (20000*g*, 15 min), and washed twice in the same buffer at 4 °C. Sealed ghosts were loaded with 10 mM ANTS in 100 mM sucrose and 5 mM sodium phosphate, pH 7.4, by an 18–24-h incubation at 4 °C; extracellular ANTS was removed prior to experiments by three washes in >25 volumes of 100 mM sucrose and 5 mM sodium phosphate, pH 7.4 at 4 °C, not containing ANTS. To prevent traumatic cell lysis, ANTS-loaded cells were spun at 10000*g* for 15 min and resuspended by gentle swirling.

Liposomes were prepared by bath sonication as described previously (Carruthers & Melchior, 1983). PC (50 mg) and cholesterol (2.45 mg) were mixed in chloroform and dried under an N_2 stream. The mixture was dissolved in 2 mL of diethyl ether, dried, and suspended in 2 mL of 100 mM sucrose and 5 mM sodium phosphate, pH 7.4, containing 10 mM ANTS. The lipid suspension was sonicated for 12 min in a bath sonicator (Laboratory Supply Co., Hicksville, NY) at 23 °C under flowing N_2. External ANTS was removed by passage of liposomes down a Sephadex G-25M exclusion column (Pharmacia, Piscataway, NJ); the first 10 drops of cloudy eluate were pooled for subsequent experiments. Negative-staining electron micrographs showed that liposomes were single walled with an average diameter of 0.3 μm, similar to results reported previously (Kasahara & Hinkle, 1977). Gramicidin D (0.1 μg/mg of PC) was incorporated into liposomes prior to exclusion chromatography from a DMSO stock solution (0.3 mg of gramicidin D/mL) followed by bath sonication for 15 s.

Stopped-Flow Experiments. Stopped-flow measurements were performed on a Hi-Tech SF51 stopped-flow apparatus (Wiltshire, England) having a dead time of ~1.5 ms with >99% mixing efficiency within 1 ms. The temperature was controlled by a circulating water bath and monitored by an indwelling thermistor. Samples were illuminated by using a 100-W tungsten-halogen lamp powered by a stabilized DC supply (Oriel) and a 380 ± 10 nm six-cavity interference filter (Omega Optical; Brattleboro, VT) in series with a KG-3 infrared blocking filter (Schott Glass, Duryea, PA). The signal was detected with two Hamamatsu 9798Q photomultipliers (Middlesex, NJ). One photomultiplier was oriented at 90° from the illumination axis and contained a GG450 cut-on filter (Scott Glass) for measurement of ANTS fluorescence. The other photomultiplier was oriented at 0° or 90° with no filter for measurement of cell/liposome volume changes by light transmittance or scattering. The signal was amplified and recorded by an IBM PC/AT computer through a Labmaster analog-to-digital interface board (Scientific Solutions Inc., Solon, OH). The maximum rate of data acquisition used was 10 points/ms.

Stopped-flow experiments were performed by mixing 0.075 mL of ghosts (~0.2 mg of protein/mL) or liposomes (~0.3 mg of PC/mL) containing 10 mM ANTS, 100 mM sucrose, and 5 mM sodium phosphate, pH 7.4, in H_2O with an equal volume of 100 + x mM sucrose and 5 mM sodium phosphate, pH 7.4, in D_2O to give specified inward sucrose gradients. P_f (cm/s) was determined from the time course of scattered or transmitted light intensity [$I(t)$] by a three-parameter fit to the equation

$$\frac{dI(t)}{dt} = \frac{P_f(S/V)\bar{v}_w}{A}\left(C_o - \frac{C_i}{AI(t) + B}\right) \quad (1)$$

where S/V is surface-to-volume ratio (taken to be 2×10^5 cm^{-1} for liposomes and 2.5×10^4 cm^{-1} for ghosts), $\bar{v}_w$ is the partial molar volume of water (18 cm^3/mol), C_o and C_i are external and internal osmolarities immediately after mixing, and A and B are fitted parameters related to instrument gain and offset (Verkman et al., 1985). The validity of the assumption in eq 1 that I varies linearly with volume was confirmed in sealed ghosts and liposomes under experimental conditions (maximum volume decrease of 50%). P_d (cm/s) was calculated from a single exponential time constant (τ_{ex}, exchange time) fitted to the time course of ANTS fluorescence by using the relation

$$P_d = 1/[\tau_{ex}(S/V)] \quad (2)$$

It is notable that the quantities $P_f(S/V)$ and $P_d(S/V)$ in eq 1 and 2 may be grouped so that the ratio P_f/P_d can be determined without knowledge of cell geometry.

