INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book. These are also available as one exposure on a standard 35mm slide or as a 17" x 23" black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600
Raman spectroscopic studies of the antitumor drugs adriamycin and mitoxantrone, 1,4-dihydroxyanthraquinone, methylamine dehydrogenase and monoolein/water system

Lee, Bao-Shiang P., Ph.D.
The Ohio State University, 1988
Raman Spectroscopic Studies of the Antitumor Drugs Adriamycin and Mitoxantrone, 1,4-Dihydroxyanthraquinone, Methylamine Dehydrogenase and Monoolein/Water System

Dissertation

Presented in Partial Fulfilment of the Requirements for the Doctoral Degree of Philosophy in the Graduate School of The Ohio State University

by

Bao-Shiang Lee, B. Sc., M. Sc.,

The Ohio State University

1988

Reading Committee:
Professor Prabir K. Dutta
Professor Daniel L. Leussing
Professor Richard L. McCreery

Approved by

Professor Prabir K. Dutta
Department of Chemistry
Dedication

To my family
ACKNOWLEDGMENTS

I would like to thank Dr. Prabir K. Dutta for his encouragement and support throughout the course of this research. The member of the research group, including B. Del Barco, J. Hutt, D.-C. Shieh, R. Zaykoski, J. Incavo, J. Twu, C. Bowers, W. Turbeville, S. Cooper, D. Robins and M. Borja are acknowledged for their cooperation and fruitful discussion. A special gratitude is expressed to H. Hatfield for his normal coordinate analysis software, to C. Bowers for proof-reading this manuscript and to S. Wu for lending his computer to me.
VITA

April 18, 1957

1980

B.S., Chemistry

Chinese Culture University

Taipei, Taiwan, R. O. C.

1982

M.S., Chemistry

National Taiwan University

Taipei, Taiwan, R. O. C.

1982-1984

Obligatory Military Service

1984-1988

Teaching Associate,

Research Associate

Department of Chemistry

The Ohio State University

PUBLICATIONS


3. "Optical Spectroscopic Studies of the antitumor Drug 1,4-Dihydroxy-5,8-
Bis[[2-[(2-Hydroxyethyl) Amino] Ethyl ] Amino-9,10-Anthracenedione

Major Field: Analytical Chemistry

Studies in Drug, Enzyme, Lipid and Raman spectroscopy.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>DEDICATION</th>
<th>ii</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>VITA</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
</tbody>
</table>

1. GENERAL INTRODUCTION 1

1–1 The Antitumor Drugs Adriamycin and Mitoxantrone 1
1–2 Methoxatin Containing Methylamine Dehydrogenase 5
1–3 Monoolein/Water System 7
1–4 Raman Spectroscopy 8
References 16

2. THE MODEL CHROMOPHORE 1,4-DIHYDROXY-9,10-ANTHRAQUINONE AND THE INTERACTION BETWEEN ADRIAMYCIN AND CARDIOLIPIN 22

2–1 Introduction 22
2–2 Experimental 26
2–3 Results and Discussion 29
2–4 The Interaction between Adriamycin and cardiolipin 48
2–5 Conclusions 50
References 53

3. THE ANTITUMOR DRUG MITOXANTRONE 57
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1 Introduction</td>
<td>57</td>
</tr>
<tr>
<td>3-2 Experimental</td>
<td>59</td>
</tr>
<tr>
<td>3-3 Results and Discussion</td>
<td>61</td>
</tr>
<tr>
<td>3-4 Conclusions</td>
<td>111</td>
</tr>
<tr>
<td>References</td>
<td>113</td>
</tr>
<tr>
<td>4. RESONANCE RAMAN SPECTROSCOPIC STUDIES OF</td>
<td></td>
</tr>
<tr>
<td>METHOXATIN CONTAINING METHYLAMINE DEHYDROGENASE (MADH)</td>
<td>118</td>
</tr>
<tr>
<td>4-1 Introduction</td>
<td>118</td>
</tr>
<tr>
<td>4-2 Experimental</td>
<td>120</td>
</tr>
<tr>
<td>4-3 Results and Discussion</td>
<td>122</td>
</tr>
<tr>
<td>4-4 Conclusions</td>
<td>141</td>
</tr>
<tr>
<td>References</td>
<td>142</td>
</tr>
<tr>
<td>5. TEMPERATURE-COMPOSITION-MESOMORPH DIAGRAM OF MONOOLEIN-WATER</td>
<td></td>
</tr>
<tr>
<td>LYOTROPE LIQUID CRYSTAL SYSTEM</td>
<td>146</td>
</tr>
<tr>
<td>5-1 Introduction</td>
<td>146</td>
</tr>
<tr>
<td>5-2 Experimental</td>
<td>148</td>
</tr>
<tr>
<td>5-3 Results and Discussion</td>
<td>149</td>
</tr>
<tr>
<td>5-4 Conclusions</td>
<td>157</td>
</tr>
<tr>
<td>References</td>
<td>162</td>
</tr>
<tr>
<td>Bibliography</td>
<td>164</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1. Crystal data for Form I and Form II of 1,4(OH)₂AQ</td>
<td>29</td>
</tr>
<tr>
<td>2.2. The Observed and Calculated Frequencies for 1,4-(OH)₂AQ (done by Mr. H. Hatfield)</td>
<td>44</td>
</tr>
<tr>
<td>3.1. The Observed and Calculated Frequencies for 1,4-(NH₂)₂AQ</td>
<td>87</td>
</tr>
<tr>
<td>4.1. Summary of IR and R data of PQQ and R data of MADH (Q), MADH-PH and semiquinone forms of MADH (QH⁺)</td>
<td>126</td>
</tr>
<tr>
<td>5.1. Band assignments of the monoolein</td>
<td>155</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
</tr>
<tr>
<td>1.1. Structures of adriamycin and daunomycin</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Mitoxantrone</td>
<td>5</td>
</tr>
<tr>
<td>1.3. Methoxantin</td>
<td>6</td>
</tr>
<tr>
<td>1.4. 1-Monoolein</td>
<td>8</td>
</tr>
<tr>
<td>1.5. Raman phenomenon</td>
<td>9</td>
</tr>
<tr>
<td>1.6. Layout of the CW Raman system</td>
<td>10</td>
</tr>
<tr>
<td>1.7. Layout of the CW UV-Raman system</td>
<td>11</td>
</tr>
<tr>
<td>1.8. Layout of the pulse Raman system</td>
<td>12</td>
</tr>
<tr>
<td>1.9. Raman sample cells</td>
<td>13</td>
</tr>
<tr>
<td>2.1. 1,4-Dihydroxy-9,10-anthraquinone</td>
<td>22</td>
</tr>
<tr>
<td>2.2. X-ray diffraction patterns of the two crystalline polymorphic forms of 1,4(OH)₂-anthraquinone (a) Form I and (b) Forms I &amp; II</td>
<td>30</td>
</tr>
<tr>
<td>2.3. Structure of 1,4-(OH)₂AQ</td>
<td>31</td>
</tr>
<tr>
<td>2.4. Absorption spectra of 1,4(OH)₂-anthraquinone in its two polymorphic forms at (a) room temperature and (b) 77K</td>
<td>33</td>
</tr>
</tbody>
</table>
2.5. Infrared spectra of 1,4(OH)$_2$-anthraquinone in its two
polymorphic forms (a) Form II, (b) Forms I & II and (c) Form I 36

2.6. Resonance Raman spectra of 1,4(OH)$_2$-anthraquinone in its
two polymorphic forms (a) Form I and (b) Forms II. Excitation -
457.9 nm. Laser power -10 mW. Slits -6 cm$^{-1}$ 37

2.7. Resonance Raman spectrum of a 5x10$^{-4}$ M solution of
1,4(OH)$_2$-anthraquinone in (a) methanol and (b) acetone.
Excitation -457.9 nm. Laser power -10 mW. Slits -6 cm$^{-1}$ 38

2.8. Resonance Raman spectrum of 10$^{-3}$ M 1,4(OH)$_2$-
anthraquinone in an aqueous solution (pH 12). Excitation -
457.9 nm. Laser power -10 mW. Slits -6 cm$^{-1}$ 40

2.9. Resonance Raman spectra of 1,4(OH)$_2$-anthraquinone (Form
I; 50%) in Na$_2$SO$_4$ at (a) 413.1 nm, (b) 457.9 nm and (c)
647.1 nm. Band marked with an asterisk is due to the internal
standard SO$_4^{2-}$. Laser power -10 mW. Slits -6 cm$^{-1}$ 42

2.10. Resonance Raman spectra of 1,4(OH)$_2$-anthraquinone
(Form II; 50%) in Na$_2$SO$_4$ at (a) 413.1 nm, (b) 457.9 nm and
(c) 647.1 nm. Laser power -10 mW. Slits -6 cm$^{-1}$ 43

2.11. Resonance Raman spectra of 1,4(OH)$_2$-anthraquinone in its
two polymorphic forms (a) Form I and (b) Forms II. Excitation -
350.7 nm. Laser power -10 mW. Slits -6 cm$^{-1}$ 46
2.12. Curve deconvolution in the region 1357 - 1522 cm\(^{-1}\) of the resonance Raman spectra of adriamycin (a) mixed with DPPC vesicles (1 : 10), (b) mixed with cardiolipin vesicles (1 : 1), (c) mixed with cardiolipin vesicles (1 : 10) and (d) embedded in DPPC vesicles. (5 mM Tris buffer, pH = 7.4, drug concentration = .25 mM, 457.9 nm, dotted lines = experimental data, solid lines = simulated spectra)

2.13. Infrared spectra of cardiolipin (a) and cardiolipin-adriamycin complex (b)

3.1. Mitoxantrone

3.2. Absorption spectrum of a 1x10\(^{-6}\) M solution of mitoxantrone in (a) water, (b) methanol, (c) acetone and (d) chloroform

3.3. Absorption spectrum of a 1x10\(^{-5}\) M solution of mitoxantrone in [H\(^+\)]\(=\) (a) 5.7 M, (b) 3.2 M, (c) 2.0 M, (d) 0.8 M, and (e) 1x10\(^{-7}\) M

3.4. Absorption spectrum of a 1x10\(^{-5}\) M solution of 1,4(NH\(_2\))\(_2\)-anthraquinone in (a) acetone, (b) methanol, (c) 80% methanol + 20% water (d) 50% methanol + 50% water and (e) 20% methanol + 80% water

3.5. Plot of -hc(\(\sigma_f - \sigma_a\)) (see text) versus \([2(D - 1)/(2D + 1)] - [2(n^2 - 1)/(2n^2 + 1)]\) (see text)
3.6. Emission spectrum of a $1 \times 10^{-4}$ M solution of mitoxantrone in
(a) water, (b) methanol, (c) acetone and (d) chloroform

3.7. Absorption spectra of mitoxantrone in aqueous solution (a) $5 \times 10^{-4}$ M, (b) $1 \times 10^{-6}$ M (multiplied by a factor of 85) and (c) dimer, obtained by subtracting (b) from (a)

3.8. Emission spectra of aqueous solution mitoxantrone (a) $1 \times 10^{-5}$ M, (b) $1.2 \times 10^{-4}$ M, and (c) $5 \times 10^{-4}$ M. Excitation -457.9 nm. The peak of 551 nm is due to the Raman scattering of water

3.9. Relative emission quantum yield ($\Phi_{rel}$) vs. total dye concentration (T)

3.10. Plot of $F_2$ (see text) versus monomer concentration of mitoxantrone (m) calculated from Figure 3.9

3.11. Model of mitoxantrone dimer

3.12. Resonance Raman spectra of mitoxantrone (0.2%) and 9% NaNO$_3$ in KBr at (a) 350.7 nm, (b) 457.9 nm and (c) 514.5 nm. Bands marked with an asterisk are due to the internal standard NO$_3^-$. Laser power -10 mW. Slits -6 cm$^{-1}$

3.13. Resonance Raman spectra of 1,4(OH)$_2$-anthraquinone in its two polymorphic forms (a) Form I and (b) Forms II. Excitation -457.9 nm. Laser power -10 mW. Slits -6 cm$^{-1}$
3.14. Resonance Raman spectra of 1,4(NH$_2$)$_2$-anthraquinone (15% in NaClO$_4$) at (a) 350.7 nm, (b) 413.1 nm, (c) 488 nm and (d) 647.1 nm. Bands marked with an asterisk are due to the internal standard ClO$_4^-$, Laser power -10 mW, Slits -6 cm$^{-1}$

3.15. Parallel and perpendicularly polarized resonance Raman spectra of 1x10$^{-4}$ aqueous solution of mitoxantrone. Laser excitation -496.5 nm. Band marked with an asterisk is due to the internal standard NO$_3^-$ (0.1 M).

3.16. Resonance Raman spectra of (a) 1x10$^{-4}$ M solution of mitoxantrone and (b) with calf thymus DNA. Laser excitation 514.5 nm. Band marked with an asterisk is due to the internal standard NO$_3^-$ (0.1 M).

3.17. Excitation profiles of the Resonance Raman bands of (a) 1x10$^{-4}$ M solution of mitoxantrone and (b) bound to calf thymus DNA.

4.1. Methoxantin

4.2. Infrared spectrum of the molecule POQ (2%) in KBr

4.3. Resonance Raman spectra of POQ (20%) in KBr at (a) 457.9 nm and (b) 350.7 nm. Laser power -10 mW, Slits -6 cm$^{-1}$

4.4. Resonance Raman spectrum of 2x10$^{-3}$ M MADH. Laser excitation -457.9 nm. Laser power -10 mW, Slits -6 cm$^{-1}$
4.5. The absorption spectra of the enzyme MADH (0.92 mmol) titrated with (a) 0 mmol, (b) 0.025 mmol, (c) 0.05 mmol, (d) 0.075 mmol, (e) 0.1 mmol and (f) 0.125 mmol phenylhydrazine (done by Professor Michael H. Klapper)

4.6. The phenylhydrazine (Ph.) titration curve of the enzyme MADH monitored at (a) 524 nm, (b) 444 nm and (c) 310 nm. (done by Professor Michael H. Klapper)

4.7. Resonance Raman spectra of MADH-phenylhydrazine complex at (a) 413.1 nm, (b) 457.9 nm, (c) 514.5 nm and (d) 568.2 nm. Laser power -10 mW. Slits -6 cm⁻¹

4.8. Resonance Raman spectra of the semiquinone forms of the enzyme MADH reduced by dithionite (a) and by propanylamine (b). Excitation -413.1 nm. Laser power -10 mW. Slits -6 cm⁻¹

5.1. Monoolein

5.2. Resonance Raman spectra (low frequency region) of monoolein in (a) Lc, (b) Fl, (c) CP4 and (d) Lα phase. Laser excitation -413.1 nm. Laser power -10 mW. Slits -6 cm⁻¹

5.3. Resonance Raman spectra (high frequency region) of monoolein in (a) Lc, (b) Fl, (c) CP4 and (d) Lα phase. Laser excitation -413.1 nm. Laser power -10 mW. Slits -6 cm⁻¹
5.4. Resonance Raman spectra (low frequency region) of monoolein in (a) methanol, (b) chloroform and (c) CCl₄. Bands masked with an asterisk are due to solvents. Laser excitation -413.1 nm. Laser power -10 mW. Slits -6 cm⁻¹

5.5. Resonance Raman spectra (high frequency region) of monoolein in (a) methanol, (b) chloroform and (c) CCl₄. Bands masked with an asterisk are due to solvents. Laser excitation -413.1 nm. Laser power -10 mW. Slits -6 cm⁻¹

5.6. The capillary sample tube and the positions for the Raman experiments

5.7. Resonance Raman spectra (low frequency region) along the sample capillary tube (see Figure 5.6) (a) 0.0 cm, (b) 1.0 cm, (c) 2.0 cm, (d) 3.9 cm and (e) 5.4 cm from the interface. Laser excitation -413.1 nm. Laser power -10 mW. Slits -6 cm⁻¹

5.8. Resonance Raman spectra (high frequency region) along the sample capillary tube (see Figure 5.6) (a) 0.0 cm, (b) 1.0 cm, (c) 2.0 cm, (d) 3.9 cm and (e) 5.4 cm from the interface. Laser excitation -413.1 nm. Laser power -10 mW. Slits -6 cm⁻¹

5.9. Water concentration (w/w) along the sample capillary tube (see Figure 5.6) after 1296 hr (asterisk points = experimental data, solid line = theoretical calculation (done by Professor Caffrey))
CHAPTER I

GENERAL INTRODUCTION

1 - 1 THE ANTITUMOR DRUGS ADRIAMYCIN AND MITOXANTRONE

Anthraquinone derivatives are widely distributed in nature and are commonly found in bacteria, molds, fungi, plants and insects [1]. Their medicinal applications include use as antiviral agents[2], and recently, as antitumor drugs toward a wide variety of cancers [3]. More than 500 compounds of this type have been synthesized or isolated and tested for anticancer activity [4]. The correlation of the molecular structure of these drugs with activity is a major area of research. The three mechanisms of antitumor action by the drug include DNA intercalation, free radical activation and direct interaction with the membrane. The systems that we have focused on are (a) adriamycin (NSC-123127, Figure 1.1) and (b) mitoxantrone (NSC-279836, Figure 1.2).

Figure 1.1 Structures of adriamycin (R=OH) and daunomycin (R=H).
ADIAMYCIN- Adriamycin is currently the most powerful antitumor drug isolated from mutant cultures of Streptomyces peucetius [5]. Its structure has been shown to be the 14-hydroxy derivative of the antibiotic daunorubicin (Fig. 1.1) [6]. Unfortunately, because of its cumulative cardiotoxicity its use as a treatment of cancer is limited [7, 8, 9].

Adriamycin brings about its antineoplastic effect by interfering with DNA transcription and replication. Fluorescence and radiochemical data suggest that the drug is primarily taken up by the cell nucleus [10]. Various physicochemical changes have been observed and show that a complex is formed between adriamycin and DNA [10, 11]. There are three types of interactions which can cause complex formation. First, electrostatic interactions may occur between the positively charged amino group on the drug and the negatively charged phosphates of the DNA backbone. Second, hydrogen bonding may form between the adriamycin hydroxyl groups and the DNA phosphates and / or base pairs. Third, and most likely, is the intercalation of the drug into the DNA [10].

X-ray diffraction data [12, 13, 14, 15] and NMR spectroscopic studies [16, 17, 18] on adriamycin-DNA complexes shows that the intercalation model is of primary importance. Also electrostatic interactions were found between the cationic amino group and the DNA phosphates. Spectrophotometric analysis, equilibrium dialysis [19] and viscosity measurements [20] all support the intercalation model. The large binding constant of adriamycin, $2 \times 10^6$ l/mol, is thus attributed primarily to intercalation, with the other two binding modes contributing to a lesser degree [21]. More recent crystallographic studies have shown that the long axis of the planar portion of the drug is perpendicular to the
DNA base pairs, with the B and C rings extensively overlapping the DNA base pairs, the D ring protruding into the center of the helix, and the A ring protruding out away from the complex, allowing the amino group to bind to the phosphate backbone [18, 22, 23].

THE CYTOTOXIC EFFECTS- Several pathways have been proposed for the cytotoxic effects of adriamycin. The three major mechanisms proposed include binding to cellular membranes [24, 25], intercalation into DNA [26, 27] and DNA strand scission caused by adriamycin free radicals [28, 29, 30, 31]. There are two ways the strand scission may occur. First of all, DNA interacts directly with the reduced form of adriamycin [32, 33]. Secondly, DNA interacts indirectly with superoxide radicals produced by the adriamycin free radical [29, 34].

CARDIOTOXICITY-The action of adriamycin on cell membranes has been a topic of considerable interest. It has been shown that adriamycin will affect many membrane properties when it binds to the cell membrane, including glycoprotein synthesis [35], phospholipid structure and organization [36] and membrane fluidity [37]. Goormaghtigh et al. [38, 39] studied in detail the interaction between cardiolipin and adriamycin. This work has shown that adriamycin binds to cardiolipin with a stoichiometry of two adriamycins to one cardiolipin with an affinity constant of $1.6 \times 10^6$ l/mol. This constant is similar in magnitude to that observed for the binding of adriamycin to DNA. These authors agree with the proposal of Tritton et al. [36] which states that the ability of adriamycin to bind to cardiac mitochondrial cardiolipin is closely related to the cardiotoxicity. Evidence suggests that the mitochondrial membrane could be
the target responsible for the Adriamycin cardiotoxicity. The formation of a very stable complex between Adriamycin and cardiolipin, a phospholipid found almost exclusively in the inner mitochondrial membrane, has been shown to inhibit numerous mitochondrial membrane enzymes whose activities depend on the presence of cardiolipin [40, 41, 42]. The knowledge of the structure of the Adriamycin-cardiolipin complex at the molecular level is essential for understanding the effect of the Adriamycin on the mitochondrial membrane and the development of heart failure. Furthermore, such an understanding would allow a modulation of those effects by subtle changes of the molecular structure that could in turn be of pharmacological interest.

That the anthracycline moiety of the Adriamycin molecule does not penetrate deeply into the hydrocarbon chain region of the bilayer is supported by surface potential data obtained on monolayers and fluorescence measurements performed on liposomes [39]. Also, differential scanning calorimetry measurements indicate that the Adriamycin-cardiolipin complex segregates in a separate phase in the plane of the membrane [40]. Furthermore, $^{31}$P NMR demonstrates that the phosphorus motion of the cardiolipin remains typical of a bilayer structure and that the bilayer structure is stabilized in the complex in the presence of $\text{Ca}^{2+}$[43]. The stacking of the anthracycline rings of the Adriamycin molecules concentrated at the surface of cardiolipin liposomes was established by absorption spectroscopy [38].
Figure 1.2 Mitoxantrone.

MITOXANTRONE - The synthetic agent mitoxantrone (Figure 1.2), which was first synthesized by C. C. Cheng in 1977 [44], is a clinical anticancer drug [45,46] developed as an alternative to the apparently more cardiotoxic doxorubicin.

