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**MITOXANTRONE
AND ITS INTERACTION WITH
LIPIDS, DNA AND CELLS
AS STUDIED BY
PRESSURE-TUNING FOURIER-TRANSFORM
INFRARED SPECTROSCOPY**

by

Myra Nichole Michelin, BSc (Carleton)

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the degree of**

**Master of Science
Department of Chemistry**

Carleton University

Ottawa, Ontario

April 26, 1993

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MITOXANTRONE
AND ITS INTERACTION WITH
LIPIDS, DNA AND CELLS
AS STUDIED BY
PRESSURE-TUNING FOURIER-TRANSFORM
INFRARED SPECTROSCOPY

submitted by Myra Nichole Michelin, BSc (Carleton)
in partial fulfilment of the requirements for
the degree of Master of Science

Thesis supervisor

Chairman, Department of Chemistry

Carleton University

May 13, 1993

ABSTRACT:

Through inspection of the barotropic behavior of the bands of the charged lipid 1,2-dimyristoyl-*sn*-glycero-3-[phospho-L-serine] (DMPS) and the uncharged lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), with and with the chemotherapeutic agent mitoxantrone (MX) up to pressures of 15kbar, MX was found to bind to the phosphatidylserine head group of DMPS and partition into the interior of the lipid bilayer of DMPC.

The barotropic behavior of the DNA bands in the region 1300 to 900cm⁻¹, with and without the drug MX were examined. Examination of the bands to 15kbar revealed the intercalation of the drug and electrostatic interaction of the charged side arms of the drug with the phosphate groups on the exterior of the helix at all pressures.

The interaction of the drug with two cultured cell lines, colon adenocarcinoma cell line HT29 and epithelial ovarian cultured cell line 2008, were barotropically studied at pressures up to 15kbar for their interaction with the drug MX. Passive uptake of the drug occurred and the structural binding of the drug was examined. The drug caused a closer packing and increased hydrogen bonding of the nucleic acids and caused more disorder amongst the membrane lipids.

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"...to the only God our Saviour be glory, majesty, power and authority, through
Jesus Christ our Lord, before all ages, now and for evermore! Amen." Jude 25, NIV

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LIST OF SYMBOLS:

RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
MX	Mitoxantrone hydrochloride
IR	Infrared (spectroscopy)
cm ⁻¹	wavenumber
FT-IR	Fourier-transform infrared spectroscopy
DMPC	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphocholine
DMPS	1,2-dimyristoyl- <i>sn</i> -glycero-3-[phospho-L-serine]
PBS	phosphate-buffered saline
He-Ne	helium-neon
MCT	mercury-cadium-telluride

CHAPTER ONE

1.1 INTRODUCTION

1.1.1 Definition of cancer

The word cancer is derived from the Greek word for crab, *karkinos*¹. However, a more clinical definition of cancer or tumour may be found in R Willis's Pathology of Tumours: "A tumour is an abnormal mass of tissue, the growth of which exceeds, and is unco-ordinated with, that of normal tissues, and persists in the same excessive manner after cessation of the stimuli which evoked the change."²

Cancer has been with mankind for millennia. (They may have even been around before then. There is pathological evidence for the occurrence of bone tumours in dinosaurs.) One of the earliest documented reports of cancer came from Hippocrates who blamed an excess of black bile on cancer. It is an age-old battle for one author even states, "...think of cancer as a struggle for survival between a population of aberrant, aggressive neoplastic cells and the host..."³ Peyton Rous, a pioneer in viral oncogenesis, eloquently stated, "Tumours destroy man in an unique and appalling way, as flesh of his own flesh, which had somehow been rendered proliferative, rampant, predatory and ungovernable."⁴

To classify the more than 200 diseases comprising cancer, one must consider four factors: the organ (especially the tissue) of origin, the cellular appearance and arrangement (histology), the gross overall appearance and the behaviour of the tissue⁵.

The nomenclature of cancer may be confusing, even with these criteria. Precise definitions of cancer have been used indiscriminately. For example, the accurate

definition for a tumour is a solid neoplasm including benign neoplasms and some malignant neoplasms. However, in time the term cancer has been extended to describe all neoplasms⁶. (All tumours fall into one of two categories, benign or malignant, although sometimes there is a gradual spectrum between the two extremes.) The suffix -oma denotes a tumour. The name of the tumour comes from the tissue of origin. For example, a sarcoma is a malignant connective tissue tumour while carcinomas are malignant epithelial cells. The carcinomas are further specified adeno or squamous cell, depending on whether the neoplasm is glandular or of keratin formation. Some specialized tissues are not so easily categorized. Leukemias are haemopoietic neoplasms and while lymphomas are malignancies of lymphoid tissue.

1.1.2 Chromosomal mutations and cancer

On a gross overall appearance, neoplastic cells have large, irregular nuclear outlines. Even the number of chromosomes may be different than the standard 46 chromosomes. For example, the number of nuclei may be greater than normal⁷. These changes in the nucleus common to neoplastic cells do reflect an alteration in the 'control centre' of the cell. Neoplastic cells have a statistically higher incidence of chromosomal defects than normal cells. The main types of chromosomal aberrations in cancer cells comprise the following: deletion (a missing segment of the chromosome), gain (an extra chromosome, resulting in a hyperploidy cell), loss (a missing chromosome, resulting in a hypoploidy cell), translocation (a switch of one segment of one chromosome for another segment of another chromosome), or inversion (a rearrangement of sections of the same chromosome)⁸.

Some actual examples of chromosomal aberrations are given in the following. A loss of a gene or genes from the long arm of chromosome 13 in region 1, band 4 has been found to be critical for the development of retinoblastoma, both familial and sporadic^{9,10}. Another example of chromosomal aberration linked to cancer is the Philadelphia chromosome (Ph chromosome). This chromosome is a small chromosome and is derived from the following mutation: $t(9;22)(q34;q21)$. This genetic notation describes the switch of band 4 in region 3 of the long arm of chromosome 9 with band 1 of region 2 of the long arm of chromosome 22. This chromosomal mutation has been found in the dividing marrow or peripheral blood blasts of 85% of patients with chronic myelogenous leukemia¹¹. Also it has been found that 10% of acute lymphoblastic leukemia patients also have the Ph chromosome¹².

Another prominent discrepancy between normal cells and tumour cells is the occurrence of chromosomal aberrations called double minutes and homogeneously staining regions, found only in neoplastic cells. Double minutes (self-replicating, small bodies containing chromosomal material) and homogeneously staining regions (large, even staining regions of chromosomes) are usually the sites of amplified genes¹³. (This amplification of genes will be discussed shortly.) These are just a few examples of the established chromosomal mutations observed in some human malignancies.

But what actually causes a normal cell to transform and then form a mutiny upon one's own body? For a long time, researchers of cancer debated whether cancer is really a genetic disease. In this last century, great strides have been taken in answering this question, although serendipitously at first.

1.1.3 Viruses, oncogenes and cancer

Peyton Rous, who received a Nobel prize for his research, discovered that if filtrate from a chicken tumour was given to other chickens, the other chickens would also develop the same type of cancer. He was the first person to prove that a cancer could be caused by a viral agent¹⁴. The infectious agent in the chicken tumour filtrate, called the Rous sarcoma virus, is a retrovirus meaning the virus contains only RNA and uses the host's cellular apparatus to translate the RNA to DNA. The translated DNA is then used to make many copies of the RNA viral material. Further studies have shown that certain other retroviruses and some DNA viruses are also able to induce cancer.

The study of retroviruses has been linked to cancer by two different ways. Firstly, the actual insertion of the viral DNA may alter the host's own genes (insertional mutagenesis). Secondly, the retroviruses carry oncogenes (genes whose expression may result in neoplastic growth)¹⁵.

However, it was discovered that the oncogenes carried by the viruses made no contribution to viral replication! This realization that the genetic information was useless baggage to the virus then pointed to the existence of cellular copies of oncogenes. Clues as to the origin of the viral oncogenes were uncovered when a team of researchers found that sequences closely related to the RSV were already present in the DNA of normal chicken cells¹⁶. Subsequently, analogs of viral oncogenes were being discovered in cells of other species. Thus it appears that viral oncogenes are likely just cellular oncogenes in disguise! It seems that in the course of transduction of the viral DNA, the oncogenes were picked up and carried along by the virus by mistake. Table 1 shows the variety of

proteins that are encoded by retroviruses and the biochemical targets in the cells that the products affect¹⁷. The transformation of the cells seem to be brought by the activation of the oncogenes which mimic growth factors, hormone receptors and other important factors which regulate cell growth¹⁸.

Table 1: Some examples of proteins encoded by retroviral oncogenes
(excerpts from Bishop, JM (1988). *Oncogenes*. In JB Wyngaarden & LH Smith (Eds.). Cecil Textbook of Medicine, 18th ed. (pp. 1089-1092). Toronto: WB Saunders Co.)

Oncogenes	Tumorigenicity	Biochemical properties	Cellular location
<i>myc</i>	carcinoma	DNA binding	nucleus
<i>src</i>	sarcoma	protein binding	plasma membrane
<i>sis</i>	sarcoma	growth factor	cytoplasm

Some associations between viral infection and the incidence of cancer have been well-established. For example, some patients who had contracted the hepatitis B virus and who later developed hepatocellular carcinoma were found to contain some of the viral genome integrated into the cancerous liver cellular DNA¹⁹.

Proto-oncogenes (the cellular equivalent of viral oncogenes) are found in every member of every vertebrate species studied, suggesting that these genes play a crucial role for the organism²⁰. These genes code for growth factors and growth factor receptors and protein kinases, thus regulating cellular growth²¹. One widely accepted theory suggests that normally proto-oncogenes are needed to help regulate cellular growth and development of the organism. Unfortunately, the proto-oncogenes may be inappropriately activated by insertional mutagenesis or by chemical carcinogens or radiation, thus

transforming the cell from a normal one which is responsive to other cellular boundaries to one which is insensitive to crowding and reckless in proliferation. From a chicken tumour filtrate to human proto-oncogenes, the workings of cellular transformation are slowly being uncovered.

Recently, evidence has emerged for the existence of tumour suppressor genes. In the early 1960's, Ephrussi and their colleagues fused highly malignant cells with lesser malignant cells to discover the hybrid cells were highly malignant^{22,23}. From the experiments in these two laboratories, the concept of "dominantly acting oncogenes" arose where malignancy behaves as a dominant trait.

However, other researchers later showed that if malignant mouse cells were fused with non-malignant mouse cells, the resulting hybrids were unable to form tumours, indicating that tumour formation was being suppressed^{24,25}. These hybrids were unstable, and chromosomes were lost and malignant cells then arose. Through much research, it was found that tumour suppression is associated with the transfer of certain chromosomes using microcell fusion²⁶. Many studies now indicate that the inactivation of both alleles carrying the tumour suppressor genes is a prerequisite for neoplastic expression. One hypothesis suggests that the products of tumour suppressor genes may function as transcriptional factors which regulate the expression of genes that are critical in the control of carcinogenesis²⁷. A good review of this new and exciting field of research may be found in *The Annual Review of Genetics*, volume 24, pages 615-657.

To summarize, there is a close relationship between genetics and cancer, as chromosomal aberrations, retroviral oncogenes and cellular oncogenes have shown.

1.1.4 Cancer and lifestyle

Not only are trends in the incidence of cancer found through histology and molecular genetics but patterns have been seen from lifestyles as well. There are some established links between carcinogenesis and certain lifestyles. Table 2 contains excerpts from the book *The Causes of Cancer* by R Doll and R Peto²⁸. Certainly lifestyle plays a role in the occurrence of cancer as revealed by cigarette smoking, exposure to ionizing radiation and even excess alcohol.

Table 2: Established carcinogens
(excerpts from Doll, R & Peto, R. The Causes of Cancer,
Oxford University Press, Oxford, 1980, p. 1203.)

AGENT	SITE OF CANCER
aflatoxin	liver
asbestos	lung, pleura, peritoneum
benzene	marrow
alcoholic beverages	mouth, pharynx, larynx, esophagus, liver
ionizing radiation	marrow and probably all other sites
tobacco smoke	mouth, pharynx, larynx, lung, liver, esophagus, bladder
hepatitis B virus	liver

It also seems there are differences in the incidence of cancer depending upon the part of the world in which one lives, as Table 3 shows²⁹.

Table 3: Incidence of cancer among males (except where indicated)
(excerpts from Doll, R & Peto, R. The Causes of Cancer, Oxford University Press, Oxford, 1980, p. 1199.)

