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Role of Bound Water in Biological Membrane Structure: Fluorescence and Infrared Studies

Allan S. Schneider, C. Russell Middaugh, and Mary D. Oldewurtel

Sloan-Kettering Institute for Cancer Research and Cornell University Graduate School of Medical Sciences, New York, New York 10021

Bound water is a major component of biological membranes and is required for the structural stability of the lipid bilayer. It has also been postulated that it is involved in water transport, membrane fusion, and mobility of membrane proteins and lipids. We have measured the fluorescence emission of membrane-bound 1-anilino-8-naphthalenesulfonate (ANS) and the infrared spectra of membranes, both as a function of hydration. ANS fluorescence is sensitive to polarity and fluidity of the membrane-aqueous interface, while infrared absorption is sensitive to the hydrogen bonding and vibrational motion of water and membrane proteins and lipids. The fluorescence results provide evidence of increasing rigidity and/or decreasing polarity of the membrane-aqueous interface with removal of water. The membrane infrared spectra show prominent hydration-dependent changes in a number of bands with possible assignments to cholesterol (vinyl CH bend, OH stretch), protein (amide A, II, V), and bound water (OH stretch). Further characterization of the bound water should allow its incorporation into current models of membrane structure and give insight into the role of membrane hydration in cell surface function.

Key words: membrane hydration, membrane-bound water, ANS fluorescence, infrared spectra, water-membrane interactions

In one of his recent books, Albert Szent-Györgyi marvels at the ubiquitous role of water in biology and lyrically notes that "life is water dancing to the tune of solids" [1]. This observation may be particularly relevant to current models of biologic membrane structure, which recognize that membrane components arrange themselves according to their amphipathic nature, such that polar sites can interact with the aqueous medium while nonpolar sites are excluded from it [2]. The physicochemical assumption that a lipid bilayer would form as a hydrophobic response to an aqueous environment was confirmed by

C.R. Middaugh is now at the Department of Biochemistry, University of Wyoming, Laramie.

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Finean and co-workers, who demonstrated, by X-ray diffraction, that the structure of myelin and erythrocyte membranes was disrupted upon removal of water below the 20% hydration level [3].

Membrane-bound water has been measured by a variety of methods and generally found to comprise some 20%–30% by weight of the hydrated membrane [3–9]. Chapman and co-workers have shown that the bound water is required for mixing of cholesterol and phospholipids in the bilayer, and that below 20% hydration, these lipids separate and exhibit their individual thermal transitions [4]. We have previously found membrane-associated water to be very tightly bound with binding energies comparable to and greater than water molecules in ice [5]. Parsegian, Rand, and co-workers have also found high energies required to remove the water between lipid bilayers [10]. Using proton magnetic resonance measurements, we and others have demonstrated that the mobility of membrane-bound water is much reduced compared to liquid water [8, 9]. In the present work, we extend our studies of membrane-bound water and begin to examine the nature of the membrane-water interface by means of infrared and extrinsic fluorescence spectroscopy.

Infrared (IR) spectroscopy is exquisitely sensistive to water vibrational motion (usually a problem in nonhydration studies, but an advantage here); it is capable of recognizing different water hydrogen bonding environments, and can distinguish the various phases of water [11]. It therefore should be useful in identifying various classes of membrane-bound water. The IR absorption spectrum can also provide a "fingerprint" of most structural groups within membrane proteins and lipids [12, 13] and thus may be capable of monitoring hydration effects on their conformation.

The dye molecule 1-anilino-8-naphthalenesulfonate (ANS) is perhaps one of the most studied and best characterized fluorescent probes of biologic membranes. It is generally thought to bind to membranes at the bilayer-aqueous interface [14–16]. ANS is also known to be sensitive to both the polarity and rigidity of its solvent microenvironment through a mechanism of excited-state solvent relaxation [17, 18]. Therefore, ANS fluorescence should provide a useful probe of hydration effects on the polarity and rigidity of the membrane aqueous interace.

