Inverse Raman Spectroscopy of Bilirubin and its Ditauride

Kathy J. Dien Hillig and Michael D. Morris
Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109, USA

The inverse Raman spectrum of bilirubin and bilirubin ditauride were measured under pre-resonant conditions. Spectra in chloroform and dimethyl sulfoxide were assigned by comparison with model compounds and on the basis of the known effects of the solvents on the internal hydrogen bonding in bilirubin. The band assignments were used to interpret the spectra of bilirubin ditauride in aqueous solution. Evidence is presented for strong hydrogen bonding to the pyrrole N—H bonds and to the lactam rings. It is proposed that hydrogen bonding to water is facilitated by photoactivation and, in turn, stabilizes photobilirubin.

INTRODUCTION

The structures of bilirubin and two of its conjugates are given in Fig. 1. These are naturally occurring compounds found in humans as breakdown products of hemoglobin. Bilirubin is produced by the reduction of biliverdin, which in turn is formed by the oxidation of heme in the blood. Bilirubin is not soluble in water and is transported through blood serum as the albumin complex of its carboxylate dianion. In the liver, bilirubin is conjugated (esterified) to sugars. In humans, bilirubin diglucuronide is the predominant product. The conjugate is water soluble and therefore easily excreted from the body.

An increase in serum bilirubin concentration causes jaundice, a build-up of bilirubin in fatty tissue just beneath the skin. Jaundice is especially common in newborn infants.[1] Treatment of jaundice consists in irradiating the patient with visible light. It was long thought that elimination of bilirubin from tissue was due to photodecomposition.[2,3] It has recently been found

R = OH   Bilirubin

R = [structure] Bilirubin Diglucuronide

R = -NHCH$_2$CH$_2$SO$_3$H   Bilirubin Ditaurine

Figure 1. Bilirubin IXα and its conjugates.

that irradiation causes geometric isomerization of bilirubin, which enhances its diffusion from tissues into the blood.[4,5]

Although bilirubin is most stable as the $4Z,15Z$ isomer, as drawn in Fig. 1, it can be photoisomerized to the $4Z,15E$ or $4E,15Z$ configurations, with a cis configuration about one of the exo double bonds. These, in turn, can be further photoisomerized to the $4E,15E$ isomer.[6] These three unstable forms are collectively known as photobilirubin, while the naturally occurring $4Z,15Z$ isomer is conventionally referred to as bilirubin. At photoequilibrium, the predominant fraction is the $4Z,15Z$ form. Less than 20% exists as the $4Z,15E$ and $4E,15Z$ isomers. An even smaller fraction of the $4E,15E$ isomer is formed.[7]

Although phototherapy of newborn infants causes only a 2.6–8.3% increase in serum photobilirubin after several hours of irradiation, levels of photobilirubin in duodenal bile and urine are significantly increased.[8] Photobilirubin is more hydrophilic than bilirubin and thus is stabilized when bound to albumin.[9,10] Photobilirubin bound to albumin is readily transported to the bile without first being conjugated in the liver.[11] Recent work on the physical chemistry of phototherapy has focused mainly on the structure of photobilirubin.[12–14]

The structure of crystalline bilirubin was determined by Bonnett et al.[15] by x-ray diffraction. In non-polar solvents, bilirubin retains its crystalline conformation and is in equilibrium with its enantiomer.[16,17] The internal hydrogen bonds between the propionic acid groups and the lactam groups in rings A and D cause a folding of the molecule which prevents internal rotation about the C$_9$—C$_{10}$ or C$_{10}$—C$_{11}$ bonds. It is this internal hydrogen bonding which is responsible for the low water solubility of bilirubin.

Kaplan and Navon have carried out extensive NMR studies on the conformation of bilirubin and its conjugates in various solvents.[18–20] Their work demonstrates that bilirubin remains internally hydrogen bonded in chloroform solution, as proposed by Brodersen et al.[21] In dimethyl sulfoxide, however, intramolecular hydrogen bonding does not occur to any significant extent.

Bilirubin conjugates are not able to hydrogen bond internally in the same way as bilirubin. The carboxylic acid functions are esterified, usually with bulky groups. The vibrational spectra of free and conjugated bilirubin
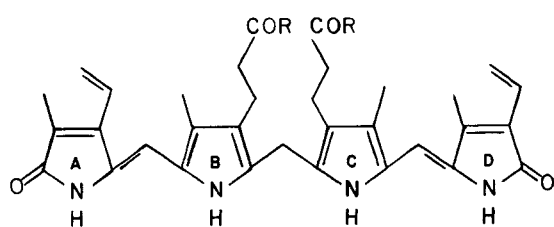
should reflect changes in both internal hydrogen bonding and interactions with various solvents.