Site of origin of cancer	High incidence area	Low incidence area	Ratio of high incidence to low incidence
skin (melanoma)	Queensland, AUS	Bombay, IND	>200
esophagus	northeast Iran	Nigeria	300
stomach	Japan	Uganda	11
breast (♀)	British Columbia, CAN	Israel, non-Jewish	15
liver	Mozambique	England	100
colon	Connecticut, USA	Nigeria	10
ovary (♀)	Denmark	Japan	6

To determine the role that the environment plays in the incidence of cancer, various studies were performed comparing the incidence of cancer on migrant groups to the cancer rate both in the homeland and in the chosen homeland. From studies between negroes and caucasians in America and negroes in Ibadan, Nigeria during the 1960's and early 1970's, it was found that the incidence of cancer for American negroes were more comparable to those of American caucasians as opposed to negroes in Ibadan. Another study in a later time period compared the incidence of cancer between caucasians and those of Japanese descent living in Hawaii and Japanese living in Japan. Once again, the incidence of cancer among Japanese in Hawaii had more similarity to those of caucasians

in Hawaii than the Japanese in Japan. Both of these experiments were published by the International Agency for research on cancer in 1976³⁰.

There are some criticisms of these experiments. For example, there are no factors taking into account the time of immigration, whether it be years or even generations. Also, one's lifestyle will change to some extent, depending upon where one moves so that exact correlations cannot be drawn solely by one's environment. However, it is very interesting and important to see the effect that environment and lifestyles play in the occurrence of cancer.

To bring the issues of the incidence of cancer closer home, Tables 4, 5, 6 and 7 show some statistics of the incidence of cancer among males and females in Canada in 1984³¹.

Table 4: Incidence of cancer in Canada, ages 0-24
(excerpt from Statistics Canada, Health Division, Vital
Statistics and Disease Registries section, July 1989)

RANK	MALE	FEMALE
1	brain	Hodgkin's disease
2	lymphoid leukaemia	brain
3	Hodgkin's disease	lymphoid leukaemia
4	testis	thyroid gland
5	myeloid leukaemia	ovary

Table 5: Incidence of cancer in Canada, ages 25-44
 (excerpt from Statistics Canada, Health Division, Vital
 Statistics and Disease Registries section, July 1989)

RANK	MALE	FEMALE
1	testis	breast
2	malignant melanoma	cervix uteri
3	lung	malignant melanoma
4	brain	thyroid gland
5	colon	ovary

Table 6: Incidence of cancer in Canada, ages 45-64
 (excerpt from Statistics Canada, Health Division, Vital
 Statistics and Disease Registries section, July 1989)

RANK	MALE	FEMALE
1	lung	breast
2	prostate	lung
3	colon	body of uterus
4	bladder	colon
5	rectum	ovary

Table 7: Incidence of cancer in Canada, ages 65 and over
(excerpt from Statistics Canada, Health Division, Vital
Statistics and Disease Registries section, July 1989)

RANK	MALE	FEMALE
1	prostate	breast
2	lung	colon
3	colon	lung
4	bladder	body of uterus
5	rectum	rectum

Some trends in the age ranges are quickly apparent: the top three cancer for males between the ages of 45 and 64 are lung, prostate and colon and this pattern is unchanged in the age group for men 65 and older (although not necessarily in that particular order). For women in the same age groups, cancer of the breast and lung are in the top three. For the group up to 24 years of age, both male and female, the most common incidences of cancer (but not in order) are brain, Hodgkin's disease and lymphoid leukemia. Malignant melanoma of the skin is in the top three for the age group 25 to 44 years of age for both males and females.

Therefore, it can be seen that cellular transformation of a normal cell to a neoplastic one involves a complicated interaction of molecular genetics and lifestyle.

1.1.5 Treatment of cancer

What would be the ideal cure for cancer? The perfect treatment would destroy all of the neoplastic cells with no harmful effects to the patient. However, this certainly is not a perfect world, so the side effects of the treatment have to be monitored along

with the benefits. The effectiveness of a treatment is usually assessed by the following criteria: (1) how long the patient survives, (2) how long the patient is clinically free of cancer and (3) the quality of life enjoyed by the patient³³.

Various approaches have been taken in the treatment of the cancer, such as (1) surgery, (2) radiative therapy, (3) immunotherapy and (4) chemotherapy . In any event, time is the crucial factor. The detection limit of a tumour is roughly 1cm. For a tumour to reach that size, it will have undergone approximately 30 doublings. Only ten more doublings will produce a tumour of 1kg, which is usually lethal³³.

Historically, surgery has longest been the method of choice, although some conditions are impossible to correct by surgery, such as deeply embedded brain tumours. Unfortunately, surgery can severely damage the surrounding tissue and, in case of malignant tumours, any metastatic spread of the disease is not eradicated by local surgery³⁴. Surgery is a good choice for those tumours which are benign (encapsulated) and accessible. Another advantage to "debulking" is that a reduced tumour causes the tumour cells to grow rapidly again, making them susceptible to the other two treatment options, radiation and chemotherapy.

Radiation therapy applies ionizing radiation to neoplastic cells. The source of the electromagnetic radiation must have the energy to remove electrons from the atoms or molecules. It is a bastion of cancer treatment in the sense that up to 70% of all cancer patients may have radiation at some point in their treatment regime³⁵.

The sources of the ionizing radiation are either γ -rays (from a radioisotope, especially cobalt-60) or x-rays (usually from the acceleration of electrons into a metal)³⁶.

Since water comprises roughly 80% of the total content of the cell, neoplastic or otherwise, the breakdown products of ionized water play an important role in irradiation. The two major radicals are electrons and the hydroxy radical³⁷. In case an organic molecule is ionized, the main products are organic free radicals (RH• and ROH•) which react rapidly via oxidation, reduction, etc³⁸. It appears that nucleic acids, particularly DNA, are vulnerable to the radicals resulting from radiation. The DNA strand may open and lose its hydrogen bonding, and the ribose sugars and pyrimidines are easily damaged.

The third means of cancer treatment is immunotherapy. This treatment involves stimulation of the immune system to recognize and attack any cancerous cells. A famous example of this treatment is interferon, single chain polypeptides which stimulate the immune system and inhibit tumour growth.

The fourth major treatment of cancer, and the one most relevant to this study, is chemotherapy. Chemotherapy involves the selective and careful use of chemicals to damage and/or kill the neoplastic cells while minimizing the damage to normal cells. There are two types of chemotherapeutic agents: cytotoxic drugs (those which kill cells) and cytostatic drugs (those which arrest the development of a cell)³⁹. In 1949, the first anti-cancer drug, nitrogen mustard, was introduced⁴⁰. Since then, approximately 50 or more drugs have been approved for clinical treatments in the United States. This is a select group of chemicals, reduced from the million or so potential compounds that have been screened for anticancer activity!

Chemotherapy drugs generally fall into the following categories: alkylating agents (compounds such as nitrogen mustard which target DNA base pairs resulting in

mutagenesis), hormones (such as tamoxifen in breast cancer treatments), anti-metabolites (compounds which interfere with a cellular metabolite, such as the well-known methotrexate which inhibits the enzyme dihydrofolate reductase) and platinum complexes (such as cisplatin which mainly targets DNA).

1.1.6 Mitoxantrone

The discovery of mitoxantrone (MX), an intercalator, was brought about by independent research of three institutions, the American Cyanamid Company, the National Cancer Institute and the Midwest Research Institute. In 1977, Zee-Chang and Cheng of the Midwest Research Institute⁴¹ announced the synthesis of a bis-substituted compound (MX) which was active against leukemia P-388 in mice. They followed a lead which had been announced earlier that year by the National Cancer Institute that a similar compound (ametantrone) had some antitumour activity⁴². (The only difference between MX and ametantrone is the hydroxylation of the first aromatic ring in MX.) In 1979, Murdock et al⁴³ announced the synthesis of mitoxantrone and its activity against tissue culture cells P-399 leukemia, L-1210 leukemia, B-16 melanoma and colon tumour 26. The synthetic path from Murdock's group is given in Appendix 1.

This drug is a significant one because it is one of the first entirely synthetic DNA binders to be used in cancer chemotherapy, along with ametantrone and amsacrine⁴⁴. Most other anticancer agents are from microbial sources or are derivatives from plant extracts. Therefore there is no danger that a plant might become extinct and thereby ending the starting product supply.

During these discoveries, doxorubicin (also called Adriamycin®), a powerful

chemotherapy drug of the anthracycline family, was in clinical use against leukemia and other solid tumours. However, this compound (and possibly the metabolites) were found to exhibit severe irreversible cardiotoxic side effects. In 1974, R.H. Adamson suggested that the amino sugar portion of the drug may be responsible for this nasty side effect⁴⁵. Therefore, the introduction of MX was viewed enthusiastically because this compound has no amino sugar portion but still retained potential as an anticancer agent.

1,4-Dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]-ethyl]-amino]-9,10-anthracenedione dihydrochloride, also known as mitoxantrone and as its trademark name Novantrone®, is made of a planar quinoid ring system with two alkyl side chains. See Figure 1. The aliphatic amino groups in the side chains are most likely protonated under physiological conditions⁴⁶.

Figure 1: Structure of mitoxantrone

MX was used in clinical trials in 1979⁴⁷. Since then, MX has been used singly

and in combination with other drugs for treatment of breast cancer, ovarian cancer and certain types of leukemias and lymphomas. It is mainly used as a treatment for advanced breast cancer with other drugs such as cyclophosphamide, 5-fluorouracil, mitomycin and doxorubicin^{48,49,50}. It is also used for treatment against acute myeloid leukemia and adult acute lymphoblastic leukemia with combinations of drugs^{51,52}.

The average dose of MX for an adult is 12-14mg/m² every three weeks for solid tumours and 8-20mg/m² for acute leukemia per day for a five day stretch. For children, 18mg/m² every four weeks is the average dose for solid tumours and 5-8mg/m² per day for five days for leukemia.

The success of the drug varies, depending upon the combination of drugs used and upon the disease. As a single agent for treatment of advanced breast cancer, MX has a response rate of roughly one-third⁵³. A particularly 'good' combination for treatment of breast cancer is cyclophosphamide, mitoxantrone, fluorouracil (CNF) where the response rate is 42%⁵⁴. MX has also been used for treatment of ovarian epithelial cancer where patients had previously been exposed to cisplatin and alkylating agents and the response rate in a phase II trial was 25%⁵⁵.

The cause of the drug's potency seems to lie in its ability to intercalate the DNA (slip between the nitrogenous bases of the DNA strand). It can even act as a staining agent for cellular preparations to highlight the nucleic acids. Its structure is favourable for intercalation due to the planar aromatic ring system⁵⁶. However, MX can only intercalate into the DNA partially because of the interference of the alkyl side arms. It is thought that the charged side arms bind to the phosphate groups on the exterior of the

macromolecule, thus stabilizing the attachment of the drug to the DNA^{57,58}. Some other effects the drug has on the cell are that it causes G2 phase arrest in mitosis⁵⁹ and inhibits DNA and RNA synthesis in certain cells in culture⁶⁰. Whether these effects of the drug is a direct consequence of intercalation is unknown (but likely --nm).

Another interesting aspect of this particular chemotherapy drug is its lessened cardiotoxicity. It is not as cardiotoxic as doxorubicin⁶¹. It is the oxygen free radicals of doxorubicin caused by reducing enzymes (such as flavoenzymes) that damage the heart tissue, yet MX is not as susceptible to enzymatic activation to free radical semiquinone forms as doxorubicin. (Since cardiotissue has inherently less antioxidants, it is highly susceptible to free radical attack^{62,63}.) It has been found that mitoxantrone is activated by peroxidases to form a nitrogen-centred free radical^{64,65,66}. The free radical reacts with hydroperoxy fatty acids and therefore inhibits lipid peroxidation in the cellular propagative stages⁶⁷. However, the amount of glutathione S-transferase, an enzyme which has been implicated with drug resistance, does show an increase upon exposure to MX in certain tumour cell lines, indicating that the entry of MX into the cell is not ignored by the cell's defence system⁶⁸.

To summarize, some attractive features of this drug is that fact that it is synthetic and therefore unreliant upon plant or microbial sources; it is not as cardiotoxic as some other chemotherapeutic agents; it can be used for a variety of cancers, including leukemias, lymphomas and advanced breast cancer.

In this research, the interaction of mitoxantrone with lipids, DNA and cells will be studied.

It is important to know whether the charge on a lipid will affect the uptake of a charged drug. After all, as previously stated, some lipids (especially phospholipids) form the membrane of cells and the membrane acts as a gate as to what may enter the cell. If the charge on a lipid does affect the cellular uptake of the cell, then the drug may be modified to be more efficiently transported.

There is a school of thought in the literature that states that if an anthracycline drug, like the powerful chemotherapeutic agent doxorubicin, is able to bind to the exterior of the cell membrane through electrostatic interaction, rather than partition into the lipid bilayer, then it is likely to be most active against malignancies^{69,70}. Therefore, a drug which simply partitions into the bilayer (as found with the interaction of DMPC and mitoxantrone) would be less active clinically since the binding to the membranes (whether cellular membranes or subcellular membranes) could possibly affect the activity of membrane enzymes⁷¹. Therefore, the study of this drug and its interaction with the two types of phospholipids would be valuable information pertaining to its uptake, and whether it is possible for the drug to actually diffuse into the cell or whether active transport is required.