MATERIALS AND METHODS

Membranes were prepared from normal human red cells as described previously [19]. Films were prepared by depositing a few drops of membrane pellet on AgCl windows for the IR measurements or on glass or quartz slides for the fluorescence measurements. Immediately prior to film casting, the membranes were given a distilled water wash to prevent high salt concentrations in the partially dried films [5]. The different levels of hydration were achieved by equilibrating the films in desiccators containing saturated salt solutions to yield the appropriate relative humidity (rh) [5].

Samples were considered to be equilibrated for the IR when transmission at 3,400 cm^{-1} for H_2O films or 2,500 cm^{-1} for D_2O films was constant for 24 hs. The films were sealed with a second AgCl window and oriented in a reproducible manner in the beam for each spectral measurement. The spectra were obtained by a Perkin-Elmer Model 621 double beam grating spectrophotometer. The spectra were scanned at 100 cm^{-1} intervals and re-equilibrated between intervals to insure that samples were not altered by heating. The difference spectra were determined by subtracting absorption spectra measured at different rh (as indicated in figures) at 10 cm^{-1} intervals.

The films for the fluorescence measurements were prepared from membranes that had been preincubated for 30 min at room temperature with ANS (Eastman Organic Chemicals No.T 484) in water solution. The ANS concentrations used were 0.4 and 0.8 mg/per ml red cell membrane pellet (1.25×10^{-3} M ANS and 2.49×10^{-3} M ANS, respectively). The slides used for these films were cut to fit diagonally in a 1-cm fluorescence cuvette.

Fluorescence spectra were obtained using a Perkin-Elmer MPF 4 spectrofluorometer. An exciting wavelength of 350 nm was used and emission was measured at a right angle to the beam. Recording of reflected light was minimized by placing the slide in the cuvette diagonally and positioning the cuvette so that the film faced away from the emission monochromator. The films were oriented so that the same portion of the film was consistently sampled during a series of measurements. The films were considered to be equilibrated at a given level of hydration when the emission peak intensity was constant for 24 hs. The sequence of hydration levels used was varied between films, and a film of red cell membranes without ANS was used as a control to insure that membrane aging did not affect the observed results.

RESULTS

Sorption isotherms for water-vapor binding to erythrocyte membranes are shown in Figure 1 [5]. These are used to determine the level of membrane hydration at a particular relative humidity. The relative humidities used in our experiments were 95, 66, 33, 11, and 0%, and these correspond to membrane hydrations of ~ 40, 11, 5, 3, and 0%, respectively.

The fluorescence emission of ANS in red cell membranes is shown in Figure 2 for several different relative humidities. A 2.5-fold increase in fluorescence intensity, accompanied by a 10-nm blue shift in emission peak wavelength, occurs with membrane dehydration between 95% and 0% rh. The majority of the intensity increase appears to occur at lower hydration levels (below 66% rh), while the main portion of the blue shift occurs

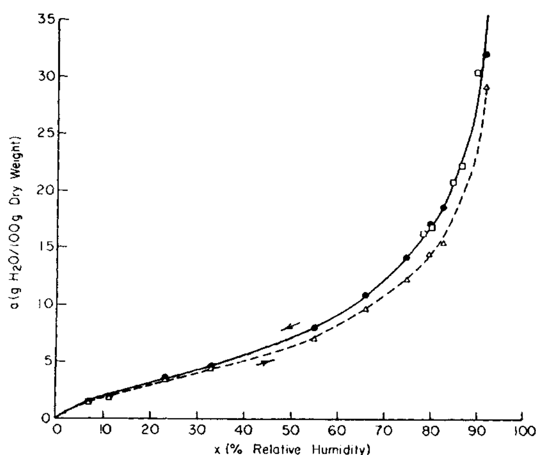


Fig. 1. Sorption isotherms of water on human red cell membranes at 20°C. Upper curve desorption; lower curve adsorption. From Schneider and Scheneider [5], with permission.