THE CYTOTOXIC EFFECTS - Biochemical evidence indicates that nucleic acids are among the targets of this drug in living cells similar to that of adriamycin. Mitoxantrone binds to DNA by intercalation and inhibits nucleic acid synthesis. Clinical results revealed that mitoxantrone had significant activity against breast cancer. Responses have been observed in Hodgkins disease, non-Hodgkins lymphoma, melanoma, renal cell carcinoma, cylinderona of the oral cavity, acute lymphoblastic leukemia, non-lymphocytic leukemia, blastic chronic myogenous leukemia, non-oat cell lung cancer, prostate cancer, soft tissue cancer. There were no significant therapeutic responses against gastric cancer and cancers of kidney and pancreas [47, 48].

1 - 2 METHOXATIN (PQQ) CONTAINING METHYLAMINE DEHYDROGENASE

In addition to the well-known pyridine nucleotide-dependent and flavin nuclotide-dependent dehydrogenases, it has been clear that there is a third
class of dehydrogenases in which pyrroloquinoline quinone (PQQ; Figure 1.3) is involved as the coenzyme [49]. Such a novel coenzyme was originally discovered and identified in methanol dehydrogenases of methylotrophic bacteria. Since the structural determination of PQQ by Salisbury et al. [50] who gave it the trivial name of methoxatin, it has been a topic of considerable interest.

![Figure 1.3 PQQ: Methoxatin; Pyrroloquinoline quinone.](image)

STRUCTURE AND REACTION OF PQQ CONTAINING METHYLAMINE DEHYDROGENASE (MADH) - It has been found that several bacterial [51] and mammalian [52] dehydrogenases and oxidases contain the PQQ as the organic cofactor. Nevertheless, the organic cofactors of the methylamine dehydrogenases have not been conclusively identified and the nature of the covalent binding is unclear. From sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the MADH, a molecular weight of 45,000 Dalton is obtained for the large subunit and 15,500 Dalton for the smaller one (containing one PQQ.) Gel chromatography of the native enzyme yields a molecular weight of
127,000 Dalton. These data suggest that the enzyme has a tetrameric structure of type $\alpha_2 \beta_2$. However, the structure and function of these enzymes are unclear.

The methoxatin dependent enzyme MADH from the methylotrophic bacterium W3A1 catalyzes the oxidation of amines to aldehydes and ammonia:

$$RCH_2NH_2 + \text{MADH}(Q) + H_2O \rightarrow RCHO + QH_2 + NH_3$$

(1-1)

$$QH_2 + NH_3 + 2A \rightarrow Q + 2AH + NH_3$$

(1-2)

with little specificity for the 1-electron acceptor A [53]. The reaction first proceeds to QH$_2$ in a few minutes without releasing ammonia, then slowly (several hours) proceeds to QH and Q together with ammonia. However, the nature of this reaction is largely unclear at this moment.

1 - 3 MONOOLEIN/WATER SYSTEM

TEMPERATURE-COMPOSITION-MESOMORPH (T-C) DIAGRAM OF MONOOLEIN/-WATER SYSTEM - Some of the biological phenomena in some of the organelles is believed to be linked to the structure of the lipids in the membrane., e. g., the maintenance of the resting potential, membrane fusion, the transmission of the action potential in nerve membranes, selective permeability for plasma membranes and energy transformation for mitochondria and chloroplasts. The studies of the structure of the lipid-water systems might provide important information which will help in understanding the biological
membranes [54]. And, the construction of the phase diagram is essential in understanding the structure of the multicomponent system. With more data available a correlation could perhaps be sought between the physiological function of cell organelles and the phase diagram of the lipids.

It was established that most lipid (polar)/water systems possess different phases which can be recognized and each exists over a defined region of the concentration-temperature diagram. Monoolein (Figure 1.4.)-water system possesses mesomorphic phases with one-, two- and three-dimensional periodicity and a fluid isotropic phase in an accessible temperature range (20-120°C). Besides being very stable and available in high purity at low price, it is an important emulsifier, intermediate in lipid digestion and compound in black lipid membrane studies [55, 56, 57, 58]. All these factors make the investigation of the monoolein/water system very attractive.

\[\begin{align*}
  &\text{H}_3\text{C} &\text{H}_{2} \text{CH}_2 \text{CH}_2 \text{CH}_2 \text{CH}_2 \\
  &\text{CH}_2 \text{CH}_2 \text{CH}_2 \text{CH}_2 \text{CH}_2 &\text{H} = \text{C} \text{H} \\
  &\text{CH}_2 \text{CH}_2 \text{CH}_2 \text{CH}_2 \text{CH}_2 &\text{C} - \text{O} \text{CH}_2 \\
  & &\text{CH}_2 \text{OH}
\end{align*}\]

Figure 1.4 1-Monoolein.

1-4 RAMAN SPECTROSCOPY

When monochromatic radiation of wavenumber \(v_0\) is incident on systems like dust-free gases, liquids and solids, most of it is reflected, but, in addition, some scattering of the radiation occurs. The scattering of radiation with change of frequency is called Raman scattering or Raman shift, after the Indian scientist C. V. Raman [59], which corresponds to the vibrational energy of a molecule.
A molecule is excited by incident light to a virtual state (Figure 1.5). There are two ways that the molecule can relax back to the ground state. If the molecule goes back to a higher vibrational level than the original one it emits a light with a longer wavelength \((v_o-v_m)\), at a lower energy, and these form the Stokes bands. On the other hand if the molecule goes back to a lower vibrational level than the original one it emits a light with lower wavelength \((v_o-v_m)\), at a higher energy, and these form the anti-Stokes bands. The Raman phenomenon is shown in Figure 1.5.

![Figure 1.5 Raman phenomenon.](image)

The scattering of radiation without change of frequency which arises from molecules is called Rayleigh scattering [60]. The scattering of radiation without change of frequency which arises from larger scattering centers like dust particles is called Mie scattering [61]. The scattering of radiation with change of
frequency originating from acoustic phonons is called Brillouin scattering and is of the order of $< 2 \text{ cm}^{-1}$ \[62\]

A basic Raman spectrophotometer system consists of the following components: (I) A light source, usually a laser. (II) An optical system including premonochromator, focussing and collection optics. (III) Sample holders for gas, liquid and solid. (IV) A monochromator (single, double or triple ). (V) A control system to collect, store and process data and drive the monochromator, usually a computer. (VI) A signal processing system including a photomultiplier tube, photo-diode array, an amplification system, and an output device. Figures 1.6, 1.7 and 1.8 show the schematic of our Raman spectrometer setups.

![Figure 1.6 Layout of the CW Raman system.](image)
Figure 1.7 Layout of the CW UV-Raman system.
THE SAMPLE COMPARTMENT-The cells for liquid samples (Figure 1.9) are (a) capillary tube (ca. 4 μl needed) (b) NMR tube and (c) cylindrical quartz cell. The samples were prepared anaerobically and sealed in NMR samples tubes (ca. 0.2 ml) and spun while recording the spectra to avoid decomposition. The cylindrical quartz cell is connected to a shaft capable of a controlled rotation from 0 to 800 rpm. The liquid sample needed is about 2-3 ml. The laser beam must be focused on a point near the wall for all cases to minimize the
Figure 1.9 Raman sample cells (a) and (b) for liquid and (c) for solid
reabsorption of the Raman light by the sample itself. Also, the laser beam must not focus on the cell wall to avoid spurious peaks and Raman lines originating from the wall.

The solid samples are made in a pellet and packed in a disk-like holder (Figure 1.9) which can be connected to a rotating shaft directly. The sample holder is arranged with a ~ 60° from the incident laser beam in order to prevent collecting direct reflection light. Sodium nitrate or sodium perchlorate as internal standard whenever it is necessary. The power at the sample was as low as possible to avoid sample decomposition.

**RESONANCE RAMAN SPECTROSCOPY** - Resonance Raman (RR) spectroscopy is a useful tool for studying the vibrational nature of chromophoric groups. Concentrations of chromophore in the $10^{-4}$-$10^{-6}$ M range in H$_2$O may be used. Moreover, optical homogeneity is usually a less stringent requirement for RR experiments. The intensity enhancement associated with the RR effect allows one to be selective and to "pick out" the vibrational spectrum of the chromophore when the latter is just one component in an extremely complex biological situation. In addition, the positions of peaks in RR spectra are a property solely of the electronic ground state. However, the intensity behavior of peaks in RR spectra is a source of information regarding the electronic excited state. To date, resonance Raman studies have been done on adriamycin \[63, 64\], adriamycin-DNA complex \[65, 66, 67\], 1,4-dihydroxyanthraquinone \[68\] and hydroxyanthraquinone \[69\].

**WORK DONE IN THIS DISSERTATION** - This work presents a vibrational investigation of the polymorphic forms of 1,4-dihydroxyanthraquinone (model
chromophore for adriamycin), adriamycin and cardiolipin interactions, 1,4-diamino-anthraquinone (model chromophore for mitoxantrone), mitoxantrone and calf thymus DNA interactions, methoxatoenzyme methylamine dehydrogenase and monoolein/water system. And, we have demonstrated that resonance Raman and normal Raman spectroscopy is a very powerful tool and it can provide important complementary information.
REFERENCES


60. A. Smekal, Naturwiss., 11, 873 (1923).

61. L. Rayleigh, Phil. Mag., XVI, 274, 447 (1871).


CHAPTER II

THE MODEL CHROMOPHORE 1,4-DIHYDROXY-9,10-ANTHRACQUINONE AND
THE INTERACTION BETWEEN ADRIAMYCIN AND CARDIOLIPIN

2 - 1 INTRODUCTION

(a) STUDIES OF MODEL CHROMOPHORE 1,4-DIHYDROXY-9,10-
ANTHRACQUINONE

1,4-DIHYDROXY-9,10-ANTHRACQUINONE - 1,4-Dihydroxyanthraquinone

(1,4-(OH)₂AQ; Figure 2.1) is a useful model chromophore of several
compounds of biological and pharmaceutical interest, including the family of
anthracycline drugs, which are widely use as antitumor antibiotics [1, 2]. These
drugs exert their biological effects by interaction between the chromophore
portion and the physiological receptors, such as

Figure 2.1 1,4-Dihydroxy-9,10-anthraquinone.
nucleic acid and lipids, and this interaction is an area of current research [3]. Much research has been carried out in order to elucidate the interaction mechanism, and in particular resonance Raman spectroscopy has been widely used because of its selectivity which permits the observation of only bands due to the vibrations of the chromophoric framework [4, 5, 6, 7]. Vibrational and electronic spectra of the 1,4-(OH)2AQ chromophore exhibit significant changes upon interaction with receptors and therefore a detailed spectroscopic analysis of this molecule is necessary in order to obtain structural information on the interaction.

PREVIOUS STUDIES AND THIS WORK- 1,4-(OH)2AQ (quinizarin) has been extensively studied previously. There are fluorescence spectra in Shpol'skii matrices (8, 9, 10, 11), electronic spectra and solvent effects (12, 13), resonance Raman spectra [14, 15, 16], infrared spectra of a polycrystalline film, solution in CCl4 and gas [14, 17, 18], normal mode calculation of planar and non-planar vibrations [8, 17, 19], and carbon-13 nuclear magnetic resonance spectra in DMSO-d6 of the 1,4-(OH)2AQ [20]. Also, photochemical hole burning spectra [21], ab initio quantum mechanical characterization of the electronic states [22], Stark effect on the S0→S1 transition [23] and luminescence studies in supersonic beams of the 1,4-(OH)2AQ [11, 24]. Thought it had been reported that quinizarin existed in two crystalline polymorphic forms [25], it was not recognized that these two polymorphic forms have very distinct spectral properties [16]. Moreover, one of the two polymorphic forms crystallizes in space group P_C (form II), is a noncentrosymmetric crystal capable of doubling the frequency of a laser beam (second harmonic generation (SHG)). On the contrary, another form (form I) crystallizes in space group P2_1/c, which is a
centrosymmetric crystal without SHG ability [26]. We report here the electronic, FT-IR, resonance Raman, powder x-ray diffraction spectra and normal coordinate analysis (done by H. Hatfield in this group) of 1,4-(OH)₂AQ in its polymorphic forms and provide an explanation for the differences in the spectral properties between these two forms. Finally, we comment on the differences in the vibrational spectra between the free chromophore and that present in the drug adriamycin.

(b) THE INTERACTION BETWEEN ADRIAMYCIN AND CARDIOLIPIN

Goormaghtigh et al. had noted earlier that doxorubicin binds tightly to the phospholipid via electrostatic forces [27]. They had shown that there was a two-step mode of interaction. The first appeared to be the formation of an electrostatic complex. The second stage was the formation of an irreversible binding between cardiolipin and doxorubicin which required doxorubicin reduction. The reduction of the cardiolipin-doxorubicin complex occurred because the drug complex was able to insert itself into the mitochondrial electron transport chain between NADH-CoQ oxidoreductase and CoQ-cytochrome c oxidoreductase. Also, the chloroform extract of the doxorubicin-exposed mitochondria exhibited new bands in the infrared spectra which in their opinion are best explained by formation of a covalent bond between doxorubicin and cardiolipin [28].

Ruysschaert et al. [29] using attenuated total reflection infrared spectroscopy found a small but significant increase in the ordering of the cardiolipin hydrocarbon chains upon interaction with adriamycin. They
concluded that there is no penetration of the adriamycin molecule into the hydrocarbon chain region of the bilayers and adriamycin neutralizes the negative charges of the cardiolipin polar head groups, allowing a closer packing of the molecules. And, it is likely that the positively charged amino group of the adriamycin interacts electrostatically with the negatively charged phosphate groups of the cardiolipin. Moreover, dichroism values indicate a slight reorientation of the fatty acyl C=O bond toward a normal to the membrane plane upon interaction with adriamycin. It could be related to an overall straightening of the hydrocarbon chains or merely induced by a specific interaction with some parts of the adriamycin molecule. The IR spectra also reveal important modifications of the hydrogen bonds of adriamycin owing to the interaction between adriamycin and cardiolipin.

On the contrary, Farnier-Suillerot et al. had shown that in the presence of cardiolipin-containing small unilamellar vesicles, the antitumor compound adriamycin loses its ability to catalyze the flow of electrons from NADH to molecular oxygen through NADH dehydrogenase. So, this strongly suggests that in the presence of cardiolipin the dihydroxyanthraquinone moiety is embedded in the phospholipid bilayer and thus inaccessible to the enzyme [30]. They suggest that there are two types of binding can take place between adriamycin and cardiolipin containing vesicles. The first type (I) involves the fixation of the amino group of the sugar residue of adriamycin to the ionized phosphate residue of cardiolipin with the dihydroxyanthraquinone moiety lying outside the bilayer. The second type (II) involves also the fixation of adriamycin to cardiolipin through interaction on the amino group with the phosphate residue but, in addition, the dihydroxyanthraquinone is embedded in the
bilayer. When the molar ratio of adriamycin to cardiolipin is high the binding site I is predominant but when this molar ratio decreases the binding site II then becomes predominant.

THIS STUDY- In this study, we report the resonance Raman spectra of adriamycin, adriamycin mixed with dipalmitoylphosphatidylincholine (DPPC; molar ratio 1:10), adriamycin mixed with cardiolipin (1:1), adriamycin mixed with cardiolipin (1:10) and adriamycin embedded in DPPC vesicles. Also, FT-IR spectra of cardiolipin (solid form) and cardiolipin-adriamycin complex (solid form) are reported. Form these data, we describe the mode of interaction between adriamycin and cardiolipin.

2-2 EXPERIMENTAL

1,4-(OH)₂AQ was obtained either from Aldrich (red-orange) or Fluka (dark red). The purity of the chemical from both these source was > 99.9% as confirmed by mass spectrometry analysis at the Ohio State University Chemical Instrumentation Center, on a Kratos MS-30 mass spectrometer. X-ray diffraction studies indicated that the Aldrich chemical was purely of Form I whereas the Fluka compound was a mixture of forms I and II. Recrystallization of Form I from acetone at 13-20 °C will produce large portion of Form II (> 70%). Form II of this material is the unstable form and converts to Form I readily at high temperatures (> 100 °C), and some crystals revert to Form I at room temperature. We could not achieve the crystallization of pure form II by any means, either by changing temperature or organic solvent (benzene, acetic acid, acetone and mixture of above solvents), since x-ray diffraction always showed some contamination with Form I. The optical spectroscopic experiments on Form II were carried out on
the Fluka compound. We estimate from the infrared and Raman intensities of
the ca. 1400 cm\(^{-1}\) band that the amount of Form I in these samples was less
than 20\%. Pure Form I was easily obtained by heating the crystals of Form II to
170\(^{\circ}\) C and rapid cooling. The pure resonance Raman and FTIR spectra of Form
II were obtained by subtracting the portion contributed from Form I from the
spectra of the mixture.

Adriamycin was obtained from Adria Laboratories and used as such. The
cardioliopin (bovine heart; eighty-seven percent of the cardioliopin chains has cis
double bonds in the 9-10 and 12-13 positions of each of the lipid chains) and
DPPC were received from Avanti Polar Lipids and used as such. Lipids were
dissolved in chloroform and then the solvent was evaporated to dryness under a
nitrogen stream and the lipid film so obtained was further dried under vacuum
for 3 hr. to remove traces of solvent. The lipids multilayers were prepared by
mechanical stirring (vortex mixing) of the lipid film with triple-distilled water for
10-20 min. with occasional heating to 45-50\(^{\circ}\) C. The bilayers of approx. 250-
300 Å in diameter [31] were prepared by sonicating samples of DPPC and
cardioliopin multilayers in a regular Branson ultrasonic bath until optical clarity
was obtained. The adriamycin embedded DPPC bilayers were prepared by using
the above procedure, except that the lipids and the adriamycin were dissolved
in ethanol at the first step.

Powder x-ray diffraction patterns were obtained using a Rigaku
Geigerflex D/Max-3B diffractometer with nickel filtered Cu K\(_{\alpha}\) source. X-rays are
irradiated from the Cu filament at 35 KV and 25 mA. Diffracted x-rays are
collected by scintillation counter, SC-30, with both divergence and scatter slits
of 0.6 degree and 1 degree for the receiving slit. The measurement is taken at a scan speed of 2 degrees per minute with 0.02 degree per sampling step. The solid samples for the Raman spectra, electronic spectra and infrared spectra were prepared by grinding 1-2 mg of the sample with 300 mg KBr. The IR spectroscopic experiments were carried out on a Mattson Cygnus 25 FT-IR spectrometer with a spectral resolution of 1 cm\(^{-1}\). The Fourier transforms were taken after 100 scans. Spectra were calibrated against polystyrene and carbon dioxide. The Raman spectra of the various samples were obtained by excitation with a Spectra-Physics Model 171 Ar\(^+\) laser which provides lines at 514.5, 501.7, 496.5, 488, 476.5, 472.7, 465.8 and 457.9 nm, a large frame Kr\(^+\) laser (Coherent K100) which provides lines at 676.4, 647.1, 568.2, 530.9, 520.8, 413.1, 406.7 and 350.7 nm, a Spectra-Physics Model 375 dye laser (Rhodamine 6G) which provides lines from 560-650 nm and the scattered light was dispersed with a Spex 1403 double monochrometer and detected with a RCA C31034 photomultiplier. The power at the sample was of the order of 6-10 mW. Data were collected on a Spex Datamate computer. All samples were prepared anaerobically and spun while recording the spectra to avoid decomposition. Sodium sulfate had been added for the Raman excitation profile experiment as an internal standard. Slit widths were of the order of 6 cm\(^{-1}\) and collection timed varied from 1 to 3 s/wavenumber.

The solution Raman spectra (in methanol, ethanol and acetone) of 1,4-(OH)\(_2\)AQ was obtained by quenching the fluorescence with saturated KI. The fluorescence interference in the solid state and in the deprotonated forms of 1,4-(OH)\(_2\)AQ was minimal and Raman spectra were obtained directly on these samples. The low temperature electronic spectra were obtained with the help of
a liquid nitrogen Dewar vessel using a Cary 17D UV-visible spectrometer (thanks for the help of Professor B. Bursten in obtaining the low-temperature electronic spectra). Room temperature electronic spectra were recorded on a Pye-Unicam 8-500 UV-visible spectrophotometer.

2-3 RESULTS AND DISCUSSION

(a) STUDIES OF MODEL CHROMOPHORE 1,4-(OH)2AQ POLYMORPHIC FORMS

X-RAY POWDER DIFFRACTION- The two polymorphic forms of 1,4-(OH)2AQ crystallize in space groups P2₁/c (Form I) and Pc (Form II) [25]. Crystal data for Form I and Form II are listed in Table 2.1. These forms can be readily distinguished by

Table 2.1. Crystal data for Form I and Form II of 1,4-(OH)₂AQ [37].

<table>
<thead>
<tr>
<th></th>
<th>Form I</th>
<th>Form II</th>
</tr>
</thead>
<tbody>
<tr>
<td>formula</td>
<td>C₁₄H₈O₄</td>
<td>C₁₄H₈O₄</td>
</tr>
<tr>
<td>M. W.</td>
<td>240.22</td>
<td>240.22</td>
</tr>
<tr>
<td>M. p.</td>
<td>195 °C</td>
<td></td>
</tr>
<tr>
<td>Crystal system</td>
<td>monoclinic</td>
<td>monoclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁/c</td>
<td>Pc</td>
</tr>
<tr>
<td>a</td>
<td>10.40 Å</td>
<td>3.77 Å</td>
</tr>
<tr>
<td>b</td>
<td>6.04 Å</td>
<td>9.49 Å</td>
</tr>
<tr>
<td>c</td>
<td>16.65 Å</td>
<td>15.70 Å</td>
</tr>
<tr>
<td>β</td>
<td>95.2°</td>
<td>94°</td>
</tr>
<tr>
<td>Z</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>D*</td>
<td>1.52</td>
<td>1.5</td>
</tr>
<tr>
<td>D**</td>
<td>1.53</td>
<td>1.51</td>
</tr>
</tbody>
</table>

D* The measured density; D** the x-ray calculated density; Z molecules per unit cell.
Figure 2.2 X-ray diffraction patterns of the two crystalline polymorphic forms of 1,4(OH)₂-anthraquinone (a) Form I and (b) Forms I & II.
their powder x-ray patterns. The prominent d spacing values for Form I are at 8.42, 4.91, 3.49 and 3.13 Å, whereas those for Form II are at 9.63, 8.08, 7.48, 5.89, 3.37 and 3.29 Å (Figure 2.2). Form II is readily converted to Form I by increases in temperature or pressure. At 150 °C or pressures of 5 x 10^4 lb in^-2, complete conversion to Form I is observed within 30 min.

