RECEIVED

JUL 29 1997

OSTI

PHYSICAL MEASUREMENTS OF Cu^{2+} COMPLEXES OF BILIRUBIN*

J. R. Ferraro¹, J.-G. Wu², R. D. Soloway³, W.-H. Li²,

Y.-Z. Xu², D.-F. Xu², and G.-R. Shen²

Argonne National Laboratory, Argonne, IL 60439¹; Peking University, Chemistry Department, Beijing 100871, China²; and University of Texas Medical Branch, Division of Gastroenterology, Galveston, TX 77555-0764³

ABSTRACT

Copper is known to form complexes with bilirubin(H_2BR). Such complexes have received increased attention due to their clinical significance as free-radical scavengers. The purpose of this study was to examine a series of Cu^{2+} BR complexes to ascertain the nature of the binding between Cu^{2+} and BR. Several physical measurements of the salts were made, such as Fourier Transform Infrared (FT-IR), Fourier Transform Raman spectroscopy (FT-R), and Electron Paramagnetic Resonance (EPR). The complexes were prepared by dissolving protonated BR in NaOH, and adding different ratios of aqueous CuCl_2 . At ratios of $\text{Cu}^{2+}:\text{H}_2\text{BR}$ of 1:1 and 2:1, soluble complexes were formed. In solution EPR spectra demonstrated 9 hyperfine peaks, which from the splitting, is indicative of Cu^{2+} coordinated to 4 nitrogen atoms coming from 2 molecules of BR. The solid obtained from the solutions demonstrated predominant infrared absorptions at 1574 cm^{-1} and 1403 cm^{-1} (assigned¹ as COO^- vibrations, asymmetric and symmetric), whereas the 1710 cm^{-1} vibration appears only as a shoulder (assigned¹ as the free COOH vibration)

*Presented at the International Bilirubin Workshop, Trieste, Italy, April 6-8, 1995.

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, make any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

indicative that most of the COO^- groups have reacted with sodium, thus accounting for the aqueous solubility. The NH stretching vibration in the pyrrole group of H_2BR has disappeared and is replaced with the OH stretching vibration in H_2O . At ratios of 3:1 and 5:1 ($\text{Cu}^{2+}:\text{H}_2\text{BR}$), black precipitates are formed, which produce no EPR signals. Furthermore, the NH vibration disappears as in the soluble solution complexes. It can be concluded that the insoluble salts (higher $\text{Cu}^{2+}:\text{H}_2\text{BR}$ ratios) are mixed complexes containing the Cu-nitrogen chelate and Cu salts involving the COOH groups.

INDEX HEADINGS: Copper bilirubin, FT-IR, FT-Raman, EPR.

Introduction

The main component, along with protein, of black pigment gallstones is calcium bilirubinate. Copper bilirubinate also can occur in gallstones, but is a minor constituent. It has been proposed to act as a free-radical scavenger in bile, protecting phospholipids from peroxidation. The nature of the binding of Cu^{2+} to bilirubin is uncertain. Suzuki²⁻⁴ concluded, from IR studies, that Cu^{2+} is bonded to four nitrogens of one molecule of H_2BR . Verification of this conclusion has not come forth. To explore this, we have used FT-IR, FT-Raman, and EPR as diagnostic probes.

Experimental

1. Syntheses

Bilirubin (H_2BR) was obtained from Sigma Chemical Co.

- a. Preparation of soluble copper-bilirubin complex.

58mgs. of bilirubin was dissolved in 2.0 ml of 0.1 molar NaOH and diluted to 5.0 ml, to form a 0.02 molar sodium bilirubinate solution at a pH = 10.4. To this solution was added a 0.06 molar CuCl₂ solution drop-wise with stirring. The solution first turned brown and then quickly to a black color. Two solutions were prepared, where the ratio of H₂BR : Cu was 2:1 (A), and 1:1 (B).

b. Preparation of precipitated copper-bilirubin complexes.

