On the Direct Observation of Water-Fluxes in Tissues and Leaves

Ulrich-Andreas Hirth and Rüdiger Lawaczeck

Institut für Physikalische Chemie, Universität Würzburg, Marcusstraße 9/11, D-8700 Würzburg, Bundesrepublik Deutschland

Z. Naturforsch. **41c,** 923–927 (1986); received April 14/June 11, 1986

Water-Fluxes, Isotopic Absorption Spectroscopy, Near-Infrared, Plant-Leaves

Based on the different absorption spectra of H_2O and D_2O in the near infrared (NIR, 800–2200 nm) a strategy is developed to measure H_2O/D_2O-fluxes in thin sections. The physical background of the method is presented. Results on the H_2O/D_2O-fluxes in filter paper and plant leaves together with both the physical and biological limitations of the new method are discussed.

Introduction

Water plays a fundamental role in living systems both as a unique solvent and as a fluid transporting substances between different organs or in plants *e.g.* from roots to leaves. In animals the fluid (blood) circulates through the body driven by an energy consuming pump (heart). In plants water flows from roots to leaves downhill the gradient of the chemical (water-)potential. Water easily transverses the membraneous barrier of single cells or unicellular organisms surrounded only by a plasma-membrane. In these cases, extracellular and intracellular water is rapidly exchanged, with exchange-times on the order of some milliseconds [1–7]. Though a variety of methods are available [8–13] and the number of experimental observations on the passive water-permeation is increasing, we are far from understanding the water-permeation on a molecular or mechanistic basis. In tissues and plants the situation is even more complex because one must discriminate between different pathways such as long, medium and short distance transport [14] or apoplasmic, symplasmic and transcellular pathways (*e.g.* [15]). In addition most methods suitable for measuring the water-permeation across lipid-bilayers or plasma-membranes cannot easily or cannot at all be extended from measurements on single cells to those on organized systems.

In the following we describe a method to observe water-fluxes in thin (s. below) systems or sections. The same method can further be extended to allow measurements on single cells. In some respect the new method adheres to our previously developed strategies and relies also on "spectroscopic differences" of H_2O and D_2O. The previously developed techniques make use of the D_2O-sensitive fluorescence quantum-yield of some fluorescent compounds [8] and of the different refraction-indices of D_2O and H_2O [16]. The later difference leads to a solvent-isotope-effect of light scattering [16]. We here describe a third attribute of the D_2O/H_2O system which can be used to directly observe water-fluxes in tissues and plants. The principle together with the limitations and first results will be presented below.

Principle

The strategy is based on the different absorption spectra of D_2O and H_2O in the near infrared (NIR) region; these spectra are reproduced in Fig. 1. The visible H_2O-bands are due to overtone or combination vibrations of the OH-group [17]. For D_2O the spectral range between 800 nm and 1900 nm is prac-

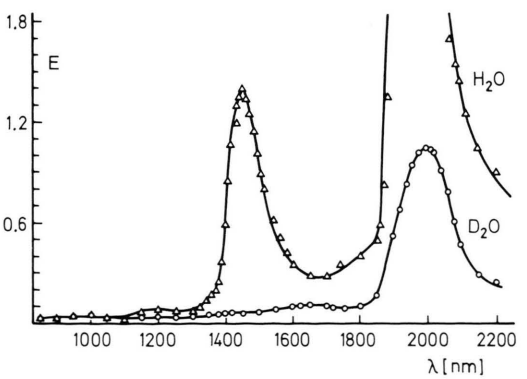

Fig. 1. Near infrared (NIR) absorption spectra of H_2O (△) and D_2O (○). Absorption E versus wavelength λ. Slit 0.02 mm, cuvette length $l = 1$ mm, room temperature.

In memoriam Priv.-Doz. Dr. Heinrich Lachmann † 1984.