NMR Measurements. The water-exchange time was estimated independently in sealed ghosts at 25 °C by proton nuclear magnetic resonance T_1 relaxation times as described previously (Wong & Verkman, 1987). Sealed ghosts in 150 mM NaCl and 5 mM sodium phosphate, pH 7.4 (containing or not containing 10 mM ANTS), were suspended at ~30% cell crit in 105 mM NaCl, 30 mM $MnCl_2$, and 5 mM sodium phosphate, pH 7.4. T_1 relaxation times were determined by the inversion–recovery pulse sequence using a Praxis Model PR1005 NMR instrument (San Antonio, TX) operating at 10 MHz. The magnetization decay was fitted to a biexponential function from which τ_{ex} was determined (Wong & Verkman, 1987). Because the sealed ghosts were hemoglobin free, intracellular spin-exchange effects (Fabry & Eisenstadt, 1978) could be neglected.

Results

Physical Properties of ANTS. ANTS is a white crystalline powder with three negatively charged sulfonate groups at neutral pH. The ANTS excitation and emission maxima are at 380 and 520 nm, respectively. The fluorescence quantum yield and lifetime of ANTS increase 3.2-fold as solvent composition is changed from 100% H_2O to 100% D_2O (Kuwahara & Verkman, 1988); ANTS fluorescence increases in a nearly linear manner with increasing percentage of D_2O. In stopped-flow experiments, a mixture of 0.01–1 mM ANTS in PBS (H_2O solvent) with PBS (D_2O solvent) gave a time-independent signal with an intensity that is expected for ANTS dissolved in a 1:1 H_2O/D_2O mixture, indicating that ANTS fluorescence responds to changes in solvent H_2O/D_2O composition in <1 ms.

Because ANTS is polar and membrane impermeable (Ellens et al., 1984), it is suitable as an internal fluid-phase marker of H_2O/D_2O composition. In liposome experiments, ANTS was entrapped during the sonication procedure. Once entrapped, there was no significant leakage of ANTS from liposomes over 4 h at 4 °C, as judged from the constancy of the fluorescence signal amplitude (proportional to entrapped [ANTS]; see below). At 23 °C, ANTS leaked out from liposomes with a half-time of 60 min. In sealed ghost experiments, ANTS could be entrapped during the sealing procedure; alternately, sufficient quantities of ANTS diffused passively into ghosts by prolonged incubation at 4 °C. Once entrapped, ANTS leaked out from ghosts with a half-time of 25 min at 23 °C and >4 h at 4 °C.

Water Transport in Red Cell Ghosts. A series of experiments were performed to validate the accuracy of P_d measurement by ANTS fluorescence and to show that ANTS itself did not affect the properties of red cell water transport. Figure 1 shows the time course of ANTS fluorescence in response to imposed H_2O/D_2O gradients in the absence of an osmotic gradient. Mixture of ghosts in H_2O with D_2O buffer resulted in a single exponential increase in fluorescence (trace A) with a time constant of 8.3 ± 0.9 ms (mean ± SD, n = 10) and a corresponding P_d of 4.8 × 10^{-3} cm/s. There was no further change in signal intensity beyond the 50 ms shown. Mixture of ghosts in H_2O with an isosmotic H_2O buffer resulted in a time-independent signal (trace B); mixture of ghosts in H_2O (~300 mosM) with a hyperosmotic H_2O buffer (500 mosM) also resulted in a time-independent signal (not shown), indicating absence of light scattering contributions to the fluorescence signal. To examine the symmetry of ghost diffusional water transport, ghosts in D_2O were mixed with an isosmotic H_2O buffer; there was a single exponential decrease in fluorescence (trace C) with a time constant of 8.0 ± 0.6 ms (n = 10), not significantly different from that in trace A.

