Fawell, S. E., & Lenard, J. (1988) *Biochem. Biophys. Res. Commun. 155*, 59-65.
Fawell, S. E., McKenzie, M. A., Greenfield, N. J., Adebodun, F., Jordan, F., & Lenard, J. (1988) *Endocrinology 122*, 518-523.
Flawia, M. M., & Torres, H. N. (1973) *J. Biol. Chem. 248*, 4517-4520.
Garcia, J. V., Fenton, B. W., & Rosner, M. R. (1988) *Biochemistry 27*, 4237-4244.
Greenfield, N. J., McKenzie, M. A., Adebodun, F., Jordan, F., & Lenard, J. (1988) *Biochemistry 27*, 8526-8533.
Greenfield, N. J., Cherapak, C. N., Adebodun, F., Jordan, F., & Lenard, J. (1990) *Biochim. Biophys. Acta 1025*, 15-20.
Kawakami, A., Iwami, M., Nagasawa, H., Suzuki, A., & Ishizaki, H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6843-6847.
Kole, H. K., & Lenard, J. (1990) *J. Cell Biol. 111*, 472a.
Kole, H. K., Abdel-Ghany, M., & Racker, E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5849-5853.
Laemmli, U. K. (1970) *Nature 227*, 680-686.
LeRoith, D., Shiloach, J., Roth, J., & Lesniak, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6184-6188.
LeRoith, D., Shiloach, J., Roth, J., & Lesniak, M. A. (1981) *J. Biol. Chem. 256*, 6533-6536.
LeRoith, D., Shiloach, J., Heffron, R., Rubinovitz, C., Tanenbaum, R., & Roth, J. (1985) *Can. J. Biochem. Cell Biol. 63*, 839-849.
Maier, V., Steiner, P., Fuchs, J., Pfeifle, B., Mezger, M., & Pfeiffer, F. (1988) *Horm. Metab. Res. 20*, 421-425.
McKenzie, M. A., Fawell, S. E., Cha, M., & Lenard, J. (1988) *Endocrinology 122*, 511-517.
Miller, T. J. (1985) in *Molecular Basis of Insulin Action* (Czech, M. P., Ed.) pp 247-261, Plenum Press, New York.
Morgan, D. O., Ho, L., Korn, L. J., & Roth, R. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 328-332.
Petruzzelli, L., Herrera, R., & Rosen, O. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3327-3331.
Pluskal, M. G., Przekop, M. B., Kavonian, M. R., Vecoli, C., & Hicks, D. A. (1986) *Biotechniques 4*, 272-283.
Robitzki, A., Schröder, H. C., Vgarkovic, D., Pfeifer, K., Uhlenbruck, G., & Müller, W. E. G. (1989) *EMBO J. 8*, 2905-2909.
Rosen, O. M. (1987) *Science 237*, 1451-1458.
Rubinovitz, C., & Shiloach, J. (1985) *FEMS Microbiol. Lett. 29*, 53-58.
Sanders, M. M., Groppi, V. E., & Browning, E. T. (1980) *Anal. Biochem. 103*, 157-165.
Scarborough, G. A. (1985) *Exp. Mycol. 9*, 275-278.
Schulte, T. H., & Scarborough, G. A. (1975) *J. Bacteriol. 122*, 1076-1080.
Shii, K., Baba, S., Yokono, L., & Roth, R. A. (1985) *J. Biol. Chem. 260*, 6503-6506.
Shii, K., Yokono, K., Baba, S., & Roth, R. A. (1986) *Diabetes 35*, 675-682.
Soderlin, T. R., & Sheorain, V. S. (1985) in *Molecular Basis of Insulin Action* (Czech, M. P., Ed.) pp 235-245, Plenum Press, New York.
Switzer, R. C., Merril, C. R., & Shifrin, S. (1979) *Anal. Biochem. 98*, 231-237.
Tellez-Inon, M. T., Terenzi, H., & Torres, H. N. (1969) *Biochim. Biophys. Acta 191*, 765-768.

# Resonance Raman Spectroscopy of Bilirubins: Band Assignments and Application to Bilirubin/Lipid Complexation†

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ABSTRACT: Resonance Raman spectra of bilirubins IX$\alpha$, III$\alpha$, and XIII$\alpha$ and mesobilirubin XIII$\alpha$ in alkaline aqueous and chloroform solutions are reported. Partial band assignments of bilirubin IX$\alpha$ are proposed. The model compounds confirm assignments of bands of the Raman spectrum of bilirubin IX$\alpha$ to each of the two different pyrromethenones. Resonance Raman spectra of mesobilirubin IV$\alpha$, vinylneoxanthobilirubinic acid, and vinylisoneoxanthobilirubinic acid in alkaline aqueous solution and of the tetra-*n*-butylammonium salt of bilirubin IX$\alpha$ are used to define markers for the presence or absence of internal hydrogen bonds. Interaction of bilirubin dianion and sphingomyelin liposomes is studied. The Raman evidence suggests that in the bilirubin dianion/liposome complex the intramolecular hydrogen bonds between the propionate groups and the lactam NH/CO are ruptured. It is proposed that in the complex the bilirubin propionates form ion pairs with the quaternary ammonium ion of the choline moiety of sphingomyelin.

(4*Z*,15*Z*)-Bilirubin IX$\alpha$ (BR IX$\alpha$), commonly called bilirubin (BR), is formed in vivo from enzyme-catalyzed ring opening of heme proteins. The molecule generally adopts an internally hydrogen-bonded conformation as shown in structure **1a**. The neutral molecule is sparingly water soluble, although the dipropionate anion (structure **1b**) is soluble in alkaline solution.

