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Water (H_2O and D_2O) Molar Absorptivity in the 1000–4000 cm^{-1} Range and Quantitative Infrared Spectroscopy of Aqueous Solutions[1]

Sergei Yu. Venyaminov[2,3] and Franklyn G. Prendergast
Department of Pharmacology, Mayo Foundation, Rochester, Minnesota 55905

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Water (H_2O and D_2O) molar absorptivity was measured by Fourier transform infrared transmission spectroscopy in the 1000–4000 cm^{-1} range at 25°C. A series of assembled cells with path lengths from 1.2 to 120.5 μm was used for these measurements. The optimal path length (the path length of aqueous solution at which the IR spectrum of solute, corrected for water absorbance, has the highest signal-to-noise ratio) was calculated for all water absorbance bands. The results presented here show that the optimal path length does not depend on solute properties and is inversely proportional to the solvent (water) molar absorptivity. The maximal signal-to-noise ratio for measurements of IR spectra of aqueous solution in the 1650 cm^{-1} spectral region, of primary interest in biological applications, can be obtained at an optimal cell path lengths of 3–4 μm (H_2O) and 40–60 μm (D_2O). As an example, the signal-to-noise ratio was calculated as a function of the cell path length for the amide I (H_2O) and amide I′ (D_2O) bands of an aqueous lysozyme solution. The molar absorptivities of water bands are several orders of magnitude weaker than those of the strongest bands of biological macromolecules in the same spectral regions. High net water absorbance in aqueous solutions is due simply to the very high molar concentration of water. A method is proposed for the quantitative measuring of the path length of the cell which exploits the molar absorptivity of the strongest water bands (stretching vibrations) or of bands which do not overlap with solute absorbance. A path length in the range from ~0.01 μm to ~1.0 mm can be determined with high precision using this technique for a samples of known concentration. Problems involved in the proper correction of strong water absorbance in IR spectra of aqueous solutions of biomolecules are discussed, including multiple reflections within the cell, the effects of pH, temperature, and perturbation of water spectral properties by polar solutes, as well as the selection of optimal spectral regions in which one may obtain the most precise absorbance corrections.

Infrared spectroscopy is one of the most sensitive physical techniques for studying biomolecular structure. Water serves as the native medium for most biomolecules. Consequently, knowledge of its IR spectral characteristics is important for choosing appropriate solute concentrations and cell path lengths for optimal measurement of IR spectra of aqueous solutions, for quantitative determination of water content in samples such as tissues and cells, and for proper subtraction of water absorbance from the IR spectra of aqueous solutions of biomacromolecules. In the last case, it is important to choose proper spectral range(s) and use absorbance in this range(s) as the criterion of precision for water absorbance subtraction. Also, the possibility of artifacts is inherent in such subtraction techniques and it is thus very important to know how different physicochemical parameters, like pH, ionic strength, temperature, and solute properties, affect the IR spectral characteristics of water.

The IR spectra of H_2O and D_2O have been published many times (Refs. (1) and (14) and citations therein), but disagreements regarding molar absorptivity of water exist among the different sets of data, especially for the high wavenumber range. If one compares the intensity of the strong bands, identified as stretching and bending water vibrations, obtained from different data sets, the disagreement is about 10%. However, for weaker bands, composed of combination and overtone

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[2] On leave from the Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow Region, Russia 142292.

[3] To whom correspondence should be addressed at the Department of Pharmacology, Mayo Foundation, Rochester, MN 55905. Fax: (507) 284-9349. E-mail: venyaminov.sergei@mayo.edu.

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water vibrations, the data disagree by as much as 100% in extreme cases (1). In many instances, these earlier measurements were made using ATR–FTIR[4] techniques (1, 14).

In this work, we used FTIR transmission spectroscopy performed with a series of 28 assembled CaF_2 cells with path lengths between 1.2 and 120.5 μm which allowed us to optimize the path length needed for measurements of weak and strong water absorbance bands. The molar absorptivities measured in this work coincide very well with those published in Ref. (1) for all bands of H_2O and D_2O, except that of the bending vibration of D_2O at 1209.4 cm^{-1}. Using these values of water molar absorptivity, we then calculated the optimal cell path length for IR spectral measurements of aqueous solutions for all water bands in the 1000–4000 cm^{-1} spectral range. Since accurate knowledge of cell path length and solute concentration are essential, we developed a method to determine the amount of water if the path length of the sample is known or the path length of the sample if the solute concentration is known. The influences of several factors which interfere with and/or perturb water IR spectral properties are quantitatively evaluated and discussed. We show that the absorbance of the stretching vibration of water in the high wavenumber range must used to indicate the effectiveness of correction for water absorbance.

MATERIALS AND METHODS

Materials

All water (H_2O) employed in this studies was passed through Barnstead (U.S.A.) NANOpure series 550 system. The resistivity of water at 25°C was equal to 18.3 Mohm-cm. Water samples were kept in a closed polyethylene bottle and were used within 1 week of purification. The D_2O was a product of CDN isotopes (Canada), No. D-2757 and was "99.9 atom% D, low conductivity" grade. The lysozyme from egg white was a product of Boehringer (Germany), No. 1423153, and was used without further purification. A molar absorptivity of 4.03×10^4 M^{-1} cm^{-1} at 281 nm was used for lysozyme concentration determination (2).

Methods

IR spectra were measured with a resolution of 4 cm^{-1} on a Digilab FTS-40 FTIR spectrometer continuously purged with N_2 and equipped with a DTGS detector. The wavenumber scale of the spectrometer was calibrated using the water vapor fundamental (3). The precision of calibration in the 3700 and the 1600 cm^{-1} spectral ranges was equal to ± 0.4 and ± 0.2 cm^{-1}, respectively. A homemade shuttle system was used for all measurements. The shuttle was equipped with a thermostated brass block which holds both sample and reference. The software allowed regulation of the number of mechanical steps between sample and reference as well as the number of coadded interferograms for each. After Fourier transformation, spectra in absorbance or transmittance scale were obtained. The solute absorbance is measured as small difference between the large absorbances of sample (solution) and reference (solvent). Any time-dependent changes in cell temperature, in atmospheric CO_2 or water vapor concentration, or in baseline drift of the spectrometer can lead to changes in the sample and/or reference absorbance, resulting in large uncertainties in the value of the solute absorbance. This problem becomes even more acute if the measurements of absorbance for solution and solvent are widely separated in time. A shuttle system essentially eliminates these influences. The time interval between sample and reference measurements decreases in proportion to the number of shuttle steps. For example, after opening the cell compartment of the FTS-40 spectrometer for 30 s, it takes approximately 36 h of continuous nitrogen flushing to obtain a spectrum without H_2O water vapor absorbance in the 6-μm spectral region. A similar result can be achieved in one hour using a shuttle system at 32 steps with 32 spectra accumulated per step for each sample and reference at a resolution of 4 cm^{-1}. The noise level for the FTS-40 instrument in the 1400–1800 cm^{-1} range, is equal to 5×10^{-5} absorbance units (AU) under these conditions. For all water absorbance measurements, the shuttle system was programmed for 12 steps with 12 spectra accumulated per step. For measurement of cell path length (interference fringe method), because the amplitude of a fringe pattern of an empty cells was very small, equal to or less than 50 mAU, the shuttle system was programmed for 32 steps with 32 spectra accumulated per step (1024 coadded spectra for sample and for reference).