As mentioned earlier, mitoxantrone is an intercalator and that the drug appears to bind electrostatically to the phosphate groups of the DNA helix. However, confirmation of the external binding would help provide understanding in the activity of this drug and its toxic effect against neoplastic cells. An understanding of the interaction of the drug and the target is essential for the design and improvement of further chemotherapeutic agents.

Studying the uptake of a drug into the cell is not simplistic. The drug may be passively taken up (diffusion) or actively transported by a membrane protein. Mitoxantrone's affinity for the nucleic acids is so strong that it could even be used as a cellular stain for the nucleolus⁷². By using cells exposed to mitoxantrone, one could study the structural changes that occur to learn more about how this drug affects the cell overall.

CHAPTER TWO

2.1 VIBRATIONAL SPECTROSCOPY

2.1.1 Background in vibrational spectroscopy

Spectroscopy is the study of the interaction of electromagnetic radiation with matter. For example, infrared spectroscopy is the study of the interaction of light in the specific region of 0.8 to 500 μm but spectroscopists more commonly use wavenumbers to define the region of light. The relationship between wavelength λ (μm) and wavenumbers ν (cm^{-1}) is $\nu = 10^4/\lambda$. The infrared region is usually groups into three regions: the near infrared comprising the 12,500 to 4000 cm^{-1} range, the mid-infrared from 4000 to 400 cm^{-1} and the far infrared from 400 to 20 cm^{-1} . The research involved in this thesis is concerned with the mid-infrared region.

Every molecule possesses three inherent types of internal energy and they are, in order of decreasing energy, electronic, vibrational and rotational⁷¹. (Translational energy can be ignored in these discussions because it has little effect on molecular spectra.) Even at absolute zero, molecules undergo vibrational motion. The vibrations are not chaotic but ordered.

A molecular spectrum is very different from an atomic spectrum. A single atom can exist at various electronic energy levels. Upon returning to a lower energy level, the atom may emit a photon of frequency corresponding to the energy difference between the two levels. In contrast, a molecular spectrum is richer in levels. Not only are there electronic levels as in atoms, but the molecules are able to absorb energy causing vibration of the nuclei with respect to one another and the molecule may even rotate as

a whole. As in electronic levels, the vibrational and rotational levels are quantized and are coupled to the electronic energy levels⁷⁴. Figure 2 is an example of molecular energy levels⁷⁵.

Figure 2: Example of molecular energy levels (From Reference 75)

Since the vibrational transitions are quantized, the frequency of the infrared light coincides with the energy necessary to make the vibrational transitions. An electronic transition would require more energetic photons for excitation, as found in the visible and ultraviolet regions, for example.

To visualize a molecular vibration, it is helpful to consider say a diatomic molecule as two vibrating masses (the atoms) joined by a spring (the bond) and the energy of the bond varying from kinetic energy to potential energy as the molecule vibrates. Although the bond distance continually changes, an equilibrium distance for the average bond length can be found. According to Hooke's law for vibrating springs, the natural frequency of vibration of a bond is given by the equation:

$$\nu = \frac{1}{2\pi c} \sqrt{\frac{K}{\mu}} = 4.12 \sqrt{\frac{K}{\mu}}$$

where ν is the frequency of vibration [cm^{-1}],

c is the velocity of electromagnetic radiation [cm/sec],

K is the force constant between the two atoms [dynes/cm],

and μ is the reduced mass of the system

where $\mu = (m_1 m_2) / (m_1 + m_2)$ where m_1 and m_2 are the masses of the atoms [g].⁷⁶

From Hooke's law then, it can be seen that stronger bonds have a large force constant and therefore will vibrate at higher frequencies than weaker bonds and also that bonds between heavier atoms vibrate at lower frequencies⁷⁷.

In order for a molecule to absorb IR energy, a net change in the dipole moment must occur when the molecule vibrates or rotates. During the vibration, there occurs a change in the distribution of electrical charges, an oscillating charge which may absorb the incoming radiation. This separation of charges (electric distortion) of the molecule is called an electric dipole. The molecule returns to its normal state when the electric field is removed, therefore the electric dipole of the molecule which temporarily formed is called an induced dipole. In order to obtain a vibrational spectrum, there must be a change in the dipole moment of the molecule⁷⁸.

In 1912, Peter Debye suggested some molecules possess a permanent dipole moment. Thus, even without the presence of an electric field, there exists a separation l of the opposite charges, giving a permanent dipole μ of magnitude zl where z is the absolute value of the charge⁷⁹.

The absence of dipole moments in molecules such as O_2 and H_2 indicate there is an equal distance between electron pairs bonding the atoms together. These homonuclear diatomic molecules are said to be non-polar. For heteronuclear diatomic molecules such as HCl and HBr , there is an unequal distribution of the electron density between the atoms, where the Cl and Br atoms have a slightly greater electron density than the hydrogens, for example. This imbalance of charges is an electric dipole.

The understanding of the electric dipole moments is crucial to vibrational spectroscopy because there is no infrared spectrum without a change in the electric dipole in the course of a molecular vibration. The absorption of infrared light is brought about by the interaction of the infrared light with the molecule, and the electric dipole moment

is changed.

Both infrared light and Raman scattering techniques may be used to examine vibrational modes of molecules. An important distinction must be made between these two techniques. As stated previously, IR spectra requires a change in the electric dipole state. Therefore, it is impossible to observe infrared spectra from molecules such as N_2 and H_2 because these molecules do not possess a permanent dipole moment. In contrast, Raman spectroscopy causes an *induced* dipole moment in molecules and therefore N_2 and H_2 may be examined using Raman spectroscopy. Also, a molecule which possess a centre of inversion cannot be both infrared and Raman active, for complex group theoretical reasons. Therefore, both IR and Raman spectroscopy are need to form a complete picture of a molecule's vibrational levels.

Some basic spectroscopic terminology must be introduced at this point. A *mode* is a type of vibration of the molecule. For an N-atomic molecule, there are 3N number of coordinates (x, y, z) needed to specify the location of the entire molecule. Yet, since each atom may change its location by simply changing one of the coordinates, there are 3N degrees of freedom in the molecule. Additionally, three of the 3N degrees of freedom relate to the translational motion of the molecule, giving 3N-3 non-translational degrees of freedom. As well, for a linear molecule only two coordinates (x and y) are required to identify its spatial orientation and all three coordinates (x, y, z) are needed to specify the location of a non-linear molecule. Accounting then for both translational and rotational movement, a linear molecule has 3N-5 degrees of freedom while a non-linear molecule has 3N-6 degrees of freedom⁶⁰. Each degree of freedom corresponds to a

vibrational mode.

A *normal* mode is an independent motion of a group of atoms that may be excited on a vibrational level without affecting the other normal modes⁸¹. Some examples of types of vibrational modes are stretching modes (symmetric and antisymmetric) and bending modes. For example, in the case of H₂O, a symmetric stretch of the O-H causes the oxygen atom to be buffeted back and forth between the two hydrogens. However, the antisymmetric stretch in this molecule would be the stretching of the hydrogen bonds where one hydrogen nears the oxygen atom while the other hydrogen atom retreats. These are normal independent modes of vibration because if one mode is excited, the other is not. A bending mode may be in the plane of the functional group (scissoring or rocking) or out of the plane of the functional group (twisting or wagging)⁸².

Spectral parameters such as frequencies, intensities, band widths and band shapes reveal information about the system under examination⁸³. Groups of molecules with an inherent dipole moment have characteristic absorptions in the infrared spectrum. (Recall that the vibrational transitions are quantized.) Therefore, IR spectroscopy has been used in the identification of molecular species, especially of organic compounds. Solids, liquids and gases may be examined through infrared spectroscopy.

2.1.2 Spectrometers

But, as in all scientific advances, scientists are always limited by the sensitivity of the equipment! The infrared spectrometer has been evolving since the nineteenth century. For example, Abney and Festing in 1882 reported absorptions in the infrared for many organic compounds and for the identification of characteristic molecular groups⁸⁴.

Efficient and high resolution grating spectrometers were in use by 1918⁸⁵. The single-beam spectrometers measures energy versus wavelength. But in the 1940's, double-beam spectrometers were introduced. These spectrometers measure the ratio between energy transmitted by sample and energy incident on the sample and then plot transmittance (or absorbance) as a function of wavelength⁸⁶.

A further improvement of the double-beam spectrometer was the application of a mathematical function, Fourier transform, to measure vibrational spectra. A Fourier-transform infrared spectrometer uses an interferometer, a device which splits the infrared beam into two halves and later recombines them, producing a curve of constructive and destructive interference. The resulting curve is called an interferogram.

The conversion from an interferogram to the more familiar frequency spectrum (intensity versus frequency) is made using Fourier cosine transformation. See Appendices 2^{87,88} and 3^{89,90} for a more detailed description of Fourier transform spectroscopy and the mathematical equations involved. An advantage of the Fourier-transform technique is that instead of the IR range being slowly scanned using prisms or gratings, the range can be scanned almost instantaneously. With computers connected to infrared spectrometers, many spectral scans may be taken and then averaged for a more accurate spectrum⁹¹.

However, no matter how sophisticated or basic an IR spectrometer is, the following elements are needed: a source, an optical system, a detector and an amplifier.

The source is typically a bonded silicon carbide rod (called a Globar) or a mixture of zirconium, thorium and yttrium oxides (called a Nernst glower)⁹².

As mentioned earlier, the IR region of the electromagnetic spectrum is inherently low in energy. Therefore, the optical system must minimize energy loss as it channels the IR radiation towards the sample. As a general rule, the larger the aperture of the optical system, the greater the quality and adjustments of the mirrors and slits. Since the optical path is shorter compared to component sizes, then more energy is available for the spectral slit width⁹³.

There are generally two types of detectors, photon detectors and thermal detectors. Since IR radiation is invisible, detecting the radiation brings its own unique difficulties. The energy is low, and the heat energy from random thermal motions of electrons must be distinguished from the true signal⁹⁴. Therefore, photon detectors are the detector of choice due to increased sensitivity over thermal detectors. Although the range may be limited, cooling the detector to liquid nitrogen temperatures improves the sensitivity and range. The photon detectors detect electrons which are ejected from the surface of a photoelectric cell. Therefore, incoming photons need to have sufficient energy to propel the electrons across the energy gap. Since the energy gap is temperature-dependent, it will decrease if the temperature is lowered⁹⁵.

Presently the preferred type of amplifier for the signal is an alternating current amplifier receiving chopped radiation so that the zero reference due to the discarded light reduces any drift from the detector, thereby producing accurate spectra⁹⁶.

2.1.3 Pressure-tuning Fourier-transform infrared spectroscopy

A further refinement of infrared spectroscopy, apart from Fourier-transform, is the use of pressure as a variable. A great deal of information may be obtained through

inspection of the barotropic behavior of spectral parameters such as frequency, intensity, band shapes and band splitting. Application of high pressures reduces the volume in a sample. Therefore, any molecular interaction which is weak at low pressures, may be amplified at higher pressures.

The instrumentation for pressure-tuning IR spectroscopy merely include an optical pressure cell and a spectrophotometer. The windows of the high-pressure cell are of paramount importance and must meet the following criteria: transparency in the IR range and be able to withstand great pressures.

A typical high pressure cell is the opposed diamond anvil cell, shown in Figure 3⁹⁷. In this cell type, the sample is placed in the hole of a gasket between the parallel faces of two diamonds so that when the two diamonds are pushed together pressure comes down upon the sample.

Figure 3: Opposed diamond anvil cell

Diamonds are a good material for high pressure work for several reasons⁹⁸. One, the hardness of a diamond enables it to withstand pressures in these cells up to 100kbar.

Two, a type IIa diamond absorbs little in the IR range of the electromagnetic spectrum. Three, only small samples are necessary for diamond anvil cells. Four, liquid and solid samples may be used. And five, opposed anvil cells are compact and are easily used in spectrometers. There are some disadvantages to the opposed diamond anvil cells. Large amounts of sample cannot be used. The diamonds may absorb in the region of interest, although spectral subtraction may easily remedy this. (Calcium fluoride⁹⁹ and glass¹⁰⁰ have also been used as window material for the opposed anvil cells but the pressure range is limited, from 6 to 13kbar respectively.)