BR has two similar planar pyrromethenone groups, A-B and C-D. Lactam ring A and pyrrole B are in the *Z* con-

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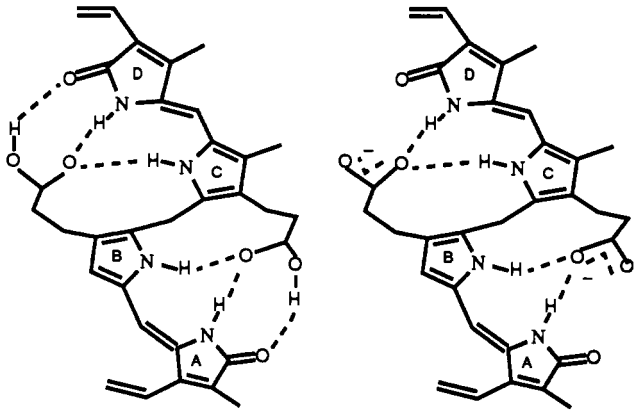


Structure 1a Structure 1b

figuration with respect to the C=C bond at C4, while pyrrole C and lactam D are in the *Z* configuration with respect to the C=C bond at C15. Each pyrromethenone has a propionic acid group which is in the proper position (C8, C12) to form internal hydrogen bonds with the lactam C=O/NH moieties and the pyrrole NH moiety in the opposing pyrromethenone. Collectively, these features define the unusual BR solubility and complexation properties [Lightner & McDonagh, 1984; Ostrow (1986) and references cited therein].

The visible electronic spectra of BR and related pyrromethenones are well understood (McDonagh, 1979; Lamola, 1985). A pyrromethenone has a single $\pi-\pi^*$ transition near 22 000 $cm^{-1}$, depending on the substituents and the medium. In BR the transitions of the two pyrromethenones are about 300 $cm^{-1}$ apart. In various solvents, the transition of the CD chromophore occurs at lower energy than that of the AB half. Exciton splitting gives rise to a broad, unresolved band.

BR can cross the blood–brain barrier, leading to irreversible brain damage or encepholopathy (Schenker et al., 1986). However, transport of BR into the brain and the mechanism of its neurotoxicity are poorly understood (Schenker et al., 1986; Nagaoka & Cowger, 1978). It has been reported that many membrane-bound enzymes are inhibited by BR, but a specific enzyme target has not been identified (Blauer & King, 1968, 1970; Mustafa et al., 1967, 1968, 1969, 1970; Karp, 1979; Kashiwamata et al., 1979).

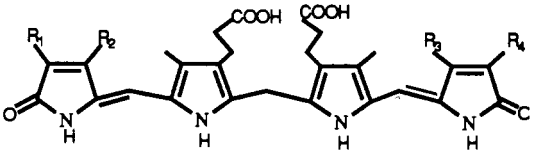
Mustafa and King (1970) found that BR is bound to lipid structures in mitochondrial and other membranes. These authors, as well as Cowger (1971), suggested that the enzymatic changes are a result of BR binding to multiple membrane structures. Sato and Kashiwamata (1983) have examined the interaction of BR with human erythrocyte membranes. Their results confirm that the BR binding sites are lipids and suggest that membrane proteins may actually inhibit the binding of BR. Association constants of bilirubin binding to lipids and liposomes have been measured (Nagaoka & Cowger, 1978; Leonard et al., 1989).

Eriksen and co-workers have identified the products of BR/phospholipid liposome interaction (Eriksen et al., 1981). At pH 8.2 the major species is a BR dianion/phospholipid complex. At pH 6–8 BR in the complex is protonated and self-aggregated to form a large BR particle containing a small amount of phospholipid. Recently, Vázquez and co-workers have also identified three separate steps in the interaction of BR with synaptosomal plasma membrane vesicles (Vázquez et al., 1988).

Although the stoichiometry of some BR/lipid complexes is now known, there is still little information on their structures. It has been suggested (Leonard et al., 1989) that neutral BR fits into voids in the apolar regions of the membranes. From perturbations to lipid IR bands, Zakim and Wong (1990) have proposed that neutral BR is in the lipid apolar region, but quite close to the head groups. The details of H-bonding of BR in the liposome complexes remain largely unknown.

Resonance Raman spectroscopy is a useful tool to study such structural questions. By examination of the spectra in water and several solvents where hydrogen-bonding patterns were known, Hsieh and Morris (1988) used this technique to propose rupture of the propionate/lactam H bonds in the BR/human serum albumin complex. Preliminary and partial normal coordinate analyses of bilirubin have been published (Margulies & Toporowicz, 1988; Wang et al., 1989). Further empirical and theoretical work is necessary before a complete assignment of the BR Raman spectrum is possible.

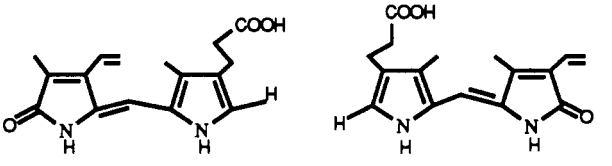
In this paper, we report the resonance Raman spectrum of several BR analogues and a BR/liposome complex. We employ bilirubins III$\alpha$ (structure **2a**) and XIII$\alpha$ (structure **2b**) and mesobilirubin XIII$\alpha$ (structure **2c**) to distinguish between AB and CD pyrromethenone chromophores in bilirubin IX$\alpha$. We use the monochromophoric pigments vinylneoxanthobilirubinic acid (structure **3**) and vinylisoneoxanthobilirubinic acid (structure **4**), bilirubin IX$\alpha$ tetra-*n*-butylammonium salt, and mesobilirubin IV$\alpha$ (structure **5**) to obtain insights into the conformational changes caused by complexation of bilirubin with sphingomyelin liposomes. Of the phospholipid/bilirubin complexes which have been studied, the sphingomyelin complexes have the largest formation constants (Nagaoka & Cowger, 1978). For this reason and because sphingomyelin is an important membrane component, we report the spectra of BR/sphingomyelin complexes.