Spectroscopic experiments in the past have always used recrystallization from glacial acetic acid and sublimation as a purification step and have therefore investigated Form I exclusively [14]. From the single crystal data on Form I, it appears that there are four molecules per unit cell with both intramolecular and intermolecular hydrogen bonds (Figure 2.3) [32].

Figure 2.3 Structure of 1,4-(OH)₂ AQ (a) Form I (b) Form II.

The molecules form both asymmetric intramolecular and intermolecular hydrogen bonds. There are remarkably short intermolecular approach distances of 2.644 Å and 2.778 Å between neighboring oxygen atoms. From the single
crystal data on Form I [25] and on Form II of similar compound 2,3-dichloroquinizarin [26], it appears that there are two molecules per unit cell with intramolecular hydrogen bonds (Figure 2.3). A major difference in the packings of the two polymorphic forms is that Form I is centrosymmetric and Form II is noncentrosymmetric. According to equation (2-1), where \( w \) is the frequency of the incident beam; \( P \) is the nonlinear polarization which determines the intensity of the second harmonic beam generated; \( E \) is the electric field of the incident beam, noncentrosymmetry is a prerequisite condition for nonvanishing \( d_{ijk} \) (nonlinear susceptibility or quadratic hyperpolarizability tensor of rank 3). This follows from the requirement that in a centrosymmetric crystal a reversal of the signs of \( E_j \) and \( E_K \) must cause a reversal in the sign of \( P_i \) and not affect the amplitude (33, 34). Another simple explanation is that the quadratic hyperpolarizability transforms as cubic functions of the vector components \( x, y \) and \( z \), and is therefore always zero for systems having inversion symmetry.

\[
P^{2w}_i = d^{2w}_{ijk} E^w_j E^w_k \quad (2-1)
\]

ELECTRONIC SPECTROSCOPY- Figure 2-4 shows the visible spectra of Forms I and II at 77 K and at room temperature. The room temperature absorption spectra of both forms are similar, with little fine structure. The lowering of temperature produces a dramatic difference, especially for Form I. The room temperature absorption spectrum of 1,4-(OH)₂AQ in hexane also shows a structured envelope with reported maxima at 19380 (516 nm; 0-0), 19881 (503 nm), 20387 (490.5 nm), 20704 (483 nm), 21231 (471 nm) and 21954 cm⁻¹ (455.5 nm) [14]. This electronic transition can be assigned to the intramolecular charge transfer transition from the C-OH group to the carbonyl.
Figure 2.4 Absorption spectra of 1,4(OH)$_2$-anthraquinone in its two polymorphic forms at (a) room temperature and (b) 77K.
group or anthraquinone nucleus [12]. Form II at 77 K shows a similar pattern, except that the spectrum appears red-shifted by about 1000 cm\(^{-1}\) with the solution spectrum. The maxima are at 18300 (546 nm), 18835 (531 nm), 19344 (517 nm), 19608 (510 nm), 20302 (492.6 nm) and 21390 cm\(^{-1}\) (467.5 nm). This shift in transition frequency must arise from solid-state effects which alter the ground-state and excited-state energies. However, the fact that the vibronic transitions in Form II and the solution follow a similar pattern must indicate that the electronic environment of the 1,4-(OH)\(_2\)AQ molecule in both these states is similar. In dilute solutions the molecules exist as intramolecularly hydrogen bonded species with little intermolecular interaction. It is suggested that similar structures exist in the crystals of Form II. In comparison, however, Form I exhibits a very different spectral pattern, indicating an alteration in the electronic environment. As evidenced from single crystal x-ray data, two 1,4-(OH)\(_2\)AQ molecules in Form I interact very strongly with one another through bifurcated hydrogen bonds. This allows very effective interaction between the transition dipoles of these molecules. The \(\pi\rightarrow\pi^*\) nature of this allowed electronic transition and an ab initio calculation [22] suggests that the transition dipoles lie in the molecular plane and this is indeed confirmed by polarized absorption measurement [35]. The in-phase arrangement of transition dipoles is attractive and leads to a lowering of energy (\(E^*\)), and the out-of-phase arrangement of transition dipoles is repulsive and causes a raising of the excited state energy (\(E^*\)) [36]. The exciton splitting energy (Davydov splitting), corresponding to the separation \(\Delta E = E^* - E^*\), is given by:

\[
\Delta E = \frac{2DG}{\hbar c R^3} \quad (3-2)
\]
where \( h \) is Planck constant, \( c \) is speed of light, \( D \) is the dipole strength, \( G = \cos \alpha \) (\( \alpha \) is the angle between the transition dipoles) and \( R \) is the intermolecular distance. Our interpretation of the spectrum of Form I in Figure 2.4 is based on transitions from the ground state to the doublet excited state. The 0-0 transitions appear at 17456 and 18181 cm\(^{-1}\) (\( \Delta E = 725 \) cm\(^{-1}\)) and the vibronic transitions are similarly split into 18668 and 19323 cm\(^{-1}\) (\( \Delta E = 455 \) cm\(^{-1}\)), 19971 and 20657 cm\(^{-1}\) (\( \Delta E = 686 \) cm\(^{-1}\)), 21118 and 21797 cm\(^{-1}\) (\( \Delta E = 679 \) cm\(^{-1}\)), 22512 and 23223 cm\(^{-1}\) (\( \Delta E = 711 \) cm\(^{-1}\)) and 23775 and 24390 cm\(^{-1}\) (\( \Delta E = 615 \) cm\(^{-1}\)). The vibronic levels, while not regularly spaced, lie between 1100 and 1400 cm\(^{-1}\), which is in contrast to both the solution spectra and absorption spectra of Form II. The intermolecular hydrogen bonding interactions must alter the normal modes involving C–OH and C=O groups and, hence, the different vibronic structure in Form I.

**VIBRATIONAL SPECTROSCOPY** - The mid-infrared spectra of both crystalline forms (Figure 2.5) are similar except for some changes in band positions and in band intensities. Namely, in Form I bands are observed at 790, 1212 and 1392 cm\(^{-1}\), whereas in Form II bands are observed at 768, 1220 and 1404 cm\(^{-1}\) and the intensities of the bands at 1273, 1069 and 1170 cm\(^{-1}\) have increased but the intensities of the bands at 1257, 1311 and 1147 cm\(^{-1}\) have decreased. The infrared spectra of 1,4-(OH)\(_2\)AQ in CCl\(_4\) or gas phase resembles that of form II [14]. In the resonance Raman spectra (Figure 2.6), a similar effect involving these bands except the 790 cm\(^{-1}\) band (not observed in Raman spectra) is observed together with changes in the intensities of the bands at 1225, 1239, 1303, 1333, and 1570 cm\(^{-1}\). The resonance Raman
Figure 2.5 Infrared spectra of 1,4(OH)₂-anthraquinone in its two polymorphic forms (a) Form II, (b) Forms I & II and (c) Form I.
Figure 2.6 Resonance Raman spectra of 1,4(OH)$_2$-anthraquinone in its two polymorphic forms (a) Form I and (b) Forms II. Excitation -457.9 nm. Laser power -10 mW. Slits -6 cm$^{-1}$. 
Figure 2.7 Resonance Raman spectrum of a $5 \times 10^{-4}$ M solution of 1,4(OH)$_2$-anthraquinone in (a) methanol and (b) acetone. Excitation -457.9 nm. Laser power -10 mW. Slits -6 cm$^{-1}$. 
spectrum of 1,4-(OH)₂AQ in methanol, acetone (Figure 2.7) and ethanol resembles that of Form II, with prominent bands at 1224, 1238 and 1402 cm⁻¹. The change in Raman intensities is most dramatic for the bands at 1225 and 1239 cm⁻¹ in Form II, since these bands are absent from the spectrum of Form I. The fact that 1239 cm⁻¹ band is present in both forms is confirmed by the infrared spectra, suggesting that its appearance in Form II is due purely to a resonance effect (excited-state effect) and not to any alteration in ground-state frequencies. This band has been assigned to the ring mode and which agrees with the deprotonated spectrum of 1,4-(OH)₂AQ (Figure 2.8) which shows a strong peak at 1238 cm⁻¹. However, the 1225 cm⁻¹ band comes from the shifting of the 1206 cm⁻¹ (1212 cm⁻¹ in infrared spectrum) in-plane O-H bending mode in form I which is very sensitive to hydrogen bonding. Such an effect is commonly observed in hydrogen bonding systems, e.g., the in-plane OH bending mode in the vapor phase occurs at 1346 cm⁻¹ and that in the liquid phase is at 1420 cm⁻¹ [37]. The Raman excitation profile shows different enhancement for these two peaks indicating that they are two different peaks and not a split peak (shown below). The similarity of the electronic, infrared and resonance Raman spectra of Form II with 1,4-(OH)₂AQ in solution suggests that in this crystalline form the molecule exists as a intramolecularly hydrogen bonded species like a free molecule with little intermolecular interaction. This is also supported by the fluorescence spectrum of 1,4-(OH)₂AQ in supersonic beams and in heptane Shpol'skii matrices at 12K [24, 38]. The vibrational structure of the fluorescence envelope shows strong bands at 1235, 1243, 1267 and 1408 cm⁻¹ and, in general, resembles closely the resonance Raman spectrum of Form II shown in Figure 2.6. The change in the ground-state
Figure 2.8 Resonance Raman spectrum of $10^{-3}$ M 1,4(OH)$_2$-anthraquinone in an aqueous solution (pH 12). Excitation -457.9 nm. Laser power -10 mW. Slits -6 cm$^{-1}$. 
frequencies and the hypochromic effects (decrease in intensity of the band) observed in the resonance Raman spectrum of Form I arises from the intermolecular hydrogen bonds and the coupling of the transition dipoles, respectively. Changes in Raman intensities through the latter effect are well recognized in the spectra of stacked base pairs in nucleic acids and may be as large as 40% [38]. The effect of exciton interaction and dispersion force interaction on the oscillator strength can account for the observed hypochromism. What is novel about the 1,4-(OH)$_2$AQ molecule in Form I is the magnitude of this effect, as manifested by the disappearance of the bands at 1239 cm$^{-1}$. The intermolecular hydrogen bonding interaction between adjacent molecules, which makes the transition moment axes rigidly positioned and closely spaced, leads to strong hypochromic effects. Based on the Raman and infrared spectroscopic data of Form I and polarized infrared spectroscopic data of Form I and its deuterated derivative [14], the band at ca. 790 cm$^{-1}$ (not observed in Raman spectra) has been assigned to an out-of-plane O–H bending mode, that at 1206 cm$^{-1}$ to an in-plane O–H bending mode, that at 1397 cm$^{-1}$ to a ring mode. The reason for the shifting of the 790 and 1206 cm$^{-1}$ in Form I to 768 and 1225 cm$^{-1}$ in Form II could be the increase in hydrogen bonding in Form II and in turn the in-plane O–H bond is more difficult to deform [39]. However, the out-of-plane O–H bond is more difficult to deform in Form I since the hydrogen is held tightly in the plane by intermolecular hydrogen bonding. The 1397 cm$^{-1}$ band which has been assigned to a ring mode is believed to have considerable O–H bending character, and shifts to higher frequency in Form II.

**RAMAN EXCITATION PROFILE** - Raman excitation profile (REP), the dependence of the Raman intensity on the wavelength of exciting light, has
Figure 2.9 Resonance Raman spectra of 1,4(OH)₂-anthraquinone (Form I; 50%) in Na₂SO₄ at (a) 413.1 nm, (b) 457.9 nm and (c) 647.1 nm. Band marked with an asterisk is due to the internal standard SO₄²⁻. Laser power -10 mW. Slits -6 cm⁻¹.
Figure 2.10 Resonance Raman spectra of 1,4(OH)$_2$-anthraquinone (Form II; 50\%) in Na$_2$SO$_4$ at (a) 413.1 nm, (b) 457.9 nm and (c) 647.1 nm. Laser power -10 mW. Slits -6 cm$^{-1}$.
been performed on both forms from both long wavelength side (676-611 nm) and short wavelength side (350.7-488 nm) (Figures 2.9 and 2.10) of the first electronic transition. The set of 25 A₁ normal modes and their assignments are listed in Table 2.2 (done by Mr. H. Hatfield).

Table 2.2 The Observed and Calculated Frequencies for 1,4-(OH)₂AQ (done by Mr. H. Hatfield)

<table>
<thead>
<tr>
<th>Observed</th>
<th>Calculated</th>
<th>P.E.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>240 (cm⁻¹)</td>
<td>1405 - 1167</td>
<td></td>
</tr>
<tr>
<td>319</td>
<td>304 (cm⁻¹)</td>
<td>ring mode</td>
</tr>
<tr>
<td>331</td>
<td>655 - 319</td>
<td></td>
</tr>
<tr>
<td>370</td>
<td>1235 - 876</td>
<td></td>
</tr>
<tr>
<td>410</td>
<td>1478 - 1067</td>
<td></td>
</tr>
<tr>
<td>419</td>
<td>369</td>
<td>ring mode</td>
</tr>
<tr>
<td>440</td>
<td>442</td>
<td>70% δC-C-C; 26% vC=C</td>
</tr>
<tr>
<td>460</td>
<td>457</td>
<td>56% δC-C-C; 20% vC-O; 14% vC=C</td>
</tr>
<tr>
<td>502</td>
<td>1561 - 1067</td>
<td></td>
</tr>
<tr>
<td>526</td>
<td>541</td>
<td>40% vC=C; 27% δC-O; 14% δC-C-C</td>
</tr>
<tr>
<td>587</td>
<td>876 - 319</td>
<td></td>
</tr>
<tr>
<td>655</td>
<td>675</td>
<td>67% δC-C-C; 24% vC=C</td>
</tr>
<tr>
<td>876</td>
<td>912</td>
<td>57% vC=C; 17% δC=O</td>
</tr>
<tr>
<td>1031</td>
<td>2x526</td>
<td></td>
</tr>
<tr>
<td>1067</td>
<td>1053</td>
<td>72% vC=C; 9% δC-H</td>
</tr>
<tr>
<td>1145</td>
<td>460+655</td>
<td></td>
</tr>
<tr>
<td>1167</td>
<td>1140</td>
<td>36% vC=C; 12% δC-H; 9% δC-H</td>
</tr>
<tr>
<td>1206 (Form I)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1223</td>
<td>1229</td>
<td>56% δC-H; 33% vC=C</td>
</tr>
<tr>
<td>1235</td>
<td>1244</td>
<td>58% vC=C; 42% δO-H</td>
</tr>
<tr>
<td>1265</td>
<td>1274</td>
<td>60% δC-H; 32% vC=C</td>
</tr>
<tr>
<td>1305</td>
<td>419+876</td>
<td></td>
</tr>
<tr>
<td>1319</td>
<td>440+873</td>
<td></td>
</tr>
<tr>
<td>1330</td>
<td>1331</td>
<td>60% vC=C; 24% δC=O; 16% δC-H</td>
</tr>
<tr>
<td>1343</td>
<td>1348</td>
<td>65% vC=C; 29% vC-O</td>
</tr>
</tbody>
</table>
Unfortunately, wavelengths between 488 and 611 nm cannot be used as excitation sources because of strong fluorescence interference. By exciting in the 600 nm region the 457 cm\(^{-1}\) band gets strongly enhanced because the laser line is approaching 457 cm\(^{-1}\) vibronic peak. Also, the peaks at 317 and 524 cm\(^{-1}\) show up as strong peaks (very weak peak in other spectra) which provides evidence for it to be regarded as a fundamental mode. By exciting with the 350.7 nm line (Figure 2.11) the 1660 cm\(^{-1}\) (in Form I and at 1627 cm\(^{-1}\) in Form II due to increase hydrogen bonding) C=O stretching mode gets strongly enhanced because the laser line is approaching the second electronic transition at 320 nm. Also, the peak at 1035 cm\(^{-1}\) shows up as a strong peak (very weak peak in other spectra) which provides evidence for it to be regarded as a fundamental mode. Finally, Raman excitation profile shows different enhancement for the peaks at 1225 and 1235 cm\(^{-1}\) in Form II indicating that they are two different peaks and not split peaks.
Figure 2.11  Resonance Raman spectra of 1,4(OH)$_2$-anthraquinone in its two polymorphic forms (a) Form I and (b) Forms II. Excitation -350.7 nm. Laser power -10 mW. Slits -6 cm$^{-1}$. 
1,4-(OH)<sub>2</sub> AQ AS A MODEL FOR ADRIAMYCIN- There is much interest in the use of vibrational spectra of adriamycin as a tool for monitoring drug-receptor interactions [40, 41]. 1,4-(OH)<sub>2</sub> AQ forms the chromophoric part of this drug and is a good model in terms of UV-visible and resonance Raman spectra. However, since the resonance Raman spectrum was only available for Form I and differed considerably from that of the drug, comparisons could not be made. On the other hand, the spectrum of Form II resembles that of the drug in both intensities and frequencies and can serve as a useful model. The major differences lie in the 1400 - 1500 cm<sup>-1</sup> region and 800 - 1000 cm<sup>-1</sup> region, with three bands in adriamycin at 1418 (m), 1443 (s) and 1447 cm<sup>-1</sup> (m-w), whereas in Form II bands are observed at 1407 (s), 1450 (w) and 1480 cm<sup>-1</sup> (w). Based on the intensity of the strongest resonance enhanced band and the similar origin of the electronic bands, we suggest that the 1407 cm<sup>-1</sup> band in Form II (C-OH stretching mode) corresponds to the 1443 cm<sup>-1</sup> band of adriamycin. Such a correlation would also explain the sensitivity of the 1443 cm<sup>-1</sup> towards deprotonation [42]. Figure 2.8 shows the resonance Raman spectrum of 1,4-(OH)<sub>2</sub> AQ with two of the phenolic protons deprotonated and closely resembles the completely deprotonated spectrum of adriamycin in both frequencies and intensities [42]. The strong band at 1407 cm<sup>-1</sup> in 1,4-(OH)<sub>2</sub> AQ assigned to C-OH stretch shifts to 1438 cm<sup>-1</sup> [43] in adriamycin in the solid state and to 1443 cm<sup>-1</sup> in adriamycin in an aqueous medium reflects an increase in the strength of the intramolecular hydrogen bond or the formation of stronger hydrogen bonds with water. This band further shifts to 1450 cm<sup>-1</sup> on intercalation of adriamycin into DNA, suggesting further changes in the hydrogen bonding of the hydroxy group with the base pair of the DNA.
2-4 THE INTERACTION BETWEEN ADRIAMYCIN AND CARDIOLIPIN

The resonance Raman spectra of adriamycin mixed with DPPC vesicles (1:10), cardiolipin vesicles (1:1), and (1:10) and embedded in DPPC vesicles are presented in Figure 2.12. In addition, the curve deconvolution results of these spectra in the region 1357-1522 cm\(^{-1}\) are presented also in Figure 2.12. Straight base line, Gaussian curve, three components and curvefit method have been used in doing the curve deconvolution. According to the adriamycin Raman spectra, there are three peaks in the region 1357-1522 cm\(^{-1}\) which are 1418, 1443 and 1477 cm\(^{-1}\), and this is the reason for choosing three peaks in conducting curve deconvolution. The straight base line has been chosen by carefully examination of these spectra. The results show that among these three curve deconvoluted peaks (1420, 1438 and 1459 cm\(^{-1}\)) and the band at 1459 cm\(^{-1}\) is sensitive to the interaction. Adriamycin interacting with DPPC vesicles does not change the resonance Raman spectra of the adriamycin, but when adriamycin interacts with cardiolipin vesicles or adriamycin embedded in DPPC vesicles, the 1459 cm\(^{-1}\) peak increases in intensity. This means that the interactions between cardiolipin and adriamycin or adriamycin embedded in DPPC has perturbed the chromophore of the adriamycin (increasing the intensity of the ring stretching mode 1459 cm\(^{-1}\) [43]), and it is an excited state effect. Moreover, since adriamycin interacting with cardiolipin shows the same effect as adriamycin embedded in DPPC and the hydrophobic tail of both lipids is very similar, we conclude that the dihydroxyanthraquinone moiety of the adriamycin is embedded in the phospholipid bilayer as shown by Garnier-Suillerot [29]. The increases in the intensity of the ring stretching mode 1459
Figure 2.12 Curve deconvolution in the region 1357 - 1522 cm$^{-1}$ of the resonance Raman spectra of adriamycin (a) mixed with DPPC vesicles (1 : 10), (b) mixed with cardiolipin vesicles (1 : 1), (c) mixed with cardiolipin vesicles (1 : 10) and (d) embedded in DPPC vesicles. (5 mM Tris buffer, pH = 7.4, drug concentration = .25 mM, 457.9 nm, dotted lines = experimental data, solid lines = simulated spectra).
cm\(^{-1}\) is due to the interactions between the dihydroxyanthraquinone moiety of the adriamycin and the hydrophobic tail of the lipids.