Homemade cells were used for water IR spectral measurements. Each cell was formed between a perfectly flat, optically clear CaF_2 disc and another CaF_2 disc, the center of which had been deepened to form a recessed parallel surface surrounded by a groove. This groove served as a barrier between the sample area and the outer seal, ensuring that the sample did not come into contact with the sealing area. The seal was made by the upper flat disc pressing onto the outer ring of the lower disc. The reproducibility of the cell path length after assembly–disassembly was equal to 0.1 μm, which is about the same as the precision of the cell path lengths measured by the interference fringe method. The cells can be very easily assembled and disassembled, filled with solution, and washed between

[4] Abbreviations used: FTIR, Fourier transform infrared; ATR, attenuated total reflection; DTGS, deuterated triglycine sulfate; AU, absorbance unit.

measurements. They can also be used for circular dichroism and absorption spectra measurements in the visible, near, and far UV spectral ranges. Detailed construction[5] was described in Ref. (4). The seal of the sample cell prevents the evaporation of water for about 24 h at room temperature in the desiccated area of the FTIR spectrometer. For longer times, or for measurements conducted at elevated temperatures, and especially for D_2O solutions, prior to cell assembly, it is better to lubricate the sealing surface with mineral oil dissolved in any volatile organic solvent. This procedure practically does not change the cell path length and permits work with one sample at temperatures up to 95°C for several weeks if necessary, without any change in sample concentration or isotope content (D_2O). In the work reported here, 28 cells with path lengths from 1.2 up to 120.5 μm were used. A volume of 74 μl is required to fill the cell of path length 120.5 μm. For other cells, the volume decreases in proportion to cell path length.

Measurements were made at 25°C, except for studies of temperature dependence. For the determination of cell path lengths or water absorbance, the cell was placed in the shuttle sample position with a 10-mm-thick CaF_2 disc in the reference position. The reference CaF_2 disc was used to compensate for the reflections from the outer cell surfaces and the absorbance of the cell body in the low wavenumber spectral range. The molar absorptivity E^λ in M^{-1} cm^{-1} at wavelength λ was calculated from

$$E^\lambda = A^\lambda/lC, \qquad [1]$$

where A^λ is the absorbance, l is the cell path length in centimeters, and C is the concentration (M). The concentration value of H_2O [D_2O] at 25°C was taken as 55.34 M [55.14 M], assuming that molecular weight is equal to 18.0153 [20.0275] and density is equal to 996.93 g/liter [1104.4 g/liter].

To measure the molar absorptivity of weak water bands at 2127.5 cm^{-1} (H_2O) and 3840, 1650,[6] and 1555 cm^{-1} (D_2O), 21 cells with path lengths from 4.5 to 120.5 μm were used. For bands with intermediate intensity, 1643.5 cm^{-1} and 1550 (see footnote 6) cm^{-1} (H_2O) and 1209.4 cm^{-1} (D_2O), 16 cells with path lengths from 3.4 to 20.3 μm were used. For the strongest bands, namely stretching vibrations of water molecules at 3404 cm^{-1} (H_2O) and 2504 cm^{-1} (D_2O), 7 cells with path lengths from 1.2 to 3.6 μm were used. In the last case, because the precision of cell path length measurements by the interference fringe method was equal to ± 0.1 μm, equivalent to an error of 3–8%, the molar absorptivity of bands belonging to the bending vibration was used as an internal standard for path length determination to improve precision. The values of molar absorptivities presented in this work are average ± standard deviation for the measurements at different cell path lengths.

[5] This design is that of S. Yu. Venyaminov and has been used for more than two decades in the Spectroscopy Group of the Institute of Protein Research Russian Academy of Sciences.

[6] Position of amide I′ (D_2O) and amide II (H_2O) bands for proteins.

RESULTS AND DISCUSSION

Assignment of Water Bands

The isolated water molecule has three vibrational modes. These correspond to the symmetrical (ν_1) and asymmetrical (ν_3) stretching vibrations of the O–H [O–D] bond, and (ν_2) to the bending vibration involving the H–O–H [D–O–D] angle (5). Because of intermolecular interactions, the IR absorbance spectrum of liquid water is much more complex than that of isolated molecules. For H_2O in the low wavenumber range, 500–800 cm^{-1}, a broad band (ν_L) is observed which arises from so-called librations, a collective normal mode involving many water molecules. Another band, belonging to the combination of libration and bending vibrations ($\nu_2 + \nu_L$), occurs at 2127.5 cm^{-1} (see Fig. 1). The absorbance band at 1643.5 cm^{-1} is a bending vibration (ν_2). The strongest band, centered at 3404 cm^{-1}, has three components: the low wavenumber component (seen on Fig. 1 as shoulder at ~3250 cm^{-1}) is an overtone of bending vibration ($2\nu_2$); it is enhanced by Fermi resonance with the second component, a (ν_1) stretching vibration at ~3450 cm^{-1}; finally, the third component is the (ν_3) stretching vibration at ~3600 cm^{-1}. IR spectra of D_2O (Fig. 2) show the (ν_2) bending vibration at 1209.4 cm^{-1}; the combination of ($\nu_2 + \nu_L$) at 1555 cm^{-1}; a complex band at 2504 cm^{-1} with two shoulders at ~2400 cm^{-1} ($2\nu_2$) and at ~2600 cm^{-1} (ν_3) and a band at ~2500 cm^{-1} (ν_1); a weak band at 3404 cm^{-1}, which is due to O–H stretching vibrations of HOD (from traces of H_2O in D_2O); and a band at 3840 cm^{-1} due to the ($\nu_1 + \nu_2$) combination.

It is seen from Fig. 1 [2] for H_2O [D_2O] that the ratio of the intensities of bending to stretching vibrations is equal to 0.22 [0.24], and the ratio of the intensities of bending to combination ($\nu_2 + \nu_L$) vibrations is equal to 6.23 [9.11]. The bending vibration of HOD molecules at 1462 cm^{-1} which strongly overlap with a combination band of D_2O at 1555 cm^{-1} has an absorbance which is a factor of 3 lower than that at 3404 cm^{-1}.