2a. Bilirubin III $\alpha$ $R_1=R_4=C_2H_3$, $R_2=R_3=CH_3$

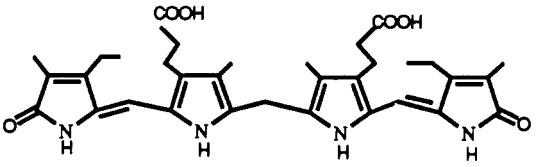
2b. Bilirubin XIII $\alpha$ $R_1=R_4=CH_3$, $R_2=R_3=C_2H_3$

2c. Mesobilirubin XIII $\alpha$ $R_1=R_4=CH_3$, $R_2=R_3=C_2H_5$



Vinylneoxanthobilirubinic acid Vinyl isoneoxanthobilirubinic acid

Structure 3 Structure 4



Mesobilirubin IV $\alpha$

Structure 5

## Experimental Procedures

Bilirubin IX$\alpha$ and sphingomyelin were obtained from Sigma. The bilirubin IX$\alpha$ was 94% pure, 4% bilirubin XIII$\alpha$, and 1% another unknown impurity by TLC (McDonagh & Assisi, 1971). Bilirubin III$\alpha$, bilirubin XIII$\alpha$ mesobilirubin XIII$\alpha$,

Table I: Electronic Absorption Maxima of Bilirubin Derivatives

| | 0.05 M Tris buffer, pH 9 | | chloroform | |
|---|---|---|---|---|
| | $\lambda_{max}$ | $\epsilon_{max}$ | $\lambda_{max}$ | $\epsilon_{max}$ |
| BI IX$\alpha$ | 443 | 65 700 | 450 | 62 400 |
| BR XIII$\alpha$ | 442 | 42 100 | 451 | 40 500 |
| BR III$\alpha$ | 443 | 37 000 | 455 | 57 200 |
| mesoBR XIII$\alpha$ | 418 | 36 700 | 431 | 58 400 |
| mesoBR IV$\alpha$ | 393 | 32 200 | insol | |
| BR IX$\alpha$ tetra-*n*-butylammonium salt | 442 | 47 300 | 459 | 48 100 |
| vinylneoXBR acid | 412 | 12 400 | insol | |
| vinylisoneoXBR acid | 413 | 19 100 | insol | |

and mesobilirubin IV$\alpha$ were prepared as described earlier (Ma & Lightner, 1984; Trull et al., 1987). The bis(tetrabutylammonium salt) of bilirubin IX$\alpha$ was prepared as described (Lightner et al., 1986). Vinylneoxanthobilirubinic acid and vinylisoneoxanthobilirubinic acid were prepared from their methyl esters isolated following treatment of biliverdin IX$\alpha$ dimethyl ester with thiobarbituric acid (Manitto & Monti, 1980). All other reagents were ACS reagent grade and were used as received. Water used to prepare all solutions was purified to ASTM type I standards.

The electronic spectra of $1 \times 10^{-5}$ M solutions of the anions were obtained in pH 9 buffers. Spectra of the free molecules were obtained in chloroform. All spectra were recorded on a Perkin-Elmer 3840 diode array spectrometer.

Most Raman spectra were obtained at a bilirubin concentration of $5 \times 10^{-4}$ M in each solvent or solution. Aqueous solution spectra were obtained at pH 9. Spectra were measured with a Spex 1877 triple spectrograph fitted with a 1200 groove/mm grating in the spectrograph stage, 200-$\mu$m spectrograph slit width, and a PAR OMA III intensified diode array detector. The diode array views an 800-cm$^{-1}$ window with 9.2-cm$^{-1}$ resolution. The instrument was calibrated at least once during each sequence of measurements, by use of the Raman spectrum of indene (Strommen & Nakamoto, 1984). Argon ion laser excitation at 514.5 nm was used in all experiments. An optical fiber system was used to irradiate the sample and collect scattered light. Most spectra were acquired with 100–120-s integration time. The samples were continuously stirred during exposure to laser light.

The BR/sphingomyelin liposome complex was prepared by a modification of Eriksen and co-workers' procedure (Eriksen et al., 1981). Sphingomyelin and bilirubin were suspended in Tris buffer, 0.05 M, pH 9. The suspension was sonicated with an Artek sonic dismembrator for 30 min, with the tube immersed in an ice–water mixture. The tube was wrapped with aluminum foil to prevent illumination of BR. The sonicated suspension was centrifuged at 10000 rpm for 1 h. The supernatant was used without further treatment.

## Results and Discussion

The electronic spectra of BR and the various model compounds in alkaline aqueous solution and chloroform are shown in Figure 1. The maxima and molar absorptivities are tabulated in Table I. These spectra are typical bile pigment electronic spectra and are in good agreement with spectra from other laboratories. For BR III$\alpha$ and BR XIII$\alpha$, the 514-nm wavelength used for Raman excitation is in resonance with the lowest electronic transition of the chromophore. For BR IX$\alpha$ the excitation wavelength is in resonance with the lowest electronic transitions of both chromophores. However, for mesoBR IV$\alpha$ and XIII$\alpha$, in which vinyl groups have been replaced by ethyl groups, as well as in the monochromophoric xanthobilirubinic acids, the electronic transitions are shifted to lower wavelengths. In these cases our spectra are more

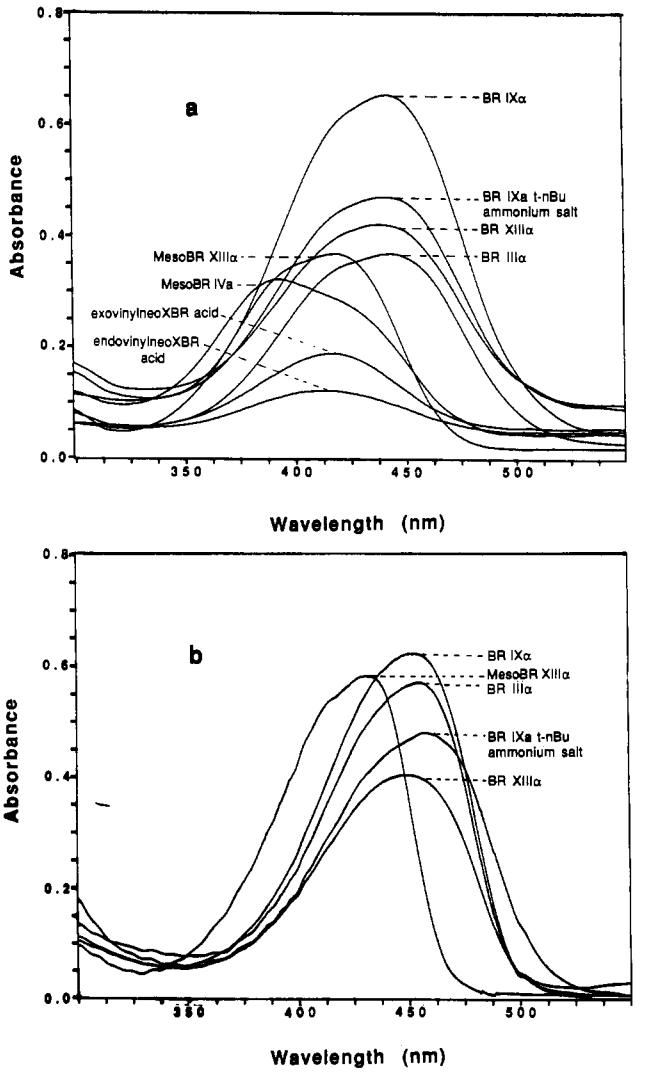



FIGURE 1: Electronic absorption spectra of bilirubin derivatives, $1 \times 10^{-5}$ M in (a) Tris buffer, pH 9, and (b) chloroform.