To further prove this conclusion, we have studied the adriamycin and cardiolipin complex by FT-IR in the C–H stretching mode region (2800-3100 cm\(^{-1}\)), and the results are shown in Figure 2.13. The band assignments are as follows: the peak at 2853 cm\(^{-1}\) arises from the CH\(_2\) symmetrical stretch, the peak at 2923 cm\(^{-1}\) arises from the CH\(_2\) asymmetrical stretch, the peak at 2890 cm\(^{-1}\) arises from the CH\(_3\) symmetrical stretch, the peak at 2955 cm\(^{-1}\) arises from the CH\(_3\) asymmetrical stretch and the peak at 3008 cm\(^{-1}\) arises from the C–H stretch of the double bond moieties [44]. The 3008 cm\(^{-1}\) peak is considerably reduced in intensity when cardiolipin interacts with adriamycin, compared to its intensity in the pure cardiolipin, and may be shifted. The decrease in the acyl chain double bond C–H stretching mode intensity at 3008 cm\(^{-1}\) in the complex demonstrates the involvement and possibly a change in the surrounding environment of the cis double bonds at the 9-10 and 11-12 positions of the lipid chains. The participation of these regions of the lipid chains in complex formation strongly suggests that this lipid binding involves substantial conformational changes within the acyl chains and that the binding consists of more than a simple attraction of the negatively charged lipid head groups with the positively charged amino group of adriamycin.

2-5 CONCLUSIONS
Figure 2.13 Infrared spectra of cardiolipin (a) and cardiolipin-adriamycin complex (b).
Resonance Raman spectroscopy has been used to probe the interaction between adriamycin and cardiolipin. The model compound 1,4-dihydroxyanthraquinone also has been investigated by optical spectroscopy in order to provide useful data for understanding the spectroscopic properties of adriamycin. It was found that the vibrational and electronic spectra and diffraction patterns of the two polymorphic forms of 1,4-(OH)$_2$AQ show significant differences, arising mainly from the nature of inter- and intramolecular hydrogen bonding interactions. Moreover, Form II of the two polymorphic forms of 1,4-dihydroxyanthraquinone is a better model for adriamycin than Form I. Upon binding to cardiolipin, the ring stretching 1445 cm$^{-1}$ peak (Raman spectra) of the adriamycin gets enhanced in intensity and the 3005 cm$^{-1}$ peak (infrared spectra), which is the acyl chain double bond C–H stretch, of the cardiolipin decreases in intensity and may be shifted. This indicates that the binding between adriamycin and cardiolipin involves substantial conformational changes within the acyl chains and that the binding consists of more than a simple attraction of the negatively charged lipid head groups with the positively charged amino group of adriamycin.
REFERENCES


34. Y. Amnon, Quantum Electronics, John Willey & Sons, New York (1975).


CHAPTER III

THE ANTITUMOR DRUG MITOXANTRONE

3 - 1 INTRODUCTION

1,4-Dihydroxy-5,8-bis [[2-[(2-hydroxyethyl) amino ] ethyl ] amino-9,10-anthracenedione (mitoxantrone; Figure 3.1) belongs to the family of substituted anthraquinones and is an effective antitumor drug presently undergoing clinical trials [1, 2, 3].

![Figure 3.1 Mitoxantrone](image)

This drug will bind to DNA very strongly ($K_D = 6 \times 10^6 \text{ M}^{-1}$) and considerable interest exists in understanding the nature of drug-nucleic acid interaction and
its role in antitumor activities [4, 5, 6]. Various forms of optical spectra of the
drug provide excellent probes for monitoring its interaction with its
environment. Optical spectroscopy of mitoxantrone has been mostly used in the
literature in a bioanalytical fashion. Mitoxantrone is characterized by a visible
absorption band, changes in which has been used to study the aggregation of
the drug and its interaction with nucleic acids [7, 8]. Kapuscinski et al. has
shown that when mitoxantrone interacts with single-stranded poly (rC) and poly
(rU) the spectra display hypochromic effect and a small (1-3 nm) red shift for this
visible absorption band. Also, as opposed to double-stranded natural DNAs, no
evidence of a significant change in the intensity pattern of the absorption
spectra is apparent and no red shift was observed. These results indicated that
when mitoxantrone interacts with single-stranded poly (rC) and poly (rU) the
states of aggregation is unperturbed, the environment that surround the
mitoxantrone doesn't change, but with double-stranded natural DNAs
mitoxantrone exists as a monomer and the hypochromic effect is due to
interaction between mitoxantrone and the base pair.

Fluorescence excitation and emission spectra of the drug as a function of
pH and in the presence of DNA have been reported [9]. This study analyzes the
absorption and emission spectra of mitoxantrone, including solvent and
centration effects. Lown et al. [10] from NMR studies of the 1 : 1
intercalation complex of the mitoxantrone and the duplex
d[C(1)pG(2)pC(3)pG(4)] have suggested that the chromophore, approximately
perpendicular to the base pair axis, is in the center of two base-pairs with
protrusion of the periphery of the chromophore beyond the area of the adjacent
base pairs. Moreover, the terminal OH groups of the side chains bind to the
central phosphate groups such that the methylene groups are adjacent to C (1) 6, C (3) 6 and G (2) 8 base protons all of which are in the major groove. In addition, the base proton C (3) 5 is not spatially close to any drug proton. This model indicated that strong interaction occurs between the base pair and central ring of the chromophore.

In the present study, the resonance Raman spectra of mitoxantrone are reported for the first time and vibrational assignments with the help of normal coordinate calculation of the model compound 1,4-diaminoanthraquinone (1,4-(NH₂)₂AQ) are proposed. Based on these spectroscopic studies, the structure of the drug - nucleic acids complexes is also discussed.

3–2 EXPERIMENTAL

1,4-dihydroxyanthraquinone was obtained either from Aldrich or Fluka and 1,4-diaminoanthraquinone was obtained from Aldrich and used as such. Mitoxantrone and calf thymus DNA were obtained from National Cancer Institute and Sigma Chemical Co., respectively and used without further purification. Aqueous solutions of DNA and mitoxantrone were prepared in solutions of 1 x 10⁻² NaCl, 0.1 M NaNO₃ and 5 x 10⁻³ M Tris buffer (pH = 7). In the solution of the mitoxantrone-DNA complex the ratio of nucleotide to mitoxantrone was 30:1.

The electronic spectra of mitoxantrone in various solvents and the mitoxantrone - DNA complex were recorded with a Shimadzu UV-265 spectrometer interfaced to an IBM computer.
The fluorescence spectra of mitoxantrone in different organic solvents and in aqueous solution were obtained on the experimental apparatus used for Raman spectroscopy (described below) with slit widths of ~ 6 cm\(^{-1}\) (457.9 nm exciting line). The exciting line was chosen at the blue end of the absorption band to ensure that only a small fraction of the exciting light is absorbed by the system and the linearity in the fluorescence intensity is maintained. Fluorescence intensity was measured at the tail-end of the emission band (702 nm in this case) to avoid errors due to the reabsorption of the emission. Relative emission quantum yields (\(\phi_{rel}\)) were calculated from the relation \(\phi_{rel} = \text{constant} \times (F / A)\) where \(F\) and \(A\) are the fluorescence intensity at 702 nm and absorbance at 457.9 nm [11].

The Raman spectra of the various samples were obtained by excitation with Spectra-Physics Model 171 Ar\(^+\) or Coherent K100 Kr\(^+\) laser, and the scattered light was dispersed with a Spex 1403 (f number = 7.8) double monochromator and detected with a RCA C31034 Ga-As Photomultiplier. The power at the sample was of the order of ~ 10 mW. Slit widths were of the order of 6 cm\(^{-1}\). All the liquid samples were prepared anaerobically and sealed in NMR sample tubes and spun while recording the spectra to avoid decomposition. No changes in the absorption spectra were noted before and after the Raman experiment. For solid samples, KBr and NaNO\(_3\) or NaClO\(_4\) were mixed with the samples. The intensity measurements were performed using either NO\(_3^\text{-}\) (1070 cm\(^{-1}\) in the solid state (9%)) and 1052 cm\(^{-1}\) in aqueous solution (0.1 M)) or ClO\(_4^\text{-}\) (954 cm\(^{-1}\) in the solid) as the internal standard.
The Raman excitation profiles of the aqueous solutions of mitoxantrone and mitoxantrone-DNA complex were constructed using the 1052 cm\(^{-1}\) band of NaNO\(_3\) as the internal standard. The Raman intensities were corrected for the \(\nu^4\) scattering dependence using wavelength sensitivity curve of the detector and self-absorption using known path length and extinction coefficient.

All the spectra shown in this study were baseline corrected and the solutions were prepared right before the experiments.

3 - 3 RESULTS AND DISCUSSION

ABSORPTION SPECTRA OF MITOXANTRONE: Figure 3.2 shows the visible absorption spectrum of a micromolar solution of mitoxantrone in a series of solvents - water, methanol, acetone and chloroform of dielectric constants 80.4, 33.6, 20.7 and 4.8, respectively. The spectra are characterized by a progression of peaks, three of which can be readily distinguished from the figure. The spacings between the adjacent peaks in all of the solvents examined are between 1275 - 1300 cm\(^{-1}\). This constancy in a series of solvents of such differing polarities is a good indication that this absorption band represents a single electronic transition. The major influence of the solvent appears to be the shifting of the absorption peaks to higher wavelengths as the solvent polarity decreases e.g. the lowest energy transition occurs at 660, 668, 677, and 682 nm in H\(_2\)O, CH\(_3\)OH, CH\(_3\)COCH\(_3\) and CHCl\(_3\), respectively. Shifting of the same magnitude for the 0-1 vibronic bands (~ 610 nm) has been observed.
The electronic spectra of anthraquinones and substituted anthraquinones have been studied extensively, but no detailed discussion of the band assignments for mitoxantrone has been reported [12, 13, 14]. In the ultraviolet region, four bands are typically observed in all the solvents examined e.g. in aqueous solution, bands at 225, 241, 280 and 380 nm are observed. The absorption spectrum of the anthraquinone chromophore is characterized by four $\pi \rightarrow \pi^*$ bands which have the same magnitude of extinction coefficient as in mitoxantrone in the ultraviolet, and the above mentioned bands in this region for mitoxantrone are therefore readily assigned to the same bands [12]. Namely, the bands at 380 and 241 nm are assigned to the benzenoid chromophore (polarized along the C$_2$ axis of the molecule) and the bands at 280 and 225 nm are assigned to quinoid chromophore (polarized along the C=O axis). It is well established that a fifth new band, usually in the visible region is observed upon substitution on the anthraquinone ring by electron donating substituents such as amino, hydroxyl and alkoxy groups [13, 14]. Several interpretations exist as to the origin of this band. These include an interchange in the positions of $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ bands of anthraquinone due to intramolecular H-bonding [15, 16], charge transfer from the substituent to the carbonyl group or to the ring or to the intramolecularly H-bonded carbonyl chelate ring [12, 13, 14, 17, 18]. Ab-initio calculations of 1,4-(NH$_2$)$_2$- and 1,4-(OH)$_2$-anthraquinone suggest that the principal effect of substitution is to extend the conjugation, leading to a substituent induced $\pi \rightarrow \pi^*$ transition in the visible region. In mitoxantrone, both amino and hydroxyl groups are present, and the band at ~680 nm is clearly linked to the presence of these substituents. However, the general pattern of this band, including the 1250 - 1300 cm$^{-1}$ spacing between
Figure 3.2 Absorption spectrum of a $1 \times 10^{-6}$ M solution of mitoxantrone in (a) water, (b) methanol, (c) acetone and (d) chloroform.
the maxima bear a strong resemblance with the absorption spectra of 1,4-(NH$_2$)$_2$AQ, which occurs at ~ 590 nm. In contrast, the absorption spectrum of 1,4-(OH)$_2$AQ exhibits a band at ~ 516 nm with five vibronic spacings at 501, 1007, 1324, 1851 and 2574 cm$^{-1}$[19]. An amino to ring charge transfer transition, with the 1250 -1300 cm$^{-1}$ band due to a C-N vibrational progression has been considered as an assignment for the visible band of 1,4-(NH$_2$)$_2$anthraquinone [20]. As will be shown later, this assignment is supported by the resonance Raman studies. The visible band of mitoxantrone, by analogy is also assigned to an amino (N 1, N 4) to ring charge transfer transition with the red shift in the absorption band arising due to two effects. The first is the extended conjugation due to the phenolic groups. A comparison of the visible transitions of 1,4-(NH$_2$, NHCH$_3$)-anthraquinone (625 nm) and 1,4-(OH, NHCH$_3$)-anthraquinone (645 nm) also support the conjugation extending effect of the -OH as compared to the -NH$_2$ substituent [14]. Also, the substitution on the amino group (N 1, N 4), e.g. 1,4-(NHCH$_2$CH$_2$OH, NHCH$_2$CH$_2$OH) anthraquinone (650 nm), has been shown to shift the transition to lower energies, presumably due to increased electron donating ability [21]. The decrease in intensity and a red shift of this band upon increasing the H$^+$ concentration and subsequent protonation of the amino nitrogens (Figure 3.3) gives further support that the amino group is involved in the electronic transition.

As shown in Figure 3.2, the visible band of mitoxantrone exhibits a red shift in increasingly nonpolar solvents. Typically, monosubstituted anthraquinones, such as 1-NH$_2$AQ show significant blue shifts with increasing nonpolarity of the solvents [20]. This is due to an increase in dipole moment of
Figure 3.3 Absorption spectrum of a $1 \times 10^{-5}$ M solution of mitoxantrone in $[H^+] =$
(a) 5.7 M, (b) 3.2 M, (c) 2.0 M, (d) 0.8 M and (e) $1 \times 10^{-7}$ M
the excited state, thus leading to its stabilization by polar solvents. The solvent effect in 1,4-(NH$_2$)$_2$AQ is not as pronounced as in the monosubstituted derivative, but still exhibits a blue shift in going from hydroxylic to nonpolar solvents [14]. Ab-initio calculations of 1,4-(NH$_2$)$_2$AQ predict a significant increase in the dipole moment of the excited state (~ 5 D) corresponding to the visible transition, in agreement with the solvent effects on the spectrum [22]. However, another factor is the decreased ability of the amino nitrogen (N1, N4) for H-bonding to solvents in the excited state since the nature of the visible transition involves a loss of an electron from the amino substituent and this will cause a red shift. Because solvent induced H-bonding stabilization will only be present in the ground state, this leads to stabilization of this state by polar solvents. Figure 3.4 shows that when the polarity of the solvents is high enough (water + C$_2$H$_5$OH system in this case) the visible absorption bond exhibits a red shift when the polarity of the solvents increase and this is a compromise of the two effects mentioned above.

The opposite trend observed with mitoxantrone clearly indicates that the presence of -OH groups is having an effect. It is likely that strong intramolecular H-bonding between the carbonyl and phenolic groups would influence the polarity of the excited state, thus minimizing the effect of polar solvents. An estimate of the change in dipole moment between the ground and excited states can be made with the Lippert equation [23, 24, 25, 26], based on the knowledge of the
Figure 3.4 Absorption spectrum of a $1 \times 10^{-5}$ M solution of $1,4(\text{NH}_2)_2$-anthraquinone in (a) acetone, (b) methanol, (c) 80% methanol + 20% water (d) 50% methanol + 50% water and (e) 20% methanol + 80% water.
transition frequencies in the absorption and emission spectra. We have obtained both the emission and absorption spectra of mitoxantrone in a variety of solvents including water, methanol, acetone, n-propanol, dichloromethane, chloroform and bromoform (we did not consider any specific solvent effects). Assuming that mitoxantrone is spherical and the angle between the dipole moment of the ground and excited state is zero and using this data and a radius of 4.5 Å for the molecule, we calculate a change of 1.2 D in the excited state from the Lippert equation (which is experimentally simple and hence the most widely used; Figure 3.5). Namely,

\[-hc (\sigma_f - \sigma_a) = \text{Const.} + \left[ \frac{2(D-1)}{2D+1} - \frac{2(n^2 - 1)}{2n^2 + 1} \right] \frac{(\mu_e - \mu_g)^2}{a^3} \]

Where, D, n : dielectric constant and refractive index of the solvent, \(\sigma_f\); \(\sigma_a\) : the 0 - 0 bands of the absorption and emission, a : cavity radius in Onsager's theory of reaction field, \(\mu_e\), \(\mu_g\) : dipole moment in the excited and ground state, h : plank's constant, c : speed of light. This value is considerably smaller than that calculated for 1,4 - (NH₂)₂AO ( ~5 D), indicating that H bonding may play a role in decreasing the polarity of the excited state. Another factor contributing to the observed shift in nonpolar solvents could be the decreased ability of the amino nitrogen (N1, N4) to H-bond to solvents in the excited state which would cause the corresponding blue shift shown in Figure 3.2. Such effects have been well documented for \(n \rightarrow \pi^*\) transitions [27]. The difference
Figure 3.5 Plot of \(-hc(\sigma_f - \sigma_a)\) (see text) versus \(\left[\frac{2(D - 1) \cdot 2(n^2 - 1)}{2D + 1 \cdot 2n^2 + 1}\right]\) (see text).
between the lowest energy transitions in H$_2$O and CHCl$_3$ correspond to 1.39 kcal/mole, which would be the energy associated with the H-bond of the amino group in water.

Upon binding to double stranded nucleic acids, the absorption spectrum of mitoxantrone undergoes a red shift, and resembles the spectrum shown in Figure 3.1 for CHCl$_3$. Based on the above discussion, this would indicate that upon binding to DNA, the amino substituent on the anthraquinone ring is in an environment in which it is unable to H-bond with the solvent water molecules or DNA backbone. The accepted model of binding of the drug to the nucleic acid is through intercalation between the base pairs [4, 5, 6, 7, 8, 9, 10] which could result in shielding of the amino (N1, N4) substituents.

EMISSION SPECTROSCOPY - Figure 3.6 shows the emission spectrum of a 1 x 10$^{-4}$ M solution of mitoxantrone in water, methanol, acetone and CHCl$_3$. These spectra are characterized by a red shift with decreasing solvent polarity, in contrast to the generally observed blue shift [26]. This again illustrates that specific solvent effects are important in mitoxantrone. Also, the mirror image relation with the absorption spectrum is absent indicating a large change in the nuclear configuration between the ground and excited states of the molecule upon electronic excitation [28] and the vibrational spacing is of the order of ~ 1100 cm$^{-1}$. The low intensity of emission of the aqueous solution is due to aggregation effects and is discussed below.

An important factor in the blue shift of the emission in polar solvents is due to the stabilization of the ground state by H-bonding of the amino (N1, N4) group on the anthraquinone ring, similar to that observed in the absorption
Figure 3.6 Emission spectrum of a $1 \times 10^{-4}$ M solution of mitoxantrone in (a) water, (b) methanol, (c) acetone and (d) chloroform.
spectra. Upon binding to DNA, the emission spectrum of mitoxantrone exhibits a red shift, and resembles the spectrum taken in a CHCl₃ solution. This can also be explained by lack of H-bonding of the amino substituents on the anthraquinone ring.

AGGREGATION OF MITOXANTRONE - Both the absorption and emission spectra of mitoxantrone in aqueous solution depend on the drug concentration. Figure 3.7 compares the absorption spectrum of mitoxantrone at $5 \times 10^{-4}$ M with a $1 \times 10^{-6}$ M solution. The appearance of a new band at ~ 580 nm along with decrease of the 660 nm band is clearly evident. A dependence of the absorption spectrum on concentration has been noted before and assigned to aggregation effects that occur in aqueous solution [8]. These authors determined a dimerization constant ($K_2$) of $3 \times 10^4$ M$^{-1}$ by computer fitting a series of absorption spectra between concentration ranges of $4.9 \times 10^{-7}$ M to $1.4 \times 10^{-5}$ M, the variables in the fit being the extinction coefficients of the monomer and dimer [8]. They also calculated an absorption spectrum of the dimer, which was characterized by a strong band at ~ 580 nm and a weaker band at ~ 680 nm. These bands can be seen on Figure 3.3a. Such a change in electronic spectra upon dimerization is best understood by applying the molecular exciton theory [29, 30]. The transition moment dipoles responsible for the visible absorption band of the two monomers can interact electrostatically in the dimer leading to a splitting which depends on the oscillator strength of the monomer band and the geometry of the dimer, including the orientation of the transition dipoles and the distance between them [31, 32]. The two exciton energy levels in the dimer have either the transition dipoles in phase (leads to an electrostatic attraction) or out-of-phase
Figure 3.7 Absorption spectra of mitoxantrone in aqueous solution (a) $5 \times 10^{-4}$ M, (b) $1 \times 10^{-6}$ M (multiplied by a factor of 85) and (c) dimer, obtained by subtracting (b) from (a).
(causes an electrostatic repulsion). The fluorescence emission from such a dimeric molecule will be quenched, since rapid internal conversion to the lower forbidden excited state will occur [29]. Such is also the case with mitoxantrone. Figure 3.8 shows the emission spectra of aqueous solutions of mitoxantrone at concentrations of $1 \times 10^{-5}$ M to $5 \times 10^{-4}$ M excited with 457.9 nm radiation. The band at the left of the spectrum (551 nm) is due to the Raman scattering from the O–H stretch of water and is used as an internal standard. It is clear from Figure 3.8 that even though the concentration is increasing 50 fold, and the general appearance of the emission is unchanged, the emission intensity only increases by a factor of $\sim 5 \cdot 10^2$. At higher concentrations of the drug, increasing fractions of the dye are present as aggregates and the fluorescence from the monomeric form is proportionately decreased. Since the fluorescence intensity is a direct measure of the concentration of the monomer, it has been used to calculate the association constants of dyes, and we have adapted the same procedure for mitoxantrone [33, 34]. Figure 3.9 is a plot of the relative emission quantum yield ($\phi_{\text{rel}}$) which were calculated from the relation $\phi_{\text{rel}} = \text{constant} \cdot (F/A)$ at 702 nm standardized with respect to the water Raman band at 551 nm as a function of the total concentration of drug. The choice of the emission wavelength was dictated by the fact that minimum reabsorption occurs at this energy. The water Raman band is also at the tail end of the absorption band and no corrections for its reabsorption were made.

