ABSTRACT

Biosystematic Studies of the genus *Betula* in North-west Europe.

M.D. Atkinson

Using canonical discriminant analysis, a discriminant function was derived which enabled 97.1% of trees to be correctly classified as either *B. pendula* or *B. pubescens*, the criterion used against which to test the classification being chromosome number. This function used three leaf measurements: leaf apical segment width, leaf tooth factor and distance to the first tooth on the leaf base. Another discriminant function was developed which, using nine leaf measurements, allowed 98.1% correct classification. Stomatal guard cell length was found to be very useful as a supplementary character in distinguishing between the diploid (*B. pendula*) and the tetraploid (*B. pubescens*).

A pair of discriminant functions was derived which could be used to distinguish interspecific hybrids from both *B. pendula* and *B. pubescens*. The overall correct classification by these functions was 94.7%. Hybrids were shown to be morphologically more similar to *B. pubescens* than to *B. pendula* but on stomatal guard cell length, they were closer to *B. pendula*.

Comparative studies were made of mixed and single species birch populations from Great Britain and Europe. Each population was scored for nine leaf characters and, together with data from two reference populations chosen as a result of their single species status, were subjected to principal components analysis and cluster analysis. By this means (i) A group of plants equivalent to *B. pubescens* subsp. *tortuosa* could be distinguished; these were morphologically similar to certain hybrids between *B. pubescens* and *B. nana*. (ii) A large-leaved variant of *B. pendula* could be distinguished which was similar to *B. pendula* var. *lappponica* Lindq.

A preliminary study of isoenzymes was made at the species level. Extracts were made from leaves, pollen and wood cambium. These were run on polyacrylamide tube gels and vertical slab gels and were stained for a-naphthyl esterase, peroxidase and malate dehydrogenase. Cambial peroxidases showed population specific rather than species specific banding patterns. It was suggested that this may be linked to their involvement in lignification and regulation of plant growth substances. Pollen esterases and peroxidases showed considerable variation between individual trees but no population or species specificity. Malate dehydrogenase extracted from leaves showed a species specific differentiation with *B. pendula* having a greater activity and number of bands than *B. pubescens*. Leaf peroxidases showed a similar differentiation in some populations but this pattern was obscured in other populations by physiological effects.

DEVELOPMENT OF POTENTIAL ANTITUMOUR AGENTS BASED ON A CONSIDERATION OF THE MODE OF ACTION AND PHARMACOKINETICS OF DAUNOMYCIN AND ADRIAMYCIN.

A THESIS

PRESENTED FOR THE DEGREE OF

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by

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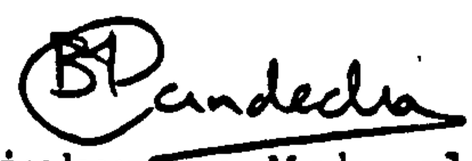
LIST OF FIGURES AND TABLES

TO MY FAMILY

Jai Jalaram.

The work reported in this thesis is original except where due reference is made, and has not been submitted in whole or in part for any other degree of the C.N.A.A., or for any degree of any other degree awarding body or institution.



Bijukumar Mohanlal GANDECHA.

DEVELOPMENT OF POTENTIAL ANTITUMOUR AGENTS BASED ON A CONSIDERATION OF THE MODE OF ACTION AND PHARMACOKINETICS OF DAUNOMYCIN AND ADRIAMYCIN.

Bijukumar Mohanlal GANDECHA.

ABSTRACT

Cancer is a major cause of death in Western countries. Although chemotherapy is widely used in the treatment of cancer, complete cure for the majority of tumours has not been achieved. The anthracyclines daunorubicin (Daunomycin) and doxorubicin (Adriamycin) have played a significant role in the treatment of cancer. Doxorubicin has a broad spectrum of antitumour activity; however its use is limited by its cumulative cardiotoxicity. One of the modes of antitumour action of doxorubicin and other anthracyclines, is intercalation (insertion) of the anthraquinone chromophore between successive base-pairs of DNA thereby inhibiting nucleic acid synthesis.