Water Molar Absorptivity

Table 1 presents water absorption parameters with numerical values for band position, molar absorptivity, absorbance of water at 10 μm path length, and the optimal cell path length for IR measurements of aqueous solution. Also given in Table 1, relevant to biologi-

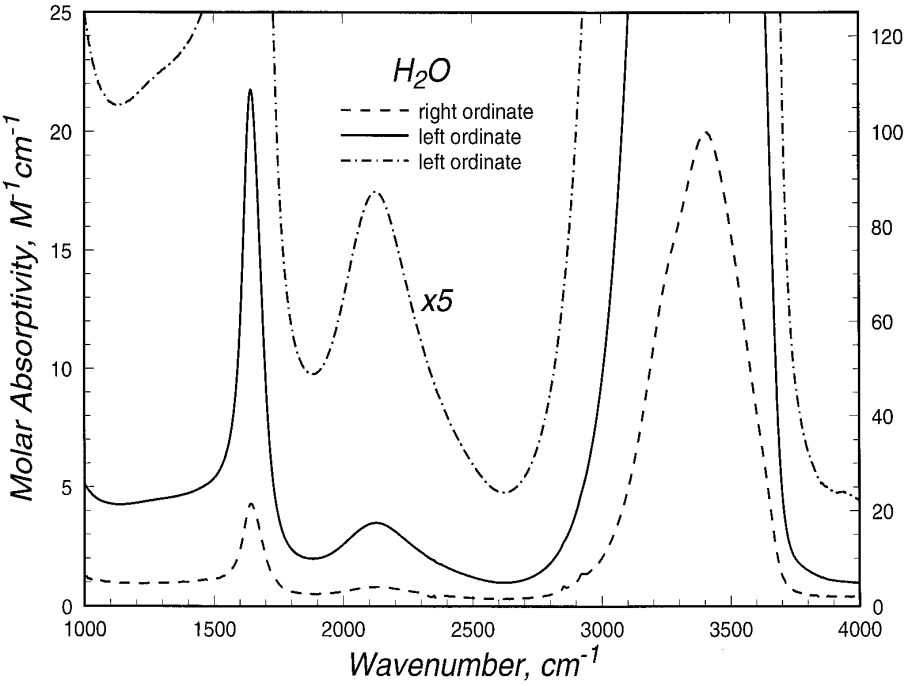

FIG. 1. Molar absorptivity of H_2O in the 1000–4000 cm^{-1} spectral range at 25°C.

cal studies, are the water absorptions at 1550 and 1650 cm^{-1}, the characteristic wavenumbers of protein amide II (H_2O) and amide I′ (D_2O) bands. D_2O never is 100% pure and usually includes HOD molecules from the traces of H_2O. The absorbance bands at 3404 cm^{-1} (intensity is proportional to content of OH groups) and at 3840 cm^{-1} (intensity is proportional to D_2O content) can be used for quick and precise determination of D_2O

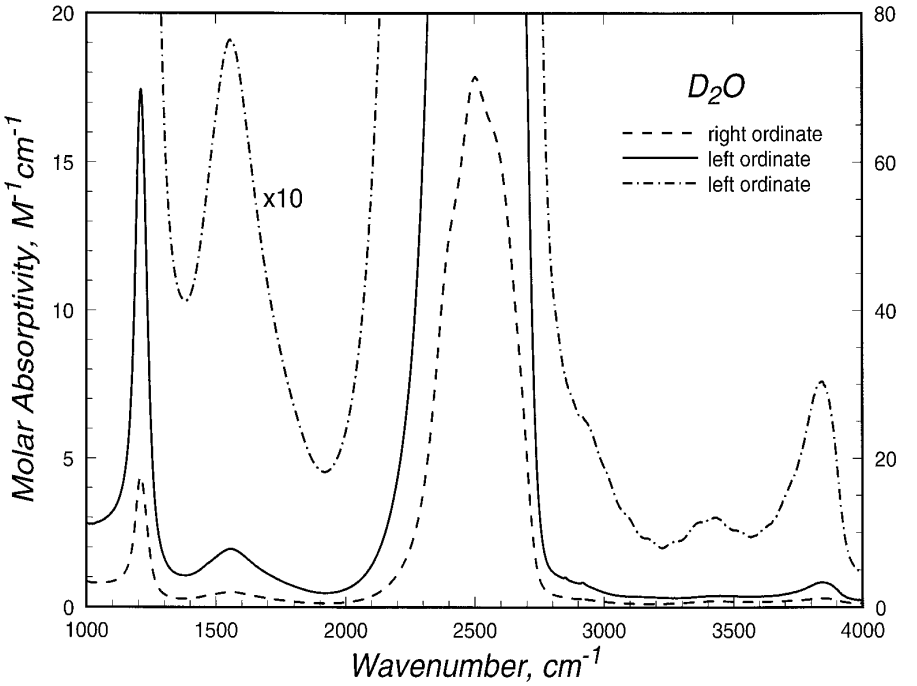

FIG. 2. Molar absorptivity of D_2O in the 1000–4000 cm^{-1} spectral range at 25°C.

TABLE 1

Absorption Parameters[a] of Water (H_2O and D_2O) Bands in the 1000–4000 cm^{-1} Range at 25°C

Type of vibration	H_2O				D_2O			
	ν_0 (cm^{-1})	E_0 (M^{-1} cm^{-1})	$A_{10\mu m}$ (AU)	l_{opt} (μm)	ν_0 (cm^{-1})	E_0 (M^{-1} cm^{-1})	$A_{10\mu m}$ (AU)	l_{opt} (μm)
Combination of stretching and bending[b]					3840.0	0.74 ± 0.05	0.0408	106.4
Stretching (asymmetrical + symmetrical) + overtone of bending	3404.0	99.9 ± 0.8	5.53	0.8	2504.0	71.5 ± 0.4	3.94	1.1
					1650[c]	1.41 ± 0.05	0.0778	55.9
Combination of bending and libration	2127.5	3.50 ± 0.10	0.194	22.4	1555.0	1.91 ± 0.05	0.105	41.2
Bending	1643.5	21.8 ± 0.3	1.206	3.6	1209.4	17.4 ± 0.2	0.962	4.5
	1550[c]	6.55 ± 0.13	0.362	12.0				

[a] ν_0, band position; E_0, molar absorptivity; $A_{10\mu m}$, absorbance of water at 10 μm path length; l_{opt}, optimal cell path length (at this path length of aqueous solution, the IR spectrum of the solute corrected for solvent absorbance has a maximal signal-to-noise ratio).

[b] The absorption parameters of combination of stretching and bending vibration of H_2O at ~5160 cm^{-1} were not measured in this work and can be found in Refs. (1) and (14).

[c] Position of amide II (H_2O) and amide I′ (D_2O) bands for proteins.

isotope content. The following empirical equation has been used by one of us (S.Y.V.) for many years for this latter purpose:

$$D_2O(\text{atom\% D}) = 100 - [(A_{3404} - A_{4000})/2(A_{3840} - A_{4000})], \quad [2]$$

where A_{3404}, A_{3840}, and A_{4000} are the absorbances at indicated wavenumbers. The validity of this formula has been checked in the range 96.0–99.9% of atom% D. The optimal cell path length for evaluation of the D_2O purity, according to Eq. [2], depends on the intensity at 3404 cm^{-1} and usually is between 20 and 100 μm.