The solid line through the experimental points in Figure 3.9 represents the trend in the experimental data. The intercept of this line on the y-axis represents the relative emission quantum yield at infinite dilution ($\phi_0$). The uncertainty in these calculation arises from the number of data points and the
Figure 3.8 Emission spectra of aqueous solution mitoxantrone (a) $1 \times 10^{-5}$ M, (b) $1.2 \times 10^{-4}$ M, and (c) $5 \times 10^{-4}$ M. Excitation $-457.9$ nm. The peak of 551 nm is due to the Raman scattering of water.
Figure 3.9 Relative emission quantum yield ($\Phi_{\text{rel}}$) vs. total dye concentration (T).
large errors in the estimation of the fluorescence intensity at dilute solution. Using $\phi_{\text{ref}} / \phi_0 = m / T$, where 'm' and 'T' are the molar concentrations of monomer and total drug, a parameter $F_2 = (T - m) / m^2$ can be calculated. Since $T = m + 2 K_2 m^2 + 3 K_3 m^3 + \ldots \ldots \ldots$, the parameter $F_2 = (T - m) / m^2 = 2 K_2 + 3 K_3 m + \ldots \ldots \ldots$ Where $K_2$ and $K_3$ are the dimerization and trimerization association constants for mitoxantrone [33, 34]. Figure 3.10 is a plot of $F_2$ versus 'm'. From a linear fit ($r = 0.99$) at the low concentration range of the plot shown in Figure 3.6, we calculate $K_2 = 2.7 \times 10^4$ M$^{-1}$ and $K_3 = 7 \times 10^4$ M$^{-2}$. We estimate the errors of this method to be in the 20 - 30% range. The value for $K_2$ previously determined by the simulation of the absorption data was $3 \times 10^4$ M$^{-1}$ [8], in reasonable agreement with the present value derived from emission studies.

The exciton model can also provide information about the geometry of the dimer, if the absorption spectrum of the dimer can be resolved from the total spectrum [31, 32]. We approached this problem by assuming that the absorption spectrum at $10^{-6}$ M is only due to the monomeric form (actually, it is of the orders of $\sim 95\%$) and from the $K_2$ determined above, calculated the concentration of the monomer in a $5 \times 10^{-4}$ M solution of drug ($\sim 8.5 \times 10^{-5}$ M; Figure 3.7b). Figure 3.7c shows the absorption spectrum of the $5 \times 10^{-4}$ M solution after subtracting the contribution of the monomer as determined from $K_2$. Figure 3.7c, then, is the absorption spectrum of the dimer. The contribution of trimers and higher aggregates at $5 \times 10^{-4}$ M is small and was neglected. The energy difference in wave numbers between the two bands in
Figure 3.10 Plot of $F_2$ (see text) versus monomer concentration of mitoxantrone (m) calculated from Figure 3.9.
Figure 3.7c and the angle between the transition dipoles are derived as follows [29]:

The ground state wave-function of the dimer is given by

\[ \Psi_g = \Phi_1 \Phi_2 \]

Where \( \Phi_1 \) and \( \Phi_2 \) represent the ground state wave-function of molecule 1 and 2 (all wave-functions assumed real).

The Hamiltonian operator for the dimer is

\[ H = H_1 + H_2 + V_{12} \]

Where \( H_1 \), \( H_2 \) and \( V_{12} \) are the Hamiltonian operator for molecule 1, molecule 2 and the intermolecular interaction energy.

The energy of the ground state of the dimer is

\[ E_g = \int \int \Psi_g^* H \Psi_g d\tau \]

which factors into

\[ E_g = E_1 + E_2 + \int \int \Phi_1 \Phi_2 (V_{12}) \Phi_1 \Phi_2 d\tau_1 d\tau_2 \]

The last term represents the columbic binding energy \( W \) for the pair, which is negative for dimers but positive for excimers. \( E_1 \) and \( E_2 \) denote the ground state energies of the molecules 1 and 2.

The wave-functions of the excited state of the dimer (exciton wave-functions) may be written as

\[ \Psi_e = r \Phi_1^* \Phi_2 + s \Phi_1 \Phi_2^* \]

where \( \Phi_1^* \) and \( \Phi_2^* \) represent excited state wave-functions for a particular excited state (with energy \( E_1^* \) and \( E_2^* \)) under study of molecules 1 and 2.

The Schrödinger equation for the excited state in question is:
\[ H(r \Phi_1^* \Phi_2 + s \Phi_1 \Phi_2^*) = E_\theta (r \Phi_1^* \Phi_2 + s \Phi_1 \Phi_2^*) \]

Multiplying both sides of this equation by \( \Phi_1^* \Phi_2 \) and then integrating over coordinates for molecules 1 and 2, and repeating this process for \( \Phi_1 \Phi_2^* \) leads to two simultaneous equation. The determinant of the coefficients \( r \) and \( s \) in this equations is set equal to zero for non-trivial solution:

\[
\begin{vmatrix}
H_{11} - E_e & H_{12} \\
H_{21} & H_{22} - E_e
\end{vmatrix} = 0
\]

where \( H_{11} = H_{22} = \int \int \Phi_1^* \Phi_2 \ H \Phi_1^* \Phi_2 \ d\tau_1 \ d\tau_2 \), \( H_{12} = H_{21} = \int \int \Phi_1^* \Phi_2 \ H \Phi_1 \Phi_2^* \ d\tau_1 \ d\tau_2 \).

The roots are

\[ E_\theta (+) = H_{11} + H_{12} \text{ with } \psi_\theta (+) = \frac{1}{\sqrt{2}} (\Phi_1^* \Phi_2 + \Phi_1 \Phi_2^*) \]

\[ E_\theta (-) = H_{11} - H_{12} \text{ with } \psi_\theta (-) = \frac{1}{\sqrt{2}} (\Phi_1^* \Phi_2 - \Phi_1 \Phi_2^*) \]

\[ E_\theta (+) = E_1 + E_2 + \int \int \Phi_1^* \Phi_2 (V_{12}) \Phi_1^* \Phi_2 d\tau_1 d\tau_2 + \int \int \Phi_1^* \Phi_2 (V_{12}) \Phi_1 \Phi_2^* d\tau_1 d\tau_2 \]

\[ E_\theta (-) = E_1 + E_2 + \int \int \Phi_1^* \Phi_2 (V_{12}) \Phi_1 \Phi_2^* d\tau_1 d\tau_2 - \int \int \Phi_1^* \Phi_2 (V_{12}) \Phi_1 \Phi_2^* d\tau_1 d\tau_2 \]

The exciton splitting term is

\[ \Delta E = \int \int \Phi_1^* \Phi_2 (V_{12}) \Phi_1 \Phi_2^* d\tau_1 d\tau_2 \]

which in the point - dipole approximation becomes

\[ \Delta E = E_\theta (-) - E_\theta (+) = 2 l M l^2 / R^3 (\cos \alpha + 3 \cos^2 \Omega) \]
Where \( M \) is the transition moment of the monomer, \( R \) is the center to center distance between molecules 1 and 2, \( \alpha \) is the angle between the transition dipoles and \( \Omega \) is the angle made by the transition dipole axes of the unit molecule with the line of molecular centers.

The geometry factor \( G = (\cos \alpha + 3 \cos^2 \Omega) \) becomes

\[
G = \cos \alpha \quad \text{(parallel-plane model; } \Omega = 90^\circ) \\
G = \cos \alpha + 3 \sin^2 (\alpha / 2) \quad \text{(in-plane model)}
\]

The transition moments to the exciton states \( E_e (+) \) and \( E_e (-) \) are given by:

\[
M (+) = \sqrt{2}M \cos \alpha \\
M (-) = \sqrt{2}M \sin \alpha
\]

Since \( f = \text{constant} \cdot M \cdot \nu \) (where \( f \) and \( \nu \) are oscillator strength and position of a absorption band in wavenumbers), \( \alpha = 2 \tan^{-1} \sqrt{\frac{\nu (-) f (+)}{\nu (+) f (-)}} \).

The energy difference (in wavenumbers) between the two maxima of the band can be rewritten as \( \Delta E = 2 \frac{D G}{h c R^3} \), where \( D \) is the dipole strength of the monomeric form \( \left( 10^{-6} \text{ M; } D = 9.173 \times 10^{-39} \left( \int \epsilon d\nu / \nu \right) \right) \), where \( \epsilon \) is the molar absorptivity \( [31] \). For all of the substituted anthraquinones on which polarized absorption measurements have been made, the transition dipole for the visible absorption band is in the plane of the molecule and oriented along the long axis of the anthraquinone nucleus \([12, 35]\). From the data in Figure 3.7c we calculate \( \alpha = 22^\circ \) and \( R = 6.2 \text{ Å} \). The orientation of the molecules is based on
the fact that the long alkyl chains would tend to be the farthest from each other. The geometry of the dimer is shown in Figure 3.11.

VIBRATIONAL SPECTRA OF MITOXANTRONE - Figure 3.12 shows the resonance Raman spectra of solid mitoxantrone with excitation at 350.7, 406.7, 457.9 and 514.5 nm. Two different patterns of enhancement are observed to the blue and red of the 406.7 nm excitation. At 406.7 nm, only one band at 1657 cm\(^{-1}\) is observed. With 350.7 nm excitation into the \(\pi \rightarrow \pi^*\) band at 380 nm a band at 1627 cm\(^{-1}\) becomes very prominent, along with two other bands at 1330 and 1370 cm\(^{-1}\). Excitation at wavelengths to the red of 406.7 nm are in resonance with the charge transfer band at 660 nm and this brings about strong enhancement of a band at 1300 cm\(^{-1}\). It was only possible to obtain spectra until excitation wavelengths of 570 nm, because of the increase in the emission background at lower energies. The resonance enhanced bands and the assignments of mitoxantrone are listed in Table 3.1. These bands can only be appropriately assigned based on a normal coordinate calculation, which necessitates a choice of normal mode frequencies, geometry and force constants. Since the resonance Raman spectrum involves only on the vibration of the anthraquinone chromophore, a considerable simplification in calculation could be made by choosing a model system. The simplest choices would be either 1,4-(OH)\(_2\) anthraquinone or 1,4-(NH\(_2\))\(_2\)-anthraquinone. Figure 3.13 shows the resonance Raman spectra of the two polymorphic forms of 1,4-(OH)\(_2\)-anthraquinone and Figure 3.14 that of 1,4-(NH\(_2\))\(_2\)-anthraquinone. There is a striking similarity in both the frequencies and intensities for mitoxantrone and the aminoanthraquinone for most of the bands.
Figure 3.11 Model of mitoxantrone dimer.
Figure 3.12 Resonance Raman spectra of mitoxantrone (0.2%) and 9% NaNO$_3$ in KBr at (a) 350.7 nm, (b) 457.9 nm and (c) 514.5 nm. Bands marked with an asterisk are due to the internal standard NO$_3^-$.

Laser power 10 mW. Slits 6 cm$^{-1}$. 

<table>
<thead>
<tr>
<th>Raman shift (cm$^{-1}$)</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 - 1900</td>
<td></td>
</tr>
<tr>
<td>350.7</td>
<td></td>
</tr>
<tr>
<td>457.9</td>
<td></td>
</tr>
<tr>
<td>514.5</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.13 Resonance Raman spectra of 1,4(OH)_2-anthraquinone in its two polymorphic forms (a) Form I and (b) Forms II. Excitation -457.9 nm. Laser power -10 mW. Slits -6 cm\(^{-1}\).
Figure 3.14 Resonance Raman spectra of 1,4(NH$_2$)$_2$-anthraquinone (15% in NaClO$_4$) at (a) 350.7 nm, (b) 413.1 nm, (c) 488 nm and (d) 647.1 nm. Bands marked with an asterisk are due to the internal standard ClO$_4$$. Laser power - 10 mW. Slits - 6 cm$^{-1}$. 
Table 3.1
Observed and Calculated Frequencies for Mitoxantrone and 1,4(NH$_2$)$_2$-anthraquinone

<table>
<thead>
<tr>
<th>Mitoxantrone</th>
<th>1,4(NH$_2$)$_2$-anthraquinone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Observed (cm$^{-1}$)</strong></td>
<td><strong>Observed (cm$^{-1}$)</strong></td>
</tr>
<tr>
<td>3270</td>
<td>3252</td>
</tr>
<tr>
<td>3170</td>
<td>3180</td>
</tr>
<tr>
<td>3060</td>
<td>3060</td>
</tr>
<tr>
<td>3055</td>
<td>3055</td>
</tr>
<tr>
<td>3050</td>
<td>3051</td>
</tr>
<tr>
<td>1627</td>
<td>1670</td>
</tr>
<tr>
<td>1656</td>
<td>1650</td>
</tr>
<tr>
<td>1569</td>
<td>1590</td>
</tr>
<tr>
<td>1476</td>
<td>450 + 1042</td>
</tr>
<tr>
<td>1649</td>
<td>1447</td>
</tr>
<tr>
<td>1401</td>
<td>1400</td>
</tr>
<tr>
<td>1341</td>
<td>1330</td>
</tr>
<tr>
<td>1326</td>
<td>1318</td>
</tr>
<tr>
<td>1299</td>
<td>1305</td>
</tr>
<tr>
<td>1268</td>
<td>1274</td>
</tr>
<tr>
<td>1177</td>
<td>1185</td>
</tr>
<tr>
<td>1165</td>
<td>515 + 654</td>
</tr>
<tr>
<td>1124</td>
<td>1136</td>
</tr>
<tr>
<td>1098</td>
<td>1042</td>
</tr>
<tr>
<td>993</td>
<td>1014</td>
</tr>
<tr>
<td>820</td>
<td>866</td>
</tr>
<tr>
<td>778</td>
<td>736</td>
</tr>
<tr>
<td>698</td>
<td>654</td>
</tr>
<tr>
<td>515</td>
<td>515</td>
</tr>
<tr>
<td>491</td>
<td>480</td>
</tr>
<tr>
<td>471</td>
<td>450</td>
</tr>
<tr>
<td>434</td>
<td>411</td>
</tr>
<tr>
<td>320</td>
<td>319</td>
</tr>
<tr>
<td>271</td>
<td>271</td>
</tr>
</tbody>
</table>

*derived from fluorescence data.

1Mitoxantrone also has a band at 1370 cm$^{-1}$, which has no counterpart in the aminoaanthraquinone, and is not listed on this table.
Table 3.1 lists the frequencies for 1,4-(NH$_2$)$_2$-anthraquinone. The enhancement patterns at different wavelengths are also similar for the drug and the amino compound e.g. in the ultraviolet, for the amino compound, a band around ~ 1650 cm$^{-1}$ is strongly enhanced, whereas in the red, the band at 1300 cm$^{-1}$ and other bands in this region are enhanced. The major difference between the drug and 1,4-(NH$_2$)$_2$-anthraquinone in the intensity of the bands at 1400 and 1443 cm$^{-1}$ which though present in mitoxantrone, are not as strongly enhanced. This similarity with the aminosubstituted anthraquinone supports the earlier observation that the visible band in both the drug and 1,4-(NH$_2$)$_2$-anthraquinone arise from aminosubstituent to ring charge transfer [20].

Several advantages exist in the choice of 1,4-(NH$_2$)$_2$-anthraquinone for normal coordinate studies. First, the geometry is simplified (C$_{2v}$). Second, high resolution fluorescence and far IR studies (at 4.2 K in n-octane) of this compound aids the selection of normal mode frequencies [36]. Third, force constants from 9,10-anthraquinone and aniline can be readily transferred [37, 38]. Group theoretical analysis predicts the presence of 27 A$_1$ + 13 A$_2$ + 12 B$_1$ + 26 B$_2$ modes for 1,4-(NH$_2$)$_2$-anthraquinone among these 78 normal modes 27 A$_1$ + 26 B$_2$ are in plane modes others are out-of-plane modes. Since the transition moment of this band lies in the plane of the molecule, only in plane modes will be resonance enhanced. The first problem is the choice of the 27 A$_1$ normal modes. There are a few weak bands in the solid state spectrum that complicates this analysis. A resonance Raman excitation profile employing 16 different excitation lines between 350.7 nm and 676.4 nm was obtained, all the bands observed are tabulated in Table 3.1. It is absolutely essential to obtain Raman spectra at different excitation wavelengths in order to observe all these
bands e.g. the 515 and 736 cm\(^{-1}\) band are observed with excitation at 501.7 nm, 1650 and 1670 cm\(^{-1}\) bands at 457.9 nm and 1136 cm\(^{-1}\) band at 350.7 nm. Two of the bands listed in Table 1 at 271 and 480 cm\(^{-1}\) were included from the previous fluorescence data [36]. There is a very good correlation between the fluorescence and the present Raman data [36] since the totally symmetric modes should be the most intense peaks in both cases and it is adequate to regard the peaks as A\(_1\) modes. Two of the weak bands at 1165 and 1476 cm\(^{-1}\) are considered as combination modes. The bands in N–H and C–H stretching region were obtained from the infrared spectrum [39] the set of 27 A\(_1\) normal modes are listed in Table 3.1.

Since the crystal structure of 1,4-(NH\(_2\))\(_2\)-anthraquinone is not available, the geometrical parameters necessary for constructing the G matrix were transferred from an analogue molecule, pyridinium 1-amino-4-bromo-9,10-dioxaanthracene-2-sulphonate [40]. A total of 89 force constants, including 62 interaction force constants were transferred from previously reported calculation of 9,10-anthraquinone and aniline [37, 38]. Since only 27 frequencies are being fitted, the normal coordinate problem is seriously overdetermined. In order to minimize artifacts in the calculation, only the diagonal force constants were altered. The calculated frequencies and the potential energy distribution are shown in Table 3.1. The 27 diagonal force constants were changed from the original values by more than 15 %. Most of them (7 of them) included the force constants on the ring containing the aminosubstituents (which were transferred from aniline). They all were decreased by 20 - 40 % from the aniline values. This is understandable since the extended conjugation of the anthraquinone will reduce the bond order.
Also, the C=O stretching and bending force constant were reduced by 20-25 % compared to 9,10-anthraquinone. The justification lies in the possible H-bonding with the amino group and thereby a reduced bond order. This calculation should be considered approximate till further isotopic data is obtained. There appears to be a reasonable correlation between the observed and calculated frequencies, which could definitely be improved by altering the interaction force constants. We decided that such an effort would be inappropriate until more experimental data are obtained.

The strongest band in the Raman spectra upon excitation into the visible band is at 1305 cm⁻¹ and has a significant contribution from C–N stretching motion. This is not unexpected considering that this electronic band is assigned to an amino to ring charge transfer. In other words, because there is a change in C–N bond length, the C–N stretching mode should have the greatest enhancement in resonance Raman spectra according to Tsuboi's empirical rule [41]. Upon resonance excitation into the π→π* band at 320 nm the most strongly enhanced Raman band is at 1670 cm⁻¹, which is primarily assigned to the C=O stretch. This π→π* electronic transition must involve the carbonyl groups.

NORMAL COORDINATE ANALYSIS- We have used Wilson's method of F and G matrices [42]. All of the required relation are combined in the equation |FG-EX| = 0 where F is a matrix of force constants, G is a matrix which involves the mass and certain spatial relations of the atoms and thus brings the kinetic energies into the equation, E is an unit matrix and λ is a term relating to the
frequency. The relationships among cartesian \( r \), internal \( R \), symmetry \( S \) and normal \( Q \) coordinates are as follows:

\[ R = Br \] where \( B \) is a matrix, \( S = UR \) where \( U \) is a matrix, and \( R = LQ \) where \( L \) is eigenvector of \( GFL = L\lambda \).

In general it is found that the normal mode \( Q_i \) is made up of contributions from the internal or symmetry modes \( q_j \), that is, \( Q_i = \sum_j L_{ij} q_j \)

where the summation runs over all the internal coordinates \( q_j \) or over all the symmetry coordinates which have the same symmetry description.