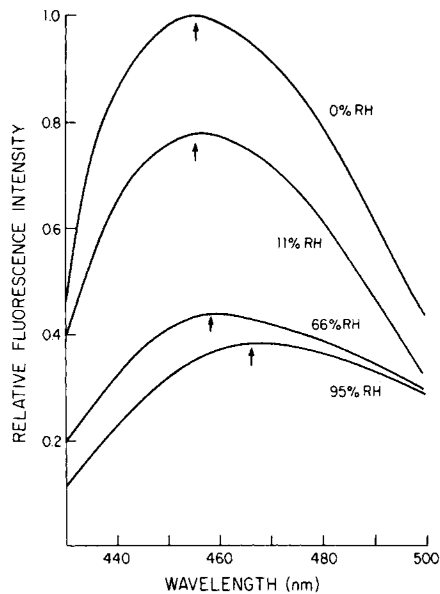


Fig. 2. ANS fluorescence emission spectra as a function of membrane hydration (rh). Excitation wavelength = 350 nm. Arrows indicate location of emission peak.

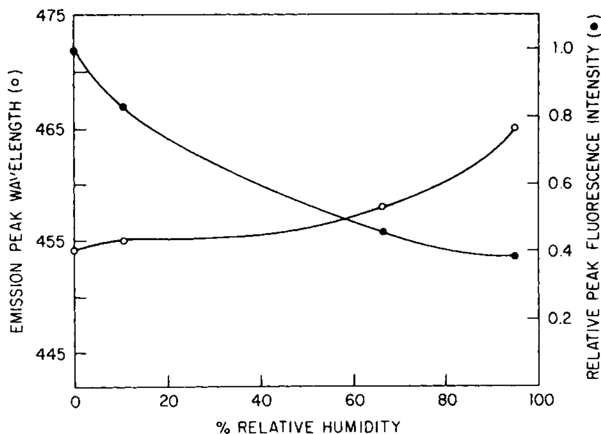


Fig. 3. ANS fluorescence emission parameters as a function of membrane hydration (rh). ○) Emission peak wavelength; ●) relative peak fluorescence intensity.

between 95% and 66% rh. These changes in ANS fluorescence parameters with membrane hydration are more clearly shown in Figure 3.

Figure 4a shows the infrared spectra of erythrocyte membranes equilibrated at 95% and 0% rh, together with that of liquid water for comparison. Upon dehydration, major membrane spectral changes are observed in the 3,800–3,200 cm^{-1} region and below 1,000 cm^{-1}. The water bands overlap several of the prominent membrane bands, and to eliminate this problem, D_2O-equilibrated film spectra were also obtained. Such complimen-

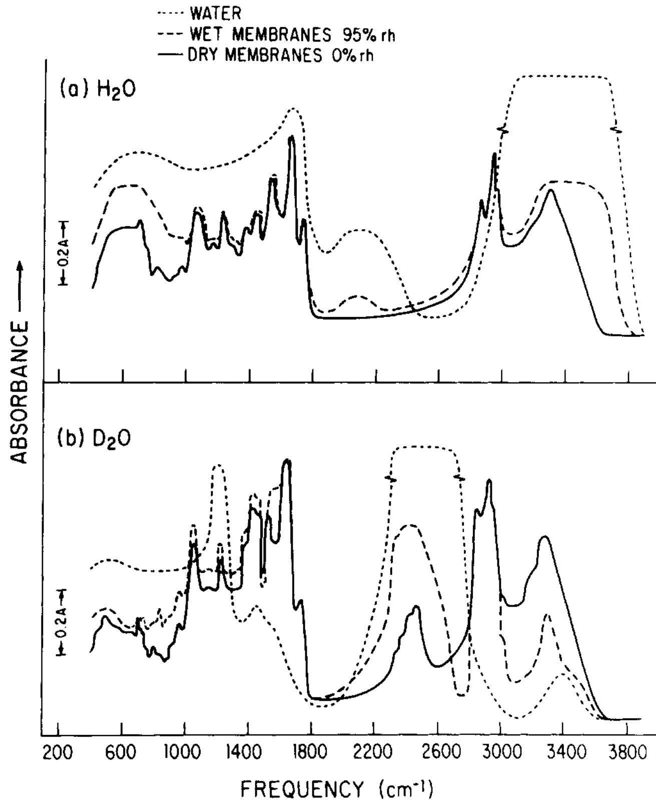


Fig. 4. Infrared spectra (4,000–400 cm^{-1}) of red cell membranes at 95% and 0% relative humidity. a) Membranes equilibrated against H_2O; b) membranes equlibrated against D_2O. Spectra of liquid water also shown for comparison.

tary spectra and that of liquid D_2O are illustrated in Figure 4b. Major hydration dependent changes are observed in the 2,700–2,100 cm^{-1} region and between 900 and 700 cm^{-1}. Greater peak resolution is available in the D_2O films than in the H_2O-equilibrated films, and several of the spectral regions are shown in detail below.