The Optimal Path Length for Measurements of IR Spectra of Aqueous Solutions

When one measures IR spectra of aqueous solutions, the signal S^λ at wavelength λ is equal to the sample absorbance A_s^λ and is directly proportional to sample concentration C_s and cell path length l. The proportionality coefficient is equal to the value of the molar absorptivity E_s^λ (see Eq. [1]).

$$S^\lambda = A_s^\lambda = E_s^\lambda \times C_s \times l. \quad [3]$$

The noise N^λ is directly proportional to the intrinsic spectrophotometer noise N_{sp}^λ and inversely proportional to solvent (water) transmittance and given by

$$N^\lambda = N_{sp}^\lambda \times 10^{A_w^\lambda} = N_{sp}^\lambda \times 10^{E_w^\lambda \times C_w \times l}. \quad [4]$$

Thus, the signal-to-noise ratio $(S/N)^\lambda$ is given by

$$(S/N)^\lambda = E_s^\lambda \times C_s \times l \times 10^{-E_w^\lambda \times C_w \times l}/N_{sp}^\lambda. \quad [5]$$

This quantity is thus a complex function of a path length. The condition for the determination of the optimal cell path length l_{opt}^λ, at which $(S/N)^\lambda$ has maximal value, is given by $d(S/N)^\lambda/dl = 0$, or

$$[(1 - l_{opt}^\lambda \times E_w^\lambda \times C_w \times ln\ 10) \times E_s^\lambda \times C_s \times 10^{-E_w^\lambda \times C_w \times l_{opt}^\lambda}/N_{sp}^\lambda] = 0. \quad [6]$$

From Eq. [6], l_{opt}^λ is equal to

$$l_{opt}^\lambda = 1/(E_w^\lambda \times C_w \times ln\ 10) \quad [7]$$

or, after substitution of C_w as 55.34 M for H_2O and 55.14 M for D_2O (both at 25°C),

$$(l_{opt}^\lambda)_{H_2O} = 7.85 \times 10^{-3}/E_{H_2O}^\lambda;$$
$$(l_{opt}^\lambda)_{D_2O} = 7.88 \times 10^{-3}/E_{D_2O}^\lambda. \quad [8]$$

The optimal path length for all water (H_2O and D_2O) bands in the 1000–4000 cm^{-1} spectral range was calculated from Eq. [8] and the values are shown in Table 1. To calculate the maximal signal-to-noise ratio, the value of l in Eq. [5] must be substituted by l_{opt}^λ from Eq. [7], or

$$(S/N)_{max}^\lambda = 0.160 \times E_s^\lambda \times C_s/(E_w^\lambda \times C_w \times N_{sp}^\lambda). \quad [9]$$

After substitution of C_w as 55.34 M for H_2O,

$$(S/N)^{\lambda}_{max} = 2.89 \times 10^{-3} \times E^{\lambda}_{s} \times C_s/(E^{\lambda}_{H_2O} \times N^{\lambda}_{sp}) \quad [10]$$

and C_w as 55.14 M for D_2O,

$$(S/N)^{\lambda}_{max} = 2.90 \times 10^{-3} \times E^{\lambda}_{s} \times C_s/(E^{\lambda}_{D_2O} \times N^{\lambda}_{sp}). \quad [11]$$

As seen from Eqs. [7]–[8], the optimal cell path length does not depend on the sample spectral properties and is inversely proportional to the molar absorptivity of the solvent at selected λ. The maximal signal-to-noise ratio is directly proportional only to sample concentration (see Eq. [9]). The other parameters are constant at selected λ for given sample, solvent, spectrometer, and number of spectral accumulations.

For numerical evaluation of the maximal signal-to-noise ratio, the IR spectrum of lysozyme was measured in H_2O and D_2O solution (spectra not shown). The molar absorptivity (per mole of residue) of the lysozyme amide I band at 1656 cm^{-1} is equal to 405 ± 12 M^{-1} cm^{-1} (average of several measurements at different concentrations and cell path lengths). The same value was obtained for amide I′ (D_2O solution) at 1646 cm^{-1}. The molar absorptivity of the band belonging to the bending vibration of H_2O at 1643.5 cm^{-1} is equal to 21.8 M^{-1} cm^{-1} (Table 1). We neglect the difference in positions of the amide I and H_2O absorbance bands because of their very strong overlap; the bandwidth at half height ($\Delta_{1/2}$) for lysozyme amide I is equal to 53 cm^{-1} and water (H_2O) band has $\Delta_{1/2}$ = 99 cm^{-1}. FTS-40 instruments equipped with a shuttle system (32 shuttle steps, 32 spectra accumulations per step for sample and reference) have N^{λ}_{sp} = 50 μAU in the 1400–1800 cm^{-1} spectral range (see Methods).

The last value we must define in order to calculate the maximal signal-to-noise ratio, Eqs. [10] and [11], is the sample concentration. If one takes the concentration of the lysozyme solution to be 50 mg/ml, equivalent to 3.5 × 10^{-3} M (per mole of protein), or 0.451 M (per mole of residues), it is possible to calculate the maximal signal-to-noise ratio for the amide I band of lysozyme.

$(S/N)^{AI}_{max}$ = 484 at l_{opt} = 3.6 μm in H_2O (Eq. [10]) and $(S/N)^{AI'}_{max}$ = 7515 at l_{opt} = 55.9 μm in D_2O (Eq. [11]). In Table 2 [Table 3] it is shown how the amide I [amide I′] absorbance of lysozyme at C = 50 mg/ml, the absorbance of H_2O [D_2O], and the signal-to-noise ratio for the amide I [amide I′] bands depend on the cell path length. Increasing the cell path length to two times the optimal, decreases the S/N by ~26%. Increasing the path length by three times decreases the S/N by ~60% of its maximum value. D_2O in the 1650 cm^{-1} spectral region has a 15.5-fold lower molar absorptivity than H_2O (see Table 1) and the maximal signal-to-noise ratio for amide I′ at the same protein concentration is thus 15.5 times higher in D_2O than in H_2O; the same signal-to-noise ratio can be obtained for amide I′ band in IR spectra of D_2O solution of protein at a 15.5-fold lower concentration (~3.2 mg/ml) than for protein solution in H_2O. It should be emphasized that at wavenumbers other than the position of the maximum for amide I, the protein absorbance decreases faster than that for solvent because the water band in this spectral range is about two times broader than the amide I band. Thus, the values of signal-to-noise ratios shown in Table 2 [Table 3] are the maximal values through the amide I [amide I′] bands.