The determination of pressure is a straightforward technique. An internal pressure calibrant is applied to the cell. The ruby static pressure scale for the measurement of pressure for Raman experiments is well-known where the accepted value for the ruby R_1 line frequency is 0.753cm^{-1} for every kbar¹⁰¹. A quartz calibration curve has been introduced which is applicable for infrared systems using diamond anvil cells. The equation for pressure calculation using the phonon band of α -quartz is:

$$p(\text{kbar}) = 0.015164(\Delta\nu)^2 + 1.2062(\Delta\nu) \text{ where } \nu_0 = 694.1\text{cm}^{-1} \text{ }^{102}.$$

2.2 FT-IR SPECTROSCOPY APPLIED TO BIOLOGICAL SYSTEMS

2.2.1 General applications of pressure-tuning Fourier-transform infrared spectroscopy

Through pressure-tuning IR spectroscopy, one can study hydrogen bonding of molecules. Hydrogen bonding occurs when a bond is formed between the hydrogen of A-H and B, where generally A and B are oxygen, nitrogen or fluorine¹⁰³. This attraction, which may be between different molecules or atoms of the same molecule,

causes a shift in the electron density of the proton-accepting functional group towards the proton-donating functional group, thereby weakening the bond of the proton-accepting functional group¹⁰⁴. As stated earlier, a weaker bond strength (a weaker force constant) causes a shift in the vibrational frequency to a lower frequency. Hydrogen bonding always moves the infrared band to a lower frequency for both groups participating in the hydrogen bond¹⁰⁵.

With the addition of pressure, the hydrogen bond is strengthened, because the pressure pushes the hydrogen bonding molecules closer together^{106,107}. The electron density of the proton-accepting functional group moves closer to the proton, further weakening the bond and resulting in an even lower frequency of vibration¹⁰⁸. As more and more pressure comes down upon the sample, eventually repulsion builds up between the atoms participating in the hydrogen bond and the bond is broken, and the strength of the proton-accepting function group returns to its normal strength. A return to an infrared frequency of a non-hydrogen bonded functional group is then observed¹⁰⁹. Therefore, using pressure-tuning infrared spectroscopy, one may observe the change in vibrational frequency to observe the presence of hydrogen bonding or the lack thereof¹¹⁰.

Pressure-tuning infrared spectroscopy may also be used to observe any environmental changes that occur upon the addition of high pressures to a system. For example, at high pressures applied to a lipid dispersion with an anesthetic, one can learn about the strength of its interactions with various lipids by observing at which pressures the anesthetic is expelled from the bilayers^{111,112,113}.

2.2.2 Lipids

Lipids are amphipathic molecules which are soluble in nonpolar organic solvents but are relatively insoluble in water. Lipids are an important group of biomolecules which include cholesterol and phospholipids¹¹⁴.

A phospholipid is composed of a hydrophobic section and a hydrophilic section. The backbone of a phospholipid is a glycerol molecule. One or two fatty acids attach to the backbone and these alkyl chains may be saturated or unsaturated. The water-loving section is composed of a phosphate group which also binds to the glycerol backbone. An organic group is bound to the phosphate group and together they form the phosphatidyl head group. The C=O region of the fatty acid which joins the alkyl chain and head group is called the interfacial region because at this point, the hydrophobic and hydrophilic portion of the lipid meet.

The nomenclature of phospholipids is straightforward. The fatty acids are named first in a phospholipid and the head group is last in the name¹¹⁵. For example, in the name 1-palmitoyl-2-oleoyl-*sn*-glycero-phosphocholine, palmitoyl and oleoyl describe those specific fatty acid chains and phosphatidyl choline refers to the particular head group. See Figure 4.

Due to their amphipathic nature, phospholipids having two fatty acid chains spontaneously form bilayers in water. The bilayers may be spherical or sheet formation. The polar head groups face outwards and the hydrophobic tails meet in the middle, shielded from the water. The inside of the lipid bilayer is known as the matrix. Phospholipids are important constituents of cell membranes and their abundance may

vary, depending upon the function of the cell. Even the abundance of particular phospholipids in a cell membrane may vary from one cell type to another. For example, a nerve cell has a high abundance of phosphatidylserine lipids¹¹⁶.

Figure 4: Example of a phospholipid

The study of cell membranes is essential to biology because the membrane is both a filter and a doorway for molecules entering and leaving the cell. Due to the reduced volume when pressure is applied, any weak interactions are enhanced. Therefore, pressure-tuning FT-IR spectroscopy has added to the knowledge of model membranes to observe pressure effects of the dynamic and structural properties. The first application of high-pressure vibrational spectroscopy appeared in 1982¹¹⁷.

Phospholipids exhibit phase transitions with increasing pressure. Some phases that may be observed for typical phospholipids are the transition to the liquid crystalline phase above the main transition temperature and a variety of gel phases at higher pressures. The band parameters of pressure-tuning vibrational spectroscopy (whether Raman spectroscopy or infrared spectroscopy) are sensitive to structural changes that occur with

these transitions and all of the above phase transitions may be observed spectroscopically¹¹⁸. Therefore, vibrational spectroscopy is an excellent technique to understand lipid dynamics by inspection of changes in frequency, band width, band intensity and band shape with pressure.

2.2.3 Deoxyribonucleic acid

There are two types of nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Both are essential for living systems. The structure of deoxyribonucleic acid was elucidated in 1953, garnering James Watson, Francis Crick and Maurice Wilkins a Nobel prize^{119,120,121}. The important features of this biomacromolecule is that the two helical polynucleotide chains are coiled about a common axis and run in opposite directions to the other. Pyrimidine and purine nitrogenous bases are located perpendicularly to the long chain axis on the inside of the double helix and phosphate and deoxyribose sugars are located on the outside at nearly right angles to the nitrogenous bases. Hydrogen bonds between the opposite nitrogenous base pairs hold the two strands together and adenine always pairs with thymine, and guanine with cytosine. The right-handed helical structure completes one full circle in ten base pairs. Certain proteins called histones support and externally surround the DNA¹²².

The importance of DNA is that the cellular genetic message is inscribed in the sequence of the nitrogenous bases. The message is translated into RNA and then into protein. DNA is generally located in the nucleus (headquarters) of eucaryotic cells. Due to its responsibility of passing on information built into molecular form, its integrity of replication is critical to cellular propagation.

There are actually three types of DNA, A-, B- and Z-DNA. B-DNA is the type that exists under physiological conditions. When hydration levels are decreased to below 75%, A-DNA structure appears, where the nitrogenous base pairs are tilted rather than perpendicular to the long chain axis. The helix widens and there are 11 base pairs per helix turn. Z-DNA is left-handed and occurs when the DNA sequence is composed of alternating purines and pyrimidines. However, the B-DNA is predominant under physiological conditions¹²³.

Due to the important role this molecule has in cellular propagation, it has been studied exhaustively. The first IR spectra of DNA was published in 1948, even before the structure had been discovered. Blout and Fields used thymus DNA and tried to make correlations of the absorptions frequencies with certain molecular groups¹²⁴.

Historically calf thymus DNA has been the material of choice for several reasons. The thymus gland has intrinsically low amounts of enzymes which degrade DNA; the ratio of DNA to other cell constituents (such as protein) is high; the thymus gland is cheap and easily obtained from slaughter houses¹²⁵.

It was not until the sixties that serious IR band assignments of DNA were made in the region $950-1350\text{cm}^{-1}$ ¹²⁶. In this region, the main bands are represented by sugar-phosphate backbone vibrations. The regions of the nitrogenous vibrations mainly occur in the regions $1250-1750\text{cm}^{-1}$ ¹²⁷. A good comprehensive review for the infrared and Raman spectroscopy of nucleic acids may be found in Thomas, GJ & Kyogoku, Y (1976) Biological Science. In EG Brame, Jr. & JG Grasselli (Eds.). Infrared and Raman Spectroscopy, Part C. (pp. 717-870.) New York: Marcel Dekker, Inc.

The vibrational study of the interaction of certain drugs with DNA has received much scrutiny by researchers because drug-DNA interactions may be characterized by the nature of the chemical bonds formed between them. Molecular spectroscopy may be used to study the interactions and bonds because vibrational spectroscopy can provide fingerprints of substances in general and can therefore be used as a diagnostic method illuminating these interactions. Figure 5 shows an example of the methodology behind such an approach¹²⁸.

Figure 5: Spectroscopic methodology of drug-nucleic acid interactions

2.2.4 Cells

Out of the infrared spectra of cells, some interesting differences were found between normal and cancerous cells (cancerous cells being cultured cells and actual malignant tissue). It has been shown that the following features are common to many cancers: increased hydrogen bonding of the phosphodiester groups of nucleic acids, a shift in the symmetric phosphate band from 1086 to 1082cm⁻¹, decreased hydrogen bonding of C-OH groups of proteins, reduced glycogen levels in normally glycogen-rich tissues and an increased disorder of the methylene chains of lipids, except for basal cell carcinoma of the skin¹²⁹.

2.2.5 Summary and research objectives

From this introduction to FT-IR and its application to lipids, DNA and cells, it can be seen that this branch of vibrational spectroscopy has enormous potential to expand the knowledge of biological systems and applications of cancer research, including diagnosis of cancerous tissue and drug therapy.

This thesis is concerned with the interaction of an anticancer drug, mitoxantrone, with two types of lipids, charged (dimyristoylphosphatidyl serine) and uncharged (dimyristoylphosphatidyl choline), with purified DNA and with two types of cultured cells, HT29 (colon cancer cells) and cell line 2008 (ovarian cancer cells) using pressure-tuning Fourier-transform infrared spectroscopy.

The application of pressure-tuning FT-IR spectroscopy to biological systems, such as model membranes, cells and tissues has been well-studied and established¹³⁰. The use of pressure-tuning FT-IR spectroscopy for the examination of drug-nucleic acid

interactions has never been undertaken before, nor the study of mitoxantrone with these lipids. The study of the cellular uptake will help give an overall picture of the structural changes which occur to the cell through study of the infrared bands with pressure.

CHAPTER THREE

3.1 METHODS AND EXPERIMENTAL

3.1.1 Preparation of lipids with mitoxantrone

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (also called L- α -phosphatidyl choline dimyristoyl, lot number 108C-8360) and 1,2-dimyristoyl-*sn*-glycero-3-[phospho-L-serine] (DMPS) (lot number 140PS-P47) were purchased from Sigma Chemical Company (St. Louis, MO) and Avanti Polar Lipids (Birmingham, AL), respectively. Mitoxantrone (lot number 060060) in powder form was a gift from Cyanamid Company (also called American Cyanamid Company), (Wayne, NJ).

The pure lipids and lipid:drug mixtures were hydrated in 50mM Tris-HCl H₂O buffer, pH7.0. To ensure complete dispersion of the lipids, the multi-layers were heated and vortexed two to three times. Once the lipid dispersions were complete, the drug was added and the sample was made up to a 1:1 molar ratio. Lipid:drug dispersions in an approximate molar ratio of 20:1 were also prepared. The mixtures were vortexed again for an equal dispersion of drug then the samples were left overnight at approximately 5°C.

3.1.2 Purification of DNA

Calf thymus DNA (Type I, sodium salt) was purchased from Sigma Chemical Company (St. Louis, MO, lot number 109F-9545).

The method used to purify the DNA followed standard protocols¹⁹. The DNA sample was dissolved in 50mM Tris-HCl H₂O buffer, pH7.0 and diluted to approximately 5mg/mL. The solution was then sheared three times through a 21G needle to fragment the long polymers. To purify the sample, the sample was extracted twice with an equal

volume of phenol, once with an equal volume of a 25 part phenol : 24 part chloroform : 1 part isoamyl alcohol solution and once with a 24 part chloroform : 1 part isoamyl alcohol solution. All centrifugations were performed at 4°C at 10,000rpm for 10 minutes.

After the final centrifugation, the upper aqueous layer was carefully pipetted off and the DNA was precipitated in cold absolute ethanol. The precipitate was washed in 70% ethanol, then with absolute ethanol. The pellet was resuspended in sterile 50mM Tris-HCl buffer, pH7.0. A 260/280 absorbance ratio of the purified DNA have a reading of 1.8, indicating an acceptable degree of purity.

The purified DNA was then dialyzed against Millipore-Q water to remove nucleotides and small fragments of DNA.

To reconcentrate the DNA, the sample was spun under speedy vacuum for approximately one hour at 40-45°C, bringing the sample volume to one-fifth of its original volume and the approximate concentration of the DNA was 50mg/mL. The purified DNA had a gel-like consistency. The sample was stored at -80°C and was used as needed.

The sample was run on an agarose gel to check for fragment size. The gel was 0.7% agarose and the buffer was made up of 89mM Tris, 89mM boric acid and 2.5mM EDTA at pH7.2. The voltage applied to the gel was 150V (70mA). Afterwards, the gel was stained with ethidium bromide to view the fragments. Fragment size ranged from 1 to 10kbp.