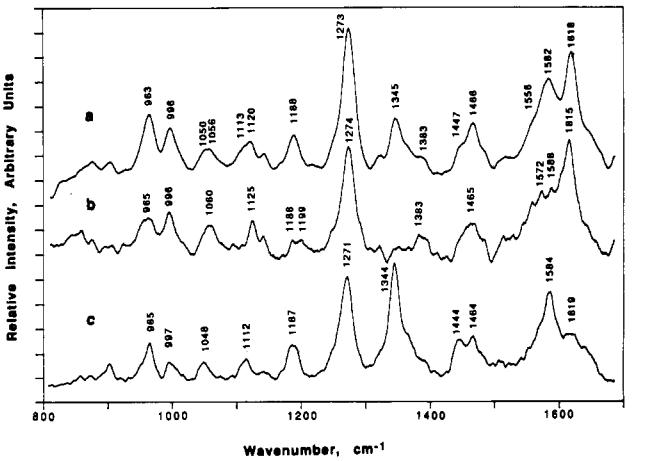



FIGURE 2: Resonance Raman spectra of $2 \times 10^{-4}$ M (a) BR IX$\alpha$, (b) BR III$\alpha$, and (c) BR XIII$\alpha$, in alkaline aqueous solution, pH 9. 514.5-nm, 25-mW excitation.

accurately preresonance rather than resonance Raman spectra.

The resonance Raman spectra of bilirubin IX$\alpha$, bilirubin III$\alpha$, and bilirubin XIII$\alpha$ in alkaline aqueous solution (pH 9) are shown in Figure 2. We were able to obtain good Raman spectra with 514.5-nm excitation using the fiber optic probe in stirred solutions. At 514.5 nm photoisomerization is inefficient, so that our spectra are of the *ZZ* isomers (McDonagh

Table II: Resonance Raman Shifts ($cm^{-1}$) of Bilirubin and Analogues in Different Environments[a]

| BR IXα assignments | BR IXα, aq | BR IIIα, aq | BR XIIIα, aq | MBR XIIIα, aq | BR IXα, chl | BR IIIα, chl | BR XIIIα, chl | MBR XIIIα, chl |
|---|---|---|---|---|---|---|---|---|
| lactam | 963 | 965 | 965 | 962 | 949 | 950 | 948 | 962 |
| | 996 | 996 | 997 | | 994 | 995 | 992 | 985 |
| | 1050 | | 1048 | 1054 | 1057 | 1059 | 1046 | 1048 |
| | 1056 | 1060 | | | | | | |
| | 1113 | | 1112 | 1107 | | | | |
| | 1120 | 1125 | | | 1126 | 1126 | | 1116 |
| | | | 1139 | | | | | 1133 |
| pyrrole | 1188 | 1188 | 1187 | 1190 | 1191 | 1188 | 1191 | 1192 |
| | | 1199 | | | | 1199 | | 1220 |
| | | | | 1253 | 1248 | 1247 | 1252 | 1253 |
| lactam | 1273 | 1274 | 1271 | 1284 | 1264 | 1270 | 1269 | |
| | 1321 | | | 1316 | 1287 | 1288 | 1283 | 1293 |
| | 1345 | | 1344 | 1368 | 1344 | | 1343 | 1369 |
| | 1383 | 1383 | | 1409 | 1382 | 1382 | | 1406 |
| | 1447 | | 1444 | | 1443 | | | |
| lactam | 1466 | 1465 | 1464 | 1463 | 1454 | 1454 | 1451 | 1456 |
| | | | | 1507 | 1503 | 1513 | 1501 | 1507 |
| | 1556 | 1572 | | | | 1557 | | |
| lactam A | 1582 | 1588 | 1584 | | 1572 | 1571 | 1576 | |
| lactam D | 1618 | 1615 | 1619 | 1606 | 1613 | 1614 | 1609 | 1597 |
| | | | | 1646 | | | 1634 | 1634 |

[a] Abbreviations defined in the text.

& Lightner, 1988). With few exceptions our measured frequencies and relative intensities are in good agreement (±3 $cm^{-1}$) with those of Hsieh and Morris (1988).

In the Raman spectra there are shoulders on many bands, as well as several broad bands which are composed of two or more unresolved components. Overall, the spectrum of BR IXα (Figure 2a) appears to consist of overlapping spectra of the AB and CD chromophores. To sort out the contributions of the two pyrromethenones, we have examined several synthetic bilirubin derivatives.

Figure 2b is the Raman spectrum of bilirubin IIIα, which has two CD chromophores separated by a methylene bridge. Figure 2c is the spectrum of bilirubin XIIIα, which possesses two AB chromophores. The spectra were taken under same conditions used to obtain the spectrum of BR IXα (Figure 2a). We have listed the strongest bands in Table II. From these data it is clear that many of the vibrational frequencies of AB and CD pyrromethenones differ by only a few wavenumbers. In the Raman spectrum of BR IXα the two sets of pyrromethenone bands are superimposed and appear as unresolved or partially resolved broad bands.

An example of incomplete resolution is the medium-strong broad band at 1056 $cm^{-1}$. The contribution from the AB (BR XIIIα) chromophore appears at 1048 $cm^{-1}$, while the contribution from the CD (BR IIIα) is at 1060 $cm^{-1}$. Similarly, the 1120-cm-1 broad band in BR IXα is the unresolved sum of a contribution from the AB system at 1112 $cm^{-1}$ and one from the CD system at 1125 $cm^{-1}$.