Comparison of Water Molar Absorptivity with That for Biomolecules

Examination of Table 1 shows that water molar IR absorptivity for all types of vibrations in the 1000–4000 cm^{-1} spectral range is very low. The authors of two reviews (8, 9) inadvertently increased water molar absorptivity values by a factor of 1000 when they cited the correct data of review (10) but used different units (1000 cm^2 mol^{-1} = 1 M^{-1} cm^{-1}). For example, H_2O molar absorptivity at 3404 cm^{-1} is equal to 99.9 M^{-1} cm^{-1} and at 1643.5 cm^{-1} is equal to 21.8 M^{-1} cm^{-1}. The molar absorptivity of different biomolecules in the 1500–1800 cm^{-1} spectral range, even if calculated per residue, is significantly higher; the molar absorptivity for the amide I band of proteins and polypeptides being in the range of 300–1000 M^{-1} cm^{-1} (2, 11), for the principal IR bands of nucleic acids and polynucleotides the maximal molar absorptivity is in the range of 400–800 M^{-1} cm^{-1} (12), and for the carbonyl band of lipids maximal IR molar absorptivity is at ~700 M^{-1} cm^{-1} (Venyaminov, unpublished data). Nevertheless, the net water absorbance is very high because of high intrinsic water molar concentration. Because this simple issue frequently causes misunderstanding, let us provide an example. In Table 2, the water (H_2O) absorbance is 6.6 times higher in the 1650 cm^{-1} spectral region than lysozyme absorbance at the same path length and at a protein concentration of 50 mg/ml. This is due solely to the water concentration being 55.34/(3.5 × 10^{-3}) or 1.58 × 10^4 times higher than the protein concentration, and is in spite of the protein molar absorptivity (lysozyme has 129 amino acids) being 405 × 129/21.8 or 2.4 × 10^3 times higher than water.

Determination of Path Length for a Cell Filled with Aqueous Solution

In an IR spectrum of any water solution it is possible to select the spectral region(s) where the water absorbance band(s) either has significantly stronger intensity than the solute bands or does not overlap at all with the latter. The water absorbance in the region of

TABLE 2

Dependence of Amide I Absorbance for Lysozyme at C = 50 mg/ml, H_2O Absorbance at 1643.5 cm^{-1}, and Signal-to-Noise Ratio (S/N) for Amide I Band on Cell Path Length (l)

	l (μm)						
	2	3	3.6 (l_{opt})	4	6	8	10
A_{amideI} (AU)	0.037	0.055	0.065	0.073	0.110	0.146	0.183
A_{H_2O} (AU)	0.241	0.362	0.434	0.483	0.724	0.965	1.206
S/N	419	476	484	480	414	317	227

strong stretching vibrations of H_2O [D_2O] at 3404 cm^{-1} [2504 cm^{-1}] is much stronger than that of any biomacromolecules at reasonable concentrations of the latter. The water absorbance in the region of the relatively weak combination of stretching and bending vibration of H_2O [D_2O], i.e., at ~5160 cm^{-1} [3840 cm^{-1}] or at wavelength where there is a combination of bending vibration and libration of H_2O at 2127.5 cm^{-1}, practically does not overlap with bands belonging to biomacromolecules. So, for IR spectra of the aqueous solutions which is corrected generally only for reflections from the window surfaces, one can take the absorbance value(s) in the just mentioned spectral range(s) and calculate from Eq. [1] the product of a concentration and a path length for water, or water weight per surface unit, using the molar absorptivities from Table 1. If the path length is known, one can thus calculate the water concentration in the sample.

On the other hand, if the concentration of solute is known from independent measurements, it is possible to calculate the sample path length. For this let us assume that the response of an FTIR spectrometer with a signal-to-noise ratio of a 100 is linear between ca. 5 mAU and 2.5 AU. The water molar absorptivity from the Table 1 for the strong stretching vibration should be used in the determination of shorter effective path lengths, as short as 0.01 μm. The weakest bands from Table 1 should be used in the determination of the longer path lengths, ~0.1 mm (2127.5 cm^{-1} band of H_2O), ~0.6 mm (3840 cm^{-1} band of D_2O), or ~1.0 mm (~5160 cm^{-1} band of H_2O, Ref. 14). When calculating the path length using a known sample concentration, the "dilution" of water by the solute must be taken into account as follows. As stated above, if the IR spectrum of a sample is measured at a small path length (less then 4 μm), the very strong water band at 3404 cm^{-1} (H_2O) or 2504 cm^{-1} (D_2O) is used for quantitative determination of cell path length with high precision. For a cell with an unknown path length and filled with lysozyme solution at 5% concentration, the total absorbance at 3404 cm^{-1} corrected for reflection from window surfaces is equal to 1.89 AU. The path length of this cell can be calculated as l = 3.60 μm = 1.89/(0.533 × 0.95), where 1.89 is the absorbance of the cell at 3404 cm^{-1}; $A_{3404}^{1\mu m}$ = 0.533 AU (see Table 1); and the factor 0.95 shows that the 5% dilution of water by protein has been taken into account. At the same time, the absorbance of the amide I band in lysozyme solution at 5% concentration and path length 3.6 μm is equal to 65 mAU (see Table 2), and the absorbance of amide A at ~3300 cm^{-1} is equal to ~40% of amide I, which is ~26 mAU. The contribution of the protein amide A band to the total absorbance of an aqueous solution of a protein at 3404 cm^{-1} (where the H_2O band has its maximum intensity) is only about 13 mAU, which is less then 1% of the water absorbance 1.89 AU. After having measured the IR spectrum of a sample (solute + water) with known concentration, the correction of this spectrum for reflection from the window surfaces will allow determination of the path length of the sample. The correction of the same sample spectrum for the solvent absorbance will allow determination of the solute absorbance. Thus, one can calculate the quantitative IR

TABLE 3

Dependence of Amide I′ Absorbance for Lysozyme at C = 50 mg/ml, D_2O Absorbance at 1650 cm^{-1}, and Signal-to-Noise Ratio (S/N) for Amide I′ Band on Cell Path Length (l)

	l (μm)						
	20	40	55.9 (l_{opt})	60	80	100	120
$A_{amideI'}$ (AU)	0.365	0.730	1.019	1.096	1.461	1.826	2.191
A_{D_2O} (AU)	0.156	0.311	0.434	0.467	0.622	0.778	0.933
S/N	5100	7138	7515	7476	6976	6089	5113

spectrum (in units of the solute molar absorptivity) from Eq. [1]. IR spectroscopy can be used to determine the cell path length of aqueous samples for any type of cells and for other types of techniques (not only IR), the only requirement being cell window transparency in the IR spectral range.

Because CaF_2 windows are transparent in the far UV spectral region, we use our cells for measurement of protein circular dichroism at concentrations 30–50 mg/ml. The consistency of the molar absorptivities of amide I and II bands or molar ellipticities in the far UV, measured at different cell path lengths, is significantly improved when this kind of path length measurement is applied, particularly for cells with path lengths of several micrometers. If the path length is determined by IR for use in other experiments, such as circular dichroism, the same cell holder with the cell which had been used for the path length determination by IR spectroscopy must be used unaltered.