Reprint requests to Dr. R. Lawaczeck.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0900–0923 $ 01.30/0

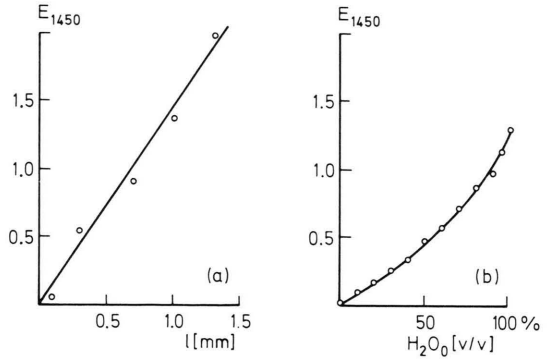

Fig. 2. a) Absorption E at 1450 nm of H_2O versus optical path-length l. b) Absorption E at 1450 nm of H_2/D_2O mixtures versus the stoichiometric H_2O_0 content (l = 1 mm).

tically transparent. The optical density (OD) around 1450 nm is a monotonic function of the H_2O/D_2O-content and linearily depends on the optical path-length as demonstrated in Fig. 2a, b. In agreement with results by Luck [18] it was further observed that salts at moderate concentrations have only a minor effect on the spectra.

By comparing the measured OD at 1450 nm for thin layers with reference to the same system in pure H_2O and pure D_2O it is possible according to Fig. 2 to calculate the H_2O/D_2O-content or at constant isotope-composition of the solvent to deduce the depth of the absorbing water-layer. The differences of the H_2O-D_2O NIR absorption signals can further be used to measure transport phenomena by changing the H_2O/D_2O composition and following the time-dependent signal, *i.e.* performing an isotope-jump experiment. The method is limited to thin samples where the unspecific background absorption of the supporting (dry) material (proteins, cellulose) is still transparent to the measuring light. It will further be possible to visualize directly the H_2O/D_2O content or the H_2O/D_2O-fluxes in dynamic studies by appropriate video techniques with NIR-sensitive targets.

Experiments and Results

All spectra were obtained with a single beam Zeiss PMQ2-spectrometer using the PbS-cell in the range between 800 and 2200 nm at room temperature. For the work on thin layers with high background absorption, the band-width of the reference (air or D_2O (Merck, Darmstadt)) and of the sample were set to differ by a constant factor to allow a convenient OD-reading for the sample. Usually the slit-width for the reference was 1/10 to 1/25 the slit-width for the sample. Thus the actual meter-readings correspond to apparent OD-values under the chosen experimental conditions.

Objects to be studied were placed between the monochromator exit and the PbS-photocell. Care was taken that the objects stayed in contact with the aqueous reservoir (H_2O or D_2O). For kinetic studies an interference filter (Oriel, Darmstadt, 1450 nm Type 3) was mounted in front of the photo-cell, allowing the observation at 1450 nm without any interference of the day-light.

Fig. 3 reveals three spectra for filter-paper (Machery-Nagel, Düren, MN 615) in the spectral-range between 800 and 2200 nm. The spectra correspond from top to bottom to the dry filter, the filter soaked in H_2O and D_2O, respectively. As a consequence of the different indices of refraction of air and H_2O or D_2O, the light scattered and therefore the background-absorption are reduced by going from the dry to the wet filters. The absorption decreases in the series dry, wet (H_2O), wet (D_2O).

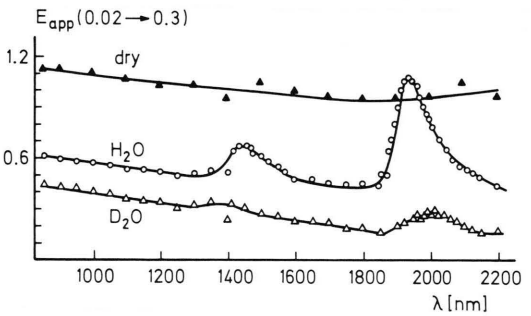

Fig. 3. NIR absorption spectra of dry, wet (H_2O) and wet (D_2O) filter-paper (from top to bottom). Apparent absorption E_{app} (reference slit 0.02, measuring slit 0.3 mm) versus wavelength λ. The filter-papers were placed between two supporting quartz-plates.