50 mgs. of H₂BR were dissolved in a 0.02 molar NaOH aqueous solution to form a Na₂BR solution. A 0.1 molar CuCl₂ solution was added to the Na₂BR solution at ratios of H₂BR : Cu, 1:3 (C) and 1:5 (D). Black precipitates formed when the pH was adjusted with a HCl solution. The final pH of the solution was 5.5 and 5.2. The precipitates were washed several times to remove excess CuCl₂, and unreacted H₂BR, and the precipitates then desiccated.

2. Instrumentation

a. Infrared Measurements

The infrared spectra were obtained using an Nicolet Magna 750 FT-IR interfaced with a microscope. Sampling for the soluble solutions (A and B) was undertaken by placing a drop of the solution and placing it on a BaF₂ window, drying it using dry air and the spectra obtained on a thin film. The solids from (C and D) were measured using the microscope FT-IR interface. A Bruker High-Vacuum Model #98 was also used for some infrared measurements, with KBr disks.

b. Raman Measurements

Raman measurements were made using a Spex #1403 spectrophotometer, with Ar⁺ laser excitation at 514.5 nm; a Renishaw Raman imaging spectrophotometer #2000, with a HeNe laser (633 nm) operating at 10 mW power, and interfaced with a microscope; a Bio-Rad-Digilab Raman spectrophotometer using a Nd:YAG laser (1064 nm), and a FT-Raman Nicolet 910 using a Nd:YAG laser.

c. EPR Measurements

Spectra of the copper-bilirubin complexes were obtained in quartz tubes at room temperature and liquid nitrogen temperatures in the solid state, as well as in NaOH solution. The spectra were obtained using a Bruker ER200 D-SRC Electron Paramagnetic Spectrometer.

Results and Discussion

In attempting to characterize the nature of the binding of copper to bilirubin we have employed infrared and electron paramagnetic spectroscopy. We have also used Raman spectroscopy, but for the CuBR case the technique was not diagnostic.

1. Infrared Spectroscopy

In analyzing the infrared data we have utilized the assignments for the major functional groups in uncomplexed bilirubin made by Yang and coworkers,¹ and Wang, et al.⁵ Figure 1 demonstrates a Fischer's structure of bilirubin. There are 5 frequencies, which have proven useful in characterizing the bonding in Cu:H₂BR. These are the NH

stretch in the two pyrrole groups, NH stretch in the two lactam groups, the unreacted carboxylate vibration (COOH), the COO⁻ asymmetric and the COO⁻ symmetrical stretches.

a. Soluble Solutions (A and B)

If we examine the solid obtained from the soluble solution of copper and bilirubin (A and B) - see Table I, it is observed the ν_{NH} stretching vibration in the pyrrole groups (Band C rings, Fig. 1) has disappeared, whereas the ν_{NH} stretching vibration in the lactam groups (A and D rings, Fig. 1) remains. Although in some cases masked by the OH stretch in H₂O, the lactam ν_{NH} vibration is only slightly changed in frequency from Na₂BR and is unchanged in intensity. We can infer that the copper is bound to the nitrogen of the two central pyrrole groups and the two hydrogens are displaced. The spectra of A and B are dominated by 2 intense peaks at $\sim 1570\text{ cm}^{-1}$ and $\sim 1400\text{ cm}^{-1}$, which are assigned to the COO⁻ asymmetrical and COO⁻ symmetrical stretching vibrations, and are indicative of salt formation (See Fig. 2). Since the solution is alkaline (pH = 11.0 and 10.4), the salt formation involves the Na⁺ reaction with the carboxyl groups in bilirubin (see Table I). The results indicate that four nitrogens are coordinated to the Cu²⁺ ion and that the sodium salt formation with the COO⁻ groups of HBR, provides the solubility found for A and B.