Most of the infrared spectra of bilirubin found in the litarature have been taken using solid samples or in mulls. Infrared spectra of bilirubin in nujol mulls[22-24] and KBr disks[25,26] confirm the presence of intramolecular bonding. The high concentrations needed for infrared spectroscopy and the low solubility of bilirubin in many solvents preclude easy observation of infrared spectra of bilirubin in polar media.

There have been very few band assignments outside the C–H, N–H and O–H stretching regions. Many authors have not even reported bands below 1600 cm^{-1}. For those which have been assigned, a great deal of disagreement exists.

A major question in past infrared studies has been whether bilirubin would form a lactim in sufficient amounts to affect the spectrum. It has now been determined that the lactam form is more stable by at least 10^4 relative to the lactim.[26,27] Thus the lactim configuration cannot contribute to the spectrum and the assignments by Suzuki and Toyoda[29] and Newbold and LeBlanc[24] based on the lactim form are incorrect. Information about the C=C stretching region is not very detailed. Assignments by McDonagh[25] and by Suzuki and Toyoda[29] do not differentiate between the various C=C bonds in the molecule. Other workers did not assign the bands observed in this region or have not reported such bands.

The resonance Raman spectrum of biliverdin dimethyl ester has been reported earlier.[30] No band assignments were reported, although spectral changes were noted when the solution was changed from neutral to strongly acidic. Lippitsch[31] noted that the fluorescence of aetiobiliverdin was strong enough to obscure spontaneous Raman bands. He obtained surface-enhanced Raman spectra (SERS) of this compound adsorbed to silver colloids. Again, no band assignments were reported. Other than the present work, we know of no reports of the Raman spectra of any other bile pigments.

Inverse Raman spectroscopy, like other forms of coherent Raman spectroscopy, is generally known to be useful for the observation of the Raman spectra of luminescent biomolecules.[32] However, IRS is also useful for the investigation of photosensitive compounds, such as bile pigments. Low average and peak powers of the pump beam at ω_1 can be used in IRS to avoid photoisomerization or decomposition. A high pulsed probe beam power at ω_2 can still be used to give high signal-to-noise ratios. To obtain good sensitivity, ω_1 can be made nearly resonant with an electronic transition on the low-energy side. Because ω_2 will be 500–1800 cm^{-1} further to the red, photodecomposition or photoisomerization can be minimal.

Even though bilirubin and its ditauride are only weakly fluorescent, with a quantum yield of 0.001 or less, they are extremely photosensitive. Attempts to observe spontaneous resonance Raman spectra at 488 nm using 50–200 mW power have yielded only one or two major bands accompanied by rapid photodecomposition. Attempts to measure pre-resonant Raman spectra using 514.5 nm excitation were equally unrewarding.

We report here solvent effects on the spectra of bilirubin and bilirubin ditauride. The ditauride has properties similar to those of the diglucuronide,[33] which is not commercially available. The frequency shifts and changes in band intensities observed provide information about interactions occurring between bilirubin and various solvents. These results can be useful in examining the binding of bilirubin to albumin and in understanding the mechanism of photoisomerization. In this study bilirubin was examined in dimethyl sulfoxide (DMSO) and chloroform. The ditauride was examined in DMSO and aqueous phosphate buffer (pH 7.4). DMSO was chosen as a solvent because of the good solubility of both bilirubin and the ditauride in it. Chloroform provides a different bilirubin environment than DMSO. However, the ditauride is not soluble in chloroform. The ditauride is water soluble and readily dissolves in buffer.

EXPERIMENTAL

The optical system for a.c.-coupled inverse Raman spectroscopy has been described previously.[34] A CW argon laser is used as the pump beam (ω_1). A pulsed dye laser provides the probe beam (ω_2). Because a nanosecond pulsed dye laser is used, only a small time fraction of the argon laser output is needed to provide apparent CW behavior. A chopper with a 0.3% duty cycle is placed in the argon beam. In this way, the sample is only irradiated by the argon laser when the dye laser is ready to fire. The low duty cycle of the chopper pulse and the intensity losses of about 50% due to insertion of optical elements reduces the average pump power delivered to the sample to about 0.25 mW. Insertion losses of about 40% reduce the probe dye laser average power to 3–4 mW, delivered as 6 ns pulses of 200–250 μJ.