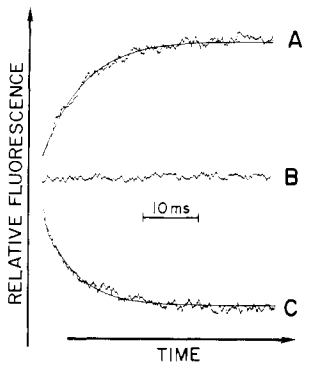

FIGURE 1: Time course of diffusional water permeability in red cell ghosts measured by ANTS fluorescence. Ghosts containing 100 mM sucrose, 5 mM sodium phosphate, and 10 mM ANTS, pH 7.4, were mixed with an isosmotic buffer in a stopped-flow apparatus as described under Materials and Methods. Experiments were performed at 21 °C. Fluorescence was excited at 380 nm and detected at >450 nm. (A) Ghosts in an H_2O buffer mixed with a D_2O buffer. (B) Ghosts in an H_2O buffer mixed with an H_2O buffer. (C) Ghosts in a D_2O buffer mixed with an H_2O buffer. Fitted time constants were 8.2 ms (A) and 7.8 ms (C).

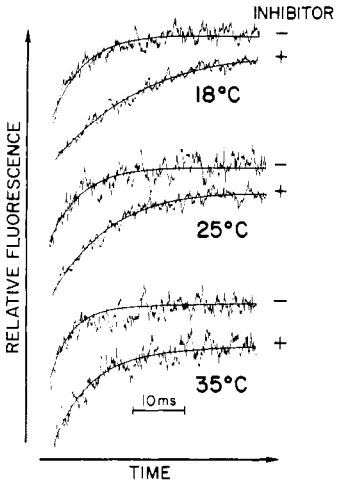

FIGURE 2: Effect of $HgCl_2$ and temperature on red cell ghost diffusional water permeability. Experiments were performed as in Figure 1, trace A, in the presence and absence of 0.5 mM $HgCl_2$ added to ghosts 5 min before the experiment. In two sets of experiments, each performed ten times, fitted time constants were as follows: 9.3 ± 0.8 ms ($-HgCl_2$) and 21 ± 1 ms ($+HgCl_2$) (18 °C); 7.7 ± 0.4 ms ($-HgCl_2$) and 13 ± 1 ms ($+HgCl_2$) (25 °C); and 5.7 ± 0.9 ms ($-HgCl_2$) and 8.1 ± 0.3 ms ($+HgCl_2$) (35 °C).

The ANTS fluorescence method was used to examine two characteristics of red cell diffusional water permeability that have been studied previously by ^3H_2O and NMR methods: inhibition by mercurials and temperature dependence. Figure 2 shows the inhibition of ghost P_d by 0.5 mM $HgCl_2$ at three temperatures. P_d was inhibited by 50%, 41%, and 30% at 18, 25, and 35 °C, respectively. The activation energy of P_d, calculated from the temperature dependence of the exchange time, increased from 5.1 ± 0.7 kcal/mol ($-HgCl_2$) to 10 ± 1 kcal/mol with maximum $HgCl_2$ inhibition, consistent with closure of a water channel. The higher activation energy after $HgCl_2$ inhibition probably represents residual water movement by a noninhibitable lipid pathway.