The potential energy distribution (PED), the potential energy for a given normal vibration express as a weighted sum of the potential energy contributions from all the internal modes comprising the normal mode, for the \( K \)th normal mode is calculated according to \( 2V = \sum_{ij} f_{ij} L_{ik} L_{jk} \). The \( G \) matrix is calculated by

\[ G_{tt'} = \frac{1}{m_k} \sum_{k=1}^{N} S_{tk} S_{t'k} \]

where \( stk \) is calculated from \( R_t = \sum_{k=1}^{N} S_{tk} e_k \). The potential energy can be expressed by a sets of variables as \( V = f(q_1, q_2, \ldots, q_p) \) where \( q \)'s and \( p \) are the internal parameters and degree of freedom. However, in general valent force field the potential energy can be written in terms of the internal displacement coordinates \( \Delta q_1, \Delta q_2, \ldots, \Delta q_p \) as \( 2V = \sum_{ij} f_{ij} (\Delta q_i) (\Delta q_j) \)

The procedure for doing normal coordinate analysis of \( 1,4-(NH_2)\_2 \)-anthraquinone is described below.
Group theoretical analysis of the 1,4-diaminoanthraquinone

Normal modes $3 \cdot 28 - 6 = 78$

In-plane modes $27A_1 + 26B_2$

Out-of-plane modes $13A_2 + 12B_1$
The molecular geometry of 1,4-diaminoanthraquinone
Internal valence coordinates for 1,4-diaminoanthraquinone
### Local symmetry coordinates for the 1,4-diaminoanthraquinone

<table>
<thead>
<tr>
<th>C-C Stretches</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 = 1</td>
<td>(4)</td>
</tr>
<tr>
<td>2 = 2</td>
<td>(7)</td>
</tr>
<tr>
<td>3 = 3</td>
<td>(1)</td>
</tr>
<tr>
<td>4 = 4</td>
<td>(7)</td>
</tr>
<tr>
<td>5 = 5</td>
<td>(4)</td>
</tr>
<tr>
<td>6 = 6</td>
<td>(8)</td>
</tr>
<tr>
<td>7 = 7</td>
<td>(5)</td>
</tr>
<tr>
<td>8 = 8</td>
<td>(2)</td>
</tr>
<tr>
<td>9 = 9</td>
<td>(3)</td>
</tr>
<tr>
<td>10 = 10</td>
<td>(2)</td>
</tr>
<tr>
<td>11 = 11</td>
<td>(5)</td>
</tr>
<tr>
<td>12 = 12</td>
<td>(6)</td>
</tr>
<tr>
<td>13 = 13</td>
<td>(10)</td>
</tr>
<tr>
<td>14 = 14</td>
<td>(9)</td>
</tr>
<tr>
<td>15 = 15</td>
<td>(10)</td>
</tr>
<tr>
<td>16 = 16</td>
<td>(9)</td>
</tr>
<tr>
<td>C-H Stretches</td>
<td></td>
</tr>
<tr>
<td>17 = 17</td>
<td>(27)</td>
</tr>
<tr>
<td>18 = 18</td>
<td>(27)</td>
</tr>
<tr>
<td>19 = 19</td>
<td>(26)</td>
</tr>
<tr>
<td>20 = 20</td>
<td>(25)</td>
</tr>
<tr>
<td>21 = 21</td>
<td>(25)</td>
</tr>
<tr>
<td>22 = 22</td>
<td>(26)</td>
</tr>
<tr>
<td>N-H Stretches</td>
<td></td>
</tr>
<tr>
<td>23 = 23</td>
<td>(13)</td>
</tr>
<tr>
<td>24 = 24</td>
<td>(15)</td>
</tr>
<tr>
<td>25 = 25</td>
<td>(13)</td>
</tr>
<tr>
<td>26 = 26</td>
<td>(15)</td>
</tr>
<tr>
<td>27 = 27</td>
<td>(16)</td>
</tr>
<tr>
<td>28 = 28</td>
<td>(16)</td>
</tr>
<tr>
<td>C=O Stretches</td>
<td></td>
</tr>
<tr>
<td>29 = 29</td>
<td>(14)</td>
</tr>
<tr>
<td>30 = 30</td>
<td>(14)</td>
</tr>
<tr>
<td>C-C-C Alphas</td>
<td></td>
</tr>
<tr>
<td>31 = 2·33·36·39+2·42·45·46</td>
<td>(20)</td>
</tr>
<tr>
<td>32 = 2·67·47·44+2·70·63·66</td>
<td>(21)</td>
</tr>
<tr>
<td>33 = 2·49·64·61+2·58·55·52</td>
<td>(22)</td>
</tr>
<tr>
<td>C-N-H Alphas</td>
<td></td>
</tr>
<tr>
<td>34 = 73</td>
<td>(17)</td>
</tr>
<tr>
<td>35 = 78</td>
<td>(17)</td>
</tr>
</tbody>
</table>
C-H Betas
36=34-35 (18)
37=37-38 (18)
38=50-51 (19)
39=54-53 (23)
40=56-57 (23)
41=59-60 (19)
C=O Betas
42=68-69 (12)
43=71-72 (12)
C-N Betas
44=31-32 (24)
45=40-41 (24)
N-H Betas
46=75-74 (11)
47=76-77 (11)

Note. Numbers in parentheses indicate the corresponding force constants.
Symmetry coordinates for the 1,4-diaminoanthraquinone

1 = 1
2 = 2 + 4
3 = 1 + 5
4 = 6
5 = 14 + 16
6 = 13 + 15
7 = 12
8 = 11 + 7
9 = 8 + 10
10 = 9
11 = 19 + 22
12 = 21 + 20
13 = 17 + 18
14 = 27 + 28
15 = 24 + 26
16 = 23 + 25
17 = 29 + 30
18 = 31
19 = 32
20 = 33
21 = 34 + 35
22 = 36 + 37
23 = 38 + 41
24 = 39 + 40
25 = 42 + 43
26 = 44 + 45
27 = 46 + 47
<table>
<thead>
<tr>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 6588</td>
<td>7.40</td>
<td>12%</td>
<td></td>
</tr>
<tr>
<td>2 6547</td>
<td>6.90</td>
<td>5.4%</td>
<td></td>
</tr>
<tr>
<td>3 6525</td>
<td>6.98</td>
<td>7.4%</td>
<td></td>
</tr>
<tr>
<td>4 6585</td>
<td>4.61</td>
<td>30%</td>
<td></td>
</tr>
<tr>
<td>5 6525</td>
<td>5.75</td>
<td>12%</td>
<td></td>
</tr>
<tr>
<td>6 6303</td>
<td>6.70</td>
<td>6.3%</td>
<td></td>
</tr>
<tr>
<td>7 6585</td>
<td>5.20</td>
<td>21%</td>
<td></td>
</tr>
<tr>
<td>8 6585</td>
<td>5.15</td>
<td>22%</td>
<td></td>
</tr>
<tr>
<td>9 4.617</td>
<td>4.5</td>
<td>2.5%</td>
<td></td>
</tr>
<tr>
<td>10 4.617</td>
<td>4.717</td>
<td>2.2%</td>
<td></td>
</tr>
<tr>
<td>11 0.431</td>
<td>0.26</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>12 1.100</td>
<td>0.30</td>
<td>24.5%</td>
<td></td>
</tr>
<tr>
<td>13 6.579</td>
<td>5.71</td>
<td>13%</td>
<td></td>
</tr>
<tr>
<td>14 10.651</td>
<td>8.40</td>
<td>21%</td>
<td></td>
</tr>
<tr>
<td>15 6.579</td>
<td>5.71</td>
<td>13%</td>
<td></td>
</tr>
<tr>
<td>16 5.975</td>
<td>5.20</td>
<td>13%</td>
<td></td>
</tr>
<tr>
<td>17 0.567</td>
<td>0.401</td>
<td>29%</td>
<td></td>
</tr>
<tr>
<td>18 0.497</td>
<td>0.511</td>
<td>3%</td>
<td></td>
</tr>
<tr>
<td>19 0.490</td>
<td>0.61</td>
<td>24.5%</td>
<td></td>
</tr>
<tr>
<td>20 0.983</td>
<td>0.97</td>
<td>13%</td>
<td></td>
</tr>
<tr>
<td>21 0.627</td>
<td>0.20</td>
<td>12%</td>
<td></td>
</tr>
<tr>
<td>22 0.882</td>
<td>1.33</td>
<td>33%</td>
<td></td>
</tr>
<tr>
<td>23 0.491</td>
<td>0.45</td>
<td>8%</td>
<td></td>
</tr>
<tr>
<td>24 0.894</td>
<td>9.61</td>
<td>31%</td>
<td></td>
</tr>
<tr>
<td>25 5.057</td>
<td>5.057</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>26 5.102</td>
<td>5.102</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>27 5.057</td>
<td>5.057</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>28 0.824</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29 -0.431</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 0.276</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31 -0.284</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32 -0.431</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33 0.276</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34 0.824</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35 -0.431</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36 0.824</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 -0.431</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38 0.276</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>39 0.272</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 -0.284</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41 0.372</td>
<td>0.746</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42 -0.353</td>
<td>0.051</td>
<td></td>
<td></td>
</tr>
<tr>
<td>43 0.246</td>
<td>0.243</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>47</td>
<td>-0.353</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>-0.353</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>0.746</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.243</td>
<td></td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>-0.353</td>
<td></td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>0.022</td>
<td></td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>0.115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>0.115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>0.144</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>0.102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>0.102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>0.686</td>
<td></td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>-0.686</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>0.300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>0.468</td>
<td></td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>0.468</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>-0.189</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>0.189</td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>0.189</td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>-0.189</td>
<td></td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>-0.189</td>
<td></td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>0.189</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>0.204</td>
<td></td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>-0.204</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>-0.204</td>
<td></td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>0.204</td>
<td></td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>-0.204</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>0.204</td>
<td></td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>0.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>77</td>
<td>0.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>0.163</td>
<td></td>
<td></td>
</tr>
<tr>
<td>79</td>
<td>-0.163</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>0.194</td>
<td></td>
<td></td>
</tr>
<tr>
<td>81</td>
<td>0.194</td>
<td></td>
<td></td>
</tr>
<tr>
<td>82</td>
<td>0.229</td>
<td></td>
<td></td>
</tr>
<tr>
<td>83</td>
<td>0.408</td>
<td></td>
<td></td>
</tr>
<tr>
<td>84</td>
<td>0.408</td>
<td></td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>0.408</td>
<td></td>
<td></td>
</tr>
<tr>
<td>86</td>
<td>0.434</td>
<td></td>
<td></td>
</tr>
<tr>
<td>87</td>
<td>0.434</td>
<td></td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>0.434</td>
<td></td>
<td></td>
</tr>
<tr>
<td>89</td>
<td>0.434</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes. A: number of the force constants; b: initial values of the force constants; c: final values of the force constants; d: percentage of the changes in the force constants.
Interaction force constants for the 1,4-diaminoanthraquinone
There is a good match between the observed Raman frequencies of mitoxantrone and 1,4-(NH$_2$)$_2$-anthraquinone. It is therefore reasonable to correlate the Raman bands of mitoxantrone with the normal coordinate calculation shows in Table 3.1. The strongest Raman band upon visible excitation is at 1299 cm$^{-1}$ and is assigned to the C-N stretch. Excitation with resonance in the ~380 nm band leads to strong enhancement of the band at 1627 cm$^{-1}$, which is assigned to a C=O stretch. This band in mitoxantrone is lowered by about 43 cm$^{-1}$ as compared to 1,4-(NH$_2$)$_2$-anthraquinone due to the intramolecular H-bond with the -OH group. A band at ~1370 cm$^{-1}$ which has no counterpart in the aminoanthraquinone is also strongly resonance enhanced in the ultraviolet. We assign this band to a C=O stretch of the phenol group based on our previous studies of hydroxyanthraquinones [43, 44]. The electronic band at 380 nm, though nominally assigned to a $\pi \rightarrow \pi^*$ transition involves the -OH and C=O groups. Finally, from the polarization measurement of the Raman spectra (Figure 3.15) of the mitoxantrone, all the bands observed are polarized and belong to $A_1$ modes.

Figure 3.16 a is the resonance Raman spectrum of an aqueous solution of mitoxantrone (1 x 10$^{-4}$ M) at 514.5 nm. Compared to the Raman spectra of the solid (Figure 3.12), bands at 432, 475, 1124, 1180, 1300, 1452 and 1651 cm$^{-1}$ are only observed. The excitation profile of these Raman bands are shown in Figure 3.17a. The bands at 475, 1124, 1180 and 1300 cm$^{-1}$ are all strongly resonance enhanced with ~600 nm band, whereas the 1655 cm$^{-1}$ band is enhanced both due to the visible and ultraviolet absorption band. This is due to the fact that as shown before in the solid state spectrum, there are two bands present in the ~1650 cm$^{-1}$ region, a ring stretching and a C=O stretch, which
Figure 3.15 Parallel and perpendicularly polarized resonance Raman spectra of 1x $10^{-4}$ aqueous solution of mitoxantrone. Laser excitation 496.5 nm. Band marked with an asterisk is due to the internal standard NO$_3^-$ (0.1 M).
Figure 3.16 Resonance Raman spectra of (a) $1 \times 10^{-4}$ M solution of mitoxantrone and (b) with calf thymus DNA. Laser excitation 514.5 nm. Band marked with an asterisk is due to the internal standard $\text{NO}_3^-$ (0.1 M).
Figure 3.17 Excitation profiles of the Resonance Raman bands of (a) $1 \times 10^{-4}$ M solution of mitoxantrone and (b) bound to calf thymus DNA.
are not resolved in the solution spectrum. The band due to C=O stretching is enhanced upon excitation in the blue. Figure 3.16b shows the resonance Raman spectrum of drug bound to calf thymus DNA at 514.5 nm (drug concentration = 1 x 10^{-4} M, drug to phosphate ratio = 30). There are no new bands apparent upon binding. The only effect is that the Raman bands all exhibit a lower intensity. This hypochromism is the result of the red shifting of the visible band upon binding to DNA, as well as decreased extinction coefficient of the band. Figure 3.17b shows the excitation profile of the 1302 and 1655 cm^{-1} band for the band drug. Because of the increased background emission, data for the 1655 cm^{-1} band was not obtained at wavelengths beyond 514.5 nm. The profiles are similar to that of the free drug. Unlike adriamycin, where distinct vibrational shifts are observed upon intercalation [45, 46, 47], no changes are observed in the Raman spectrum of bound mitoxantrone.

The theory of Raman excitation profiles are described below [48]:

The intensity of a Raman line (between two states |i> and |f>) is given by

\[ I_{fi} = \frac{\pi^2}{\epsilon_0} \left( v_0 \pm v_{fi} \right)^4 I_0 \sum_{j,k} |\partial_{jk}^f| |\partial_{jk}^i|^* \]

Where, \( I_0 \) is the irradiance of the incident radiation, \( v_0 \) and \( v_{fi} \) are the wave numbers of the exciting line and of the Raman transition |f> \( \rightarrow \) |i>, \( \epsilon_0 \) is the permittivity of free space and \( [\partial_{jk}]_f \) \( \rightarrow \) \( [\partial_{jk}]_i \) is the jkth element of the transition polarizability tensor. For vibrational Raman scattering excited far from resonance, the tensor is symmetric, i.e. \( \partial_{jk} = \partial_{kj} \), but it may become asymmetric for electronic Raman and RR scattering.
The transition polarizability tensor is express as

\[ [\partial_{jk}]_{fi} = \frac{1}{\hbar c} \sum_r \left[ \frac{[\mu_i]_f r [\mu_k]_r i}{v_{ri} - v_0 + i\xi_r} + \frac{[\mu_k]_f r [\mu_i]_r i}{v_{rf} + v_0 + i\xi_r} \right] \]

Where, \([\mu_i]_f r\) is the \(i\)th component of the transition dipole moment associated with the transition \(\psi_f \rightarrow \psi_r\) and \(i\xi_r\) is a damping factor, which is related to the lifetime of the state \(\psi_r\) and \(\xi_r\) is equal to half of the full width of the half maximum.

Since the vibronic states \(\psi_i\), \(\psi_f\) and \(\psi_r\) are formed by products of the vibrational and the pure electronic states \(\psi_i = \psi_{gm} \psi_i\), \(\psi_f = \psi_{gn} \psi_f\) and \(\psi_r = \psi_{le} \psi_r\). Where \(\psi_{gm}\) and \(\psi_{le}\) represent ground and excited electronic states and \(\psi_{gn}\) and \(\psi_{lu}\) represent vibrational states. This enables the transition polarizability to be rewritten in the form

\[ [\partial_{jk}]_{gn, gm} = \frac{1}{\hbar c} \sum_{e u} \left[ \frac{\langle n | [\mu_i]_g e | u \rangle \langle u | [\mu_k]_c g | m \rangle}{v_{cu, gn} - v_0 + i\xi_{cu}} + \frac{\langle n | [\mu_k]_g e | u \rangle \langle u | [\mu_i]_c g | m \rangle}{v_{cu, gn} + v_0 + i\xi_{cu}} \right] \]

Using the Born-Oppenheimer approximation one obtains

\[ [\mu_i]_{ge} = [\mu_i]_{ge}^0 + \sum_s [\mu_i]_{ge} Q_s + \ldots \]

Where \(Q_s\) is the \(s\) normal coordinate of the system.

In the case of resonance Raman scattering, using the Herzberg-Teller [49] perturbation description of vibronic coupling the derivative of the transition moment comes from the variation in the Hamiltonian with respect to the normal coordinate \(Q_s\) can mix the state \(\psi_e\) with other states \(\psi_f\) of the appropriate symmetry. We can express the transition moment as
\[ [\mu]_{g,e} = [\mu]_{g,e}^0 + \sum_t \sum_s [\mu]_{g,t}^0 \frac{h_{e,t}^s}{v_{e,t}} Q_s + \ldots \]

\[ h_{e,t}^s = \langle e | \frac{a H}{a Q_s} | t \rangle_{Q_s = 0} . \]

Plug this equation into the equation for transition polarization, we obtain

\[ [\partial_j k]_{g,n, g,m} = A + B + C + D. \]

Where

\[ A = \frac{1}{h} \mu_{g,e} [\mu_k]_{e,g} \sum_u \frac{\langle n_g | u_e \rangle \langle u_e | m_g \rangle}{v_{e,u,g,m} - v_0 + i \delta_{e,u}} \]

and \( B, C, D \) are very small. Assuming harmonic potentials for the states \( | g > \) and \( | e > \), the Franck - Condon overlap intergers may be calculated using the recurrence formulas derived by Manneback [50].

\[ \langle 0_e | 0_g \rangle = \left( \frac{2(v_s v_g)^{0.5}}{v_s + v_g} \right)^{0.5} \exp \left( -\frac{\Delta^2}{2} \right) \]

\[ \langle n_g | u_e + 1 \rangle = - \left( \frac{u}{u + 1} \right)^{0.5} \left( \frac{v_s - v_g}{v_s + v_g} \right) \langle n_g | u_e - 1 \rangle \]

\[ + \left( \frac{n}{u + 1} \right)^{0.5} \left( \frac{2(v_s v_g)^{0.5}}{v_s + v_g} \right) \langle n_g - 1 | u_e \rangle \]
\[- \left( \frac{1}{u + 1} \right)^{0.5} \Delta \left( \frac{2 v_s^g}{v_s^g + v_s^e} \right)^{0.5} \langle n_g | u_e \rangle \]

\[ \langle n_g + 1 | u_e \rangle = - \left( \frac{n}{n + 1} \right)^{0.5} \left( \frac{v_s^g - v_s^e}{v_s^g + v_s^e} \right) \langle n_g - 1 | u_e \rangle \]

\[ + \left( \frac{u}{n + 1} \right)^{0.5} \left( \frac{2 (v_s^g v_s^e)^{0.5}}{v_s^e + v_s^g} \right) \langle n_g | u_e - 1 \rangle \]

\[ - \left( \frac{1}{u + 1} \right)^{0.5} \Delta \left( \frac{2 v_s^g}{v_s^g + v_s^e} \right)^{0.5} \langle n_g | u_e \rangle \]

where $v_s^g$ and $v_s^e$ are the vibrational frequencies (wave number) of the normal modes in the ground and excited states and the dimensionless shift parameter $\Delta$ is given by ($dQ_s$ is the displacement of the excited-state potential minimum along the normal coordinate).

\[ \Delta = \left( \frac{4 \pi^2 c}{\hbar} \right)^{0.5} dQ_s \left( \frac{v_s^g v_s^e}{v_s^e + v_s^g} \right)^{0.5} \]

A program using the above equations and least square curvefit refinement method has been written and use. In addition, another program which can simulate the absorption spectra has been written too based on that the intensity of $u-0$ vibronic band is proportion to $v_{eu} \langle 0 | u \rangle |^2$. 
CONCLUSIONS

This study has examined the absorption, emission and resonance Raman spectra of mitoxantrone. The following major conclusions can be drawn.

1) The visible absorption band around ~ 660 nm is assigned to a charge transfer transition from the amino substituent on the anthraquinone ring to the ring itself. This transition is red shifted in nonpolar solvents probably and is explained by a lack of H-bonding stabilization of the ground state in nonpolar solvents. The emission band at ~ 680 nm exhibits a similar effect.

2) A quantitative study of the emission intensity as a function of mitoxantrone concentration has led to the calculation of dimerization and trimerization constants of $2.7 \times 10^4 \text{ M}^{-1}$ and $7 \times 10^4 \text{ M}^{-2}$, respectively.

3) Using molecular exciton theory, the absorption spectrum of the dimer was analyzed to obtain information about the geometry of the dimer. Assuming the model that the anthraquinone moiety of the drug molecules are stacked upon one another, the angle between the transition dipoles was calculated to be $22^0$ and the separation between them was ~ 6.2 Å.

4) The resonance Raman spectrum of mitoxantrone exhibited distinct patterns if excited into the visible or ultraviolet absorption bands. Upon visible excitation, a band at ~ 1300 cm$^{-1}$ was strongly enhanced, whereas for the ultraviolet band, a Raman band at 1620 cm$^{-1}$ was enhanced.

5) A normal coordinate calculation of 1,4-(NH$_2$)$_2$-anthraquinone was carried out as a model for mitoxantrone. The strongly enhanced Raman band in the
visible was found to have significant C–N stretching character, in agreement with the amino to ring charge transfer transition of the visible band. The Raman band enhanced in the ultraviolet was mostly of C=O stretching character.

(6) Raman excitation profiles of the drug in solution and bound to calf thymus DNA were obtained. There were no changes in the Raman frequencies upon interaction with nucleic acids, only a decrease in intensity.
REFERENCES


CHAPTER IV

RESONANCE RAMAN SPECTROSCOPIC STUDIES OF METHOXATIN CONTAINING METHYLAMINE DEHYDROGENASE (MADH)

( in collaboration with Professor Michael H. Klapper and the samples were prepared by Mr. Robert B. McWhirter )

4 - 1 INTRODUCTION

Recently, there has been significant interest in identifying the structure of the organic cofactor of the methoxatin containing methylamine dehydrogenase. Despite considerable effort, e. g. ESR studies of the semiquinone form of the methylamine dehydrogenase [1], resonance Raman studies of the derivatives of the plasma amine oxidases [2], X-ray studies of the methylamine dehydrogenases [3], the organic cofactors have not been conclusively identified. The identification of the organic cofactor as methoxatin is potentially very important to understanding the mechanism and function of MADH. Previous attempts to isolate and to identify the cofactor itself [4] or a stable derivative [5] from the plasma amine oxidase proved to be problematic.

Resonance Raman spectroscopy is a suitable technique for probing the structure of the organic cofactor in the enzyme MADH. The advantages of resonance Raman spectroscopy are as follows:
(i) The technique may be applied to intact enzymes under physiological conditions.

(ii) Molecular vibrational spectra provide important structural information.

(iii) The vibrational spectrum of a chromophore (cofactor) in a complex macromolecule (enzyme) can be selectively obtained by resonance enhancement.

(iv) Normal modes which are responsible for the distortion in the excited state will display the greatest resonance enhancement.

Thus the observed resonance Raman spectra should be structurally diagnostic for the chromophore (cofactor). In the past, because of the difficulty involved in obtaining high-quality resonance Raman spectra from the native enzymes, the experiments have been done on the derivatives of the native enzymes amine oxidase exclusively [2], and which made the identification of the organic cofactor very difficult. Here we report the resonance Raman spectra of the native enzyme MADH (from W3A1), the semiquinone form of the enzyme MADH either reduced by dithionite or by propylamine and PQQ (shown in Figure 4.1).