Fig. 4 indicates that the differences between the H_2O and D_2O spectra seen in the filter-paper are also visible in plant leaves where the water (H_2O) had been exchanged by D_2O. In both cases, filter-paper and plant leaf, there is a pronounced difference at 1450 nm and above 1900 nm between the H_2O and D_2O spectra. The water-band at 1450 nm is almost lost for the D_2O-soaked tissues (the small scatter of the D_2O spectra may be due to the residual humidity in the light path). From the difference of the H_2O/D_2O peak-heights at 1450 nm, the thickness of the

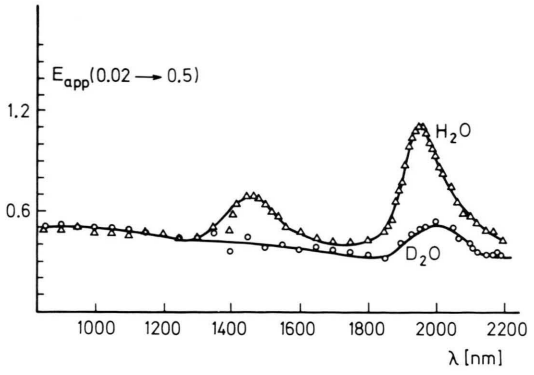

Fig. 4. NIR absorption spectra of sumach-leaflets in H_2O and D_2O, respectively. Apparent absorption E_{app} (reference slit 0.02, measuring slit 0.5 mm) versus wavelength λ. Before the D_2O-spectrum was taken the leaflet was standing in D_2O for 2 h exposed to bright sunlight.

water-layer can be approximated. For the sumach *(Rhus typhina)*-leaflets we calculated a thickness of 0.19 mm which compares well with results obtained by a micrometer (0.21 mm). The good correlation between these values is evidently due to the fact that most of the plant mass in the light beam consists of exchangeable water.

In kinetic studies the transmitted light at 1450 nm is monitored after applying a change of the isotopic solvent composition, *i.e.* by going from H_2O to D_2O. Fig. 5 reports results obtained for a filter-paper system. In the two cases considered the filter-paper was placed between quartz-plates. For system A the quartz-plates totally covered the filter-paper while for system B the filter-paper was larger than the supporting quartz-plates. Both systems were connected to the aqueous reservoir and the change of the isotopic composition of the reservoir was applied after an initial steady state was established. In system A primarily the diffusional exchange is effective. In addition, system B contains a contribution from the transpirational flow from the reservoir through the filter-paper into the air. The predominant effect of the transpiration is borne out by the experimental results. The approximated half times are 2 and 0.5 h for the system without and with transpiration, respectively.

Kinetic experiments were also carried out with plant leaves from a sumach-tree. Leaflets were cut under water from the fresh leaves and mechanically fixed so that they stood upright with their stem reaching into the aqueous reservoir. The observation light through a spherical diaphragm of 3 mm diameter could be directed through the main water vessel or through the periphery. After an equilibration period the isotopic composition of the reservoir was changed from H_2O to D_2O. The observed kinetics

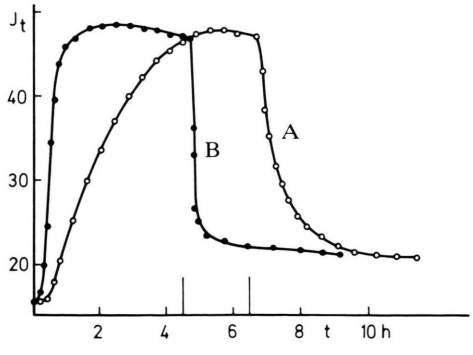

Fig. 5. H_2O/D_2O-exchange kinetics in filter-paper. Transmitted light intensity I_t (a.u.) at 1450 nm versus time t. At $t=0$ the aqueous solvent was changed from H_2O to D_2O and at the times indicated by lines the reverse change was performed. The 1450-nm-light passed through a diaphragm of 3 mm diameter located about 15 mm above the surface of the aqueous reservoir.
A: System with diffusion without or with minimal transpiration (open circles).
B: System with diffusion and transpiration (filled circles).

