WATER PERMEABILITY THROUGH BIOLOGICAL MEMBRANES BY ISOTOPIC EFFECTS OF FLUORESCENCE AND LIGHT SCATTERING

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ABSTRACT A light-scattering technique used to measure the water permeability across closed biomembranes is described, which is based on the different indices of refraction of D_2O and H_2O. This transient technique is compared with a similar method using D_2O-sensitive fluorophores in the intravesicular space. The results of both techniques are equivalent although the signal-to-noise ratio favors the light-scattering or turbidity experiment. The light-scattering method is only applicable to larger particles (no point-scatterers) and is easily extended to biological objects. Data on the H_2O/D_2O exchange across membranes of ghosts from human erythrocytes suggest two mechanisms: the D_2O and H_2O permeation through the membrane and a slower D_2O-induced conformational change of membraneous proteins.

INTRODUCTION

The majority of biochemical reactions take place in aqueous environments. However, neither the state of the cellular water nor its coupling to the extracellular milieu are well described. It is generally agreed that the transport of water proceeds in three steps: one partitioning from the extracellular solution into the membrane, a diffusion through the membrane, and a repartitioning from the membrane into the cytoplasmatic compartment. The two partitioning steps need not necessarily be equal because of possible differences of the chemical potential on both sides of membrane and/or a membrane asymmetry.

To understand the permeation processes it is necessary to measure transport phenomena under various experimental conditions. In recent publications (1, 2) a new method to study the permeation of H_2O/D_2O through vesicular lipid bilayers was described and compared with established methods. The new technique relies on D_2O-sensitive fluorescent probes that are intravesicularly encapsulated. The fluorescent molecules report on the intravesicular isotopic composition of the aqueous solvent. Rapid changes of the extravesicular milieu (H_2O vs. a H_2O/D_2O mixture) are imparted to the intravesicular compartment by the permeation of the respective components through the membrane. The exchange rate, k_{ex}, and the permeability coefficient, $P_d = k_{ex}\, r/3$, at known vesicular radius, r, can be deduced from the time dependence of the fluorescence intensity.

In the course of the above experiments it was realized that the fluorescence signal is accompanied by an equivalent change of the light scattering or turbidity. It was argued that the observed changes of the light-scattering intensity might originate from the different indices of refraction of H_2O and D_2O. In this communication the fluorescence and the light-scattering signals will be compared. It will be shown on experimental grounds that both methods register the same time dependence. The light-scattering technique is devoid of any sample manipulation and is thus easily extended to biological systems. In this sense, preliminary results for erythrocyte ghosts will be reported.

RESULTS AND DISCUSSION

For the experimental comparison of the two methods single-bilayer vesicles composed of dipalmitoylphosphatidylcholine (DPPC) (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) were prepared where 5-methoxytryptamine HCl (Sigma Chemical Co., St. Louis, MO) was intravesicularly encapsulated. This indole derivative exhibits a solvent isotope effect of the fluorescence quantum yield, and at neutral pH is only slowly permeating the lipid bilayer especially below the crystalline to liquid-crystalline phase-transition temperature (T_c). Large vesicles were used and prepared by sonifying the lipid solution below T_c, then allowing the unannealed vesicles to fuse for longer periods of time (3). Finally the vesicles were annealed and separated from the extravesicular chromophores. There are three reasons for the use of large vesicles, (*a*) at constant lipid concentration (1–2 mg/ml in the present case) the intravesicular volume is a function of the vesicular radius r. Consequently the number of encapsulated fluorescent molecules and thus the fluorescence intensity increase with increasing size of the vesicles. (*b*) The measured quantity, i.e., the exchange relaxation time $\tau_{ex} = 1/k_{ex}$, increases with the volume-

to-surface ratio and easily exceeds the instrumental dead time for larger vesicles, and (*c*) most important, in the light-scattering experiment the time-dependent signal relies on the equalization of the initially different indices of refraction for the intra- and extravesicular media, thus one does not expect any measurable time-dependent signals for point scatterers like small vesicles.