Substantiation for these conclusions are found from Electron Paramagnetic Resonance studies. Figure 3 indicates the results obtained. The soluble copper complex shows 9 hyperfine EPR peaks in solution. Based on the formula $2n_iI+1$, where $n_i = 4$ nitrogen neighbors and $I_{\text{Cu}} = 3/2$, 13 are predicted. The Cu:H₂BR complex exhibits large g values (~ 2.076) where H₂BR ($g = 2.0039$). Although 13 peaks are predicted for four coordinated nitrogens to copper, the 9 peaks detected may represent

peaks overlapping with each other, resulting in masking. Table II summarizes the EPR results.

b. Precipitated Solids

The precipitated solids C and D where the $\text{H}_2\text{BR}:\text{Cu}$ ratios are 1:3 and 1:5 respectively demonstrate that the ν_{NH} (pyrrole) stretching vibration has disappeared, indicative of Cu to N bonding. The ν_{NH} (lactam) remains unchanged in intensity and only slightly changed in frequency. A weak shoulder occurs at $\sim 1700\text{ cm}^{-1}$ indicating some small amount of unreacted COOH remains. The spectra are dominated by the 2 absorptions at $\sim 1570\text{ cm}^{-1}$ and $\sim 1400\text{ cm}^{-1}$, indicative of copper binding to the COOH group, and assigned as COO^- asym. and COO^- sym. vibrations (See Figures 4 and 5).

The precipitated solids (samples C and D) do not show any EPR spectra, as shown in Fig. 3.

Failure of Raman spectroscopy to serve as a diagnostic tool was due to black color of the solid and because it contained copper. Whether we used an Ar^+ , an He-Ne source or a Nd:YAG laser we observed fluorescence, even at low laser power intensity ($\sim 10\text{ mW}$). Another factor, particularly with FT-Raman, transition metals show electronic absorptions in the near infrared region, which also masks any Raman scattering.⁶ Only using the Renishaw imaging spectrophotometer were some scattering Raman lines observed, off the fluorescence envelope, occurring at 1612, 1466, 1389 and 1263 cm^{-1} .

Some qualitative information on the nature of binding in carboxylates (e.g., formates, acetates, trifluoroacetate can come from measuring the separation of the ν_{COO^-} (asym.) and ν_{COO^-} (sym.).⁷⁻⁹ The difference is labeled as Δ and is related to the coordination mode. Unidentate coordination demonstrates a larger Δ than an ionic structure, while a bidentate

coordination has a lower Δ . Bridging carboxyl groups show a Δ similar to the ionic form. Table IV tabulates several compounds relative to Δ . It may be observed that the inference from IR that the sodium salt of the carboxylate group in H_2BR has formed in alkaline solution, as well as the formation of the copper salt in acid solution is substantiated by Δ values in the ionic region.

For two molecules of H_2BR to be able to coordinate to one Cu^{2+} ion in the soluble copper bilirubinate complex, it is necessary for H_2BR to become flexible from the ridge-tile structure. Conformational calculations for H_2BR were made using the MM3 (Molecular Mechanism Program*).¹⁰ The AB and CD rings in H_2BR (see Fig. 1) were considered to form two planes, connected by the central CH_2 -group. The AB plane can rotate from 0-360° along the C_9-C_{10} axis, and plane CD can rotate 0-360° along the $C_{10}-C_{11}$ axis. To weaken the effects of hydrogen bonds in bilirubin, the dimethyl ester of bilirubin was synthesized and molecular mechanics calculations carried out. A conformational map was calculated in the form of a two-dimensional map (torsion angles $C_8-C_9-C_{10}-C_{11}$ and $C_9-C_{10}-C_{11}-C_{12}$) at every 10 degrees. The lowest steric energy obtained in the calculations was ~64 Kcal/mol when the torsion angles of $C_8-C_9-C_{10}-C_{11}$ and $C_9-C_{10}-C_{11}-C_{12}$ are about ~120° respectively. When the torsion angles are 0° the steric energy is at a maximum level of ~110 Kcal/mol. These calculations indicate that the H_2BR molecule can be flexible, and can deviate from the ridge-tile structure and thus accommodate two molecules of H_2BR around the Cu^{2+} ion. Further support that the H_2BR can be flexible comes from the work of Lightner, et al.¹¹ Although the most stable conformation of H_2BR is a ridge-tile structure, it can become flexible. Figure 6 illustrates this flexibility. Stability for an expanded structure for H_2BR can result from its coordination to the Cu^{2+} ion.