Figure 5a, b shows dehydration difference spectra in the OH and OD stretching regions of H_2O- and D_2O-hydrated red cell membranes, respectively. With dehydration, three difference bands appear with frequencies 3,550 cm^{-1}, 3,500 cm^{-1}, and 3,200 for H_2O-hydrated membranes and 2,650, 2,500–2,540, and 2,350 for D_2O films. The 3,550 (2,650) band is most evident at high levels of hydration, and the 2,650 band from the D_2O films completely disappears by 11% hydration.

Hydration effects are also observed for D_2O films in the 650–900 cm^{-1} region, as shown in Figure 6. The 701 cm^{-1} peak increases with dehydration while the 740 cm^{-1} band decreases. The 720 cm^{-1} peak remains constant. A band at 800 cm^{-1} becomes clearly visible at 0% rh, while the 834 cm^{-1} band has significantly decreased.

Figure 7 shows D_2O-hydrated membrane spectra in the 1,200–1,800 cm^{-1} region at different levels of hydration. The intense band at 1,650 cm^{-1} remains relatively unchanged in going from 95% to 0% relative humidity. However, the band at 1,525–1,550

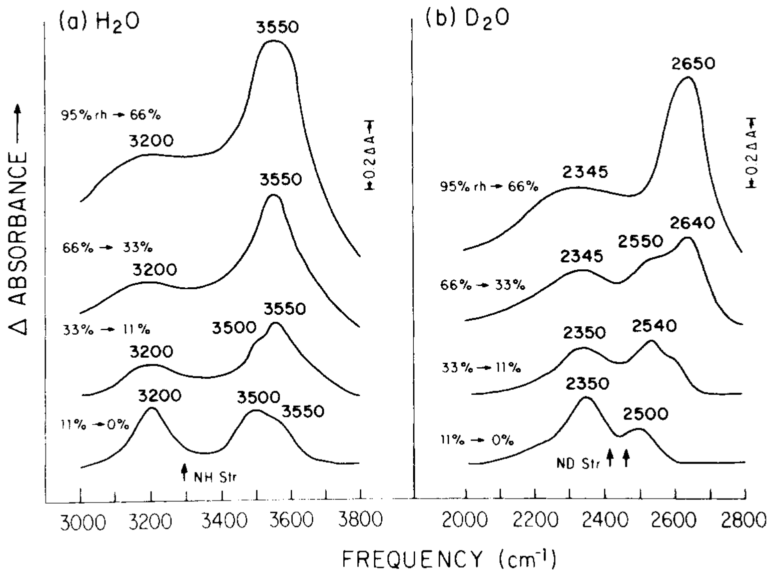
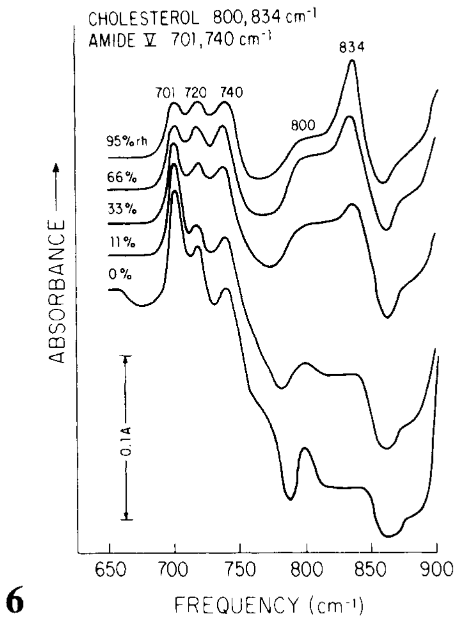


Fig. 5. Infrared dehydration difference spectra of red cell membranes: a) In H_2O (OH fundamental stretching region), b) in D_2O (OD fundamental stretching region). Difference spectra derived by subtracting spectra between the indicated relative humidities.