Three series of anthraquinones were prepared. The first and the second series consisted of 1-; 1,5-; 1,8-; and 1,4-; 2'-(diethylamino)ethylamino and 2'-(hydroxyethylamino)ethylamino-substituted anthraquinones respectively. The third series consisted of azoanthraquinones and their possible aminoanthraquinone metabolites. All compounds prepared were examined for their DNA binding properties. The methods included effect of DNA on spectral properties of drug, spectrophotometric titration, stopped-flow kinetics, competitive fluorescence polarisation, the effect of drug on thermal denaturation of DNA, and on the unwinding of cccDNA. It was shown conclusively that the anthraquinones bind to DNA by intercalation. The data obtained from this work were correlated with computer graphics modelling studies. The *in vitro* metabolism of azoanthraquinones by rat liver was also examined. Furthermore all anthraquinones were tested for their antiproliferative activity against HeLa cells, and CCRF human leukaemia cells.

The affinity for DNA, in both series was in the order 1,5-,> 1,4-,> 1,8-,> 1- substituted anthraquinones. The 2'-(diethylamino)ethylamino series had greater affinity for DNA than the corresponding 2'-(hydroxyethylamino)ethylamino anthraquinones. It was also shown that the azoanthraquinones did not intercalate into DNA; but the corresponding aminoanthraquinones to which they were metabolised by rat liver azoreductase did intercalate into DNA. These results are of use in the design of new effective antitumour agents.

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1. INTRODUCTION

1.1 GENERAL INTRODUCTION.

Cancer is a leading cause of death in Western countries. To date, complete cure for the majority of tumours using chemotherapeutic agents has not been achieved, despite an international effort. Nevertheless, since the late 1940's, many antitumour agents have been discovered, mainly by large scale empirical screening programmes. An alternative approach to discover more effective antitumour agents is to study the mechanisms by which the existing drugs act and to try and improve their activity by modification of the structure, or by selectively targetting the drugs to the tumour site.

This project is one such attempt to design antitumour agents based on the present understanding of the mode of action of doxorubicin* (1) and daunorubicin* (2), (Fig.1) (Arcamone et al., 1969; DiMarco et al., 1969). Doxorubicin and daunorubicin are members of the anthracycline group of antibiotics and both are potent antileukaemic agents. Doxorubicin also shows cytotoxicity against a broad spectrum of solid tumours (Blum and Carter, 1974; Bonnadonna et al., 1975). A major drawback of both these drugs is that they are cardiotoxic above a cumulative dose of about 550 mgM^{-2} (Carter, 1975; Wiernik, 1980).

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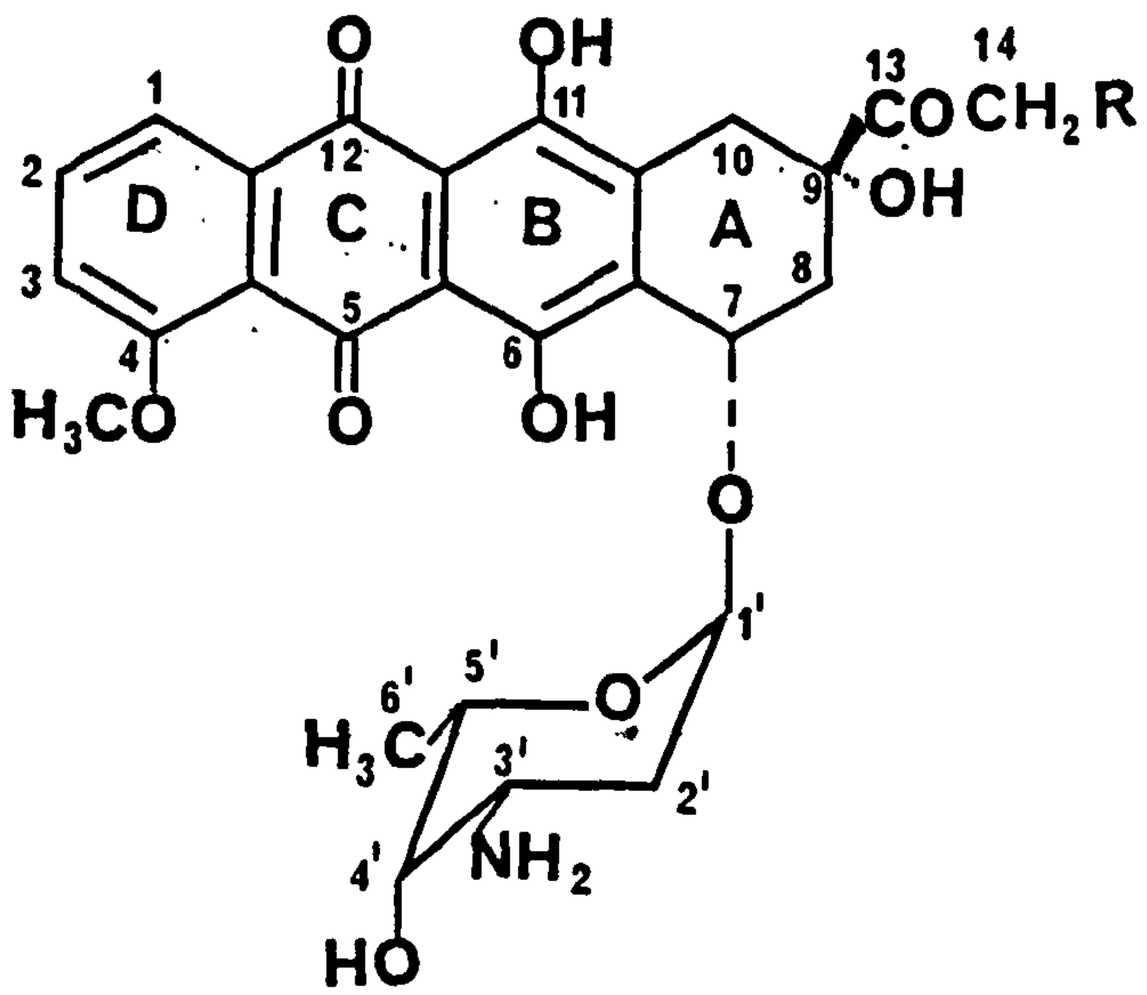
* The names doxorubicin and daunorubicin are international non-proprietary names; these drugs are also known by their trivial names adriamycin and daunomycin respectively.