![Figure 4.1 PQQ: Methoxantin; Pyrroloquinoline quinone.](image-url)
We draw several conclusions about the structure of the organic cofactor and the reduced form of the enzyme MADH. The structure of the reduced form of the enzyme MADH is very important to understanding the catalytic reaction (oxidation of amines to aldehydes and free ammonia).

4–2 EXPERIMENTAL

PREPARATION OF THE ENZYME MADH- Methylamine dehydrogenase (MADH) was isolated from the methylotrophic bacterium W3A1 (a gift by Professor W. C. Kenney) by the procedure of Chandrasekar and Klapper [6]. Namely, the bacterium W3A1 was grown on the defined culture medium of Owens and Keddie [7] at 25 - 30 °C using methylamine (0.3 %) as the sole carbon source. A large amount of MADH (ca. 100 mg / 15 L of growth culture) is produced under conditions of aerobic growth in this culture medium. The bacteria were harvested by centrifugation at 7,000 rpm when the apparent absorbance at 600 nm (a measure of turbidity) reaches one.

The bacterial pellet was resuspended in 20 mM pH 7.0 phosphate buffer (~80 mg pellet / 300 ml buffer). While cooling and stirring on ice, the suspension was sonicated at maximum intensity for 4 min with a Bronson Sonifier, and then, after waiting ~ 5 min it was sonicated again for 4 min. The broken cell suspension was centrifuged for 30 min at 15,000 rpm using a previously chilled Sorvall SS-34 head. The supernatant pH was adjusted back to 7.0 and then heated with stirring at 70 °C for 20 min. The resultant precipitate was removed by centrifugation in preparation for the column chromatographic purifications. The extract was loaded onto a Carboxymethyl - Sephadex (CM - Seph) C -50 column (1" x 7") previously equilibrated with 20 mM phosphate
buffer (pH=7.0) at 4°C. After washing the resin with 500 ml of the same phosphate buffer and then with 250 ml of 35 mM phosphate buffer (pH=7.0) to elute contaminating proteins, the column was developed with 60 mM phosphate buffer (pH=7.0) to obtain a peak of pure MADH. The success of this purification is based on the thermal stability of the enzyme, exploited to remove most of the protein and nucleic acid by heating, and on the high level of MADH (5% of the total protein) in W3A1.

The enzyme MADH as obtained by this procedure was a mixture of redox states. Ferricyanide (~7 mM) was added to oxidize this mixture at 5°C for approximately 20 min in the dark. The ferrocyanide and excess ferricyanide were removed by passing the enzyme solution over a Dowex 1-X8 column equilibrated with 0.1 M phosphate buffer (pH=7.5). The resulting oxidized MADH was desalted by passing it through a Sephadex G-25 column equilibrated with 0.1 mM phosphate buffer (pH=7.5). All the enzyme MADH prepared in this manner had a final specific activity of ~13 (Units/mg) as determined by the procedure of Eady and Large [8]. The concentrated MADH solutions were prepared by passing the solution over a column of CM-Sephal 1 x 2 cm that has been equilibrated with 20 mM phosphate (pH=7.0) and then eluting the bound protein with 200 mM phosphate (pH=7.0). The final concentration of the enzyme MADH for the Raman experiment is ~2 x 10⁻³ M.

PREPARATION OF THE SEMIQUINONE FORM OF THE ENZYME MADH - Two methods have been used to prepared the semiquinone form of the enzyme MADH. First of all, the enzyme (0.2 mM) solutions were made anaerobic by careful bubbling with O₂-free Ar and then the enzyme was reduced to its
semiquinone form with sodium dithionite at room temperature in 5 mM sodium phosphate and 0.1 M NaHCO₃ (pH=7.3). Secondly, a sufficient quantity of 0.1 M n-propylamine hydrochloride solution (O₂ free) to give the desired final concentration (2-10 mM) was added to the O₂ - free enzyme solution (final concentration 0.2 mM) in 5 mM phosphate buffer (pH=7.5) and 0.1 NaHCO₃ at room temperature. The enzyme was completely reduced within 5 min and after a period of over 7 hr it converted mostly to the semiquinone form.

All the sample solutions mentioned above were sealed in NMR tubes for the Raman experiments and the absorbance measurements were obtained with a Kontron Uvikon 820 spectrophotometer.

4 - 3 RESULTS AND DISCUSSION

VIBRATIONAL ASSIGNMENT OF PQQ - The PQQ molecule is assumed to be planar and belongs to the point group C₃. The 84 normal modes of vibration are divided among the symmetry species 57 A' + 27 A". All of the 84 normal modes are both infrared and Raman active. The infrared spectrum of PQQ taken in KBr and the resonance Raman spectra (excited at 350.7 and 457.9 nm) taken in KBr are shown in Figures 4.2 and 4.3, respectively. The assignments of the bands were based on these data and fluorescence data of 9,10-phenanthraquinone [9] and by taking aid from the vibrational assignments of the similar molecules, 1,7-phenanthrolone (C₅) [10], 9,10-phenanthraquinone (C₂ᵥ) [11, 12], phenanthrene (C₂ᵥ) [13] and quinoline (C₅) [14], and aromatic acids in general [15, 16, 17]. These assignments are summarized in Table 4.1.
According to Bellamy [15], Gregory [18] and Staiger [19], the carbonyl stretching frequencies in aryl α-diketones lie between 1690 - 1670 cm\(^{-1}\) and in aryl acids lie between 1700 - 1680 cm\(^{-1}\). However, the carbonyl stretching frequency of the 2, 4-pyridinedicarboxylic acid is at 1724 cm\(^{-1}\) [20]. Also, the carbonyl vibration frequency is easy to locate because of its intensity (strong) and because of the absence of other bands in the 1600 - 1800 cm\(^{-1}\) region. We have assigned the peak at 1678 cm\(^{-1}\) to the C=O stretching mode of the carbonyl group of the PQQ molecule and the peak at 1712 cm\(^{-1}\) to the C=O stretching mode of the two carboxylic groups of the pyridine ring of the PQQ molecule. The fact that the intensity of the 1678 cm\(^{-1}\) peak is higher than 1712 cm\(^{-1}\) peak, which contradicts the well-known fact that the C=O stretching of the carboxylic group is considerably stronger than ketonic C=O stretch [20], indicates that the C=O stretching of the carboxylic groups of the pyrrole ring of the PQQ molecule at ca. 1660 cm\(^{-1}\) (according to pyrrole - 2-carboxylic acid [21]) may be overlap with the band at 1678 cm\(^{-1}\) and causing the intensity of 1678 cm\(^{-1}\) peak to increase.

A comparison of the infrared spectra, Raman spectra and vibrational assignment of PQQ with those of 1,7-phenanthroline, 9,10-phenanthraquinone, phenanthrene and quinoline may be helpful in arriving at the correct assignment of the frequencies of these molecules, and therefore, an attempt is made here to compare these results. It has been observed that the bands at 464, 493, 519, 550, 754, 794, 935, 987, 1006, 1066, 1110, 1149, 1164, 1205, 1240, 1265, 1276, 1306, 1350, 1380, 1390, 1420, 1458, 1483, 1500, 1529, 1550, 1581, 1606 and 1624 cm\(^{-1}\) appear with considerable
Figure 4.2 Infrared spectrum of the molecule PQO (2%) in KBr.
Figure 4.3 Resonance Raman spectra of PQQ (20%) in KBr at (a) 457.9 nm and (b) 350.7 nm. Laser power -10 mW. Slits -6 cm⁻¹.
intensities in the Raman spectra. These can be correlated with the frequencies observed in 1,7-phenanthroline and 9, 10-phenanthraquinone. And, they are confidently assigned as fundamental modes. However, Neto [13], according to their infrared and Raman data of the phenanthrene, had assigned the 1624 cm\(^{-1}\) peak to be an overtone band\((2 \times 811 = 1622)\), which is excluded by our Raman data because 811 cm\(^{-1}\) does not exist in our Raman data but 1622 cm\(^{-1}\) does. The 750 cm\(^{-1}\) mode of the 9, 10-phenanthraquinone, which has been assigned by Nath to a ring breathing mode is corroborated by the appearance of the corresponding frequency at 754 cm\(^{-1}\) (m) and the high intensity in the Raman spectrum but weak peak in the infrared spectrum of the PQQ. Additional fundamental modes are identified at 316, 384, 416, 470, 537, 574, 666, 688, 721, 767, 771, 825, 834, 860, 879, 904, 950, 1025, 1049, 1125, 1049, 1125 and 1184 cm\(^{-1}\) according to the Shigorin et al. [11], Nath et al. [12], Rother et al. [10], Tripathi et al. [14] and Neto et al. [13] and are assigned correspondingly (Table 4.1).

Table 4.1. Summary of IR and R data of PQQ and R data of MADH (Q), MADH-PH and semiquinone forms of MADH (QH\(^+\)).

<table>
<thead>
<tr>
<th>PQQ</th>
<th>Q R</th>
<th>O+ PH R</th>
<th>R a</th>
<th>R b</th>
<th>QH+ R c</th>
<th>QH+ R d</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR</td>
<td>457nm</td>
<td>350nm</td>
<td>457nm</td>
<td>413nm</td>
<td>457nm</td>
<td>514nm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>568nm</td>
</tr>
<tr>
<td>a)</td>
<td></td>
<td>316w</td>
<td>307m</td>
<td></td>
<td></td>
<td>413nm</td>
</tr>
<tr>
<td>b)</td>
<td></td>
<td>386w</td>
<td>384w</td>
<td></td>
<td>380w</td>
<td></td>
</tr>
<tr>
<td>c)</td>
<td></td>
<td>416w</td>
<td>416w</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d)</td>
<td></td>
<td>453m</td>
<td>468w</td>
<td>464s</td>
<td>461w</td>
<td></td>
</tr>
<tr>
<td>e)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>470w</td>
<td></td>
</tr>
<tr>
<td>f)</td>
<td></td>
<td>499m</td>
<td>491w</td>
<td>493m</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g)</td>
<td></td>
<td>520w</td>
<td>519m</td>
<td>517w</td>
<td></td>
<td></td>
</tr>
<tr>
<td>h)</td>
<td></td>
<td>550w</td>
<td>551s</td>
<td>550w</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.1 continued

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>i)</td>
<td>574w</td>
<td></td>
</tr>
<tr>
<td>j)</td>
<td>592w 594w</td>
<td></td>
</tr>
<tr>
<td>k)</td>
<td>627w 629w</td>
<td></td>
</tr>
<tr>
<td>l)</td>
<td>666w</td>
<td></td>
</tr>
<tr>
<td>m)</td>
<td>688sh 710w</td>
<td></td>
</tr>
<tr>
<td>n)</td>
<td>721w 721w 721w</td>
<td></td>
</tr>
<tr>
<td>o)</td>
<td>742m</td>
<td></td>
</tr>
<tr>
<td>p)</td>
<td>767w</td>
<td></td>
</tr>
<tr>
<td>q)</td>
<td>794w 794w 793w</td>
<td></td>
</tr>
<tr>
<td>r)</td>
<td>825w 820w</td>
<td></td>
</tr>
<tr>
<td>s)</td>
<td>834sh 860w 859w</td>
<td></td>
</tr>
<tr>
<td>t)</td>
<td>873w 879w 880w 881w</td>
<td></td>
</tr>
<tr>
<td>u)</td>
<td>904w 895w</td>
<td></td>
</tr>
<tr>
<td>v)</td>
<td>935w 937w 932w 941m</td>
<td></td>
</tr>
<tr>
<td>w)</td>
<td>950w 946w 952w 954m</td>
<td></td>
</tr>
<tr>
<td>x)</td>
<td>987m</td>
<td></td>
</tr>
<tr>
<td>y)</td>
<td>999sh 1004w 1006m 1006m 1009m</td>
<td></td>
</tr>
<tr>
<td>z)</td>
<td>1014w 1028w 1027w 1026w</td>
<td></td>
</tr>
<tr>
<td>aa)</td>
<td>1043sh 1049w</td>
<td></td>
</tr>
<tr>
<td>bb)</td>
<td>1066w 1069w 1069m 1069w 1073w</td>
<td></td>
</tr>
<tr>
<td>cc)</td>
<td>1110w 1118w 1110m 1104w 1106w 1098w</td>
<td></td>
</tr>
<tr>
<td>dd)</td>
<td>1132w 1125w 1132w</td>
<td></td>
</tr>
<tr>
<td>ee)</td>
<td>1147w 1149sh 1144m</td>
<td></td>
</tr>
<tr>
<td>ff)</td>
<td>1184sh 1164s 1174m 1174m 1171m</td>
<td></td>
</tr>
<tr>
<td>gg)</td>
<td>1205s 1218s 1217m 1204w 1202w</td>
<td></td>
</tr>
<tr>
<td>hh)</td>
<td>1236s 1243w 1246m 1247sh 1249w</td>
<td></td>
</tr>
<tr>
<td>ii)</td>
<td>1267sh 1273w 1265s 1257m 1256m 1259m 1263w</td>
<td></td>
</tr>
<tr>
<td>jj)</td>
<td>1280m 1276s 1277m 1273m</td>
<td></td>
</tr>
<tr>
<td>kk)</td>
<td>1306m 1295w 1308m 1314sh 1291m 1309m 1306w</td>
<td></td>
</tr>
<tr>
<td>ll)</td>
<td>1355sh 1336s 1356w 1341s 1354m 1377m 1349m 1350w</td>
<td></td>
</tr>
<tr>
<td>mm)</td>
<td>1387m 1385w 1394s 1380m 1379w 1377m 1394m 1390w</td>
<td></td>
</tr>
<tr>
<td>nn)</td>
<td>1402sh 1416w 1423s 1418w</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.1 continued

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>oo</td>
<td>1445sh</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pp</td>
<td>1458m 1454w 1466s 1462s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>qq</td>
<td>1479sh 1470w 1488m 1474m 1483w 1481s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rr</td>
<td>1500m 1512m 1502sh 1495m</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ss</td>
<td>1550m 1524s 1529w 1553m 1522s 1518s 1537s 1525s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tt</td>
<td>1581s 1591s 1566s 1588m 1587s 1581s 1582m</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>uu</td>
<td>1606m 1604m 1602s 1600m 1600s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vv</td>
<td>1624sh 1620s 1620s 1620sh 1828m</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ww</td>
<td>1678s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>xx</td>
<td>1712s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A. QH* was prepared by reducing O with propylamine; b. QH* was prepared by reducing Q with dithionite. Frequencies in cm\(^{-1}\) units.
Intensities abbreviated as s = strong, m = medium, w = weak, sh = shoulder.

Table 4.1 continued

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td>skeletal deformation [3]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b)</td>
<td>C - O in plane bend [2]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c)</td>
<td>skeletal deformation [2]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d)</td>
<td>skeletal deformation + C - O in plane bend [2, 3]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e)</td>
<td>skeletal deformation [3]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f)</td>
<td>skeletal deformation + C - O in plane bend [5]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g)</td>
<td>skeletal deformation [3]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h)</td>
<td>skeletal deformation [3]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i)</td>
<td>?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>j)</td>
<td>skeletal deformation [5]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>k)</td>
<td>skeletal deformation [2]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>l)</td>
<td>skeletal deformation [2]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m)</td>
<td>skeletal deformation [2]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n)</td>
<td>skeletal deformation + C - O in plane bend [2]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>o)</td>
<td>skeletal breath [3]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p)</td>
<td>skeletal deformation [2]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>q)</td>
<td>C - H out of plane bend [3]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r)</td>
<td>skeletal deformation [2, 3]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.1 continued

s) skeletal deformation \([2, 3]\)
t) C - H out of plane bend \([3]\)
u) C - H out of plane bend \([2]\)
v) skeletal deformation + C = O in plane bend \([2]\)
w) C - H out of plane bend \([4, 5]\)
x) C - H out of plane bend \([2, 3]\)
y) C - H in plane bend \([3]\)
z) C - H in plane bend \([3]\)
aa) C - H in plane bend \([2]\)
bb) skeletal deformation \([2]\)
cc) ring stretch \([3, 5]\)
dd) C - H in plane bend \([2]\)
ee) C - H in plane bend \([5]\)
ff) C - H in plane bend \([2]\)
gg) skeletal breath \([4]\)
hh) C - H in plane bend \([2, 3]\)
ii) C - H in plane bend \([2]\)
jj) ring stretch \([2, 3, 4]\)
kk) ring stretch \([2, 3]\)
ll) ring stretch \([2, 3]\)
mm) ring stretch \([3, 4, 5]\)
nn) ring stretch \([3]\)
oo) ring stretch + C = O stretch \([2]\)
pp) ring stretch \([2]\)
qq) ring stretch \([2]\)
rr) ring stretch \([3]\)
s) ring stretch \([4]\)
tt) ring stretch + C = O stretch \([2, 3]\)
uu) ring stretch + C = O stretch \([2, 3]\)
vv) ring stretch \([2]\)
ww) C = O stretch; carbonyl group + carboxylic group of the pyrrole ring \([2, 3, 12]\)
xx) C = O stretch; carboxylic group of the pyridine ring \([6, 7, 8]\)
STRUCTURE OF THE COFACTOR OF THE ENZYME MADH (Q) - Recently, Dooley et al. [2] have employed the resonance Raman technique in trying to prove that the organic cofactor in bovine plasma amine oxidase (BPAO), pig plasma amine oxidase (PPAO) and lysyl oxidase (LO) is PQQ. However, because of the difficulty in obtaining high-quality resonance Raman spectra from the native enzyme, they investigated the 2,4-dinitrophenylhydrazine and phenylhydrazine derivatives of the enzyme. And from the similarities of the resonance Raman spectra among amine oxidase-DNPH, amine oxidase-PH and PQQ-DNPH, they concluded that the prosthetic group is PQQ. We have approached this problem in a different way. Instead of examining the derivatives, we have studied PQQ molecule and the enzyme MADH itself. Also, for the sake of comparison, we have obtained the RR spectra of the phenylhydrazine derivative of the enzyme MADH.

The absorption spectra of PQQ in aqueous solution shows three peaks at 249 (ε = 18,400 cm\(^{-1}\) M\(^{-1}\)), 275 (shoulder) and 330 nm (ε = 8,960 cm\(^{-1}\) M\(^{-1}\)) [22] and a small broad peak at 460 nm. These peaks resemble the absorption spectrum of 9,10-phenanthraquinone which shows peaks at 256 (ε = 28,840 cm\(^{-1}\) M\(^{-1}\); benzenoid π → π\(^{*}\)), 265 (ε = 28,840 cm\(^{-1}\) M\(^{-1}\); quinonoid π → π\(^{*}\)), 322 (ε = 4,168 cm\(^{-1}\) M\(^{-1}\); benzenoid π → π\(^{*}\)) and 410 nm (ε = 1,349 cm\(^{-1}\) M\(^{-1}\); quinonoid π → π\(^{*}\)) [23, 24] and the peaks are accordingly assigned. However, the absorption spectrum of the enzyme MADH shows a new peak at 440 nm (ε = 8,780 cm\(^{-1}\) M\(^{-1}\)) [25]. The 440 nm peak may be attributed either to a charge transfer complex or to a peculiarity in the link between PQQ and the enzyme. This type of behavior has been observed for several coenzyme systems such as DPN (diphosphopyridine nucleotide) and FAD (flavin adenine
dinucleotide) systems [26]. Though it has been the subject of some controversy, there is a body of evidence which suggests that these bands arise from a charge transfer complex [27]. The reason for us to believe that the cofactor is PQQ and the 440 nm is a charge transfer band is from our vibrational spectroscopic study (described below).

The resonance Raman spectrum of the enzyme MADH excited by 457.9 nm laser line is displayed in the Figure 4.4. Though the relative intensities of the bands are very different from the RR spectra (Figure 4.3) of the PQQ molecule, the positions of the peaks are very similar except for the peaks at 1445 (sh) and 1458 (m) cm\(^{-1}\), which exist in the enzyme MADH but do not exist in PQQ molecule. All the other peaks agree within 5 cm\(^{-1}\) (1566 cm\(^{-1}\) peak is within 15 cm\(^{-1}\)). This leads us to believe that the prosthetic group of the enzyme MADH is PQQ. The similarity of the RR spectra between MADH-phenylhydrazine (PH) and bovine plasma amine oxidase-PH (described below) gives further support of this point. The appearance of the peaks at 1445 and 1458 cm\(^{-1}\) may come from the charge donor of the charge transfer complex.

The absorption spectra of MADH titrated with phenylhydrazine, the titration curve and the Raman spectra of MADH-phenylhydrazine complex at different laser excitations are shown in figure 4.5, 4.6 and 4.7, respectively. The result of the titration data (monitoring the 310, 444 and 524 nm peaks) demonstrated that the stoichiometry is one to one ratio, i.e. one phenylhydrazine binds to one cofactor. This indicated that the MADH-phenylhydrazine adduct is the monohydrazone of the enzyme MADH. Similarity, the PQQ-
Figure 4.4 Resonance Raman spectrum of $2 \times 10^{-3}$ M MADH. Laser excitation - 457.9 nm. Laser power - 10 mW. Slits - 6 cm$^{-1}$. 
dinitrophenylhydrazine adduct is reported to be the monohydrazone of the PQQ [22, 28, 29]. The absorption spectrum of the MADH-PH adduct shows two peaks in the region from 320 - 600, one at 444 nm and another at 524 nm. This behavior is totally different from other enzymes, which supposedly contain PQQ as a prosthetic group, such as lysyl oxidase and bovine plasma amine oxidase [2] (only one peak around 450 nm in the absorption spectra in the region from 350-600 nm). From the RR data excited into the high frequency electronic band at 444 nm and low frequency electronic band at 524 nm (described below), we conclude that the 524 nm band is the charge transfer band of the enzyme MADH, which has been modified by a phenylhydrazine group attached and the 444 nm band is from the MADH-phenylhydrazine adduct.