The bands at 1188 and 1273 $cm^{-1}$ show a small shift to lower energy in BR XIIIα and a small shift to higher energy in BR IIIα. The bands at 1466 and 1618 $cm^{-1}$ also shift slightly in both BR XIIIα and BR IIIα. While these overlapped bands could in principle be used to identify AB or CD rings, it would be helpful to have markers which are less ambiguous.

It is the positions of the lactam vinyl groups which distinguish the AB pyrromethenone from the CD. In the regions 1340–1385 and 1580–1620 $cm^{-1}$ there are pronounced characteristic differences between the spectra of BR IIIα and BR XIIIα. These spectral regions contain many lactam moiety vibrations.

BR XIIIα has two very intense bands at 1344 and 1584 $cm^{-1}$, whereas BR IIIa does not have a 1344-$cm^{-1}$ band. The

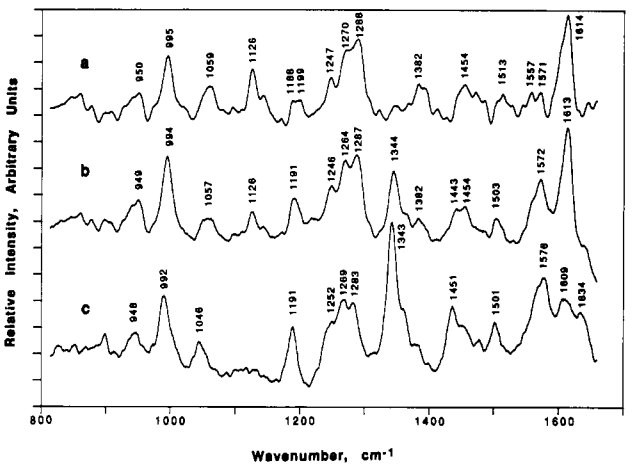



FIGURE 3: Resonance Raman spectra of $2 \times 10^{-4}$ M (a) BR IIIα, (b) BR IXα, and (c) BR XIIIα, in chloroform. 514.5-nm, 25-mW excitation.

band at 1582 $cm^{-1}$ appears as several weak bands in BR IIIα. There is a weak to medium band at 1383 $cm^{-1}$ in BR IIIα, which hardly can be observed in BR XIIIα. A band at 1615 $cm^{-1}$ is prominent in BR IIIα but is missing in BR XIIIα. Any of these bands can serve as markers to distinguish the AB chromophore contribution from the CD contribution to the Raman spectrum of BR IXα in aqueous alkaline solution.

Analogous markers exist in the resonance Raman spectra of the acid forms of BR IXα, IIIα, and XIIIα in chloroform. The spectra of these three analogues in chloroform are shown in Figure 3. Resonance Raman spectra of BR IIIα and XIIIα in chloroform are very similar except at vinyl-sensitive regions, where they exhibit the characteristic differences between AB and CD chromophores as discussed above.

Resonance Raman spectra of mesobilirubin XIIIα (MBR XIIIα) in alkaline aqueous solution and chloroform solvent are shown in Figure 4. In a MBR XIIIα molecule the vinyl groups in BR XIIIα are replaced by ethyl groups. However, the molecule still forms a intramolecularly hydrogen-bonded structure. This bilirubin analogue further identifies bands sensitive to vinyl groups.

We define vinyl group sensitive bands as those which vary over a range of at least 4 $cm^{-1}$ between BR XIIIα and MBR

Table III: Resonance Raman Shifts ($cm^{-1}$) of Bilirubin Analogues and Sphingomyelin Complex

| BR IX$\alpha$ salt, aq | BR IIX$\alpha$ salt, chl | *exo*-BR, aq (monomer) | *endo*-BR, aq (monomer) | *endo*-BR, aq (dimer) | MBR IV$\alpha$, aq | BR IX$\alpha$/sphingomyelin |
|---|---|---|---|---|---|---|
| 964 | 968 | | | | | |
| 997 | 993 | | | 990 | 993 | 994 |
| | | | | 1011 | 1013 | |
| 1050 | 1050 | 1056 | 1055 | 1056 | 1054 | 1056 |
| | | 1076 | 1076 | 1096 | 1096 | |
| 1113 | 1119 | | | 1125 | | 1121 |
| | | 1141 | 1138 | 1142 | 1142 | |
| 1189 | 1196 | 1198 | 1198 | 1198 | 1197 | |
| | | 1220 | 1221 | 1225 | 1225 | |
| | | 1244 | 1245 | 1249 | | |
| 1273 | 1268 | 1274 | 1274 | 1271 | 1275 | 1271 |
| | | 1319 | 1319 | 1322 | 1319 | |
| 1345 | | 1337 | 1341 | 1345 | 1339 | |
| | 1385 | 1378 | 1380 | 1384 | 1384 | 1384 |
| | | 1443 | 1441 | 1449 | | |
| 1466 | 1466 | 1467 | 1467 | 1471 | 1462 | 1469 |
| | | | 1481 | 1481 | 1482 | |
| 1508 | 1510 | 1512 | 1519 | 1516 | 1507 | 1516 |
| | | 1567 | | 1523 | 1552 | |
| 1581 | 1592 | 1598 | 1598 | 1596 | 1597 | 1597 |
| 1617 | | 1613 | | | | |
| | | | 1641 | | | |
| | | | 1661 | | 1659 | |

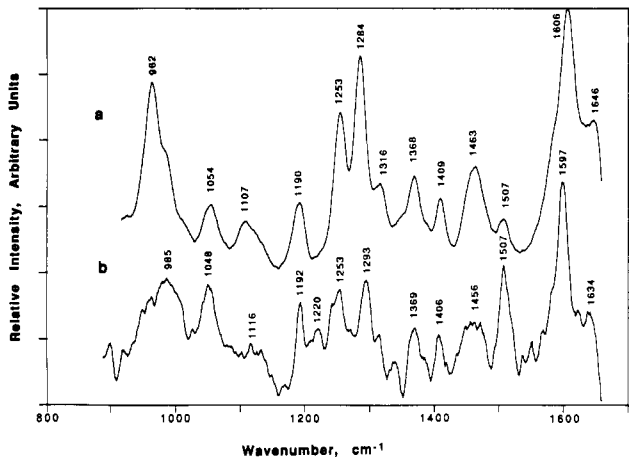



FIGURE 4: Resonance Raman spectra of $2 \times 10^{-4}$ M MBR XIII$\alpha$ in (a) alkaline aqueous solution, pH 9, and (b) chloroform, 514.5-nm, 25-mW excitation.