Both experiments (fluorescence and light scattering) start by rapidly mixing a vesicle solution bathing in H_2O with the equivalent D_2O buffer or vice versa. The deuterated buffers were prepared by lyophilization of the normal buffer and a subsequent resolution in D_2O. The mixing is performed in a Sigma (Biochem GmbH, Puchheim/München, West Germany) stopped-flow instrument where the two mixing syringes are vertically arranged minimizing the interference with unavoidable air bubbles. Instead of a stop syringe, the movement of the pistons is abruptly stopped. Except during the short piston movements, the system works almost without pressure. Monochromatic light is passed through the observation cell. Either fluorescence perpendicular to the incident beam or the transmitted light or both can be measured as function of time. 10^4 data points at 12 bit with a minimal time resolution (per point) of 0.2 ms are recorded and stored.

Just after the mixing period ($t = 0$) the intravesicular medium still contains pure H_2O while extravesicularly a 1:1 mixture of H_2O and D_2O is established. As a consequence of the permeation through the bilayer, the intravesicular solvent composition changes with time until the solvents on both sides of the membrane are finally equal. Due to the usually large excess of the extravesicular volume, the isotopic composition of the external medium remains constant. The quantum yield of the encapsulated fluorophore directly responds to the time-dependent solvent composition. Concerning the light-scattering experiments, one has to consider three indices of refraction that are n_l for the lipid membrane and n_i and n_e for the intra- and extravesicular solvents, respectively. During the time of observation n_i approaches n_e according to

$$n_i(t) = n_i(t = 0) + \Delta n[1 - \exp(-k_{ex}t)] \qquad (1)$$

with $\Delta n = n_e - n_i(t = 0)$, i.e., the difference of the initial indices of refraction, and k_{ex} the exchange relaxation rate. In a subsequent publication (H. Engelbert and R. Lawaczeck, manuscript submitted for publication) it will be deduced on the basis of the Mie theory for coated spheres (4) that the time-dependent part of the light-scattering intensity changes almost mono-exponentially at the same time, so that k_{ex} can be obtained from the measured light-scattering signal.

In Fig. 1 two typical curves for the time dependence of the fluorescence, I_{fl}, and of the forward scattered light, I_{tr}, are shown. Apart from the different signal-to-noise ratios, the time-dependent parts can be made to coincide. Consequently, both the fluorescence and the light scattering monitor the same physical change that is the permeation of

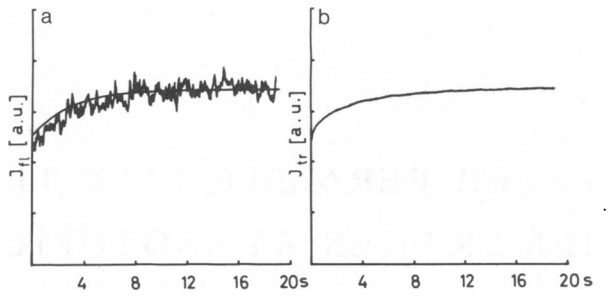

FIGURE 1 Fluorescence, I_{fl}, and intensity of the transmitted light, I_{tr}, as function of time after mixing of DPPC vesicles in H_2O with D_2O. 5-methoxytryptamine HCl is intravesicularly encapsulated. Both the normal and deuterated buffer contain 20 mM $CaCl_2$ at neutral pH. T = 22.3°C (T_c of DPPC 41.5°C). (*a*) Fluorescence of the indole-derivative: λ_{ex} 290 nm, $\lambda_{em} > 305$ nm perpendicular to the incident light. The drawn line corresponds to a single-exponential fit of *b* with $k_{ex} = 0.35\ s^{-1}$. (*b*) Light scattering, transmitted light at 400 nm; same sample as in *a*.