Correction of IR Spectra of Aqueous Solutions for Water Absorbance

If, in the spectral range of interest, absorption bands of solute and solvent overlap, in order to properly correct the water absorbance in IR spectrum of aqueous solutions, obviously it is important to select a spectral range where the water absorbance does not overlap with that of the solute and has intensity comparable with that in the range of interest. Let us now consider some numerical evaluations for the precision of such correction. If one substitutes l in Eq. [1] for optimal path length as defined in Eq. [7], the solvent absorbance at this condition does not depend on a wavelength or of any characteristics of the solvent. The value of solvent absorbance at optimal path length is constant and equal to $1/ln\ 10$, or 0.434 AU (see also, Tables 2 and 3). According to Eq. [4], this leads to an increase of the noise level of spectrophotometer by a factor of 2.72, or, in our case, to a value of 0.136 mAU. To measure the IR spectra of biomolecules in aqueous solution at optimal path length, one must correct the water absorbance (0.434 AU) with a precision equal to the noise level (0.136 mAU) or 0.031%. Only under such conditions is it possible to measure the IR spectrum of solute with maximal signal-to-noise ratio and without distortion by water absorbance. The need for such high precision of water absorbance subtraction eliminates from consideration several water bands which can be used for the path length determination with precision ca. ~1%. For example, the absorbance of H_2O band at 2127.5 cm^{-1}, which is widely used for correcting water absorbance at frequencies around 1650 cm^{-1} (15), is 6.23 times smaller than that at 1643.5 cm^{-1} (see Table 1). At a path length of 3.6 μm, which is optimal for measurements at 1650 cm^{-1}, a band at 2127.5 cm^{-1} has an IR absorbance of ~70 mAU (see Table 1). If the absorbance of this band is used for correcting water absorbance in the 1650 cm^{-1} region, it must be subtracted with a precision of ~22 μAU (0.031% of ~70 mAU), which is smaller than noise level. It is for this same reason, that the 3840 cm^{-1} band of D_2O should not be used for absorbance correction. The best spectral regions for this purpose are in the vicinity of ~3645 cm^{-1} (H_2O) and ~2770 cm^{-1} (D_2O). The water absorbance around these last-mentioned values will not overlap with those of biomolecules and in any case has a value equal to or even higher than that found in the 1550–1650 cm^{-1} range. Thus, in IR spectra of aqueous solutions corrected for absorbance of H_2O [D_2O], the residual absorbance at ~3645 cm^{-1} [~2770 cm^{-1}] can serve as a measure for precision of water subtraction at 1643.5 cm^{-1} [1555 cm^{-1}].

For correct subtraction of the D_2O absorbance in the 1300–1800 cm^{-1} range the second parameter, in addition to the path length, which must be taken into consideration is the isotope content of D_2O. The absorbance band at 3404 cm^{-1} should be used as the criterion of precision in correcting for HOD absorbance. The contribution of HOD absorbance at 1650, 1550, and 1462 cm^{-1} is equal, respectively, to ~2, ~4, and ~33% of its absorbance at 3404 cm^{-1}. The band at 3404 cm^{-1} is very well separated from any remnant band due to N–H stretching vibration of solute at ~3300 cm^{-1}, the latter being several times narrower then the former.

There are several complicating factors which interfere with water absorbance and/or can perturb water spectral properties. These factors include multiple reflections within the cell, pH, salts (or more generally, polar solutes), and temperature, and must also be taken into account through use of appropriate controls for proper water absorbance correction. Obviously, if such factors are not properly compensated for between sample and reference spectra, artifacts will be introduced into the water corrected solute spectrum. Reflection from the outer cell surface is corrected by placing a CaF_2 disc in the reference position; the reflection from the water–CaF_2 interface remains uncorrected and is usually neglected. If one measures the absorption spectrum of a smooth plane sample with refractive index n and path length l, the spectrum of this sample lying out of the absorbance bands will look like an interferogram with maxima at $\lambda = (2ln)/k$, and minima at $\lambda = (2\ ln)/(k + \frac{1}{2})$, where $k = 1, 2, 3 \ldots$. Maxima or minima in the wavenumber scale are separated by a distance equal to $\Delta\nu = 1/(2\ ln)$, and near the sample absorption band $\Delta\nu$ is not constant, because of changes in water refractive index (n_w). A good approximation of the intensity of multiple reflections is given by:

$$I^{\lambda} = \left(\frac{n_W^{\lambda} - n_{CaF_2}^{\lambda}}{n_W^{\lambda} + n_{CaF_2}^{\lambda}}\right)^2 \times \frac{1}{E_W^{\lambda}}$$

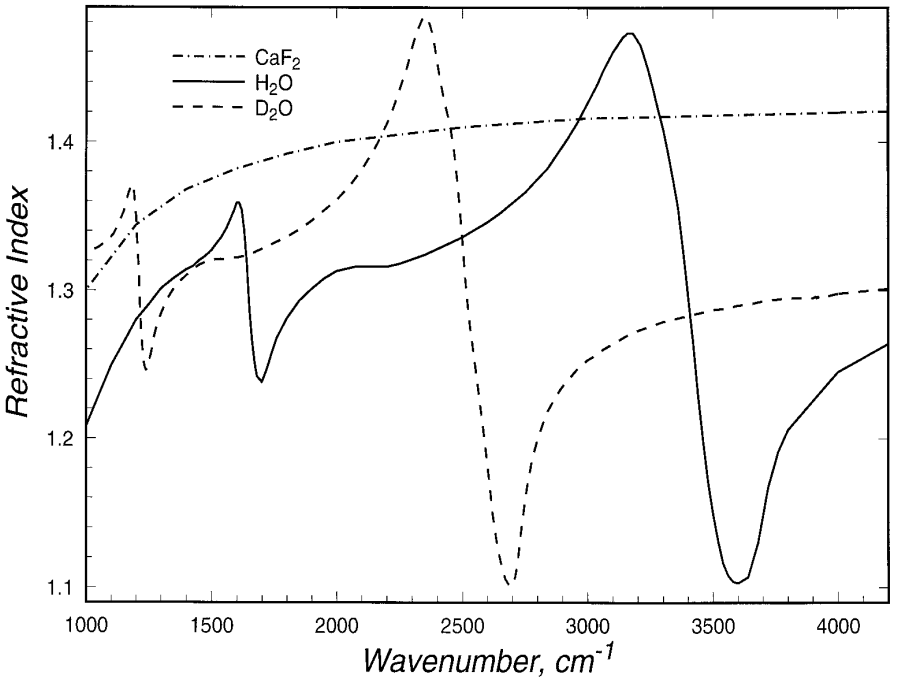

FIG. 3. Refractive index of H_2O, D_2O, and CaF_2 in the 1000–4200 cm^{-1} spectral range. The numerical data were taken from Refs. (1) and (6) for H_2O, Ref. (1) for D_2O, and Ref. (7) for CaF_2.