Bilirubin and bilirubin ditauride were obtained from U.S. Biochemical Corp. and used as received. Reagent-grade DMSO and chloroform were used to prepare solutions in the 10^{-5}–10^{-4} M range. Phosphate buffer of pH 7.4 was prepared using aqueous 0.2 M NaH$_2$PO$_4$ and Na$_2$HPO$_4$ solutions.

All solutions were used within 48 h of preparation. Samples not used immediately were refrigerated for use the next day, and were brought to room temperature slowly on the day they were used. Aliquots were filtered through 0.22 μm Fluoropore or Acropore (Millipore) membrane filters immediately prior to use.

The pump frequency was 19 436 cm^{-1}, 514.5 nm. This frequency is far enough below the electronic origin to give Lorentzian band shapes and yet still provide much of the signal enhancement seen in the rigorous resonance-enhanced region.

Spectra of bilirubin and bilirubin ditauride in DMSO with a 514.5 nm pump wavelength are presented in Fig. 2. The spectra of bilirubin in chloroform and bilirubin ditauride in aqueous phosphate buffer (pH 7) are shown in Figs 3 and 4, respectively.

BAND ASSIGNMENTS

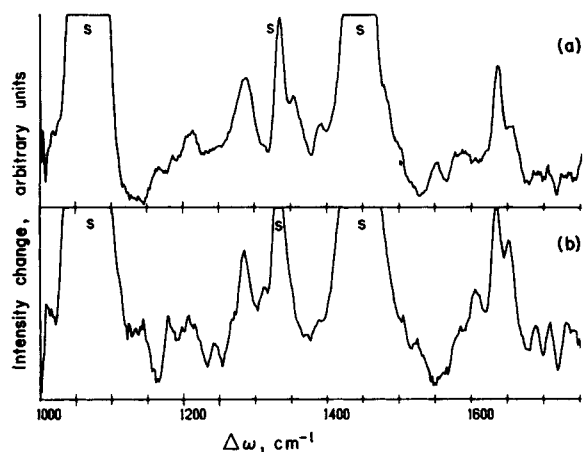Table 1 summarizes the positions and assignments of the Raman bands found in the spectra. Assignments

Figure 2. Inverse Raman spectra of bile pigments in DMSO. (a) Bilirubin, 5×10^{-5} M; (b) bilirubin ditauride, 5×10^{-5} M. S, Solvent bands.
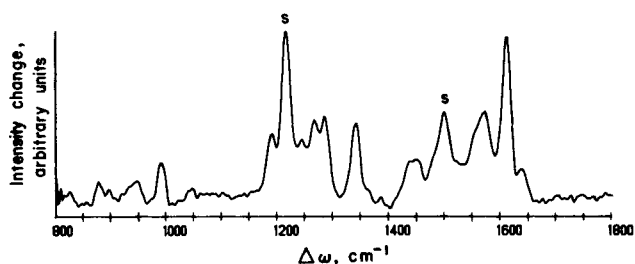
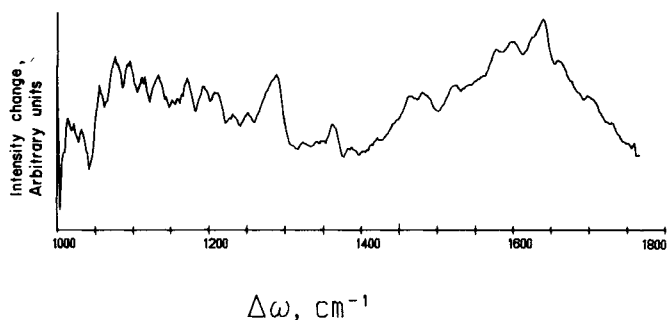Figure 3. Inverse Raman spectrum of 1×10^{-4} M bilirubin in chloroform. S, Solvent bands.

Figure 4. Inverse Raman spectrum of 1×10^{-4} M bilirubin ditauride.

were made using spectra of the two compounds in DMSO and of bilirubin in chloroform. This information was used to interpret the spectrum of the aqueous bilirubin ditauride.

The hydrogen bonding patterns of bilirubin in chloroform and DMSO are well understood. The solvent dependence of some of the bilirubin bands, therefore, can provide clues to their assignment. Shifts in frequency by as much as $16\ \text{cm}^{-1}$ were observed in our spectra. Since resonance and pre-resonance Raman intensities depend on the difference between excited- and ground-state geometries, some additional information can be deduced from a knowledge of electronic transitions.