R = OH Doxorubicin (1)

R = H Daunorubicin (2)

Figure 1 : Doxorubicin (1) and daunorubicin (2).

One of the modes of antitumour action of doxorubicin and other anthracyclines is by intercalation (insertion) between successive base-pairs of DNA thereby inhibiting the synthesis of DNA and RNA (DiMarco, 1975; Brown, 1978; Arcamone, 1981). The majority of antitumour agents used clinically are thought to exert their action by inhibition of synthesis of nucleic acids. Conversely, the cardiotoxic properties of doxorubicin (and daunorubicin) are thought to be mediated by formation of semiquinone free radicals, resulting in lipid peroxidation and disruption of cellular membranes (Bachur et al., 1977; Mimnaugh et al.,1979). Doxorubicin is thus a good candidate as a lead for the design of antitumour drugs based on its mode of action because from our present understanding there appears to be at least two distinct mechanisms of action, one related to the antitumour activity and the other to the cardiotoxic action.

The anthraquinone moiety of the anthracyclines can intercalate into DNA, and therefore several aminoalkyl-substituted anthraquinones have been synthesised to exploit this property (Müller, et al., 1971; Double and Brown, 1975; Zee-Cheng and Cheng, 1978; Murdock, et al., 1979). However only a limited range of structural variation has been investigated in these studies. Furthermore there is a lack of systematic study of the nature of interaction of anthraquinones with DNA.

In this project 1-; 1,4-; 1,5-; and 1,8-aminoalkyl-substituted anthraquinones will be synthesised and their binding to DNA, in solution, examined. The data obtained from this work will be correlated with computer graphics modelling studies (performed by S.A. Islam and S. Neidle) of the interaction of the synthesised anthraquinones, with DNA. Furthermore prodrugs of these aminoalkyl substituted anthraquinones will be prepared in an attempt to target these drugs to the liver. The metabolism of these prodrugs will also be examined.

1.2 CANCER.

Cancer is a term which describes a group of diseases embracing a multitude of unique conditions which are classified according to the tissue affected. These conditions are characterised by an uncontrolled cell division with invasion of normal non-dividing tissue; it can occur in any tissue capable of cell division. Cancer may arise in any organ of the body, in tissues of ectodermal, mesodermal, or endodermal origin. The host mounts a variable immune response to this uncontrolled cell division. Each case of cancer is thus unique, and the response to drugs and other treatment will vary and may be modified due to the variability of the health of the patient (Carter and Mathé, 1980).

Cancers can be classified into two major categories; solid tumours and haematological malignancies. Solid tumours are initially confined to a specific tissue or organ site. In time, however cancerous cells detatch from the original tumour mass, enter the circulatory system and start secondary growths, termed metastases. When this occurs the disease is said to be in a disseminated state. Conversely the haematological malignancies involve both blood and lymph systems, and are therefore frequently disseminated at the time of initial presentation (Carter and Mathé, 1980).