and the spacing in the fringe pattern is equal to: $\Delta\nu = 1/(2l_W \times n_W^{\lambda})$. For detailed discussion see Harrick (16). Figure 3 shows the dependence of the refractive index (n) of H_2O (1, 6), CaF_2 (7), and D_2O (1) on wavenumber in the 1000–4200 cm^{-1} spectral range. Two intense extrema of the refractive index exist for H_2O at 3170 cm^{-1} (n = 1.473) and 3600 cm^{-1} (n = 1.103) and for D_2O the extrema are at 2350 cm^{-1} (n = 1.484) and 2690 cm^{-1} (n = 1.101). These are clearly in the vicinity of the strongest absorbance bands belonging to water-stretching vibrations. The reflection coefficient is approximately equal to the squared difference between refractive indices divided by the squared sum of the refractive indices of water and CaF_2. The amplitude of multiple reflections from the water–CaF_2 interface is maximal at 2690 cm^{-1} (D_2O) and 3600 cm^{-1} (H_2O). These are responsible for the interference fringe patterns usually observed in the high wavenumber range in IR spectra of aqueous solution, after the correction for water absorbance. The path length of a sample must be longer then that for a solvent reference to compensate for the effective dilution of the solvent caused by the dissolved solute. If this is done properly, then the resulting fringe pattern is a superposition of two patterns (from the cell with solution and from the cell with solvent only) with slightly different periodicities. The water refractive index changes in this spectral range and, therefore, the distance between maxima or minima of the fringe pattern is not constant and decreases when it approaches 3600 cm^{-1} (H_2O) or 2690 cm^{-1} (D_2O). However, the intensity of the fringe pattern also decreases because of the increased absorbance of water. At wavenumbers lower than ~2500 cm^{-1} (D_2O) and ~3400 cm^{-1} (H_2O), differences between water and CaF_2 refractive indices become smaller than 0.1–0.15. Because of this, the reflection coefficient becomes smaller than 0.002–0.004, leading to a significant decrease in the amplitude of the interference fringe pattern in this spectral range from the water capillary layer within the cell. So, when selecting the baseline level in the range 1700–1900 cm^{-1} for spectra in the 1400–1700 cm^{-1} range, one can usually neglect the interference fringe. But it obviously must be taken into account when water absorbance at ~3645 cm^{-1} (H_2O) or ~2770 cm^{-1} (D_2O) is used as the criterion of the accuracy of the water absorbance subtraction.

Regarding the effects of pH, salt, and temperature, Kalnin and Venyaminov (4) have shown that a change in pH between 3 and 11 has no significant influence on water absorbance in the IR range. However, Figs. 4a and 4b demonstrate how salt and changes in temperature influence the IR spectral properties of water. (Similar changes are seen for D_2O, data not shown.) Three spectra are compared in this figure: the difference between spectrum of water at 50 and 25°C (path length 3.6 μm); the difference spectrum between 2.5 M NaCl solution (l = 3.6 μm) and water (l ~ 3.2 μm), both spectra at 25°C; and, for comparison, the absorbance

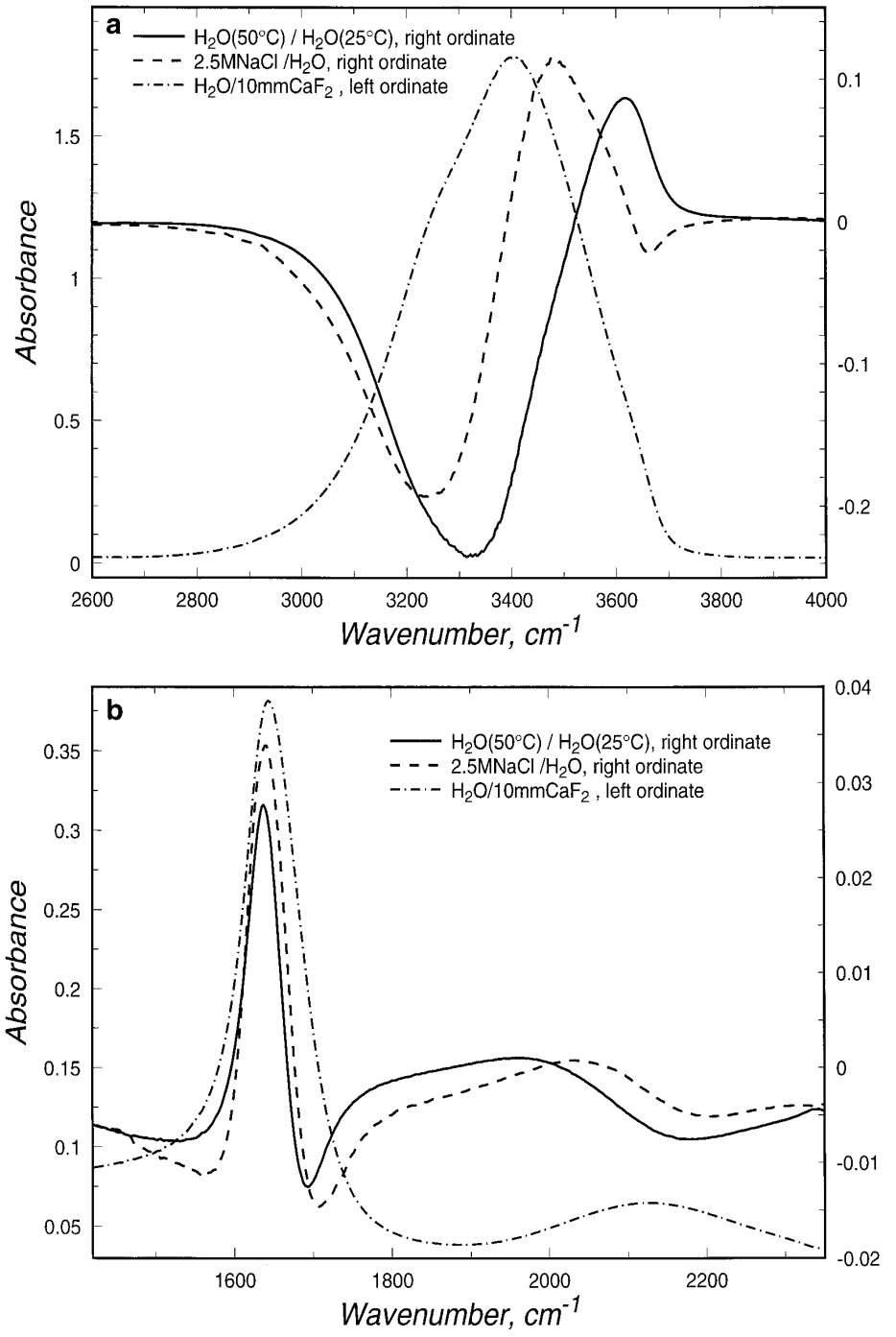

FIG. 4. Comparison of the IR absorbance spectrum of H_2O (corrected for reflections from the CaF_2 window surfaces) with the difference spectra of H_2O perturbed by temperature (ΔT = 25°C) or salt (2.5 M NaCl), see text for details, in the 2600–4000 cm^{-1} (a) and the 1430–2350 cm^{-1} (b) spectral range.

spectrum of water (l = 3.6 μm, T = 25°C). It is seen that water spectral perturbations due to the temperature difference (ΔT = 25°C) and to the presence of salt (2.5 M NaCl) are very similar. With regard to salt effect, sodium chloride does not absorb in the 1000–4000 cm^{-1} spectral range. At elevated temperatures or in the presence of salt, the absorbance band of water belonging to the bending vibration (ν_2) becomes more narrow and more intense with a slight shift to lower wavenumbers. The band belonging to the combination vibration (ν_2