From inspecting the RR spectra of the MADH-phenylhydrazine adduct, BPAO-phenylhydrazine adduct and LO-phenylhydrazine adduct, all the peaks agree very well in terms of intensity and position except that the 1400 cm\(^{-1}\) ring stretching mode which is not observed in MADH-phenylhydrazine adduct spectra. The disappearance of the 1400 cm\(^{-1}\) peak is not unexpected if we consider the variations in the microscopic environment of the chromophore (cofactor) between different enzymes. This similarity has led us to conclude that the prosthetic groups (PQQ) in these enzymes is the same.

STRUCTURE OF THE QH\(^+\) AND QH\(_2\) - According to McIntire et al. [25] and Davidson et al. [30], the anerobic titration of the enzyme MADH with dithionite proceeded through two phases. The first phase, which consumed 0.99 mole of dithionite per mole of
Figure 4.5 The absorption spectra of the enzyme MADH (0.92 mmol) titrated with (a) 0 mmol, (b) 0.025 mmol, (c) 0.05 mmol, (d) 0.075 mmol, (e) 0.1 mmol and (f) 0.125 mmol phenylhydrazine. (done by professor Michael H. Klapper)
Figure 4.6 The phenylhydrazine (Ph.) titration curve of the enzyme MADH monitored at (a) 524 nm, (b) 444 nm and (c) 310 nm. (done by Professor Michael H. Klapper).
Figure 4.7 Resonance Raman spectra of MADH-phenylhydrazine complex at (a) 413.1 nm, (b) 457.9 nm, (c) 514.5 nm and (d) 568.2 nm. Laser power - 10 mW. Slits -6 cm\(^{-1}\).
enzyme, resulted in the formation of a spectrally distinct half-reduced semiquinone intermediate. The second phase of the dithionite titration, which consumed an additional 1.06 mole of dithionite per mole of enzyme, resulted in the formation of the fully reduced species. Since methylamine dehydrogenase can accept 4 electron equiv/mole of enzyme, after the addition of 2 electron equiv, each of the cofactor contains one electron and is thus in a radical form. The semiquinone intermediate was extremely stable, which permitted resonance Raman spectrum to be taken. The reaction scheme was proposed as follows (according to the studies on reduction of ketones and various biological materials by sodium dithionite) [31, 32, 33, 34, 35]:

\[
S_2O_4^{2-} \rightarrow 2SO_2^\bullet \quad (4-1)
\]

\[
\begin{align*}
\{ & \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \ Quad...
(1) $S_2O_4^{2-}$ species disproportionates or decomposes to $SO_2^-$ species in aqueous solution (reaction 1) [31, 35]. (2) the $SO_2^-$ radical anion attacks the cofactor PQQ nucleophilically at the position 5 since this carbon is the most electrophilic center for PQQ and its analogs [31, 32, 22, 36] and forms the semiquinone (I) which is stabilized by the enzyme and is well known as an intermediate in the redox process between quinone and hydroquinone [37]. (3) the semiquinone (I) then reacts with $SO_2^-$ and forms the fully reduced form (II) which is extremely stable against reoxidation.

Whereas the titration of MADH with dithionite proceed through a distinct semiquinone intermediate, the titration of this enzyme with n-propylamine-HCl proceeded directly from the fully oxidized to the fully reduced species (first half reaction). The second half - reaction, i.e., reoxidation of the fully reduced form takes place after a few hours and reaches the semiquinone form which is extremely stable against reoxidation. Other than the fact of its existence, we presently know very little of the second half - reaction. We believe that
reoxidation proceeds through the PQQ semiquinone, since MADH both stabilized the PQQ semiquinone form and utilizes 1-electron acceptors in the reoxidation reaction. The proposed reaction scheme is as follow (McWhirter & Klapper, unpublished result):

\[
\begin{align*}
Q + RCH_2NH_2 & \longrightarrow (RCH_2NH_2)\cdot Q \longrightarrow (RCHO)\cdot QH_2\cdot (NH_2) \longrightarrow QH_2\cdot (NH_2) + RCHO \\
2 A + QH_2\cdot (NH_2) & \longrightarrow [X] \longrightarrow Q + NH_3 + 2 A^- 
\end{align*}
\]

The resonance Raman spectra of the semiquinone form of the enzyme MADH reduced by sodium dithionite (species I) and by n-propanylamine-HCl (species II) are shown in the Figure 4.8. Though the absorption spectra between these two semiquinones are almost identical (425 nm) the resonance Raman spectra show some differences as follows: (1) the strongest peak at 1525 cm\(^{-1}\) (species I) shifts to 1537 cm\(^{-1}\) (species II), (2) shifting of the peaks at 1460 and 1466 cm\(^{-1}\) (species I) to 1466, 1481 cm\(^{-1}\) (species II). All these ring stretching modes shift ~ 10 cm\(^{-1}\) and this may be due to increases in the electron density of the ring donated by the lone pair electron of the ammonia molecule. This kind of behavior has been observed for some semiquinones such as the semiquinone forms of the benzoquinone and flavins [38, 39, 40, 41, 42].

Since the absorption spectrum of the fully reduced and the semiquinone form of MADH either prepared by sodium dithionite or by n-propylamine are
Figure 4.8 Resonance Raman spectra of the semiquinone forms of the enzyme MADH reduced by dithionite (a) and by propanylamine (b). Laser excitation -413.1 nm. Laser power -10 mW. Slits -6 cm\(^{-1}\).
almost identical [30, 25] and the resonance Raman spectra of both semiquinone forms are very similar, instead of the generally believed aminoquinol structure we proposed that the structure of the semiquinone and the fully reduced form should be structure (I) and (II) in scheme I, respectively. Further support of this point is that if the species II is a semiquinone form of the aminoquinol structure, we expect to observe a NH\textsubscript{2} bending mode around 1615 cm\textsuperscript{-1} [43, 44, 45].

4–4 CONCLUSIONS

This study has examined the resonance Raman spectra of the methoxatin containing methylamine dehydrogenase (MADH), its semiquinones, its phenylhydrazine adduct and its prothetic group PQQ. Several conclusions about the structural information of the cofactor and the reduced forms of the enzyme MADH can be drawn as follows:

1. It was found that the cofactor of the enzyme MADH is methoxatin or a methoxatin-like molecule based on the similarity of the positions of the vibrational peaks.

2. The reduced form of MADH (reduced by propanyl amine) is not an aminoquinol because its absorption and resonance Raman spectra either prepared by dithionite or propanyl are very similar.
REFERENCES


CHAPTER V

TEMPERATURE-COMPOSITION-MESOMORPH DIAGRAM OF MONOOLEIN/WATER LYOTROPE LIQUID CRYSTAL SYSTEM

(In collaboration with Professor Martin Caffrey and the samples were prepared by Professor Martin Caffrey.)

5-1 INTRODUCTION

The equilibrium properties of MAG (monoacylglyceride)/water mesomorphic phases have been the subject of extensive investigations since the pioneering work of Lutton [1, 2, 3]. The studies of the structure of the lipid-water systems might provide important information which will help in understanding the biological membranes [4]. A lyotrope gradient method for liquid crystal temperature-composition-mesomorph diagram construction using time-resolved x-ray diffraction (TRXRD) has been used to study the monoolein (MO; Figure 5.1)/water system with great success [5, 6, 7].

![Figure 5.1 1-Monoolein.](image)

The monoolein/water system possesses mesomorphic phases with one-, two- and three-dimensional periodicity and a fluid isotropic phase in an accessible
temperature range (20-120°C). Besides being very stable and available in high purity at low price, it is an important emulsifier, an intermediate in lipid digestion and a compound in black lipid membrane studies [1, 8, 9]. Among the many physical techniques available for phase diagram construction, X-ray diffraction is the most powerful one since it provides direct phase identification both in single and multiple phase regions and facilitates the location of phase boundaries. Nevertheless, due to low photon flux, conventional x-ray diffraction is laborious and time-consuming. The advent of synchrotron x-radiation with its extreme brightness enables phase identification and structural characterization in a very short time (30 sec for scanning through the capillary).

The lytrope(water) concentration gradient is established by bringing the monoolein and water into contact in the capillary and allowing limited diffusion of water into the monoolein until a gradient of sufficient length (~5 cm) forms at a fixed temperature (20-120°C). Each capillary sample tube represents an isotherm in the corresponding T-C diagram with the different phases and phase boundaries localized at different points along the length of the capillary. The advantages of this new method of T-C diagram construction are as follows:

(1) It is a very simple and quick method.

(2) The continuous nature of the method prevents the possibility of missing phase fields or coexistence regions that exist over narrow ranges of lytrope concentration.

(3) The water transport properties can be determined.
(4) The method is applicable to a wide range of lyotropic liquid crystalline material.

By using TRXRD, additional advantages can be provided which are as follows:

(1) Direct Phase identification, structural characterization, quantitation and phase boundary location can be readily achieved.

(2) It is a nondestructive and extremely rapid method.

A major shortcoming of the method is that water concentration along the gradient is not known. As a result of this shortcoming, the T-C plots cannot be referred to as phase diagrams in the conventional sense. Raman spectroscopy has been used to solve this problem. It turns out that Raman spectroscopy is a sensitive and convenient technique for probing water content along the capillary tube and by constructing a standardizing curve the water content is readily calculated. Moreover, Raman spectroscopy has provided important structural information.

5-2 EXPERIMENTAL

Monoolein (C18:1c9) was purchased from Nu-Chek-Prep (Elysian, MN). It is reported to be >99% pure and was used without further purification. Water was purified by using a Milli-Q (Millipore) system.

Water and monoolein were sequentially centrifuged into 0.5 mm diameter capillaries by using the low speed on a clinical centrifuge (International Clinical Centrifuge, Model 1528 E). Capillaries were then flame- and epoxy-sealed (Harduron, Inc.). Gradients were established by incubating the samples at room
The Lc phase was prepared by cooling (~5°C) the pure monoolein and the fluid isotropic (Fl) phase was prepared by melting the Lc phase. The CP4 (cubic, primitive, space group No. 4) was prepared by saturating the MO solutions with defined composition and phases were prepared by mixing the proper amount of water and MO. Homogeneous mixing was achieved by using a home-built device consisting of two disposable 1 mL tuberculin syringes connected at the Luer tip via a 5-mm length of plastic in which a 0.5 mm diameter hole had been drilled. Lipid was added to the device as a liquid (MO at ~ 40°C) upon which was layered the suspending aqueous phase. The system was flushed with argon, the plungers were inserted into the syringe barrels, and the mixture was passed under pressure from one syringe to the other through the interconnecting constriction by working the two plungers back and forth. The samples were then transferred to capillaries. In this way, the phases such as Lα (lamellar liquid crystal phase), Fl + water, CP4 and BCC12 (cubic, body center, space group No. 12) phase with defined composition have been prepared.

5-3 RESULTS AND DISCUSSION

ASSIGNMENTS OF THE RAMAN PEAKS OF THE MO- The Raman spectra of the CP4, Lα, Fl, and Lc phase of the MO are present in Figures 5.2 and 5.3 and the assignments of the peaks based on sodium oleate [10] is listed in Table 5.1. The water peak at ~ 3400 cm⁻¹ is clearly seen in the spectrum of the CP4 phase. Also, as expected the peaks in the Lc crystalline phase are much more resolved. Three region of peaks are very sensitive to the phase changes, these

THE C=C DOUBLE - BOND STRETCHING REGION- The C=C stretch at 1658 cm⁻¹ drops dramatically in intensity when the phase changes from Fl, Lα
or CP4 to Lc. This anomalous decrease in intensity of the C–C stretch in the Lc phase is mostly likely due to a slight decrease in the C=C bond length [10]. The reason for this [10] is that the Raman intensity is proportional to bond length and bond order [16, 17, 18] and the frequencies are all the same in all the phases and this indicates that the bond order does not change. The decrease in the C=C bond length was attributed to the following [10]: (1) the balance between long-range attraction and steric repulsion forces between neighboring oleate chains, (2) the increase in the strain on the C=C bond by neighboring trans rotation in the chain (3) the increase in lateral pressure due to the order induced increase in density near the methyl end.

THE C–O DOUBLE – BOND STRETCHING REGION – Two C=O stretching bands show up in the CP4 and Fl phase but only one (1732 cm⁻¹) in Lc phase. This type of doublet has been observed previously and is demonstrated to be due to different conformations about the interface C₃C₂C₁(=O)O-ester group. The one with a bent conformation at the C₂ position of the acyl chain (gauche) is at 1721 cm⁻¹ while that of a nearly straight acyl is at 1739 cm⁻¹ [15, 19]. The possibility that the two C=O bonds are located in different environments e.g. one in a strong and another one in a weak hydrogen bonding site has been eliminated. To prove this point, Raman spectra of MO in different solvents such as methol, chloroform and CCl₄ (Figures 5.4 and 5.5) have been obtained and the data agree with above interpretation.

SKELETAL C–C STRETCHING REGION – Raman spectra in this region has been widely used to monitor the conformational changes in lipids [20, 21]. The peak height ratio of the 1121 or 1064 cm⁻¹ (trans C–C stretching band) and the
Figure 5.2 Resonance Raman spectra (low frequency region) of monoolein in (a) Lc, (b) Fl, (c) CP4 and (d) La phase. Laser excitation 413.1 nm. Laser power 10 mW. Slits 6 cm⁻¹.
Figure 5.3 Resonance Raman spectra (high frequency region) of monoolein in (a) Lc, (b) Fl, (c) CP4 and (d) Lα phase. Laser excitation -413.1 nm. Laser power -10 mW. Slits -6 cm⁻¹.
Figure 5.4 Resonance Raman spectra (low frequency region) of monoolein in (a) methanol, (b) chloroform and (c) CCl₄. Bands masked with an asterisk are due to solvents. Laser excitation -413.1 nm. Laser power -10 mW. Slits -6 cm⁻¹.
Figure 5.5 Resonance Raman spectra (high frequency region) of monoolein in (a) methanol, (b) chloroform and (c) CCl₄. Bands masked with an asterisk are due to solvents. Laser excitation -413.1 nm. Laser power -10 mW. Slits -6 cm⁻¹.
1087 cm\(^{-1}\) (gauche C-C stretching band) is a good indication of the conformation of the MO. As expected, the strongest peak in this region in the Fl, Lα and CP4 phase is 1087 cm\(^{-1}\) and that in Lc phase is 1062 cm\(^{-1}\) and this indicates that in Fl, Lα and CP4 phases MO exist mostly as the gauche conformer and entirely as trans conformer in Lc phase.

C-H STRETCHING REGION- Raman spectra in this region have been well investigated due to their widespread use in the study of model and biological membranes [21]. The peak height ratio of the 2884 cm\(^{-1}\) (asymmetric CH\(_2\) stretch) has been used as a sensitive measurement of interchain interaction (or fluidity of the lipid hydrocarbon chains) [22, 23]. The sharp band at 2884 cm\(^{-1}\) (Lc phase) disappears (more accurately, broadens [10]) in the spectra of the Fl, Lα and CP4 phases and this has been attributed to reorientational fluctuation, breakdown of the zero-wave-vector selection rule and coexistence of a variety of local structures [10].

Table 5.1 Band assignments of the MO.

<table>
<thead>
<tr>
<th>MO</th>
<th>assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3009</td>
<td>=CH symmetric stretch [10]</td>
</tr>
<tr>
<td>2962</td>
<td>CH(_3) asymmetric stretch [10]</td>
</tr>
<tr>
<td>2934 sh</td>
<td>Fermi resonance [10]</td>
</tr>
<tr>
<td>2920</td>
<td>(\alpha)CH(_2) stretch [10]</td>
</tr>
<tr>
<td>2900 sh</td>
<td>Fermi resonance [10]</td>
</tr>
<tr>
<td>2884</td>
<td>CH(_2) asymmetric stretch [10]</td>
</tr>
<tr>
<td>2875 sh</td>
<td>CH(_3) symmetric stretch [10]</td>
</tr>
<tr>
<td>2849</td>
<td>CH(_2) symmetric stretch [10]</td>
</tr>
<tr>
<td>2732</td>
<td>1296 + 1439</td>
</tr>
<tr>
<td>2718</td>
<td>1296 + 1419</td>
</tr>
<tr>
<td>1739 (Fl and CP4 phase)</td>
<td>C=O stretch (trans)[11]</td>
</tr>
</tbody>
</table>
Table 5.1 continued

<table>
<thead>
<tr>
<th>Frequency (cm$^{-1}$)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1732 (Lc phase)</td>
<td>C=O stretch (trans) [11]</td>
</tr>
<tr>
<td>1720 (Fl and CP4 phase)</td>
<td>C=O stretch (gauche) [11]</td>
</tr>
<tr>
<td>1656</td>
<td>C=C symmetric stretch [10]</td>
</tr>
<tr>
<td>1457</td>
<td>CH$_3$ scissor [10]</td>
</tr>
<tr>
<td>1439</td>
<td>CH$_2$ scissor [10]</td>
</tr>
<tr>
<td>1419</td>
<td>CO$_2^-$ symmetric stretch [10]</td>
</tr>
<tr>
<td>1366</td>
<td>CH$_2$ wagg [10]</td>
</tr>
<tr>
<td>1353</td>
<td>CH$_2$ twist [10]</td>
</tr>
<tr>
<td>1296</td>
<td>CH$_2$ twist [10]</td>
</tr>
<tr>
<td>1270</td>
<td>HC=CH twist [10]</td>
</tr>
<tr>
<td>1258</td>
<td>(C–C)CH$_3$ symmetric stretch, trans [10]</td>
</tr>
<tr>
<td>1121 (Lc phase)</td>
<td>(C–C)CO$_2^-$ symmetric stretch, trans [10]</td>
</tr>
<tr>
<td>1087 (Fl and CP4 phase)</td>
<td>C–C asymmetric stretch, gauche [10]</td>
</tr>
<tr>
<td>1062</td>
<td>C–C asymmetric stretch, trans [10]</td>
</tr>
<tr>
<td>1052 sh</td>
<td>C–C asymmetric stretch, trans [10]</td>
</tr>
<tr>
<td>996</td>
<td>=C–H out-of-plane bend [10]</td>
</tr>
<tr>
<td>969</td>
<td>=C–H out-of-plane bend [10]</td>
</tr>
<tr>
<td>943</td>
<td>rocking vibration of the terminal –CH$_3$ [10]</td>
</tr>
<tr>
<td>918</td>
<td>rocking vibration of the terminal –CH$_3$ [10]</td>
</tr>
<tr>
<td>892</td>
<td>rocking vibration of the terminal –CH$_3$ [10]</td>
</tr>
<tr>
<td>850</td>
<td>skeletal [12]</td>
</tr>
<tr>
<td>828</td>
<td>skeletal [12]</td>
</tr>
<tr>
<td>771</td>
<td>CH$_2$ rock [13]</td>
</tr>
<tr>
<td>728</td>
<td>CH$_2$ rock [14]</td>
</tr>
</tbody>
</table>

Frequencies in cm$^{-1}$ units; intensities abbreviated: sh = shoulder.

THE WATER CONTENT ALONG THE CAPILLARY TUBE—The sample capillary tube and the positions for the Raman experiments is shown in Figure 5.6. The typical spectra are shown in Figures 5.7 and 5.8. The amount of the water and MO is determined by measuring the peak height at 3400 cm$^{-1}$ (OH
stretch; no interference from MO) and 1296 cm$^{-1}$ (CH$_2$ twist; insensitive to phase change). By taking the ratio of these two, the relative amount of H$_2$O can be calculated. Moreover, assuming the point at the interface has a water content of 42%, the absolute concentration of water can be calculated and is

\[ H_2O \quad Monoolein \quad Ar \]

Figure 5.6 The capillary sample tube and the positions for the Raman experiment: shown in Figure 5.9. As expected, water content gradually decreases along the capillary tube and it agrees well with theoretical calculation (solid line in Figure 5.9).

5-4 CONCLUSIONS

A lyotrope gradient method for rapidly constructing temperature-composition-mesomorph diagram using time-resolved x-ray diffraction has been used to study monoolein/water liquid crystal system with great success. Unfortunately, a major shortcoming of this method is that lyotrope concentration along the gradient in the capillary tube is not known. In this study, Raman spectroscopy has been used to solve this problem. It turns out that Raman spectroscopy is a sensitive and convenient technique for probing water content along the capillary tube and by constructing a standardizing curve the water content is readily to be calculated. Moreover, Raman spectroscopy has provided
Figure 5.7 Resonance Raman spectra (low frequency region) along the sample capillary tube (see Figure 5.6) (a) 0.0 cm, (b) 1.0 cm, (c) 2.0 cm, (d) 3.9 cm and (e) 5.4 cm from the interface. Laser excitation -413.1 nm. Laser power -10 mW. Slits -6 cm⁻¹.
Figure 5.8 Resonance Raman spectra (high frequency region) along the sample capillary tube (see Figure 5.6) (a) 0.0 cm, (b) 1.0 cm, (c) 2.0 cm, (d) 3.9 cm and (e) 5.4 cm from the interface. Laser excitation -413.1 nm. Laser power -10 mW. Slits -6 cm⁻¹.
Figure 5.9 Water concentration (w/w) along the sample capillary tube (see Figure 5.6) after 1296 hr. (asterisk points = experimental data, solid line = theoretical calculation (done by Professor Callrey)).
important structural information. Namely, there are two conformers exist in organic solvents, BCC12 phase, Lα phase, CP4 phase and FI (without water) phase but only one conformer in Lc phase. Also, there are more trans state and less reorientational mobility of the acyl chain in Lc phase than other phases.


BIBLIOGRAPHY


60. A. Smekal, Naturwiss., 11, 873 (1923).

61. L. Rayleigh, Phil. Mag., XVI, 274, 447 (1871).


65. L. Angeloni, G. Smulevich and M. R. Marzzocchi, Spectrochim. Acta, 
   38A, 213 (1982).

66. M. Manfait, A. Alix, P. Jeanneson, J. Jardiller and T. Theophanides, 


70. A. N. Anoshin, E. A. Gastilovich and D. N. Shigorin, Zh. Fiz. Khim, 54, 
   1474 (1980).


   (1982).

73. G. Smulevich, A. Amirav, V. Even and J. Jortner, Chem. Phys., 73, 1 
   (1982).


143. C. Manneback, Physica, 17, 1001 (1951).