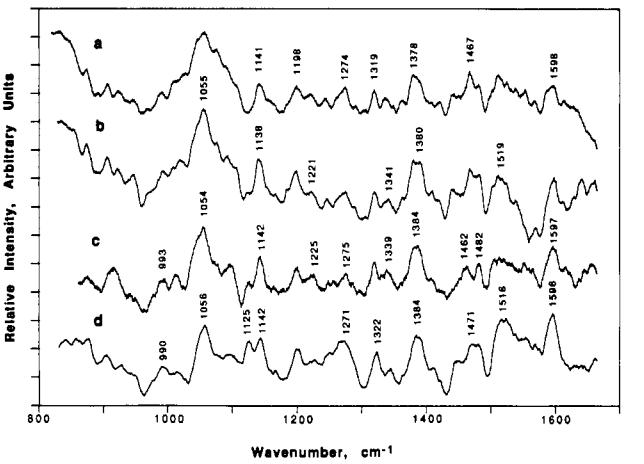



FIGURE 5: Resonance Raman spectra of $2.6 \times 10^{-5}$ M (a) *exo*-vinylneoxantho-BR acid, (b) *endo*-vinylneoxantho-BR acid, and (c) MBR IVa and (d) $5.2 \times 10^{-4}$ M *endo*-vinylneoxantho-BR acid in alkaline aqueous solution, pH 9. 514.5-nm, 25-mW excitation.

XIII$\alpha$. Comparing the spectra of BR XIII$\alpha$ and MBR XIII$\alpha$ and identifying the bands by their frequencies in alkaline aqueous solution, we find that the BR XIII$\alpha$ band at 1271 $cm^{-1}$ splits into bands at 1253 and 1284 $cm^{-1}$ in MBR XIII$\alpha$. The 1344-$cm^{-1}$ band shifts to 1368 $cm^{-1}$. The bands at 1584 and 1619 $cm^{-1}$ shift to 1606 and 1646 $cm^{-1}$, respectively. Therefore, the bands at 1271, 1584, and 1619 $cm^{-1}$ are vinyl group sensitive. They are assigned as lactam ring modes, in agreement with Hsieh and Morris (1988). In addition, since 1582 $cm^{-1}$ is a characteristic band for the AB chromophore, we assign 1582 $cm^{-1}$ as a lactam A ring mode containing coupled C=C and C=O stretches. For the same reasons, we assign the 1618-$cm^{-1}$ band as a lactam D ring mode containing C=C and C=O stretches. This conclusion experimentally supports the normal coordinate calculation of Margulies and Toporowicz (1988) although they suggest that the AB chromophore is active in protonated BR, while the CD chromophore is active in alkaline solutions of the dianions. However, Wang et al. (1989) calculate this mode to be at 1606 $cm^{-1}$ in the AB chromophore and identify it with the IR band at 1612 $cm^{-1}$.

The 1344-$cm^{-1}$ band is sensitive to the vinyl group but insensitive to hydrogen bonding on lactam carbonyl groups. It is enhanced when there is an *endo*-vinyl group in a intramolecularly hydrogen-bonded structure but disappears when the *endo*-vinyl group is absent or the intramolecularly hydrogen-bonded structure cannot form completely (see the following discussion). Therefore, we propose that 1344 $cm^{-1}$ is either an *endo*-vinyl-perturbed amide I vibration at a lactam functionality or an *endo*-vinyl-perturbed pyrrole B mode in the ridge-tile conformation.

The monochromophoric pigments vinylneoxanthobilirubinic acid/methyl ester and vinylisoneoxanthobilirubinic acid/methyl ester cannot form intramolecular hydrogen bonds. Instead, they may form intermolecular hydrogen bonds (Lightner et al., 1988, 1987; Falk et al., 1977). However at the low concentration ($2.6 \times 10^{-5}$ M) used here, dimerization is about 5% or less (Falk et al., 1977). The resonance Raman spectra of these monochromophore bilirubin analogues in aqueous alkaline solution are shown in Figure 5a,b. Their Raman shifts are summarized in Table III.

The band positions and relative intensities of these monochromophore pigments differ from those with intramolecular hydrogen bonds. In these spectra, 1380 $cm^{-1}$ becomes a pronounced band while 1344 $cm^{-1}$ disappears. Bands around

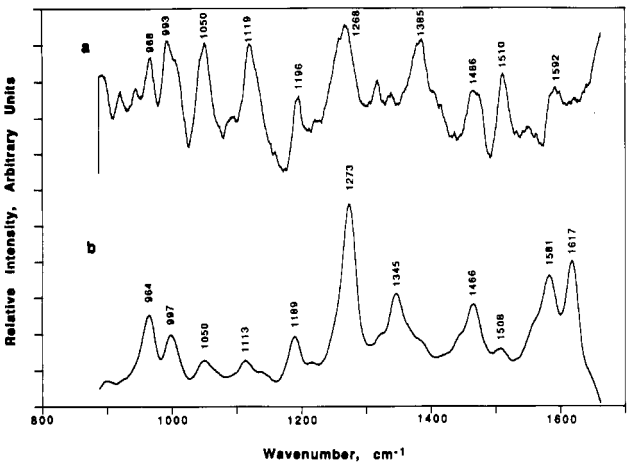



FIGURE 6: Resonance Raman spectra of $1.4 \times 10^{-3}$ M BR IX$\alpha$ tetra-*n*-butylammonium salt in (a) chloroform and (b) alkaline aqueous solution, pH 9. 514.5-nm, 25-mW excitation.