*J. J. P. Stewart, QCPE Program 455 (Version 6.0), Indiana University, 1990. J. J. P. Stewart, J. of Computer-Aided Molecular Design 4:1, 1990.

Conclusions

A summary of the results for the Cu:H₂BR complexes obtained in basic and acid solution, and is tabulated in Table III. Our conclusions for the basic and acid solutions are shown as follows.

1. Basic Solutions

- a. Neutral salts formed
- b. Most of free COOH groups in H₂BR have reacted with Na⁺ and formed the sodium salt, which generates a soluble salt.
- c. Cu²⁺ binds to the NH pyrrole groups. The central ν_{NH} vibrations disappear. The lactam ν_{NH} vibrations remain. In order to obtain a stable, 4-coordinate complex, the four nitrogens must come from two BR molecules. Since the central ν_{NH} vibration disappears, while the lactam ν_{NH} vibration remains, the nitrogen bonding to Cu²⁺ must come from the central pyrrole nitrogens of the two BR molecules. In this respect we differ from Suzuki,^{1,2} who considers only one molecule of BR is bonded to the Cu²⁺.

2. Acid Solutions

- a. Acid salts formed
- b. In absence of Na⁺ ions, more COOH groups become available for reaction with Cu²⁺.

- c. Especially in higher ratios of Cu:H₂Br this causes Cu²⁺ to react with the COOH groups, and mixed complexes form, which are insoluble and precipitate.
- d. Attachment to the nitrogens is the same as in the neutral salts.

References

1. B. Yang, R. C. Taylor, M. D. Morris, X.-Z. Wang, J.-G. Wu, B.-Z. Yu, G.-X. Xu, and R. D. Soloway, *Spec. Acta*, 49A, 1735 (1993).
2. N. Suzuki and J. Tohoku, *Exp. Med.* 90, 195 (1966).
3. N. Suzuki and M. Toyoda, *ibid* 88, 353 (1966).
4. F. Parker, "Applications of Infrared Spectroscopy in Biochemistry, Biology and Medicine," Plenum Press, NY (1971).
5. X.-Z. Wang, R. D. Soloway, J.-G. Wu, B.-Z. Yu, and G.-X. Xu, 7th International Conference on Fourier Transform Spectroscopy, June 19-23, Fairfax, VA, SPIE Vol. 1145, D. G. Cameron, Ed., p. 132 (1989).
6. J. R. Ferraro and K. Nakamoto, "Introductory Raman Spectroscopy," Academic Press (1994).
7. K. Nakamoto, "Infrared and Raman Spectra of Inorganic and Coordination Compounds," J. Wiley and Sons, 4th Ed., NY (1986).
8. K. Iton and H. J. Bernstein, *Can. d. Chem.* 34, 170 (1966).
9. G. B. Deacon and R. J. Phillips, *Coord. Chem. Rev.* 33, 227 (1966).
10. J.-G. Wu and Y.-Z. Xu, to be published.
11. D. A. Lightner, D. F. Nogales, D. L. Holmes and D. T. Anstine, "Bilirubin Structure - An Overview," International Bilirubin Workshop, Trieste, Italy, April 6-8, 1995.

Figure Captions

- Figure 1. Fischer's Structure of Bilirubin.
- Figure 2. Fingerprint region of CuBR obtained from soluble solution (Sample A ; $\text{H}_2\text{BR}:\text{Cu}$ 1:1). 1 = normal spectrum, 2 = deconvoluted spectrum.
- Figure 3. EPR results for Samples A, B, C, and D measured in solution.
- Figure 4. Fingerprint region of $\text{Cu}:\text{H}_2\text{BR}$ obtained from solid precipitating from an acid medium (Sample C, $\text{H}_2\text{BR}:\text{Cu}$ 1:3). 1 = normal spectrum, 2 = deconvoluted spectrum.
- Figure 5. Fingerprint region of $\text{Cu}:\text{H}_2\text{BR}$ obtained from an acid medium (Sample D, $\text{H}_2\text{BR}:\text{Cu}$ 1:5). 1 = normal spectrum, 2 = deconvoluted spectrum.
- Figure 6. Lepidopterous action in ridge-tile bilirubin illustrating its flexibility. Low energy rotations about the C(9)-C(10) and C(10)-C(11) bonds (also see Fig. 1) cause the dipyrinones to flutter, i.e., the ridge-tile expands or contracts ($\varnothing$ increases or decreases). Further expansion leads to high-energy stretched conformers; further contraction leads to high energy helical (porphyrin-like) conformers. Intramolecular hydrogen bonding is thereby stretched past breaking, and non-bonded steric interactions destabilize the structures. Reprinted with permission of D. A. Lightner (Ref. 11).