The C=C stretching region ($1560-1660\ \text{cm}^{-1}$) is rich in bands because of the variety of C=C environments in the molecule. The Raman spectrum of Ni-protoporphyrin dimethyl ester show similar bands in this region.[35]

Table 1. Inverse Raman band assignments for bilirubin and bilirubin ditauride

Bilirubin		Bilirubin ditauride		
In DMSO	In CHCl₃	In DMSO	In Buffer	Band assignments
	990			Lactam ring breathing
1185	1193	1183		Pyrrole ring breathing
	1248			ν_{C-O} of COOH
1260	1269	1260	1265	
	1288	1286		
1327	1343	1327	1340	Amide III
1365	1366	1363		ν_{C-N}, pyrrole
	1386			
	1443		1442	
	1457		1460	
			1500	
1525	1534			
1558	1559		1556	
1572	1579		1577	$\nu_{C=C}$, pyrrole
1610	1613	1610	1617	$\nu_{C=C}$, exo
1628	1640	1627	1638	$\nu_{C=C}$, lactam
1664	1664			$\nu_{C=C}$, vinyl
1679	1683			

These bands show some sensitivity to both the solvent and to the presence of conjugation to the taurine moiety. The *exo* double bond is assigned to the strongest band in the spectrum: $1613\ \text{cm}^{-1}$ in bilirubin in chloroform and $1610\ \text{cm}^{-1}$ in both compounds in DMSO. The high intensity is consistent with the proposed excited-state geometry of the bile pigments which is elongated and twisted about this double bond.[14,36] Since the *exo* double bond is part of the dipyrrole π system, it is slightly dependent on the molecule and the solvent. The much weaker band at $1627\ \text{cm}^{-1}$ in DMSO and $1640\ \text{cm}^{-1}$ in chloroform is assigned to the lactam double bond on rings A and D. This frequency is similar to the C=C stretch in the related compound uracil.[37] Again, this band shifts less than $5\ \text{cm}^{-1}$ between the conjugated and unconjugated forms of the molecule, but is dependent on the medium. The C=C stretch of the pyrrole rings B and C and the vinyl groups of rings A and D are assigned to $1572-1580$ and $1664\ \text{cm}^{-1}$, respectively. Their relative insensitivity to both solvent and hydrogen bonding is attributed to the distance of the nitrogen and carbonyl oxygens of these rings from the hydrogen bonding sites.

The large band in the $1327-1343\ \text{cm}^{-1}$ region is assigned to amide III of the pyrrole rings. A similar band is found in δ-valerolactam.[38] This band would be expected to be sensitive to changes in both inter- and intramolecular hydrogen bonding, since it is a combination of a ν_{C-N} and a δ_{N-H}. Thus, it is not surprising that this band shows greater variability than any of the other observed bilirubin vibrations.

The assignment of the $1363-1365\ \text{cm}^{-1}$ band to the ν_{C-N} is based on a comparable band in the spectrum of protoporphyrin. The lack of solvent sensitivity indicates that the pyrrole moiety is the major contributor to this vibration.

The intense bilirubin band at $1288\ \text{cm}^{-1}$ remains unassigned. The lower signal-to-noise ratio in the spectrum in DMSO does not allow us to say with certainty whether or not this band is present as a shoulder on the large DMSO band in the spectrum of bilirubin. Tentatively,

this band is grouped with the other solvent-insensitive vibrations.

Similarly, the assignment of the 1260–1269 cm^{-1} band is uncertain, although it is clearly solvent dependent and present in all of the spectra.

The band at 1248 cm^{-1} has been assigned by IR to the C–O stretch of the carboxylic acid.[23] We confirm this assignment, noting that this band is only seen with chloroform as the solvent and not in DMSO or buffer.

Although it is higher than most pyrrole ring breathing vibrations, we assign the 1193 cm^{-1} band of bilirubin to this mode. The lactam ring breathing occurs at 990 cm^{-1}. We cannot yet make assignments of the other bands in the spectra of either molecule.