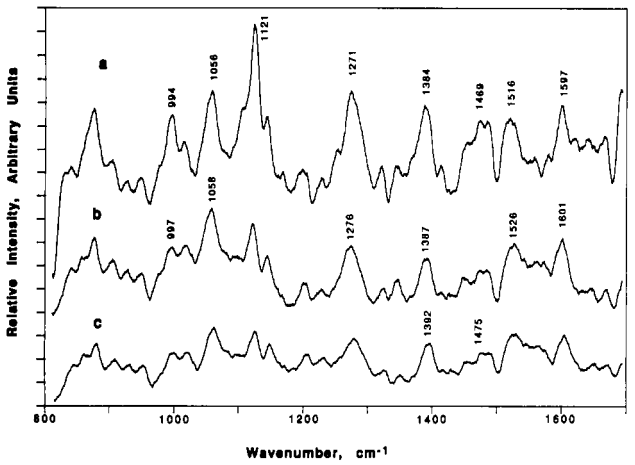



FIGURE 7: Resonance Raman spectra of (a) BR IX$\alpha$/sphingomyelin, (b) BR III$\alpha$/sphingomyelin, and BR XIII$\alpha$/sphingomyelin (1:1) complexes. 514.5-nm, 25-mW excitation.

1512 $cm^{-1}$ are strong in these molecules, while they are very weak and somewhat shifted in the intramolecularly hydrogen-bonded molecules. The 1582-$cm^{-1}$ band in bilirubin IX$\alpha$ shifts to 1598 $cm^{-1}$. These band shifts serve as markers for molecules lacking an intramolecularly hydrogen-bonded structure.

Further confirmation of these hydrogen-bond markers comes from the spectrum of mesobilirubin IV$\alpha$ (Figure 5c) in aqueous alkaline solution. The propionic acid groups of this molecule are located at C7 and C13, making it incapable of adopting an intramolecularly hydrogen-bonded structure (Lightner et al., 1988). As expected, the spectrum shows the hydrogen-bond-free Raman pattern. Most notable is the prominent band at 1597 $cm^{-1}$, which replaces the bands in the 1580-$cm^{-1}$ region in BR IIIa, BR IXa, and BR XIIIa. There is no change in the Raman spectrum as the concentration of MBR IV$\alpha$ is increased to about $10^{-3}$ M. Therefore, this dichromophoric molecule does not form a dimer as readily as monochromophoric molecules.

We were able to obtain the resonance Raman spectrum of the dimer of vinylneoxanthobilirubinic acid (Figure 5d) by increasing the concentration to $5 \times 10^{-4}$ M. Using Falk and co-workers' association constant (Falk et al., 1977), we estimate over 90% dimer at this concentration. In the spectra of the dimer, there is a prominent band at 1125 $cm^{-1}$. We propose that this band arises from the H-bonding of propionate to pyrrole rings.

There are many factors which cause solvent-dependent frequency shifts in vibrational spectra [Nyquist and Settineri (1990) and references cited therein]. Using the tetrabutylammonium salt of BR IX$\alpha$, which is soluble in both aqueous solution and chloroform, we can sort out solvent effects from the effects of changes in hydrogen bonding between acid and dianion forms of BR IX$\alpha$. The bands which shift more than 4 $cm^{-1}$ between basic to acid form are defined as H-bonding-sensitive bands. In BR IXa, they are 963, 1273, 1466, 1582, and 1618 $cm^{-1}$. These bands are lactam ring vibrations.

The resonance Raman spectra of bilirubin IX$\alpha$ bis(tetra-*n*-butylammonium salt) in both alkaline aqueous solution and chloroform solvent are shown in Figure 6. The spectrum of this BR IX$\alpha$ salt in alkaline aqueous solution (Figure 6b) is the same as that of BR IX$\alpha$ dianion, of course. Since the salt is ionized in aqueous solution to form dianions, the spectrum confirms the chemical identity of the chromophore. However, in chloroform, the two tetra-*n*-butylammonium ions remain as ion pairs with the carboxylate group. Here we observe the Raman spectrum (Figure 6a) which resembles the spectra of the monochromophoric molecule (Figure 5). In this case the 1581-$cm^{-1}$ band has been shifted to 1592 $cm^{-1}$, and the 1385- and 1510-$cm^{-1}$ bands are strong. These spectral changes occur in bilirubin systems in which intramolecular hydrogen bonding is weak or absent. At most, there may be some hydrogen bonds to pyrrole rings as indicated by the band at 1119 $cm^{-1}$. This band appears in the spectra of the dimer solution of vinylneoxanthobilirubinic acid at 1125 $cm^{-1}$ (Figure 5d). These conclusions are surprising because molecular models suggest that the quaternary ammonium ions are not large enough to disrupt intramolecular hydrogen bonding.

Although complete band assignments may not yet be possible, we have defined enough empirical markers to monitor changes in the bilirubin conformation while it complexes with liposome. The differences in the spectra among intramolecular bonded BR, bilirubin salt, and monochromophore pigments provide adequate markers for different conformations and hydrogen-bonding patterns of bilirubin IX$\alpha$.

The spectra of bilirubin IX$\alpha$, III$\alpha$, and XIII$\alpha$ complexes with sphingomyelin liposomes are shown in Figure 7. They resemble the Raman spectra of model systems which do not have lactam–propionate hydrogen bonds. These include the BR IX$\alpha$ bis(tetra-*n*-butylammonium salt) in chloroform, monochromophore bilirubinic acids, and mesobilirubin IV$\alpha$.

In the resonance Raman spectrum of the BR IX$\alpha$/sphingomyelin complex, the pattern of bands at 1384, 1516, and 1597 $cm^{-1}$ is characteristic of a BR molecule lacking intramolecular H-bonds. BR IX$\alpha$ must have strong ion pair bonds between propionate residues and the quaternary ammonium ions of sphingomyelin head groups. However, there may still be some residual hydrogen bonding to pyrrole rings, as indicated by the presence of the band at 1121 $cm^{-1}$. From the bands in the 1590–1650-$cm^{-1}$ region, we suggest that both pyrromethenones are involved in the complexes.

Further evidence for this view comes from the spectra of the BR III$\alpha$ and BR XIII$\alpha$ complexes (Figure 7b,c). BR III$\alpha$/sphingomyelin and BR XIII$\alpha$/sphingomyelin complexes show the typical patterns for their chromophores lacking intramolecular H-bonds. The similar Raman frequencies and relative intensities imply that both pyrromethenones are involved in the interaction with sphingomyelin liposomes.