To validate that P_d values obtained by the ANTS fluorescence method are accurate and that ANTS itself does not affect P_d, proton T_1 relaxation times were measured at 25 °C as described under Materials and Methods. In two sets of measurements, each performed in triplicate, the calculated diffusional water-exchange time was 8.4 ± 0.6 s, not significantly different from the exchange time determined from the

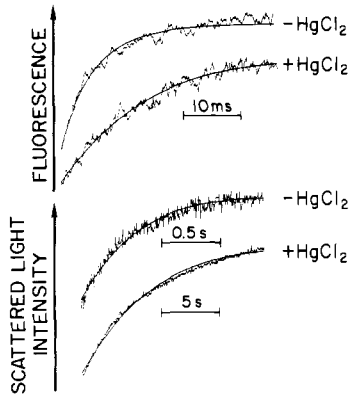

FIGURE 3: Red cell ghost P_f and P_d measured simultaneously. Ghosts containing 100 mM sucrose, 5 mM sodium phosphate, and 10 mM ANTS, pH 7.4, in H_2O were mixed in a stopped-flow apparatus with an equal volume of buffer containing D_2O to give a 100 mM inward sucrose gradient (see Materials and Methods). Experiments were performed at 25 °C. The sample was illuminated at 380 nm. Fluorescence was measured at >450 nm, and 90° light scattering was measured at 380 nm. Fitted permeability coefficients for measurements performed in triplicate were (5.1 ± 0.5) × 10^{-3} cm/s (P_d, fluorescence, $-HgCl_2$), (2.8 ± 0.4) × 10^{-3} cm/s (P_d, fluorescence, $+HgCl_2$), (2.1 ± 0.4) × 10^{-2} cm/s (P_f, scattering, $-HgCl_2$), and (2.2 ± 0.3) × 10^{-3} cm/s (P_f, scattering, $+HgCl_2$).

time course of ANTS fluorescence at 23 °C (7.7 ± 0.4 ms). The proton T_1 relaxation time determined for sealed ghosts containing ANTS (8.3 ± 0.5 ms, n = 3) did not differ from that obtained without ANTS, showing no effect of ANTS on red cell P_d. In similar control studies using stopped-flow light scattering (see below), there was no effect of ANTS on ghost membrane P_f (not shown).

Ghost membrane P_f and P_d were determined simultaneously by mixing ghosts loaded with an H_2O buffer containing ANTS with a hyperosmolar D_2O buffer (Figure 3). The sample was excited at 380 nm. The time course of ANTS fluorescence in both the presence and absence of $HgCl_2$ was similar to that in Figure 2 with exponential time constants of 7.9 ± 0.7 ms (n = 3) and 14 ± 2 ms (n = 3), respectively. Corresponding P_d values are given in the legend. Because diffusional water exchange occurs over a time much shorter than osmotically induced volume movement under the conditions of the experiment, there is no measurable effect of an osmotic gradient of the time course of H_2O/D_2O exchange. The time course of ghost volume was determined by 90° light scattering at 380 nm. In response to a 100 mosM inward sucrose gradient, ghost volume decreased over ~500 ms with a calculated P_f of 0.021 ± 0.004 cm/s (n = 3), giving P_f/P_d = 4.1 ± 0.9. In response to 25 and 100 mosM inward sucrose gradients, measured P_f values were 0.023 ± 0.003 cm/s (n = 3) and 0.022 ± 0.005 cm/s (n = 3), respectively, showing independence of P_f on osmotic gradient size, as reported for osmotic water transport in red cells (Terwilliger & Solomon, 1981). With the experimental protocol in Figure 3, addition of 0.5 mM $HgCl_2$ resulted in 90% inhibition of P_f (0.0022 ± 0.0003 cm/s, n = 3) and 45% inhibition of P_d (0.0028 ± 0.0004 cm/s, n = 3). P_f/P_d for the inhibitable component of water transport was 8.2.