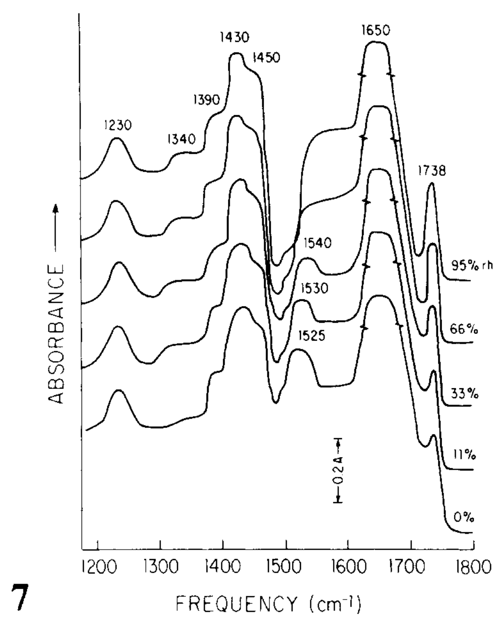


Fig. 6. Infrared spectra of red cell membranes in D_2O in the region 650–900 cm^{-1} as a function of membrane hydration (rh).

Fig. 7. Infrared spectra of red cell membranes in D_2O in the region 1,200–1,800 cm^{-1} as a function of membrane hydration (rh).

cm^{-1} changes extensively. Upon dehydration, the band narrows considerably and shifts to lower frequency. There is no apparent change in a 1,230 cm^{-1} band and there are no obvious changes in the 1,390–1,450 cm^{-1} region.

DISCUSSION

The hydration-dependent changes we have described in membrane infrared spectra and ANS fluorescence may be useful for a) identifying different classes of membrane-bound water, b) determining which structural groups within membrane proteins and lipids are affected by membrane hydration, and c) elucidating polarity and/or rigidity (microviscosisty) effects at the membrane-aqueous interface.

ANS is an appropriate probe for these investigations because its fluorescence emission is dependent on solvent microenvironment. The quenching of ANS fluorescence in water has been attributed to the ability of the polar solvent molecules to interact with the first excited singlet state (S_1). Brand and co-workers have shown that the dipole moment of S_1 is greater than that of the ground state (S_0) and that of the first excited triplet (T_1) [17, 20]. Thus polar solvents preferentially stabilize S_1 relative to S_0 and T_1. This decreases the energy gap for intersystem crossing and increases the probability of quenching by this mechanism, resulting in decreased fluorescence intensity. This stabilization of S_1 also decreases the energy gap for fluorescence and causes a red shift in the wavelength of maximum intensity.

The ability of polar solvents to preferentially stabilize S_1 depends on the capacity of the solvent molecules to reorient around the excited state. Seliskar and Brand compared the fluorescence characteristics of N-methyl-2,6-ANS in liquid water and ice [18]. They observed a much larger quantum yield in ice and a blue shift of the maximum wavelength to 455 nm, thus demonstrating the importance of solvent fluidity to the solvent relaxation quenching mechanism.

The results reported here show similar intensity changes and wavelength shifts for membrane-bound ANS as a function of membrane dehydration. At high levels of membrane hydration (40%) the ANS fluorescence is relatively low, with an emission peak near 465 nm, which is similar to its location in methanol. From the polarity standpoint this is reasonable, since the lipid polar head groups and bound water allow for some stabilization. Upon removal of the water, the membrane ANS fluorescence is markedly enhanced (2.5 times), and there is a 10-nm blue shift in emission peak. These effects could derive from an increase in rigidity and/or decrease in polarity of the membrane-ANS microenvironment. That dehydration should rigidify the membrane is plausible, since the thermal motion of the aqueous medium would no longer be imparted to the membrane. It is interesting that about 20% hydration seems to be required for membrane integrity, as discerned by X-ray diffraction [3], and for phospholipid-cholesterol mixing, as measured by differential scanning calorimetry [4]. One can suppose, therefore, that the effect of increasing hydration may be to increase membrane fluidity, since at least a certain amount of molecular motion should be required for intermixing of lipid molecules. The possibility should also be mentioned that ANS fluorescence changes with membrane dehydration might monitor similar membrane structural changes as detected by X-ray and calorimetric methods. If so, this could provide an independent marker for the hydration level at which these changes occur and furnish some information on the nature of the changes. Our fluores-

cence data cannot yet distinguish between the various possible origins of the ANS emission changes; future fluorescence polarization measurements may provide further insight into this problem.