3.1.3 Preparation of DNA with mitoxantrone

Calf thymus DNA was purified according to protocol outlined in section 3.1.2. Mitoxantrone in powder form (lot number 060060) was a gift from Lederle Laboratories

(also called American Cyanamid Company), (Wayne, NJ).

Mitoxantrone was solvated in 50mM Tris-HCl buffer, pH7.0 to a composition of 10%(w/w) drug, resulting in an inky dark blue solution.

The drug sample was then added to the purified DNA, giving a sample of 10%(w/w) drug in DNA. The ratio of DNA base pairs to drug molecules was calculated to be roughly 30. The sample was then stored overnight at approximately 5°C.

3.1.4 Preparation of cells with mitoxantrone

The following tissue cell culture cells were used: HT29 cells and cells from the well-known 2008 cell line. Clinical sample of mitoxantrone, 2mg/mL (lot number 1E1637R) was purchased from Lederle Laboratories (also called American Cyanamid Company)(Wayne, NJ).

HT29 cells (colon cancer cells), grown with small amounts of gentamycin, were washed with phosphate-buffered saline (PBS). Trypsin was added to the cells to separate them from the flask walls and from each other and the trypsin was aspirated. The cells were then incubated for 10 minutes at 8.0% CO₂ atmosphere at 37°C. Bovine serum solution was added to the cells to inactivate remaining trypsin and the cells were washed twice in PBS.

The protocol was the same for the 2008 cell line (human ovarian carcinoma cells) except that the cells were grown without antibiotics^{132,133}.

The HT29 cells and the 2008 cell line were both divided into portions of 0.1mL. One portion was not exposed to the drug while the other was exposed to roughly 0.4µmol of mitoxantrone.

All samples with the drug were left overnight at 5°C. The cells were then observed under a light microscope.

3.1.5 Loading the samples

A small amount of internal pressure calibrant α -quartz (purchased from Karl Lambrecht Corporation (Chicago, IL)) and individual samples (approximately 0.1mg) were placed in a 0.37mm diameter well of a stainless steel gasket 0.23mm thick. The gasket was mounted on a diamond anvil cell. (Samples that were used for atmospheric pressure spectra only were either mounted upon the diamond anvil cell or BaCl_2 windows were used, but no quartz was added.)

3.1.6 Measuring infrared spectra

Infrared spectra were measured at 28°C on a Bomen-Michelson series 110 Fourier-Transform spectrometer with a liquid nitrogen-cooled mercury-cadmium-telluride detector. The infrared beam was condensed onto the diamond anvil cell by a sodium chloride lens system. Before any spectra were taken, the system was purged for one minute with a nitrogen stream to remove any water vapour and carbon dioxide.

For each spectrum, 250 interferograms were averaged at a spectral resolution of 4cm^{-1} . The spectra were measured as a function of pressure up to 15kbar.

3.1.7 Light source

The source of the high intensity infrared radiation was a temperature-stabilized glowing ceramic source. Wavenumber precision was controlled by an internal He-Ne laser.

3.1.8 Detector

The detector was a mercury-cadmium-telluride (MCT) detector, cooled by liquid nitrogen.

3.1.9 Signal processing

Fourier-transform infrared spectra were processed using a mathematical algorithm. The details of conversion of the interferogram to the more familiar absorbance versus frequency spectra are outlined in Appendix 3¹³⁴.

3.1.10 Data analysis

Raw data were converted into DOS format using a translation program from the manufacturer. National Research Council software was then used to process the data. The 695cm⁻¹ phonon band of α -quartz was used to determine the hydrostatic pressure in the gasket of the diamond anvil¹³⁵. The frequency of the band was obtained using third-order derivative spectra at a breakpoint of 0.30 in the Fourier domain and converted to pressure p according to the following equation: $p = 0.015164(\Delta\nu)^2 + 1.2062(\Delta\nu)$ where $\nu_0 = 694.1\text{cm}^{-1}$ ¹³⁶.

CHAPTER FOUR RESULTS AND DISCUSSION

4.1 1,2-DIMYRISTOYL-*sn*-3-GLYCERO-PHOSPHOCHOLINE

4.1.1 Introduction to 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine

Phosphatidylcholine is one of the most abundant lipids in animal tissues^{137,138}. 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) is a lipid composed of two saturated fatty acid chains and a phosphatidylcholine head group, all bonded to a glycerol moiety. See Figure 6. Overall, its charge is neutral at physiological pH. The positively charged head group is countered by the negatively charged phosphate group.

Figure 6: 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine

For the sake of clarity, the results of the experiments will be discussed by discussing separate areas of the lipid molecule and its interaction with the drug.

4.1.2 Interior of the lipid bilayer

At ambient pressure and temperature (28°C), DMPC is in a liquid crystalline phase with many of the lipid acyl chains disordered conformationally (with a large number of *gauche* bonds)¹⁴⁰. At very low pressure (0.20kbar), DMPC is transformed into the gel phase¹⁴⁰. In the gel phase, the acyl chains are conformationally in the ordered *trans*

state, although there are still a few *gauche* conformers¹⁴¹. From Figure 7, the transition from liquid crystal to gel phase is observed from the stacked plot of spectra (in order of increasing pressure.) As the pressure is increased, the band is narrowed and the shoulders at 1455cm⁻¹ and 1440cm⁻¹ are reduced, indicating a greater degree of order amongst the lipid molecules. As pressure increases, the number of *trans* conformers increases.

Figure 7: Stacked plot of DMPC in the methylene bending region

Figure 8: Stacked plot of 1:1 DMPC:MX in the methylene bending region

When the orientation of all the methylene chains in the bilayer are highly disordered due to re-orientational fluctuations and the twisting/torsion motions, the vibrational coupling of the modes between neighbouring chains is random, resulting in a broadening of the vibrational modes. However, when the conformation of the chains is highly ordered in zig-zag *trans* formation, lateral compression is then able to dampen the re-orientational fluctuations and torsion/twisting motions of the chains, resulting in orientational order in the bilayer^{142,143}. Correlation field splitting of the methylene bending mode is the result of vibrational interactions between orientationally non-equivalent methylene chains¹⁴⁴.

This splitting of the methylene bending mode may be observed with the pressure is sufficient enough to cause the acyl chains to forfeit their random orientational fluctuations, provided the equilibrium positions of neighbouring chains are non-equivalent. As a consequence, pressure may be used to examine the order in a lipid bilayer (both conformational order and orientational order), because a more disordered membrane would require higher pressures to achieve the transition from the conformationally disordered liquid crystalline phase to conformationally ordered gel phase, to dampen the re-orientational fluctuations and other motions, and then to induce correlation field splitting than a more ordered one. Inspection of the methylene bending mode is a good method to examine the degree of disorder in a lipid dispersion^{145,146,147}.

However, an important distinction should be made at this point. Not all lipid

bilayer systems will show correlation field splitting in the methylene bending mode. In unsaturated lipid bilayers under high pressures, the restricted orientational mobility of the bent hydrocarbon chains form equivalent and parallel orientation of the acyl chains, closely packed together¹⁴⁸.

Figure 9: Stacked plot 1:1 DMPC:MX with more lipid added in the methylene bending region

The correlation field splitting of the methylene bending mode of DMPC, with and without mitoxantrone (MX) is shown in Figures 7 and 8. The correlation field splitting pressure for the lipid with the drug is 0.4kbar higher than for the pure lipid, from 4.2 to 4.6kbar. Therefore, it may be said that MX induces a disordering effect on the orientation of the methylene chains in the DMPC bilayers. When more lipid is added to the 1:1

DMPC:MX sample, the correlation field splitting pressure is reduced slightly to 4.3kbars, as seen in Figure 9. Additionally, a comparison of the stacked plot of DMPC and 1:1 DMPC:MX reveals broader correlation field component bands when the drug is added, which is also indicative of greater disorder.

The range of frequencies between the higher and lower bands of the correlation field splitting of the methylene bending band (called the magnitude of splitting) is smaller in the presence of MX, as shown in Figures 10 and 11. This is significant because the magnitude of splitting reveals the extent of interchain interactions. The greater the difference in frequency between the correlation field component bands, the greater the interchain interactions¹⁴⁹. Therefore, the addition of the drug lessened interchain interactions.

Figure 10: Pressure dependence of the frequencies of the methylene bending mode of DMPC and 1:1 DMPC:MX

Inspection of the intensity of the correlation field component bands also indicates the orientation of the chains¹⁵⁰. When the intensity of the acyl chains are equal, the alignment of the planes of the acyl chains are nearly perpendicular to one another. From Figures 7 and 8, it can be seen that the angle of the zig-zag planes of the chains do not near orthogonality to one another when MX is added to the lipids and pressure is increased.

Figure 11: Pressure dependence of the frequencies of the methylene bending mode of DMPC and more lipid added to 1:1 DMPC:MX

This observation of intensity changes along with the point of correlation field splitting and the magnitude of correlation field splitting suggest that the MX molecules reside in the interior of the lipid bilayer matrix. If the MX molecules do indeed reside in the interior of the bilayers, then some disorder would occur amongst the chains and the interactions

of the chains with neighbouring chains would be reduced (hence a lower magnitude of correlation field splitting). It is also reasonable that the drug would reside in the interior of the bilayer because the aromatic rings of the drug are hydrophobic. Although the drug may be bulky, the preferred site would be in the hydrophobic interior.

4.1.3 Interfacial region

As stated earlier, the interfacial region is the point at which the hydrophilic phosphatidyl head group encounters the hydrophobic acyl chains and the glycerol moiety. The juncture between these regions is the carbonyl ester region of the fatty acid chains. Therefore, examining the ester C=O band can provide information about the interfacial region. In hydrated lipids, hydrogen bonds may occur between the water and the interfacial region and these bonds may be observed through inspection of the barotropic behaviour of the C=O band¹⁵¹.

When third order derivatives are examined in the C=O region, it is seen that the ester carbonyl stretching band is actually composed of two bands, a low frequency band from hydrogen bonded *sn*-2 C=O groups and a high frequency band from non-hydrogen bonded *sn*-1 C=O groups¹⁵². The interfacial region is sensitive to hydrogen bonding and relative quantitative analysis of hydration may be made by inspection and comparison of intensities of the two bands.

The ester C=O band of DMPC does not change significantly in band shape or frequency as a function of pressure¹⁵³. However a plot of frequency versus pressure in Figure 12 reveals little change in hydrogen bond strength with pressure due to the similarity between the frequencies of the lipid and lipid:drug mixture. Therefore, the

strength of hydrogen bonding is virtually unchanged in this region with or without the drug.

Figure 12: Pressure dependence of the frequencies of the C=O stretching band

Band changes in DMPC and 1:1 DMPC:MX may be used then to determine any changes in the relative amount of hydration. Deconvolving the band would provide information about the amount of hydrogen bonding though inspection of the intensity of the bands. Figure 13 shows the deconvolved spectra of DMPC and 1:1 DMPC:MX at low pressures.

The addition of the drug causes a broadening and an increase in the intensity of the *sn*-2 band (the hydrogen-bonded band at the lower frequency). Thus, the addition of the drug causes a slight increase in the amount of hydrogen bonding in this region of the lipid. The broadening of the band is also significant because it indicates that the addition

of the drug causes more disorder amongst the lipid bilayers. If the drug is residing in the inner bilayer of the lipid, then it is possible that the conformation of the C=O group of the interfacial region has become slightly more accessible to water molecules.

Figure 13: Deconvolved spectra of the C=O stretching band

4.1.4 Phosphatidylcholine head group

The C-N stretching region (1010 to 940cm^{-1}) is a significant region because this region is a part of the head group of phosphatidylcholine. As Figure 14 shows, there is an increase in the frequency of this band when the drug is added for both DMPC and 1:1 DMPC:MX.

The phosphate region (1050 to 1300cm^{-1}) is also an important region to examine

in order to see an effect of the drug on the phosphatidylcholine head group. The antisymmetric phosphate stretching mode ranges from 1140 to 1300 cm^{-1} . Unfortunately, the drug has some absorbance in that area (due to a C-O stretching mode) and spectral subtraction at a 1:1 molar ratio of lipid:drug would not be reliable. The 20:1 DMPC:MX mixture taken at atmospheric pressure shows only a slight increase in intensity of the asymmetric phosphate region with addition of the drug, as shown in Figure 15. At such a low concentration of the drug, there should only be insignificant absorption by the drug, not enough to distort these results.

Figure 14: Infrared spectra of DMPC and 1:1 DMPC in the C-N stretching region

The symmetric stretch of PO_2^- of the phosphate region (1000 to 1150 cm^{-1}) is a region where the quartz band absorbs. This region, too, could not be inspected accurately using pressure. However, as shown in Figure 15, the atmospheric spectrum of 20:1

DMPC:MX shows very little change with the addition of the drug.

If the drug solely resides in the interior of the lipid bilayer, then the head group should be unaffected by the addition of the drug. From Figure 15, it is clear that the interaction of the drug with the DMPC lipids at the phosphatidylcholine head group is minimal, if at all.