Water Transport in Liposomes. An important application of the optical methodology for simultaneous measurement of P_f and P_d is the analysis of water transport in liposomes reconstituted with water channels. Liposomes that are suitable for reconstitution studies (Carruthers & Melchior, 1983) were prepared in buffer containing ANTS by bath sonication and exclusion chromatography. P_f and P_d were determined by exposure of liposomes to an inward sucrose gradient with

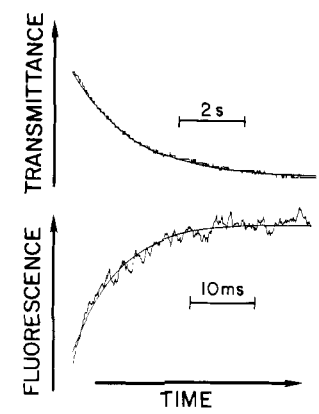

FIGURE 4: P_f and P_d measured simultaneously in liposomes. Liposomes containing 100 mM sucrose, 5 mM sodium phosphate, and 10 mM ANTS, pH 7.4, in H_2O were mixed with an equal quantity of buffer in D_2O to give a 100 mM inward sucrose gradient. Experiments were performed at 23 °C. The sample was illuminated at 380 nm. Fluorescence was measured at >450 nm, and 0° transmittance was measured at 380 nm. In three experiments, fitted permeability coefficients were (6.3 ± 0.6) × 10^{-4} cm/s (P_d, fluorescence) and (5.1 ± 0.4) × 10^{-4} cm/s (P_f, transmittance).

hyperosmotic buffer containing D_2O (Figure 4). In three sets of measurements performed in liposomes composed of 90% PC and 10% cholesterol, the H_2O/D_2O exchange time was 7.9 ± 0.7 ms, corresponding to a P_d of (6.3 ± 0.6) × 10^{-4} cm/s, assuming a 0.3-μm liposome diameter as determined by electron microscopy. The corresponding P_f determined by transmittance was (5.1 ± 0.4) × 10^{-4} cm/s (n = 3), giving P_f/P_d = 0.8 ± 0.1. There was no effect of $HgCl_2$ on liposome P_f and P_d (not shown).

Addition of gramicidin D to liposomes resulted in an increase in P_f and P_d due to parallel transport of water through membrane phospholipid and the porelike gramicidin water channel (Rosenberg & Finkelstein, 1978). P_f and P_d values measured in the presence of gramicidin D were (1.2 ± 0.1) × 10^{-3} cm/s and (9 ± 1) × 10^{-4} cm/s, respectively, consistent with incorporation of water channels with P_f/P_d >1 into the membranes.

Discussion

The goal of these studies was to develop a method to measure osmotic and diffusional water permeability simultaneously in closed membranes, including biological cells and vesicles, and liposomes reconstituted with water channels. The important design criteria for the method were as follows: (1) small sample requirement, (2) P_f and P_d measurement on the same sample without cross-interference, and (3) good reproducibility and accuracy in the determination of permeability coefficients. The optical method developed and validated in these studies satisfies the design criteria. Measurement of P_f and P_d, and the ratio P_f/P_d, provides essential information about the physical characteristics of the transmembrane water pathway. The value of P_f/P_d is particularly important because of its direct implications about the presence of "water channels" and its independence on the exact membrane geometry. In addition, for studies of protein reconstitution into liposomes and of the effects of water transport inhibitors, P_f/P_d is far less sensitive to artifacts of nonspecific membrane perturbation than is P_f or P_d alone.

The method developed was based on the simultaneous recording of scattered or transmitted light intensity and fluorescence of the entrapped indicator ANTS following rapid exposure of sealed ghosts or liposomes to an osmotic and an isotopic (H_2O/D_2O) gradient. The intensity of scattered or

transmitted light provided an instantaneous measure of cell volume and thus of transmembrane volume flow occurring in response to an osmotic driving force (P_f). ANTS fluorescence provided a measure of intracellular H_2O/D_2O content and thus the rate of diffusional water exchange (P_d).

It was found that the fluorophore ANTS had good loading and leakage properties in cells and liposomes, a visible spectral response, a 3.2-fold increase in fluorescence when dissolved in D_2O vs H_2O, and a very rapid response in fluorescence (<1 ms) to changes in solution H_2O/D_2O content. The optical properties of ANTS and its large transient signal amplitude in response to isotopic water gradients facilitated the measurement of rapid diffusional water movement (<10 ms exchange times) with good signal-to-noise ratios and reproducibility. We were unable to obtain an acceptable and reproducible time course of diffusional water exchange by the light scattering and tryptamine fluorescence methods reported by Lawaczeck (1984).