Table I.
FT-IR of H₂BR:Cu

<u>Assignment</u>	<u>A</u> (2:1)	<u>B</u> (1:1)	<u>C</u> (1:3)	<u>D</u> (1:5)	<u>E</u> Na ₂ BR
ν_{OH} in H ₂ O	3396(M)	3373(M)	3401(M)	3419(M)	3304(M)
ν_{NH} (lactam)* <-----Appears as a shoulder at ~3260 cm ⁻¹ ----->					
COOH	1710(W,Sh)	1707(W,Sh)	1711(Sh)	1712(SH)	—
$\nu_{C=C}$,					
$\nu_{C=O}, \nu_{C-N}$	1667(S)	1667(M)	1662(M)	1661(M)	1665(M)
$\nu_{C=C} \nu_{C=O}$,					
ν_{N-C}, δ_{OH}	1628(Sh)		1615(Sh)		1630(M)
COO ⁻ (Asym)	1575(VS)	1569(VS)	1558(VS)	1568(VS)	1567(S)
			1518(Sh)		
			1435(W,Sh)		1443(M)
COO ⁻ (Sym)	1404(S)	1404(M)	1398(M)	1402(M)	1401(M)

* For some CuBR₂ samples the ν_{NH} (lactam) was observed with only slight changes in frequency from H₂BR. For samples with broad, strong ν_{OH} of H₂O the band can be obscured.

Table II.

Electron Paramagnetic Resonance (EPR)

H₂BR:Cu

SAMPLES:	<u>A</u> ¹	<u>B</u> ¹	<u>C</u> ²	<u>D</u> ²
	(2:1)	(1:1)	(1:3)	(1:5)
	9 peaks*	9 peaks*	no signal	no signal

-
1. Solution from alkaline media.
 2. Precipitated solid from highly acid solutions.

* $2n_i I + 1 = 13$ predicted
 $n_i = 4$ Nitrogens
 $I_{Cu} = 3/2$

Table III.

SUMMARY: Neutral and Acid Salts of CuBR2

<u>Bilirubin Skelatal Vibrations</u>	<u>Prepared in Basic Solutions (Soluble Compounds)</u>	<u>Prepared in Acid Solutions (Precipitates)</u>
$\nu_{OH}(H_2O)$	appears	appears
$\nu_{NH}(\text{central})$	-	-
$\nu_{NH}(\text{lactam})$	masked*	masked*
ν_{COOH}	+ (weak)	+
$\nu_{C=O}, \nu_{C=C}$	+	+
$\delta_{OH}(H_2O)$	appears	appears
$\nu_{asym}(COO^-)$	+	+
$\nu_{sym}(COO^-)$	+	+

-Signifies disappearance of vibrations.

+Signifies presence of vibration.

*Vibration can be masked by δ_{OH} vibration of H_2O , although it generally is observed.

Table IV.

Tabulation of Δ for Several Compounds.

		Δ (cm ⁻¹)	Remarks
CH ₃ COONa		164	ionic
Rh(ac)(CO(PØ ₃) ₂)		228	unidentate
Rh(ac)(PØ ₃) ₂		77	bidentate
Rh ₂ (ac) ₂ (CO) ₃ (PØ ₂)		140	bridging
CaBR		151	ionic or bridging
CuBR (H ₂ BR:Cu)	1.1	165	} alkaline solution
"	1.2	171	
"	1.3	164	} acid solution
"	1.5	167	
PrBR		96-99	bidentate

$$\Delta = \nu_{\text{COO}}(\text{asym.}) - \nu_{\text{COO}}(\text{sym.})$$