D_2O/H_2O through the vesicular lipid bilayer. This permeation leads to an equalization of the isotopic compositions of the intra- and extravesicular solvents. The different signal-to-noise ratios are partly due to the experimental setup and will be described in due course. Fig. 1 *a* also includes an exponential fit of the data from Fig. 1 *b*. The single-exponential function simultaneously approximates both curves quite well except at short observation times where effects of the vesicular size distribution are most important (see reference 2).

The fluorescence and the light-scattering signals are functions of the size distribution of the vesicular ensemble in a twofold way. One contribution originates from the size-dependent exchange rate, k_{ex}, for the equalization of the intra- and extravesicular solvent compositions with $k_{ex} \sim 1/r$. The second contribution results from the size dependence of the observable signal intensity. For fluorescence it was shown that a size distribution leads to a deviation from a mono-exponential behavior only just after the short mixing period (2).

In Fig. 2 typical results by the light-scattering technique (turbidity) are reproduced for ghost from human erythrocytes. 20–40-ml blood from adult volunteers was drawn by venipuncture through a 18-gauge needle into heparinized tubes. Sealed erythrocyte ghosts were prepared according to Steck and Kant (5). 0.2 ml of the concentrated ghosts were diluted with the respective H_2O buffer (150 mM NaCl, 5 mM Na-phosphate, pH 8) and equilibrated at 37°C for 0.5 h before use. In the stopped-flow apparatus the erythrocyte ghosts were mixed with normal, deuterated, and hyperosmolar buffers, respectively, while the intensity of the transmitted light at 400 or 589 nm was recorded. In the early stage of the experiments, part of the unsealed ghosts were labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) and the fluorescence anisotropy, r, was measured. At room temperature the calculated r value of 0.238 ± 0.012 (mean ± SD) lies well between the values reported by Eisinger et al. (6) and Kutchai et al. (7).

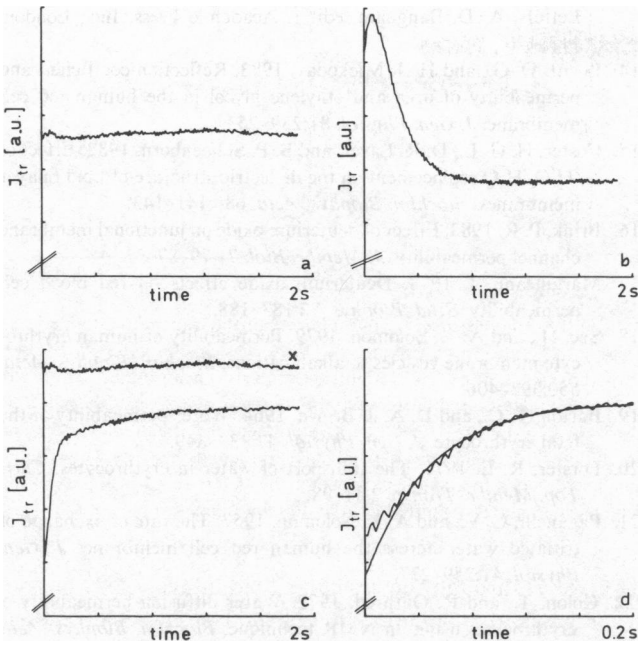

FIGURE 2 Transmitted light intensity, I_{tr} at 589 nm as function of time after mixing of sealed erythrocyte ghosts with normal buffer (150 mM NaCl, 5 mM Na-phosphate, pH 8) (*a*, control), with hyperosmolar buffer (additional 300 mM NaCl) and (*b*), with deuterated buffer (*c*, *d*). For comparison *c* contains also the control experiment, *x*. *d* is an enlarged section of *c* at short times (0.2 s full scale). The drawn line in *d* corresponds to a mono-exponential fit with $k_{ex} = 20.8\ s^{-1}$. The scales of *a* and *b* are equivalent and different from those in *c* and *d*. $T = 18°C$.