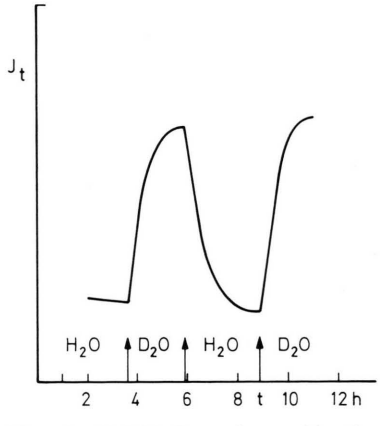

Fig. 6. H_2O/D_2O exchange-kinetics in sumach-leaflets. Transmitted light intensity I_t (a.u.) at 1450 nm versus time t. At the times indicated by arrows the solution of the reservoir was changed from H_2O to D_2O and backwards from D_2O to H_2O, respectively. Sumach-leaflets were cut under water and stood upright with their stems reaching into the aqueous reservoir. The measurements were performed through a diaphragm of 3 mm diameter located about 15 to 20 mm above the aqueous surface. Control experiments where the stem was cut or disconnected from the reservoir did not show isotope specific responses.

resembled the exchange-kinetics of the filter-paper system except that the half-times were longer. The exchange was reversible, *i.e.* the back-reaction $D_2O \rightarrow H_2O$ led to the initial value (before the application of the D_2O-jump). It was further observed that the exchange-kinetics in the periphery was slower than the kinetics in the main vessels.

Discussion and Limitation

The results obtained so far demonstrate the capability of the method. The method is versatile and can be adapted to various problems and objects under a variety of conditions. Except for unspecific light scattering we did not observe any specific absorption in the spectral range studied which resulted from the non-aqueous material. The experimental curves obtained from the kinetic studies can be fitted by a mathematical simulation.

For kinetic experiments the method relies on spectroscopic differences of H_2O and D_2O of the exchangeable water. For plant leaves it was shown by neutron scattering [19] that the bulk water comprises 80%, the remaining water is 3-times coordinated or to a few percent firmly and thus irrotationally bound [19]. Water of plant leaves has further been modelled on the basis of recent NMR data [20].

The limitations of the method are of both physical and biological origin. The material must be transparent in the applied wavelength region, which puts an upper limit on the thickness of the samples. However, most biological material at thin layers is transparent at the wavelength considered. A lower limit in thickness will certainly exist for those cases where adsorption phenomena have on overwhelming influence on the water-structure and might overshadow the described H_2O/D_2O spectra and effects. Vicinal water-layers up to 100 Å (equivalent to about 30 molecule-diameters) or more [21] are discussed in that respect. Small D_2O-induced contributions of the thickness of the water-layer, if existent, can be tolerated because D_2O is transparent at the wavelength chosen. The species HDO is at thermodynamic equilibrium with H_2O and D_2O. However, the inclusion of the species HDO and the evaluation of the data of Fig. 2b as function of the actual H_2O concentration does not lead to a straight line. Thus the Lambert-Beer-law does not strictly hold, possibly due to intermolecular interactions and the interference with HDO-vibrations. For the present considerations specific HDO-influences can be neglected.

The biological limitations result from the usual D_2O-intolerance of plants. Osmotic effects of heavy water were discussed in connection with measurements of the water-permeability of plant cells [22]. The different behaviour of living systems in H_2O and D_2O is certainly due to secondary effects, *i.e.* D_2O-induced changes in viscosity, hydrophobicity and rate constants or pK-values for protonation/deprotonation reactions. We succeeded to grow a bean from the seed up to the first leaves. However, the differences with respect to controls grown up in H_2O were rather remarkable. Difficulties arising from the D_2O-intolerance are only limiting in long term studies where one is interested in the survival of the plants or generally the object to be studied. Two possible ways to overcome these difficulties can be considered: i. the application of only low concentrations of D_2O together with an increase of the sensitivity of the measuring device, and ii. the application of short isotopic pulses. The monitoring of short D_2O-pulses should have the additional advantage of allowing a decoupling between flux and diffusion.