In red cell ghosts, P_d measured by ANTS fluorescence agreed with P_d measured by NMR; there was no effect of ANTS on P_f or P_d. P_d was measured with a "ghost crit" of <1% by ANTS fluorescence compared to ~30% by NMR. With the ANTS method, it was found that ghost P_d was 56% inhibitable by $HgCl_2$ at 18 °C; the activation energy for P_d was 5.1 kcal/mol in the absence and 10 kcal/mol in the presence of $HgCl_2$, in agreement with previous results in intact red cells and red cell ghosts (Brahm, 1982; Benga et al., 1986). P_f/P_d in red cell ghosts was 8.2 for the $HgCl_2$-inhibitable component of water transport and 0.8 for the noninhibitable pathway, consistent with the view that mercurials result in closure of a water channel (Moura et al., 1984). This interpretation is supported by the increase in P_d activation energy with $HgCl_2$ inhibition. The high P_f/P_d for the inhibitable pathway is consistent with the presence of a water pore or channel; if water moved through a right cylindrical pore, its diameter would be >10 Å (Macey, 1984; Solomon et al., 1984); if water moved in part through a single-file channel, then ~8 water molecules would be in contact within the narrow channel.

In liposomes, P_f/P_d was near unity, as predicted for water movement through membrane phospholipid (Finkelstein, 1987). The increments in P_f and P_d following gramicidin addition were $(0.7 \pm 1) \times 10^{-4}$ cm/s and $(0.3 \pm 0.1) \times 10^{-4}$ cm/s, respectively, giving P_f/P_d for the incremental permeability of 2.3 ± 0.8. These results are consistent with the insertion of gramicidin pores into the membrane; however, the incremental P_f/P_d may be artificially low if gramicidin incorporation caused nonspecific membrane leaks. These data demonstrate the feasibility of P_f and P_d measurements in liposomes reconstituted with water channels.

There are a number of potential difficulties that can confound the quantitative interpretation of P_f and P_d data, making the ratio P_f/P_d inaccurate. If sealed membranes have considerable heterogeneity in surface-to-volume ratios, then the measured apparent permeability coefficient will be weighted toward those in membranes contributing most to the optical signal. For P_d measurement by fluorescence of ANTS, an internal fluid-phase marker, the signal contribution from membranes of a given size is proportional both to the number of membranes and their internal volume. For P_f measurement by light scattering or transmittance, the signal contribution from membranes of a given size is difficult to predict a priori and depends upon detailed membrane geometry and optical properties. To minimize heterogeneity effects, P_f can be measured with a fluid-phase internal marker such as fluorescein sulfonate (Chen et al., 1988), which undergoes concentration-dependent self-quenching. Under these conditions, the relative contribution to the kinetic signal from vesicles of differing geometries is the same for both P_f and P_d determination, factoring out from the P_f/P_d ratio.[1] Inaccuracy in P_f/P_d measurement may also occur from underestimation in P_d due to unstirred layer effects (Barry & Diamond, 1984), underestimation in P_f due to nonmembrane restrictions to volume change if large osmotic gradients are used (Illsley & Verkman, 1986), or possible differential effects of D_2O on P_f and P_d (Karan & Macey, 1980).

Given these technical cautions, the data reported here show that P_f, P_d, and P_f/P_d can be determined by a single stopped-flow optical measurement on closed biological or artificial membranes. Absolute P_f/P_d and changes in P_f/P_d can be interpreted in terms of closure or insertion of functional water channels into membranes.

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[1] Parallel determination of P_f by transmittance and fluorescein sulfonate fluorescence was carried out in liposomes containing 10 mM fluorescein sulfonate. The sample was excited at 460 nm. Transmitted light and fluorescence at >520 nm were measured simultaneously. In response to a 100 mM inwardly directed sucrose gradient at 23 °C, liposome transmittance and fluorescein sulfonate fluorescence decreased with exponential time constants of 2.4 and 2.5 s, respectively, showing absence of significant heterogeneity artifacts.