Gulik-Krzywicki et al [21], in analogous ANS-phospholipid-hydration experiments, have shown that the area per lipid head group increases with hydration and this is accompanied by a decrease in ANS fluorescence and red shift in emission peak wavelength. This may allow increased water penetration to the ANS sites and/or greater exposure of ANS to the aqueous environment. Indeed, D_2O solvent perturbation measurements of ANS fluorescence in membranes indicate exposure of ANS to water at the surface of the membrane [15]. Other related studies have been reported using naphthalenesulfonate dyes to probe bound water in phospholipid [22] and detergent [23] micelles. Wells [22] has shown by fluorescence polarization measurements of dansylphosphatidylethanolamine in phosphatidylcholine micelles that an increase in micelle hydration results in increased molecular motion of the lipid-water interface [22]. He has also shown a shift in emission peak to longer wavelengths and a decrease in fluoresecence intensity with increasing amounts of bound water, – changes that are analogous to those we have found for the red cell membrane. Similar results were found by Wong et al for hydration effects on ANS in detergent micelles [23].

The IR spectrum of erythrocyte membranes also undergoes several hydration-dependent changes. In the total spectrum of H_2O-equilibrated films (Fig. 4a), well-established regions include the amide A (3,292 cm^{-1}), CH stretching (3,200–2,800 cm^{-1}), ester carbonyl stretching (1,738 cm^{-1}), amide I (1,650 cm^{-1}), amide II (1,540 cm^{-1}), P = O stretching (1,230 cm^{-1}), and POC aliphatic stretching (1,080–1,060 cm^{-1}) vibrations [12]. The H_2O IR bands overlap a few of these. The intense absorption centered near 3,400 cm^{-1} arises from OH stretch in liquid water. There is a strong OH bending mode absorbing at 1,640 cm^{-1} and the band near 2,100 cm^{-1} is thought to result from a combination of the 1,640 cm^{-1} band with an intermolecular mode or with overtones [11].

The D_2O-equilibrated spectra (Fig. 4b) are perhaps more informative owing to the lesser masking of membrane bands. In the dry-film spectrum, the nonexchangeable NH stretching vibration is visible near 3,300 cm^{-1}. OD and protein ND stretching modes absorb in the 2,200–2,600 cm^{-1} region. Also, HDO impurities may lead to absorption at 3,400 cm^{-1} and 1,450 cm^{-1} [11]. At lower frequencies, amide V bands (700, 740 cm^{-1}) [24] and cholesterol bands (800, 834 cm^{-1}) [13] can be discerned.

The difference spectra in the OH and OD stretching vibration regions give valuable information about hydration effects (Fig. 5). The 3,550 cm^{-1} (2,650 cm^{-1}) difference bands appears at relatively high hydration levels and probably represent removal of loosely bound water. The frequency of this difference band (3,550 cm^{-1} or 2,650 cm^{-1}) is higher than that of the stretching vibration of liquid H_2O or D_2O and indicates decreased hydrogen bond strength [25, 26]. Such high-frequency bands have been observed for water bound to polypeptides [26], phospholipids [22], molecular sieves [27], and polyelectrolyte membranes [28]. This band may represent removal of water molecules that are shielded from efficient hydrogen bonding. Perhaps some of these are isolated in the kink regions of the lipid hydrocarbon chains, as originally postulated by Träuble [29, 30].