Fig. 2 *a* shows the control experiments. Erythrocyte ghosts were mixed with the normal buffer. Clearly, the resultant signal of the transmitted light stays constant, thus within the time of observation, a settling of ghosts is not visible. Fig 2 *b* documents the typical changes for rapid mixing experiments with hyperosmolar NaCl or glucose solutions. Just after the short mixing period, there is a rather fast increase of the intensity of the transmitted light. This fast process is usually not seen and might be due to an enforced shrinkage of the membrane or change in shape. This fast process is not visible in the control. The amplitude of this effect seems to be directly related to the magnitude of the hyperosmolarity. ~ 70 ms after the mixing has ceased, a maximum of the signal intensity is reached. Subsequently the transmitted light decreases (1–2 s) to an almost constant value. From this plateau a rather slow process leads to an increase of the transmitted light again (data not shown). The second and third steps are well documented for erythrocytes and other cells. These steps are attributed to the hydraulic water permeability and the rather slow permeation of the components of the hyperosmolar buffer, respectively (8–12). In terms of the light scattering one has to consider the time-dependent change of the size of the erythrocytes and also of the internal and external indices of refraction. The efflux of water leads to a shrinkage of the ghosts. Consequently there is a simultaneous increase in the internal buffer concentration and in the index of refraction. An analogous interpretation was recently described for liposomes (13) and erythrocytes (14), except that in the latter case permeable substances were regarded. The tendency to equalize the chemical potential of water on both sides of the membrane is accompanied by an equalization of the initially different indices of refraction. Following the short mixing with the hyperosmolar solutions, the sequence of events might be: a shrinkage of the membrane induced by the osmotic pressure, the efflux of water molecules, and finally an inflow of slowly permeating substances.

Fig. 2 *c*, *d* contain typical data on the mixing of erythrocyte ghosts with the deuterated buffer. The curves of Fig. 2 *c*, *d* are significantly different from the control (*x*, and Fig. 2 *a*) and suggest two effects taking place due to the rapid change of the outside isotopic buffer composition. The fast increase of the transmitted light intensity at short observation times is attributed to the permeation of D_2O/H_2O across the membrane leading to the equalization of the isotopic compositions in both milieus. The exchange rate, k_{ex}, is deduced from this initial change of the transmitted light by an exponential fit of the respective data. At room temperature a k_{ex} value of $18.5 \pm 3.9\ s^{-1}$ (mean ± SD) is calculated from five different ghosts preparations of apparently healthy donors. The second part of Fig. 2 *c* might be assigned to conformational changes of membrane-associated proteins induced by the change of the solvent from H_2O to a mixture of H_2O and D_2O. Several other explanations as origin for the observed long-time effect were discussed and found to be less probable. It is known that the dielectric substructure of planar lipid bilayers is not affected by the isotopic change of the solvent (15). However, there is experimental evidence suggesting differences between D_2O and H_2O with respect to biochemical membrane phenomena including transport processes of cations (e.g., 16, 17). Whether the postulated, D_2O-induced subtle conformational changes are the sole originators of the observed effect at longer observation times is under current investigation. The second effect is not observed in pure lipid systems.

Using literature data for the volume-to-surface ratio for erythrocyte ghosts ($0.65\ 10^{-4}$ cm [18]), a permeability coefficient P_d of $1.2\ 10^{-3}$ cm/s (at room temperature) can be calculated from the measured relaxation rate k_{ex}. This P_d value is about a factor of 3–4 lower than corresponding values for erythrocytes obtained with various other techniques (19–28). Recently Brahm (28) reported P_d values for erythrocyte ghosts using the tracer-efflux technique. His values (2.4 to $3.2\ 10^{-3}$ cm/s) are similar to our values. For a direct comparison of the values one should consider that for tracer methods the ions $^3H_3O^+$ or $^3H^+$ might also contribute to the efflux of tracer. In our case not only H_2O and D_2O but also the species HDO participate in the exchange process. It is known that D_2O penetrates biological membranes slower than H_2O (29), so that the observed difference between the P_d values might be reasonable. In

transient techniques, the diffusion through unstirred layers has to be taken into account. However, estimates on external unstirred layers show only a minor influence on the value of the permeability coefficient (for discussion see 10, 20, 26, 30).

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