DISCUSSION

The presence or absence of internal hydrogen bonding strongly influences the bilirubin Raman spectrum. Bilirubin spectra in chloroform were expected to be different from those in DMSO, whereas similar spectra were expected when comparing bilirubin and the ditauride in DMSO. Our spectra from these three cases reflect the presence of these hydrogen bonding effects. For example, large shifts such as in the amide III stretch and in the lactam $\nu_{C=C}$ can be used as indicators of the existence of internal hydrogen bonding. The spectra of bilirubin ditauride in aqueous solution might have been expected to match the spectra of those compounds with no internal hydrogen bonding, since it also cannot bond intramolecularly.

However, the spectrum of bilirubin ditauride in aqueous solution closely matches that of bilirubin in chloroform. In particular, the major hydrogen bond-sensitive frequencies, the amide III and the lactam C=C stretch, are nearly identical (Table 1). The 2 cm^{-1} difference in the C=C stretch is only slightly greater than experimental error, about ±1 cm^{-1}.

Only the pyrrole C=C stretching frequency in aqueous ditauride is closer to the DMSO value than to the chloroform value, reflecting the relatively weak carboxylic acid hydrogen bonding to pyrrole. It is only in this bond that the aqueous ditauride spectra differs significantly from that of internally bonded bilirubin.

The striking similarities of the spectra indicate that ditauride in buffer is in an environment similar to bilirubin when it is internally hydrogen bonded. Hydrogen bonding between the ditauride and water molecules accounts for this congruence. In particular, the hydrogen bond of water oxygens to pyrrole hydrogens and of water protons to lactam carbonyl must resemble the internal hydrogen bonds to propionic acid. The high solubility of bilirubin ditauride in aqueous solution also suggests strong solvation effects.

The solubility of bilirubin in DMSO and the breaking of the internal hydrogen bonds in this solvent imply that DMSO has a stronger bonding effect than does a hydrogen bond in this system. It is also known that bilirubin is not very soluble in water. This indicates that the internal hydrogen bonds are stronger than any external hydrogen bonds which would be necessary for solubility. Since bilirubin ditauride can have no internal hydrogen

bonds, the frequency shift will be affected by the degree of bonding to water. If the solvent effect is as great as with DMSO, only small frequency shifts relative to those in DMSO would be expected. Larger frequency shifts would be seen as the interaction becomes weaker.

Cis–trans photoisomerization of C=C compounds is generally known. However, in most cases the *trans* isomer is the more stable. In the case of bilirubin, however, it is the *cis* isomer (Z, Z) which is the more stable form. Brown and McDonagh[7] have shown that at photoequilibrium only 20% of the bile pigment contains an *E* isomer. The Z, Z isomer is stabilized by internal hydrogen bonding. The *E*-containing isomers are actually destabilized by the presence of the bulky vinyl groups, which prevent the planar geometry, favored by the Z, Z conformation, from forming.[23,39] It is surprising that photoisomerization proceeds at all.

Our observations of hydrogen bonding patterns in aqueous bilirubin ditauride suggest that hydrogen bonding is the destabilizing force which allows photo-isomerization to take place sufficiently to be clinically useful. Although vibrational energies are not direct measures of bond strengths, the identities of both hydrogen bond markers on the lactam suggest that the hydrogen bonds to propionic acid and to water are of very similar strengths. If anything, the water hydrogen bonding to the pyrrole is stronger than the internal hydrogen bonding. We propose that this near balance of forces allows photoisomerization to occur.

The internal hydrogen bonds are disrupted by the stretching and twisting of bilirubin caused by photoexcitation. This disruption is adequate to allow the formation of hydrogen bonds with water. Hydrogen bonding certainly includes the propionic acids, although this is not demonstrable through resonance Raman spectroscopy at visible excitation frequencies. The solvated molecule can then dissolve in pH 7.4 serum as the dianion.

Photobilirubin is known to revert to the Z, Z isomer in the dark. This fact suggests that the internal hydrogen bonds, or the combination of hydrogen bonds and steric effects, may be slightly stronger than the hydrogen bonds to water. The balance between bilirubin and photobilirubin is a delicate one indeed.

Near resonant Raman spectroscopy is a generally useful probe of bile pigment conformation and chemistry. The technique is already applicable to solutions in the 10^{-4}–10^{-5} M range. With some improvement in lasers, data acquisition techniques or experimental procedures, it should be possible to study systems at the 10^{-6} M level. Many of the vibrational bands are environment sensitive and should prove to be sensitive probes for the study of the binding of bilirubin to albumin and other molecules, and also to the photobilirubin isomers. Such work should provide valuable information on the details of jaundice phototherapy. Experiments towards these ends are under way in our laboratories.

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