The difference band at 3,500 cm^{-1} (2,500–2,530 cm^{-1}) may also be due to bound water with relatively weak hydrogen bonds (Fig. 5). Such blue-shifted OH and OD stretching bands have been observed in hydrated stratum corneum (2,480 cm^{-1}, OD) [31], polyproline (3,490 cm^{-1}, OH) [26], molecular sieves (3,500 cm^{-1}, OH) [27], and phospholipid micelles (3,530 cm^{-1}, OH) [22]. This difference band appears below 66% rh and

may correspond to water somewhat more tightly bound than the 3,550 cm^{-1} (2,640–2,650 cm^{-1}) band.

The difference band (Fig. 5) observed at 3,200 cm^{-1} (2,350 cm^{-1}) appears most prominently at low hydrations and could have several sources. Amide A and B bands occur in this region. This difference band, then, may indicate protein structural changes which alter the NH hydrogen bond strengths. Similar low-frequency bands have been observed in dehydration difference spectra of proteins. Bendit measured a difference band at 3,270 cm^{-1} for β-keratin [32] and Buontempo et al observed one at 3,260 cm^{-1} for lysozyme [33]. In each case, the band was assigned, at least in part, to protein structural changes induced by dehydration.

The cholesterol hydroxyl may also be represented in this band. The OH stretch of cholesterol normally absorbs around 3,400 cm^{-1}, but Zull et al have shown that the band shifts to 3,250 cm^{-1} when lecithin is present [34]. Ladbrooke and Chapman have shown the phospholipid-cholesterol interaction requires water molecules [4]. This difference band may in part be due to lipid separation resulting in a loss of the cholesterol-phospholipid interaction.

There is yet another possible interpretation of this low-frequency difference band, namely that it is due to tightly bound water. Our IR spectra of the D_2O-equilibrated films would support this interpretation. Extensive dehydration leads to complete loss of the 2,350 cm^{-1} band. Membrane-associated water yielding this stretching frequency would be tightly hydrogen-bonded, and since it is removed only at low relative humidities, it must be a tightly bound component. Ice-like hydrogen bond strengths for water in the membrane are consistent with our previous results, yielding heats of binding of water vapor to membranes similar to and greater than those of water in ice [5]. Buontempo et al assigned his 3,260 cm^{-1} band, in part, to an amorphous ice-like water bound to lysozyme [33]. Hansen and Yellin [31] observed a 2,360 cm^{-1} OD dehydration difference band in stratum corneum, which they assigned to tightly bound water. Their band is also removed by desiccation, and hence it cannot be due to ND vibrations. The possibility of this band being an overtone of the DOD bending mode was eliminated, since the spectrum was measured with HOD in H_2O. Other workers have also assigned IR difference bands with similar frequencies to bound water on polyelectrolyte membranes [28] and molecular sieves [27].

Other interesting dehydration effects occurred in the 875-650 cm^{-1} region for the D_2O-equilibrated films (Fig. 6). This is the CH bending and NH deformation region of the IR. The 720 cm^{-1} band has been attributed to the CH rocking vibration, which is observed only when four consecutive methylene groups of the fatty acid chains are in the *trans* configuration [35]. In our spectra, the band is clearly resolved and is not effected by dehydration. Chapman et al [35] did not observe this band in the IR spectra of erythrocyte membranes but did in that of the extracted lipids. Perhaps our ability to see the band in the spectra of the total membrane is due to better instrument resolution. The presence of this 720 cm^{-1} band indicates less rotational mobility in the hydrocarbon region than was thought to exist from other data [35]. The lack of the dehydration effect on this band is surprising in view of the ANS fluorescence results. Perhaps there is a rigidifying effect at the membrane surface, sampled by the ANS molecules, which is not propagated to the hydrocarbon interior.

The 800 cm^{-1} and 834 cm^{-1} bands (Fig. 6) may be due to the vinyl CH bending vibrations of cholesterol [13]. Cholesterol in CS_2 has a medium band at 800 cm^{-1} and a weaker one at 834 cm^{-1} [36], which are similar to the bands we found in the mem-

brane at 0% rh. This result is consistent with the idea that cholesterol separates from the phospholipids at low levels of hydration and then gives a spectrum similar to that of the isolated molecule.

The 701 cm^{-1} and 740 cm^{-1} bands (Fig. 6) may be due to the amide V vibration of membrane proteins [24]. Since these are observed in the D_2O film spectra, they must

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