+ ν_L) shifts to lower wavenumbers (Fig. 4a), and the absorbance bands belonging to the stretching vibrations (ν_1, ν_3) and to the overtone of the bending vibration (ν_2) shift to higher wavenumbers (Fig. 4b). The latter changes can also be interpreted as a decrease in intensity of the $2\nu_2$ and an increase in intensity of the ν_3 water vibration. The intensity of water (H_2O) absorbance bands at 3404 and 2127.5 cm^{-1} was found to decrease ~0.4% per 1°C and the band at 1643.5 cm^{-1} increased ~0.3% per 1°C for a temperature increase over the range of 0–85°C. (The intensity of D_2O absorbance bands at 2504 and 1555 cm^{-1} was decreased ~0.4% per 1°C and the bands at 3840 and 1209.4 cm^{-1} increased ~0.35% per 1°C over the range of 4–85°C.) Because the precision for subtraction of water IR absorbance must be equal to ~0.03%, the difference in temperature between aqueous solution and solvent must be less than 0.1°C. Consequently, for accurate IR absorption spectral measurements accurate equilibration of temperature between sample and reference, and accurate temperature control are essential.

Changes in salt concentration lead to a shift of band positions and to an increase in intensity of the bending vibrations of about 1% per 0.14 M of anions or cations; thus, as small as ~5 mM concentrations of salt can cause detectable distortion of water IR spectral properties. A highly charged sample can significantly change the optical properties of the solvent, leading to artifacts when one tries to correct for water absorbance in the sample using bulk water as a reference. Such an error may not only change the intensity of solute IR spectra, but also the shape of the absorption bands. While this is not a big problem for most protein solutions (4, 13), it is very important for aqueous solutions of low-molecular-weight polar compounds (13), nucleic acids, and lipids (Venyaminov, unpublished data). For example, Venyaminov and Kalnin (13) have considered an "effective" band at 1652 cm^{-1} in order to describe uncompensated water absorbance in analysis of IR spectra of low-molecular-weight model compounds derived from amino acid. Maybe by adding salt to the reference solvent one can mimic the distortion of the water IR bands due to the presence of a highly polar solute and thereby improve correction for water absorption in IR spectra of aqueous solutions.

Comparison of Water Molar Absorptivity with Published Values

To compare the most comprehensive published data measured by multiple ATR–FTIR (1) with those determined in this work, the imaginary infrared optical constant k^ν of water must be recalculated into the molar absorptivity E^ν (M^{-1} cm^{-1}) and corrected for the difference in temperature. The following formulas were used for recalculation: $E^\nu_{H_2O} = 0.0986 \times \nu \times k^\nu$ and $E^\nu_{D_2O} = 0.0990 \times \nu \times k^\nu$, where ν is in wavenumbers (cm^{-1}). Correction for the temperature difference (3°C) was about 1% for all bands (see previous paragraph). The molar absorptivities measured in this work for H_2O bands at 3404 and 1643.5 cm^{-1} coincide with those measured in (1) within two standard deviations (see Table 1) while the value is within one standard deviation for the band at 2127.5 cm^{-1}. For D_2O, the coincidence was better than one standard deviation for bands at 3840 and 2504 cm^{-1}, and within two standard deviations for the band at 1555 cm^{-1}. However, for the D_2O band at 1209.4 cm^{-1}, the molar absorptivity measured in this work was 10 standard deviations (11%) higher than that in Ref. (1). We do not know the reason for this inconsistency between the two methods of measurement. In this work, the same set of cells was used for H_2O and D_2O measurements and the value E_0 = 17.4 M^{-1} cm^{-1} at 1209.4 cm^{-1} for D_2O (see Table 1) was used as the internal standards for measurements of the molar absorptivity at 2504 cm^{-1}, and the latter agrees very well with previously published values (1).

CONCLUSION

In summary, the molar absorptivities measured in this work by FTIR transmission spectroscopy coincide very well (±two standard deviations or better) with those published in Ref. (1) for all bands of H_2O and D_2O, except the band of D_2O at 1209.4 cm^{-1}. The molar absorptivity of water is several orders of magnitude weaker than that of the strongest bands of biomacromolecules. The very high net absorbance of water in aqueous solutions is due to its high molar concentration.

Because the signal is directly proportional to cell path length and noise is exponentially dependent on the path length, an optimal path length exists at which signal-to-noise ratio is maximal. The optimal path length is determined by optical properties of water only and does not depend on the properties of the solute. The optimal path length for measurement of IR spectra for water solutions was calculated in this work for all water bands and the maximal signal-to-noise ratio for the protein amide I band was also determined. In the 1650 cm^{-1} spectral region for H_2O [D_2O] solution at the optimal path length of 3.6 μm [55.9 μm], every 10 mg/ml of protein concentration gives a maximal signal-to-noise ratio for amide I [amide I′] equal to ~100 [~1500].

The data obtained in this work allow the determination of the amount of water if the path length of the samples is known, or of the path length of the sample if the water and sample concentration are known. The weak water bands at 2127.5 cm^{-1} (H_2O) and 3840 cm^{-1} (D_2O) can be used for determination of path length in the range of 4–600 μm. The strong absorption bands belonging to the water stretching mode at 3404 cm^{-1} (H_2O) and 2504 cm^{-1} (D_2O) can be used for determina-

tion with precision ~1% of very small path lengths, up to 0.01 μm if the sample concentration is less than ca. 7.5%.

The optimal spectral range to use for precise subtraction of the water absorbance is in the vicinity of ~2770 cm^{-1} for D_2O solutions and ~3645 cm^{-1} for H_2O solutions. This work shows that multiple reflections from the cell windows, visible as interference fringes found within the background between absorbance bands, must be taken into account at wavenumbers higher than 2600 cm^{-1} (D_2O) and 3500 cm^{-1} (H_2O) in correcting water absorbance for IR spectra of aqueous solutions. Several factors change the IR spectral properties of water. Changing the temperature between freezing point and ~85°C leads to a shift of band positions and to changes in intensity of all water bands by ~1% per 2.5–3°C. Similar changes in water spectral properties occur when cations or anions at concentrations of ~0.14 M are added to water. A very careful procedure for subtraction of solvent absorbance from IR spectra of aqueous solutions must be applied in order to prevent errors in the intensity and/or the shape of solute band(s) sufficient to meaningfully alter physical interpretations.

The methods for determination of the effective path length and for correction of solvent absorbance described in this work are universal and can be used for any type of IR cells or for different procedures of subtraction (optical or mathematical). For optical subtraction, one must have a well-matched pair of IR cells and use the shuttle system; with mathematically based subtraction it is possible to use only one cell and consequently measure IR spectra of the solution and of the neat solvent. Because solute absorbance is usually a small difference between two relatively large and time-varying absorbances, the method of optical subtraction has an advantage in the precision of correcting strong water IR absorbance over the method of mathematical subtraction, and is therefore recommended for quantitative IR spectroscopy of aqueous solutions of biomolecules.

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