Figure 15: Infrared spectra of DMPC and 20:1 DMPC:MX in the phosphate stretching region

4.1.5 Mitoxantrone

It is important to examine drug bands to see how the bands of the drug are affected when the lipid is added. For example, the stretching mode of C=O of quinone in the range 1590 to 1550cm⁻¹ does not overlap with lipid bands. See Figure 16. The band of the drug at low pressures and room temperature is approximately 1568cm⁻¹. When DMPC is added in a 1:1 molar ratio, this frequency lowers to 1565cm⁻¹. This shift

indicates that the drug undergoes an environmental change, confirming that the drug indeed interacts with the lipid. The method of IR spectroscopy is an accurate one for the detection of environmental changes. For example, detection of the expulsion of anesthetics from lipid bilayers may be observed in this way^{154,155}.

Figure 16: Infrared spectra of DMPC and 1:1 DMPC in the C=O stretching region of quinone

Another region of the spectrum of the drug showing the effect of the DMPC molecules is the stretching mode of C-N of a secondary amine aromatic group at the 1354cm⁻¹. This band is an important one to study because it is the 'hinge' linking the aromatic ring system with the alkyl side arms. Any conformational changes in the drug may be evident through inspection of this band. As Figure 17 shows, the exposure of the lipids causes a large downward shift in the frequency of this band, to 1345cm⁻¹. There is no interfering band in this region, as Figure 17 shows, so that the influence on the drug

band is entirely due to its environment. Certainly this downward shift of 9cm^{-1} indicates that the usual conformation of the drug has been altered. If the drug does indeed partition into the lipid bilayer, then movement of the side arms would be limited.

Figure 17: Infrared spectra of DMPC and 1:1 DMPC:MX in the C-N stretching mode of a secondary amine

4.2 1,2-DIMYRISTOYL-*SN*-3-GLYCERO-PHOSPHO-L-SERINE

4.2.1 Introduction to 1,2-dimyristoyl-*sn*-glycero-phospho-L-serine

Phosphatidylserine is a head group found mainly in excitable tissues^{156,157}. 1,2-dimyristoyl-*sn*-glycero-phospho-L-serine (DMPS) is a lipid which is charged at physiological pH. See Figure 18. The phosphate of the phosphatidyl head group is negatively charged, the nitrogen of the head group is positively charged and the carboxyl group of the head group is negatively charged. Therefore, overall, the lipid has a negative

charge at physiological pH.

Figure 18: 1,2-Dimyristoyl-*sn*-glycero-3-[phospho-L-serine]

As in the case of DMPC, this lipid and its interaction with MX will be discussed according to regions of the lipid and then with the drug.

4.2.2 Interior of the lipid bilayer

Figure 19: Stacked plot of DMPS in the methylene bending region

The transition from liquid crystalline to gel phase for DMPS occurs at 39°C at ambient pressures¹⁵⁸. All pressure spectra of the pure lipid occur in the gel phase. From the stacked plot of the lipid in the methylene bending mode region of Figure 19, correlation field splitting occurs at 2.9kbar, lower than the literature value of 3.7kbar¹⁵⁹. However, as Figure 20 shows, the addition of MX transforms DMPS into the liquid crystalline phase at 28°C at 0.6kbar. This indicates the drug drastically alters the conformational order in the lipid, causing a phase change roughly 10°C cooler at ambient pressure. Furthermore, from Figure 20 it is seen that the addition of MX increases the pressure required for correlation field splitting from 2.9kbar (pure DMPS) to 4.1kbar (1:1 DMPS:MX). Therefore, the drug greatly increases the disorder amongst the methylene chains. An increase of pressure more than 1kbar is required to still the twisting and wagging of the alkyl chains when the drug is added.

The magnitude of correlation field splitting reveals information about the interaction between DMPS and MX. As stated earlier, the greater the interactions between neighbouring chains, the greater the magnitude of the correlation field splitting¹⁶⁰. In the case of DMPS and MX, the magnitude of the 1:1 DMPS:MX is comparable to that of the pure lipid, as seen from Figure 21. However, if the point of splitting of the pure lipid and the mixture is superimposed upon one another, the magnitude of the splitting is similar. That is to say the addition of the drug does not affect the interaction of the neighbouring chains of DMPS, except for the pressure that causes the splitting to begin. The lipid chains are greatly disordered upon addition of the drug, yet the interaction of the acyl chains remain the same. The similar magnitudes

leads to the conclusion that the drug does not reside in the interior bilayer matrix at high or low pressures, although it does disturb the fatty acid chains.

Figure 20: Stacked plot of 1:1 DMPS:MX in the methylene bending region

Inspection of the intensity of the correlation field bands of the stacked plot shows that the addition of MX to DMPS results in correlation field bands of similar intensity. Conversely, for pure DMPS, the intensity remains consistently greater for the higher intensity band. Equal intensities of the correlation field splitting component bands means that the planes of the acyl chains become orthogonal to one another¹⁶¹. Therefore, the orientation of the neighbouring alkyl chains changes dramatically when the drug is added. At 14kbar, the neighbouring zig-zag planes of the alkyl chains in 1:1 DMPS:MX are nearly perpendicular to each other while the fatty acid chains in the pure lipid are not

perpendicular although non-equivalent at high pressures.

Figure 21: Pressure dependence of the frequencies of the methylene bending mode of DMPS and 1:1 DMPS:MX

4.2.3 Interfacial region

The pressure plot of the ester carbonyl region for DMPS and 1:1 DMPS:MX shows the separation of the hydrogen bonded and non-hydrogen bonded bands in Figure 12. The higher frequency band of DMPS drops in frequency, then the frequency increases with increasing pressure. Yet when MX is added, this higher frequency band then remains constant with increasing pressure at a consistently higher frequency. This increase in frequency upon the exposure to the drug would indicate that the addition of the drug causes a weakening of the hydrogen bonding in the system. If the drug is bound to the head group of the phosphatidylserine, then it could replace any water that would normally be in this region. Once again, the amount of hydrogen bonding that is present is examined using deconvolution to separate the hydrogen bonded and non-hydrogen

bonded bands of $\text{C}=\text{O}^{162}$. As Figure 13 shows, there is a sharp decrease in the intensity of the hydrogen bonded band while the intensity of the non-hydrogen bonded band is relatively unaffected. Therefore, the addition of the drug to the DMPS bilayers causes a noticeable decrease in the amount of hydrogen bonding in the lipid bilayers. Once again, if the MX molecules are bound to the phosphatidylserine head group, then the water molecules would be replaced by the drug, as is shown here.

4.2.4 Phosphatidylserine head group

As with DMPC, the phosphate region (1300 to 1150cm^{-1}) was difficult to examine accurately with pressure, due to interferences with the drug and quartz bands. Spectral subtraction of the drug could be misleading at a ratio of 1:1 lipid:drug.

However, the 20:1 lipid:drug dispersion at atmospheric pressure in Figure 22 shows that there is a drastic increase in the intensity of the asymmetric phosphate stretching band when the drug is added. There is also a small decrease in the symmetric phosphate band at 1086cm^{-1} when the drug is added. The asymmetric stretch of phosphate seems particularly sensitive to any changes that may occur at the head group. Therefore, the drug is residing at the phosphatidylserine head group. (The bands at 1062.9cm^{-1} and 1044.1cm^{-1} are bands of the lipid which are unidentified but which could be C-O stretches of the phosphodiester region of the lipid. These bands, too, are affected by the addition of the drug.)

Figure 22: Infrared spectra of DMPS and 20:1 DMPS:MX in the phosphate stretching region

The carboxylate symmetric stretching region occurs in the range 1420 to 1300 cm^{-1} ¹⁶³. For DMPS, the maximum absorption occurs at 1415.5 cm^{-1} , as Figure 23 shows. Yet upon the addition of MX, there is a dramatic shift to a higher frequency of 1431.0 cm^{-1} . (This shifted band of 1:1 DMPS:MX is also visible in Figure 23, which displays the methylene bending mode.) The increase in frequency with an increase in pressure of the symmetric carboxylate band indicates that hydrogen bonding strength has been lost.

Figure 23: Infrared spectra of DMPS and 1:1 DMPS:MX in the carboxylate symmetric stretching region

The antisymmetric stretch of COO⁻ ranges from 1680 to 1550cm⁻¹¹⁶⁴. Upon the addition of MX, this band in DMPS broadens and has two peaks at the top, the higher frequency band due to the asymmetric COO⁻ stretch and lower frequency band due to the presence of the drug, as shown in Figure 24. When this carboxylate stretching band is derivatized and plotted against pressure, the two components of 1:1 DMPS:MX are clearly seen and both increase with increasing pressure yet the plot of pure DMPS is not affected by pressure but remains somewhat constant with increasing pressure, as shown in Figure 25. The jump to a higher frequency and the consistent increase in frequency with pressure of the asymmetric stretch of COO⁻ show that the addition of MX causes a loss of hydrogen bonding strength about the phosphatidylserine head. As stated earlier, an increase in frequency with an increase in pressure indicates intermolecular repulsion is

occurring, while a decrease in frequency with an increase in pressure indicates hydrogen bonding.

Figure 24: Infrared spectra of DMPS and 1:1 DMPS:MX in the carboxylate asymmetric stretching region

Thus, the barotropic and frequency-related changes in the stretching of the carboxylate band, both symmetric and antisymmetric, show that a loss in hydration occurs in the phosphatidylserine head group when MX is added.

These results are reasonable because the arms of the drug are likely protonated at physiological pH^{165,166} and this protonation would enable binding of the drug to the negatively charged phosphatidylserine head group and thus a replacement of the sphere of hydration. Past studies have shown that adriamycin (an anthracycline drug) has an affinity for charged lipids^{167,168,169}.

Figure 25: Pressure dependence of the frequencies of the carboxylate asymmetric stretching mode of DMPS and 1:1 DMPS:MX

If the drug mitoxantrone also binds to negatively charged phospholipids, such as DMPS, then this would clearly explain why the orientation of the neighbouring alkyl chains is unchanged by the addition of the drug. Recall that the magnitude of correlation field splitting is comparable when the spectra are superimposed at the point of splitting. If binding of the drug occurs at the head group, the structural change in the phosphatidylserine of the lipid may affect the level of conformational disorder among the acyl chains, without residing in the matrix.

4.2.5 Mitoxantrone

As in the case of DMPC, the bands of the drug, with and without the lipids, can also be used as an indicator of the interaction between the drug and DMPS molecules. The stretching mode of C=O of quinone, normally at 1568cm^{-1} at low pressures, shifts to

1564cm⁻¹ upon exposure to DMPS, as shown in Figure 26. An environmental change in the drug could be reflected by a change in frequency. If binding does take place between the head group of the phosphatidylserine, more specifically between the charged carboxyl group and the charged side arms of the drug, then certainly there would be a change in the environment between a free drug and a bound one. The environmental change is then reflected in the change in vibrational frequencies.

Figure 26: Infrared spectra of DMPS and 1:1 DMPS:MX in the C=O stretching region of quinone

Comparison between the drug and the 1:1 DMPS:MX stretching frequency of the C-N of a secondary amine aromatic also reveals drastic and pronounced shifts in frequency. As shown in Figure 27, at low pressures, the band is at 1354cm⁻¹. Upon exposure to DMPS, there is a downward shift in frequency to 1339cm⁻¹. The binding of

the drug to the phosphatidylserine head group of the lipid is likely a strong one to decrease a vibrational mode of the drug 15cm^{-1} . A binding to the head group of the DMPS would probably act as a conformational restraint upon the molecule and would then be reflected as such in the vibrational modes.

Figure 27: Infrared spectra of DMPS 1:1 DMPS:MX in the C-N stretching mode of a secondary amine

4.3 DEOXYRIBONUCLEIC ACID

4.3.1 Introduction to DNA

The pressure-tuning vibrational spectrum of DNA has never been examined before. Although the atmospheric infrared spectra have been studied extensively to learn about DNA conformational structures and many modes have been assigned (as discussed earlier), the knowledge gained from these experiments could increase the understanding

of the hydrogen bonding of the macromolecule and how it adapts with reduced volumes. Additionally, more can be discovered about the effect of the drug upon the double helix through studying its interaction with MX under pressure to enhance weak interactions.