In contrast to IR light in the main absorption region of water (3280 cm^{-1}, 3490 cm^{-1} [23, 24]), which leads within seconds to leaf-damage, the application of the NIR light did not induce visible changes even after prolonged periods of time. Current studies are concerned with experimental refinements, so that studies on plant systems under various well defined conditions, including effects of environmental-toxins, can be pursued.

Acknowledgements

R. L. thanks the Deutsche Forschungsgemeinschaft (DFG, SFB 176) and the Centre National de la Recherche Scientifique (CNRS, France) for support. The paper was finished while R. L. was at the Centre de Biophysique Moléculaire in Orléans (France).

[1] M. C. Steward and M. J. Garson, J. Membrane Biol. **86,** 203 (1985).
[2] H. Pitterich and R. Lawaczeck, Biochim. Biophys. Acta **821,** 233 (1985).
[3] R. R. Sharp and C. F. Yocum, Biochim. Biophys. Acta **592,** 169 (1980).
[4] S. Ratkovic and G. Bacic, Bioelectrochem. Bioenerg. **7,** 405 (1980).
[5] D. C. McCain and J. L. Markley, FEBS Letters **183,** 353 (1985).
[6] G. Bacic and S. Ratkovic, Biophys. J. **45,** 767 (1984).
[7] T. J. Wydrzynski, S. B. Marks, P. G. Schmidt, Govindjee, and H. S. Gutowsky, Biochemistry **17,** 2155 (1978).
[8] R. Lawaczeck, J. Membrane Biol. **51,** 229 (1979).
[9] M. E. Fabray and Eisenstadt, Biophys. J. **15,** 1101 (1975).
[10] C. V. Paganellie and A. K. Solomon, J. Gen. Physiol. **41,** 259 (1957).
[11] S. Wendler and U. Zimmermann, J. Membrane Biol. **85,** 121 (1985); **85,** 133 (1985).
[12] R. J. Macey, in: Membrane Transport in Biology (G. Giebisch, D. C. Tosteson, and H. H. Ussing, eds.), pp. 1–57, Springer Verlag, Berlin 1981.
[13] R. I. Sha'afi, in: Membrane Transport (S. L. Bonting and J. J. H. dePont, eds.), pp. 29–60, Elsevier/North-Holland, Amsterdam 1981.
[14] U. Lüttge, Stofftransport der Pflanzen, pp. 213–219, Springer Verlag, Berlin 1973.
[15] E. Steudle and W. D. Jeschke, Planta **158,** 237 (1983).
[16] H. P. Engelbert and R. Lawaczeck, Ber. Bunsenges. Phys. Chem. **89,** 754 (1985).
[17] J. D. Worley and I. M. Klotz, J. Chem. Phys. **45,** 2868 (1966).
[18] W. A. P. Luck, in: Water and ions in biological systems (A. Pullman, V. Vasilescu, and L. Packer, eds.), pp. 95–126, Plenum Publ. Corp., New York 1985.
[19] P. Martel, Biochim. Biophys. Acta **714,** 65 (1982).
[20] D. C. McCain and J. L. Markley, Biophys. J. **48,** 687 (1985).
[21] W. Drost-Hansen, in: Water and ions in biological systems (A. Pullman, V. Vasilescu, and L. Packer, eds.), pp. 523–534, Plenum Publ. Corp., New York 1985.
[22] G. Hübner, Protoplasma **60,** 401 (1965).
[23] D. Eisenberg and W. Kauzmann, The structure and properties of water, Oxford University Press, Oxford 1969.
[24] R. E. Verrall, in: Water, a comprehensive treatise (F. Franks, ed.), **Vol. 3,** pp. 211–264, Plenum Press, New York 1973.