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Retinal Isomer Ratio in Dark-Adapted Purple Membrane and Bacteriorhodopsin Monomers†

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ABSTRACT: On the basis of data obtained by spectroscopic analysis and chromatography of retinal extracts, a consensus has been adopted that dark-adapted purple membrane (pm) contains 13-*cis*- and *all-trans*-retinal in equal amounts, whereas the light-adapted membrane contains *all-trans*-retinal only. We have developed an improved extraction technique which extracts up to 70% of the retinal in pm within 4 min. In the extracts from dark-adapted pm at room temperature, we consistently find 66–67% 13-*cis*-retinal and 33–34% *all-trans*-retinal, and more than 98.5% all-trans isomer in light-adapted samples. The spectrum obtained by reconstitution of bacterioopsin with 13-*cis*-retinal at 2 °C (to minimize isomerization) shows an absorbance maximum at 554 nm and agrees well with the spectrum for the 13-cis component calculated from the dark-adapted and light-adapted bR spectra with our extraction data. The ratio of 13-cis:all-trans isomer in dark-adapted pm is 2:1 and nearly constant between 0 and 38 °C but begins to decrease distinctly above 40 °C, and more rapidly near 70 °C, reaching 0.75 at 90 °C. The van't Hoff plot of the isomer ratio shows a nonlinear temperature dependence above 40 °C, indicating a more complex system than a simple thermal 13-cis/all-trans isomer equilibrium. We attribute the broadening, absorbance decrease, and blue shift of the visible absorption band with increasing temperature to the appearance of at least one and possibly two or three new chromophores which contain, mainly or exclusively, the all-trans isomer. The 2:1 ratio of the isomers and its small temperature dependence between 0 and 40 °C suggest that it could be determined by the trimeric association of bR in the planar protein lattice of pm and that the lowest free energy conformation in the lattice is a trimer which contains one all-trans and two 13-cis isomers. However, the lattice remains intact up to ~75 °C, and trimers of bR exist at still higher temperatures. Moreover, monomeric bR solubilized with Triton X-100 shows a similar isomer ratio near room temperature and slightly higher 13-cis isomer content (e.g., 71% 13-cis and 29% all-trans at 4 °C) at low temperature, and also a nonlinear but steeper temperature dependence in the van't Hoff plot. We argue that the lattice stabilizes the protein in a conformation which slightly favors the 13-cis chromophore but restricts its structure from assuming the conformation still more favorable to 13-*cis*-retinal which it attains in the monomer.

Bacteriorhodopsin (bR),[1] the protein in the purple membrane (pm) of *Halobacterium halobium*, functions as a light-driven proton pump [for review, see Stoeckenius et al. (1979), Birge (1981), and Stoeckenius and Bogomolni (1982)]. The bR molecules are arranged within the membrane in a hexagonal lattice, and structural analysis by electron microscopy suggests a structure where the polypeptide chain forms seven helix segments spanning the membrane. Like visual pigments, bR contains one retinylidene chromophore (Oesterhelt & Stoeckenius, 1971) bound to Lys-216 as a protonated Schiff base (Lewis et al., 1974; Katre et al., 1981; Bayley et al., 1981; Lemke & Oesterhelt, 1981; Mullen et al., 1981). The chromophore has a main absorption band near 560 nm; the more than 100-nm red-shift compared to a protonated Schiff base of retinal in solution is explained by additional noncovalent protein–retinal interactions (Blatz et al., 1972; Spudich et al., 1986; Lugtenburg et al., 1986). After exposure to light, the absorption maximum is found at 568 nm, and this light-adapted bR (bR^{LA}) contains >98% *all-trans*-retinal. In the dark, the absorption maximum shifts

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[1] Abbreviations: pm, purple membrane; bR, bacteriorhodopsin; bR^{LA}, light-adapted bacteriorhodopsin; bR^{DA}, dark-adapted bacteriorhodopsin.

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