Figure 28: Pressure profile of DNA in the range 1300 to 900cm⁻¹

Figure 28 shows the pressure profile of the FT-IR spectrum of DNA in the range 1300 to 900cm⁻¹. Surprisingly, the vibrational spectrum of the polymer is quite simple. The overall spectrum is dominated by some absorption bands which are characteristic of specific molecular groupings. As discussed earlier, the 1300 to 900cm⁻¹ region is the site of vibrational modes of the outer groups of the helix, the phosphate groups and the deoxyribose sugars. The peak near 970cm⁻¹ is probably caused by sugar pucker conformations, as well as the peak at 1015cm⁻¹ ^{170,171}. The prominent band at

1086 cm^{-1} is the symmetric stretch of the phosphate groups while the shoulder at 1055 cm^{-1} is the C-O stretch of the sugars. The band at 1225 cm^{-1} is the antisymmetric stretch of the phosphate group. The two peaks of weak intensity at 1281.6 cm^{-1} and 1295 cm^{-1} are both probably vibrational modes of the cytosine residues. (Since the DNA was examined in an H_2O buffer and not in D_2O , the other regions of DNA vibrational modes could not be examined due to water absorption.)

The pressure spectra of DNA and DNA:MX in the region 1300 to 900 cm^{-1} as shown in Figures 28 and 29 will be discussed together but only one band of the DNA IR spectrum at a time, to avoid confusion.

Figure 29: Pressure profile of DNA + MX in the range 1300 to 900 cm^{-1}

4.3.2 Pressure-tuning FT-IR of DNA and MX

From Figures 28 and 29, the addition of MX to the DNA changes the intensity of the asymmetric phosphate stretching and broadens the symmetric stretching of phosphate.

As shown in Figure 30, plots of frequency with pressure reveal that the band at 970cm^{-1} increases with increasing pressure. As stated earlier, this band is likely a normal mode of a sugar pucker. In furanose rings, the conformation of the rings are never planar. There are two conformations, either an envelope shape or half-chair (twist) twist forms. The two principal puckering modes are $C_1\text{-endo}$ and $C_2\text{-endo}$. The $C_2\text{-endo}$ conformation is found in B-DNA¹⁷².

Figure 30: Pressure dependence of the frequencies of sugar vibrational mode of DNA and DNA + MX

B-DNA has approximately 20 water molecules per nucleotide^{173,174,175}. There are water molecules located at the ribose units but the pressure plot indicates some molecular repulsion amongst the hydrogen bonds is building up as pressure increases.

Therefore, these data might indicate the addition of pressure upon the helix changes the conformation so that the hydrogen bonding oxygens of the sugar molecules are slightly less accessible to the water.

Usually when intercalation occurs, the pucker of the furanose rings of B-DNA takes the C_3' -*endo* conformation¹⁷⁶, a mode which has a slightly lower frequency of vibration¹⁷⁷. Therefore, it is important to note that the addition of the drug lowers the frequency of the vibrational mode, and this trend continues with increased pressure. From this data then, it may be seen that the drug intercalates with the DNA and if the intercalation may be observed spectroscopically. To contrast the results with the drug and the DNA, the pressure study of free mitoxantrone reveals a phase change in the drug at 8kbar in all bands, which is unidentified. Since the drug is added at a ratio of 30 basepairs of DNA per drug molecule, no phase change is observed because the amount of the free drug (if any) is likely too small to notice the change of phases.

In the pressure plot of the symmetric stretch of phosphate in Figure 31, the addition of the drug to the DNA leaves this vibrational mode relatively unaffected. The drug is also shown, having an absorbance near 1070cm^{-1} at low pressures. Since the drug seems to have vanished into the DNA:drug mixture, it would seem that most of the drug has intercalated. (Although as stated earlier, the drug signal, free or intercalated, may be too weak to be detected.)

Figure 31. Pressure dependence of the frequencies of symmetric stretch of phosphate of DNA and DNA + MX

The pressure plot of the antisymmetric phosphate stretching of the DNA, shown in Figure 32, although it does coincide with a band in mitoxantrone, shows a sharp contrast in barotropic behaviour between pure DNA, DNA:MX and MX alone. (The amount of drug in the DNA:MX mixture is small enough so that the influence of the drug on the asymmetric phosphate stretching band is minimal.) When pure DNA is subjected to high pressures, there is an initial decrease in the frequency with the addition of pressure up to roughly 4kbar, then the frequency stabilizes and remains constant at higher pressures.

However, when the drug is added, the frequency of the vibration is lowered and then remains constant with pressures to roughly 4kbar, then the frequency of the mixture increases with the application of more pressure. In contrast, the phase change of the drug by itself drops approximately ten wavenumbers in the region when pressure exceeds 8kbar

but this phase change is not seen with the DNA:drug mixture. As stated earlier, this may be because the amount of drug, free or bound, is simply not great enough to observe the change spectroscopically.

Figure 32: Pressure dependence of the frequencies of the asymmetric stretch of phosphate of DNA and DNA + MX

These findings imply that the drug affects the antisymmetric vibration of the phosphate groups, first by its lowering of the frequency of the vibration (indicating an increase in the strength of hydrogen bonding) and then by changing the pattern of hydrogen bonding with pressure. Since the addition of the drug increases the strength of hydrogen bonding in the system at ambient pressures, less pressure is required to cause intermolecular repulsion than with the pure DNA. Therefore, an increase in frequency of the DNA:drug mixture is seen at 4kbar, while at 4kbar the frequency of the DNA stabilizes with pressure.

This decrease in frequency and its behavior with pressure of DNA:MX indicates

that the drug is probably partially bound to the phosphate groups of the DNA helix. Previous studies have speculated that the positively charged arms of the intercalated drug electrostatically bind to the negatively charged phosphate groups, stabilizing the helix^{178,179}. The vibrational mode of the asymmetric phosphate stretching appears to be more sensitive to interactions with the drug than its symmetric stretching counterpart. This greater sensitivity in the asymmetric phosphate stretch than in the symmetric phosphate stretch of DNA has been demonstrated with other intercalators^{180,181,182}.

Figure 33: Pressure dependence of the frequencies of the cytosine vibrations at roughly 1280cm⁻¹ of DNA and DNA + MX

The cytosine vibrations (in the range 1280 to 1300cm⁻¹) show little change in pressure, with or without the drug, as shown in Figures 33 and 34. Although the drug favour intercalation at GC base pairs¹⁸³ and one would assume a significant change in GC bands, the absorptions in these cytosine vibrations are very small and any change with

pressure are quite noisy. The drug MX has no significant absorption in this area.

Figure 34: Pressure dependence of the frequencies of the cytosine vibrations at roughly 1290cm^{-1} of DNA and DNA + MX

4.4 CULTURED CELLS

4.4.1 Introduction to cultured cells

There are some similarities between the spectra of DNA and that of cultured cells. From the spectra in the range 1800 to 900cm^{-1} of colon cultured cells shown in Figure 35, it can be seen that as in the case of the DNA, the spectra is surprisingly simple for such a complex system. The peak at 970cm^{-1} is due to the symmetric stretching of dianionic phosphate monoester of phosphorylated proteins and cellular nucleic acids.¹⁸⁴ The band at 1086cm^{-1} is the symmetric stretch of phosphate of the nucleic acids while the antisymmetric stretch of phosphate has two overlapping bands (a hydrogen bonded band at 1220cm^{-1} and a non-hydrogen bonded band at 1240cm^{-1}). The band in the range 1155

1170 cm^{-1} is mainly due to C-O stretching of the C-OH groups of serine, threonine and tyrosine in proteins¹⁸⁵. The bending mode of CH_2 of the fatty acid chains of lipids is in the range 1460 to 1480 cm^{-1} ¹⁸⁶. Also the C=O stretching vibration of the membrane lipids are in the range 1650 to 1750 cm^{-1} ¹⁸⁷.

Figure 35: Pressure profile of cultured colon cells in the range of 1800 to 900 cm^{-1}

The other two important bands shown in Figure 36 are the symmetric and antisymmetric stretching of CH_2 of the methylene chains of the lipids in the range 2800-2950 cm^{-1} ¹⁸⁸.

Since two types of cultured cells are to be examined and then discussed (HT29 cell line, a type of colon adenocarcinoma, and type 2008 cell line, a well-known human epithelial ovarian cancer cell line), the cells will be discussed and compared for their response to the drug in order of spectral bands.

Figure 36: Stacked plot of cultured colon cells in the range 3000 to 2800cm⁻¹

4.4.2 Cultured cells and mitoxantrone

Comparing Figures 37 to 40 in the spectral range 1400 to 900cm⁻¹, the band width of the phosphate bands broaden with the addition of the drug mitoxantrone and with the application of high pressures. The antisymmetric stretch of phosphate begins to fan outwards with high pressures and is composed of two bands which become more resolved with increasing pressure.

Figure 37: Stacked plot of cultured colon cells in the range 1400 to 900 cm^{-1}

Figure 38: Stacked plot of cultured colon cells + MX in the range of 1400 to 900 cm^{-1}

Figure 39: Stacked plot of cultured ovarian cells in the range 1400 to 900 cm^{-1}

Figure 40: Stacked plot of cultured ovarian cells + MX in the range 1400 to 900 cm^{-1}

As shown earlier in the spectra for the lipids and DNA, much can be learned from the pressure plots of bands, such as environmental factors and hydrogen bonding. The

PM-1 3 1/2"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

plots for the symmetric stretch of phosphate are shown in Figures 41 and 42. The cultured colon cells and the cultured ovarian cells, with and without exposure to the drug, show an increase in frequency with an increase in pressure. In general, the normal mode frequencies of a functional group of long chain systems increase when the interchain interactions are enhanced¹⁸⁹. Of course, the nucleic acids press crowd closer together when pressure is brought down upon the system.

When mitoxantrone is added to the cells, the symmetric stretch of the phosphate groups of the nucleic acids show no significant changes with the cultured colon cells but the increase in frequency with pressure is even more pronounced. The cultured ovarian cells are more sensitive to the addition of the mitoxantrone than the cultured colon cells, as evidenced by the symmetric stretch of the phosphate of the nucleic acids.

Figure 41: Pressure dependence of the frequencies of the symmetric stretch of phosphate for cultured colon cells and cultured colon cells + MX

Figure 42: Pressure dependence of the frequencies of the symmetric stretch of phosphate for cultured ovarian cells and cultured ovarian cells + MX

The effects of the drug on the two types of cells as shown by the asymmetric stretch of phosphate is also revealing. Figures 43 and 44 shows the derivatized component bands of the asymmetric stretch of phosphate, the hydrogen bonded band at the lower frequency and the non-hydrogen bonded band at the higher frequency. With or without the drug, both cultured cell types are essentially unaffected in the strength of hydrogen bonding in this region.

Figure 43: Pressure dependence of the frequencies of the asymmetric stretch of phosphate for cultured colon cells and cultured colon cells + MX

Figure 44: Pressure dependence of the frequencies of the asymmetric stretch of phosphate for cultured ovarian cells and cultured ovarian cells + MX

Deconvolved plots of the spectra for both cell types show alterations of the nucleic acids when the drug is added. From Figures 45, 46, 47 and 48, it is seen that at ambient pressures, the hydrogen bonded band (the lower frequency band) has less intensity than the non-hydrogen bonded band. With the application of pressure the lower frequency band begins to gain intensity, indicating an increase in the amount of hydrogen bonding with pressure.

Figure 45: Deconvolved spectra of the asymmetric stretch of phosphate for cultured colon cells

However, when the cells are exposed to the drug, the lower frequency band already has a stronger intensity than the higher frequency band, indicating an increase in hydrogen bonded nucleic acids. With pressure applied to the system, the non-hydrogen bonded band gains some intensity, indicating a decrease in the amount of hydrogen bonding. The drug increases the amount of hydrogen bonding of the nucleic acids in both of the cultured cell types. These results are very interesting because non-cancerous tissue has only the non-hydrogen bonded band of the antisymmetric stretch of phosphate, with or without pressure, while malignant cells have the two components of this band^{190,191,192}.

Figure 46: Deconvolved spectra of the asymmetric stretch of phosphate for cultured colon cells + MX

The antisymmetric stretch of phosphate is very sensitive to structural changes, as with pure DNA and with the two types of phospholipids. The amount of hydrogen bonding of the nucleic acids increase in malignant cells, indicating that the environment of the nucleic acids is altered in cancerous tissues¹⁹³. As discussed previously, the genetic changes of neoplastic tissue that are even visible in electron microscopy (such as amplifications) testify to drastic alterations of nucleic acids on a gross scale.

Figure 47: Deconvolved spectra of the asymmetric stretch of phosphate for cultured ovarian cells

Figure 48: Deconvolved spectra of the asymmetric stretch of phosphate for cultured ovarian cells + MX

The C-O stretching region ($1155\text{-}1170\text{cm}^{-1}$) also exhibits changes between the cells with pressure and with the drug. Increasing frequency with increasing pressure is shown with the two cell types, as shown in Figures 49 and 50. The addition of the drug increases the frequency at lower pressures and the trend of increasing frequency with increasing pressure is maintained.