## Conclusions

Although the BR IX$\alpha$ Raman spectrum is quite complex, we have shown that it is composed of independent contributions

from the AB and CD chromophores. The spectra of the AB and CD pyrromethenones show characteristic differences, caused by the different positions of their vinyl groups in the 1344–1382- and 1582–1618-$cm^{-1}$ regions. The 1582-$cm^{-1}$ C=C, C=O stretch is a characteristic band for an AB pyrromethenone, while the 1618-$cm^{-1}$ C=C, C=O stretch is characteristic for a CD pyrromethenone. Other bands identify the presence or absence of hydrogen bonds to lactams and pyrroles.

The bilirubin IX$\alpha$/sphingomyelin liposome complex may be a good model for an early step in the transfer of bilirubin across the blood–brain barrier. Formation of carboxylate/quaternary ammonium ion pairs may be strong enough to extract the BR from its albumin complex. The usual hydrogen-bonded structure cannot be maintained because of steric hindrance from the liposome. These changes may position BR for penetration into the hydrocarbon tail group of the lipids.

**Registry No.** **2a**, 36284-06-7; **2b**, 35991-50-5; **2c**, 79719-28-1; **3**, 54620-18-7; **4**, 37983-13-4; **5**, 94732-74-8; BR IX$\alpha$, 635-65-4; BR IX$\alpha$ tetrabutylammonium salt, 107440-18-6; pyrromethenone, 77055-31-3.

## References


Blauer, G., & King, T. E. (1968) *Biochem. Biophys. Res. Commun. 31*, 678–684.

Blauer, G., & King, T. E. (1970) *J. Biol. Chem. 245*, 372–381.

Cowger, M. L. (1971) *Biochem. Med. 5*, 1–16.

Eriksen, E. F., Danielsen, H., & Brodersen, R. (1981) *J. Biol. Chem. 256*, 4269–4274.

Falk, H., Grubmayr, K., Günther, H., Hofer, O., Leodolter, A., Neufingerl, F., & Ribô, J. M. (1977) *Monatsh. Chem. 108*, 1113–1130.

Hsieh, Y.-Z., & Morris, M. D. (1988) *J. Am. Chem. Soc. 110*, 62–67.

Karp, W. B. (1979) *Pediatrics 64*, 361–368.

Kashiwamata, S., Goto, A., Semba, R. K., & Suzuki, F. N. (1979) *J. Biol. Chem. 254*, 4577–4584.

Lamola, A. A. (1985) in *Optical Properties and Structure of Tetrapyrroles* (Blauer, G., & Host, S., Eds.) pp 311–330, de Gruyter, Inc., New York.

Leonard, M., Noy, N., & Zakim, D. (1989) *J. Biol. Chem. 264*, 5648–5652.

Lightner, D. A., & McDonagh, A. F. (1984) *J. Am. Chem. Soc. 17*, 417–424.

Lightner, D. A., Ma, J.-S., & Wu, X.-X. (1986) *Spectrosc. Lett. 19*, 311–320.

Lightner, D. A., Reisinger, M., & Wijekoon, W. M. D. (1987) *J. Org. Chem. 52*, 5391–5395.

Lightner, D. A., An, J.-Y., & Pu, Y.-M. (1988) *Arch. Biochem. Biophys. 262*, 543–559.

Ma, J.-S., & Lightner, D. A. (1984) *J. Heterocycl. Chem. 21*, 1005–1008.

Manitto, B. P., & Monti, D. (1980) *J. Chem. Soc., Chem. Commun.*, 178–180.

Margulies, L., & Toporowicz, M. (1988) *J. Mol. Struct. 174*, 153–158.

McDonagh, A. F. (1979) in *The Porphyrins* (Dolphin, D., Ed.) Vol. VIA, pp 293–491, Academic Press, New York.

McDonagh, A. F., & Assisi, F. (1971) *FEBS Lett. 18*, 315–317.

McDonagh, A. F., & Lightner, D. A. (1988) *Semin. Liver Dis. 8*, 272–283.

Mustafa, M. G. (1969) *Fed. Proc. 28*, 882.

Mustafa, M. G., & King, T. E. (1970) *J. Biol. Chem. 245*, 1085–1089.

Mustafa, M. G., Cowger, M. L., & King, T. E. (1967) *Biochem. Biophys. Res. Commun. 29*, 661–666.

Mustafa, M. G., Cowger, M. L., & King, T. E. (1968) *Fed. Proc. 27*, 833.

Mustafa, M. G., Cowger, M. L., & King, T. E. (1969) *J. Biol. Chem. 244*, 6403–6414.

Nagaoka, S., & Cowger, M. L. (1978) *J. Biol. Chem. 253*, 2005–2011.

Nyquist, R. A., & Settineri, S. E. (1990) *Appl. Spectrosc. 44*, 791–796.

Ostrow, J. D., Ed. (1986) *Bile Pigments and Jaundice*, Marcel Dekker, New York.

Sato, H., & Hashiwamata, S. (1983) *Biochem. J. 210*, 489–496.

Schenker, S., Hoyumpa, A. M., & McCandless, D. W. (1986) in *Bile Pigments and Jaundice* (Ostrow, J. D., Ed.) pp 395–415, Marcel Dekker, New York.

Strommen, D. P., & Makamoto, K. (1984) in *Laboratory Raman Spectroscopy*, pp 64, 114, Wiley, New York.

Trull, F. R., Franklin, R. W., & Lightner, D. A. (1987) *J. Heterocycl. Chem. 24*, 1573–1579.

Vázquez, J., García-Calvo, Valdivieso, F., Mayor, F., & Mayor, F., Jr. (1988) *J. Biol. Chem. 263*, 1255–1265.

Wang, X.-Z., Soloway, R. D., Wang, J.-F., Xu, G.-X., Zhou, Y.-G., & Lu, K. G. (1989) *Proc. SPIE 1145*, 132–133.

Zakim, D., & Wong, P. T. T. (1990) *Biochemistry 29*, 2003–2007.