Figure 49: Pressure dependence of the frequencies of the C-O stretch of cultured colon cells and cultured colon cells + MX

Figure 50: Pressure dependence of the frequencies of the C-O stretch of cultured ovarian cells and cultured ovarian cells + MX

The addition of the drug to the two cultured cell types decreases the frequency of the CH₂ bending mode. This mode is sensitive to order amongst the lipid methylene chains and to the interchain packing of the lipid bilayers because pressure applied to lipid bilayers has an ordering effect on the lipids¹⁹⁴. For both the cultured ovarian cells and the cultured colon cells, the addition of the drug causes a smaller shift in the frequency of the mode with pressure, indicating that the drug causes a disordering effect upon the lipids of the cells, as seen in Figures 51 and 52.

Figure 51: Pressure dependence of the frequencies of the CH_2 bending mode of cultured colon cells and cultured colon cells + MX

Figure 52: Pressure dependence of the frequencies of the CH_2 bending mode of cultured ovarian cells and cultured ovarian cells + MX

The range 2800 to 2950cm^{-1} is the stretching range of the CH_2 groups of the methylene chains of the lipids. As the pressure plots in Figures 53 and 54 of the symmetric CH_2 mode shows, there is an increase in frequency with an increase in pressure for both cell types, with and without the drug. The symmetric stretch of CH_2 (2800 - 2900cm^{-1}) can be used to measure the extent of interchain interactions because when the interchain interactions are stronger, the frequency is higher^{195,196}. However, there is no significant difference in this region with or without the drug. As Figures 55 and 56 show, the antisymmetric stretch of CH_2 with pressure is similar to the symmetric stretch of CH_2 .

Figure 53: Pressure dependence of the frequencies of the symmetric CH_2 stretching mode of cultured colon cells and cultured colon cells + MX

Figure 54: Pressure dependence of the frequencies of the symmetric CH_2 stretching mode of cultured ovarian cells and cultured ovarian cells + MX

Figure 55: Pressure dependence of the frequencies of the asymmetric CH_2 stretching mode of cultured colon cells and cultured colon cells + MX

Figure 56: Pressure dependence of the frequencies of the asymmetric CH_2 stretching mode of cultured ovarian cells and cultured ovarian cells + MX

The last band to be discussed is the stretching mode of $\text{C}=\text{O}$ of the membrane lipids. For both the cultured colon cells and the cultured ovarian cells, the strength of hydrogen bonding is slightly stronger upon the addition of the drug to the cells, as shown by the lower frequencies of the bands with pressure in Figures 57 and 58.

Figure 57: Pressure dependence of the frequencies of the stretching mode of C=O of cultured colon cells and cultured colon cells + MX

Figure 58: Pressure dependence of the frequencies of the stretching mode of C=O of cultured ovarian cells and cultured ovarian cells + MX

4.5 CONCLUSIONS

As evidenced by the spectral bands of the DMPC and DMPS and their barotropic behaviour, mitoxantrone partitions into the lipid matrix of DMPC and binds electrostatically to the head group of DMPS. This is an important observation because the charge of the lipids could then determine whether the drug enters the cell by passive or active transport.

However, the mitoxantrone does not merely bind to the exterior of membranes. As stated earlier, it could even be used as a stain for nucleic acids in cells, its affinity for the biomolecule is so strong¹⁹⁷. Therefore, it does not simply bind to the outside but enters the cell. A recent patent (patent 3,825,374) in Germany used phosphatidylcholine lipids as vesicles to transport the mitoxantrone located inside to mice with leukemia, giving them an increased life span of 713%¹⁹⁸. Clearly, a neutrally charged lipid, such as DMPC, could efficiently deliver the drug to the cell in a passive manner.

The intercalation of the drug into the DNA is seen by the effect of the drug upon the sugar pucker bands of the cultured cells and DNA. The drug binds electrostatically to the phosphate groups of DNA, as shown by the asymmetric phosphate stretching mode. Mitoxantrone causes an increase in hydrogen bonding in the cellular nucleic acids, as shown by the behaviour of the asymmetric phosphate stretching band of the cells.

It is important to distinguish the difference between the cellular phosphate groups of the nucleic acids and the purified DNA. The phosphate bands of the DNA are already maximally hydrated due to that fact that even the histones of the helix had been stripped away in the purification process. They are totally exposed to the buffered solution, while

the nucleic acids in a cell are closely and efficiently packed. Any change in the cellular nucleic acids as shown in the hydrogen bonded and non-hydrogen bonded asymmetric phosphate group could be due to a change in the amount of hydration and the effect the drug has upon the packing of the nucleic acids.

The drug causes closer packing of the nucleic molecules especially for the cultured ovarian cells as shown by the symmetric stretch of the phosphate groups. The hydrogen bonding of the nucleic acids increased with the addition of the drug and the membrane lipids showed more disorder upon addition of the drug, especially the cultured ovarian cells. It should not be a surprise that the ovarian cells were more sensitive than the colon cells to the drug. Mitoxantrone has been used in clinical trials with ovarian cancer¹⁹⁹, but is not significantly active against colon cancer²⁰⁰. Therefore, the sensitivity of the drug to the cultured cells were confirmed spectroscopically.

As shown with both the colon and ovarian cultured cells, though the cells were dying when exposed to high doses of mitoxantrone, they were able to take up the drug passively and this resulted in an alteration of hydration about the nucleic acids. If the drug molecules had simply sat upon the exterior of the cellular membrane, then the nucleic acids would have been unchanged, as would the C-O stretch of the proteins. The effect of the drug upon the nucleic acids was confirmed by spectrally examining the effect of the drug upon the cells and then the purified DNA. As stated earlier, the purpose was not to study the cells through clinical dosing, but to study the binding of the drug with the cells and to learn about the physical nature of the uptake and the structural changes that arise from the drug's entry.

The drug causes closer packing of the nucleic molecules especially for the cultured ovarian cells as shown by the symmetric stretch of the phosphate groups. The hydrogen bonding of the nucleic acids increased with the addition of the drug and the membrane lipids showed more disorder upon addition of the drug, especially the cultured ovarian cells.

Therefore, from this research, the importance of using pressure-tuning Fourier-transform infrared spectroscopy to study the interaction and potency of the chemotherapeutic agent mitoxantrone with lipids, DNA and cultured cells has been demonstrated.

Appendix 1: Synthesis of mitoxantrone hydrochloride

(From Reference 43)

I leuco-1,4,5,8-tetrahydroxyanthraquinone

II 2-((2-amino-ethyl)amino)ethanol

III 1,4-dihydroxy-6,7-dihydro-5,8-bis((2-((2-hydroxyethyl)amino)ethyl)amino)-9,10-anthracenedione

IV mitoxantrone hydrochloride

Appendix 2: Instrumentation of interferometers

(from Chapman, DI (1986). Infra-red spectrophotometry. In
AC Moffatt (Ed.). Clarke's Isolation and Identification of
Drugs. (pp. 237-239.) London: The Pharmaceutical Press.
Also, Michelson Series User's Manual Bomen Easy Version
1.41, 1989. p. C-1.)

Fourier-transform infrared spectrometers use an interferometer to collect infrared absorbance spectra. An interferometer is composed of a beam splitter (a plate transmitting half the light which falls on it and reflecting half the light) and two mirrors perpendicular to each other. One of the mirrors is fixed in place while the other can be moved backward and forwards but always perpendicular to the fixed mirror. Half of the infrared radiation is passed from the source to the fixed mirror which then reflects half of the radiation given it (one quarter of the original radiation) to the detector. The other half of the radiation at the fixed mirror passes to the movable mirror via the beam splitter. The moveable mirror then reflects half of the radiation (one quarter of the original radiation) to the detector. (Therefore, at this point the detector has received half of the original radiation from the source, one quarter from the fixed mirror and one quarter from the movable mirror.) Light is a wave phenomena. When two beams originating from the same source are combined, the waves may either constructively interfere (add together) or destructively interfere (cancel each other), depending upon whether they are in phase. Therefore, when the two mirrors are both at an equal distance from the beam splitter, the two beams

interfere constructively. However, when the movable mirror is displaced by one quarter of a wavelength, destructive interference occurs.

When the light is monochromatic, the detector records intensity of the radiation which varies as a sine wave due to the moving mirror. If the light is polychromatic, the sum of all the sine waves is recorded by the detector. As before, when the mirrors are an equal distance from the beam splitter, all of the sine waves of the polychromatic light interfere constructively, resulting in a strong central peak in the interferogram.

The placement of a sample in the beam absorbs light selectively. The resulting interferogram is the sum of all the sine waves, except those absorbed by the sample.

The mathematical transformation must therefore convert the interferogram from a sum of sine waves to an absorbance versus frequency plot.

Appendix 3 Fourier-transform applied to interferometers

(from Beer, R. Remote Sensing by Fourier Transform Spectrometry, John Wiley & Sons, Inc., New York, 1992, pp. 1-14)(Also from Michelson Series User's Manual, Bomen Easy Version 1.41, 1989, pp. C-1 to C-4.)

The behavior of a wave in terms of distance is described by the following equation:

$$a = A\cos(2\pi x\nu) \text{ where } A \text{ is the amplitude of the waveform,}$$
$$x \text{ is the distance of propagation,}$$
$$\text{and } \nu \text{ is the frequency.}$$

Light wave amplitudes are additive. If two wavetrains which have the same frequency and a constant phase relationship converge, the resulting wave is a cosine wave with a different phase and amplitude. Depending upon the point of interception, the amplitude of the resulting waveform may vary from $2A$ (maximum constructive interference) to zero (maximum destructive interference).

If the waves interfere, then $a = a_1 + a_2$

$$= A[\cos(2\pi x_1\nu) + \cos(2\pi x_2\nu)]$$

$$= 2A\cos[\pi\nu(x_1 - x_2)]\cos[\pi\nu(x_1 + x_2)]$$

where a = the resulting wavetrain whose amplitude varies from $2A$ to zero, depending upon the phase difference and the distance of

propagation.

Since intensity I is the square of the amplitude a ,

$$I = a^2 = 4A^2 \cos^2[\pi\nu(x_1 - x_2)]$$

Let $A^2 =$ the incident intensity I_m .

$$\begin{aligned} I &= 4I_m \cos^2[\pi\nu(x_1 - x_2)] \\ &= 2I_m [1 + \cos[2\pi\nu(x_1 - x_2)]] \end{aligned}$$

Let $F = 2I_m$.

Therefore, $I(x) = F(1 + \cos[2\pi\nu(x_1 - x_2)])$

where x is the path difference.

Since the light is actually polychromatic and not a point source, let F be a function of ν and integrate over all frequencies:

$$I(x) = \int_0^{\infty} F(\nu) [1 + \cos(2\pi\nu x)] d\nu$$

Multiplying out the integral,

$$I(x) = \int_0^{\infty} F(\nu) d\nu + \int_0^{\infty} f(\nu) \cos(2\pi\nu x) d\nu$$

The first integral is the total incident light (a constant) while the second integral relates the cosine function of the 2 beams which converged.

Fourier transform must be applied.

Using spectral notation, an integral Fourier pair is:

$$G(x) = \int_{-\infty}^{\infty} F(v) e^{(2\pi i vx)} dv$$

$$F(v) = \int_{-\infty}^{\infty} G(x) e^{(-2\pi i vx)} dx$$

Since the spectrum is real and not imaginary, the integrals may be replaced by the cosine:

$$F(v) = \int_{-\infty}^{\infty} G(x) \cos(2\pi vx) dx$$

and

$$G(x) = \int_{-\infty}^{\infty} \cos(2\pi vx) dv$$

Note the similarity between

$$I(x) = \int_0^{\infty} F(v) dv + \int_0^{\infty} F(v) \cos(2\pi vx) dv$$

and

$$G(x) = \int_{-\infty}^{\infty} F(v) \cos(2\pi vx) dv$$

Although the limits of the integral are different, negative frequencies are meaningless

so the lower limit of zero may be replaced with $-\infty$. Thus, the $I(x)$ integral is a cosine Fourier transform integral.

$$G(x) = I(x) - C$$

where the constant

$$C = \int_{-\infty}^{\infty} F(v) dv$$

$G(x)$ is the measured output called an interferogram. However, the goal is to solve $F(v)$:

$$F(v) = \int_{-\infty}^{\infty} G(x) \cos(2\pi vx) dx$$

As stated earlier, there is a finite range of wavenumbers. The integral must be replaced by a summation over the range of x . The integral now becomes

$$F'(v) = \int_{-\infty}^{\infty} G(x) B(x) \cos(2\pi vx) dx$$

where $B(x) = 1$ for $0 \leq x \leq L$ and is otherwise zero.

NOTES:

The mirror scan range of the movable mirror is less than a centimeter, making dx small.

Additionally, when the interferogram reaches the limit of a scan, apodization

functions ("foot" removals) are applied to correct for artifacts of the interferogram. The interferogram is multiplied by a function which causes the intensity to decrease to zero at each end of the interferogram.

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