Cancer is currently the second most common cause of death in the United States (Cairns, 1978). It occurs

in all parts of the world and in all races. However different cancers develop in differing populations, for example Great Britain has the highest incidence of lung cancer in the world, whilst Japan has the highest incidence of stomach cancer (Doll, 1977a). It is notable that in general lowest incidence of most types cancer occur in Africa, Nigeria has the lowest incidence of oesophagus, lung, colon, and rectal cancers.

There are several probable causes of cancer. In general, they are caused by agents external to the body, though it is generally difficult to determine the exact nature of the agent. This is because there is a large variety of carcinogens in the environment. These include chemicals, radiation, and viruses. In addition there can be a genetic tendency toward cancer, although this is rare (Cairns, 1978; Doll, 1977b).

The malignant tumour cell has one or more phenotypic derangements, which may be expressed as alterations in cellular membranes, or in levels of certain cellular enzymes (e.g. enzymes involved in nucleic acid synthesis and metabolism), or in the appearance of inappropriate gene products (e.g., the synthesis of placental hormones and of foetal antigens not normally seen in adult cells).

The biochemical mechanisms involved in this phenotypic derangement of the malignant cell is not yet fully understood. It could be genetic mutation induced by chemicals or irradiation or both, or expression of

abnormal genetic information induced by viral "oncogenes", or gain or loss of chromosomal material by neoplastic cells, or derepression of oncogenes, such as "oncofoetal" genes that are present but normally silent in adult cells, or alterations in post-transcriptional processing of critical cellular macromolecules (Pratt and Ruddon, 1979).

Until recently, the vast size of the mammalian genome effectively prevented any direct search for the cellular genes involved in cancer. The genes were discovered, however, because they confer tumourigenicity when they are picked up by certain retroviruses (Dulbeco, 1982). The genes have come to be called "oncogenes" as if their primary role were to cause cancer. However typically they are cellular genes ("proto-oncogenes"), the function of which is sufficiently important for them to have been highly conserved through the evolutionary process. It has been shown that the certain types of bladder cancer may arise due to alteration of the cellular proto-oncogene which affects the structure of the oncogene-encoded protein (Tabin et al., 1982; Reddy et al., 1982). Recently it has been shown that amino acid sequences of certain oncogene-encoded proteins are closely related to growth factors (Downward et al., 1984; Waterfield et al., 1983). The binding of growth factors to their specific receptors can induce a number of biochemical events including changes in ion movements and in intracellular pH, stimulation of tyrosine-specific protein kinases and several other changes which can

culminate in DNA synthesis and cell proliferation.

Whatever the cause of cancer the phenotypic alteration gives the cancer cell some selective advantage for growth over normal cells. The critical change in the malignant cell is that it does not differentiate normally. The genes coding for differentiation appear to be shut off or inadequately expressed, while the genes coding for cell proliferation are expressed when they should be repressed.

1.3. TREATMENT OF CANCER.

The aim in treatment of cancer is to eradicate or remove all neoplastic cells whilst causing minimal effect to the host. This is essential because the immune response to cancer cells is weak, so that even if only a few cells survive, these will divide to form a new tumour. There are four possible forms of treatment namely surgery, radiotherapy, chemotherapy, and immunotherapy (Cohen et al., 1971). Surgery is the most frequently used method of treatment for solid tumours. Radiotherapy is also used for treatment of solid tumours either in combination with surgery or with cytotoxic agents. Chemotherapy can be used against all forms of cancer and is defined as the utilisation of cytotoxic drugs to treat cancer (Carter and Mathé, 1980); this defination also includes hormonal additive therapy. Immunotherapy, or stimulation of immune response with e.g. Corynebactrium parvum, or treatment

involving the use of antibodies to tumour cells, is useful in treating both localised and, in combination with other therapy, disseminated disease; but in terms of practical results it is still in its early phase of development, (Gutterman, 1978; Morton and Goodnight, 1978; and Carter and Mathé, 1980).

1.3.1. Factors which determine the response of tumour cells to anticancer drugs.

1.3.1.1 Growth Fraction and Mass Doubling Time.

Malignant tumours arise from the induction of a mutagenic change in a cell, resulting in daughter cells which unlike normal adult tissues are not subject to growth regulation. Once a cell is thus transformed, it may continue to divide unabated, limited only by the availability of nutrients, and by the host's ability to mount an immunological response. The time taken for the initial transformed cell to produce a clinically detectable tumour may be months or years, depending upon the type of cancer (Weisberger, 1973). Some human tumours grow more rapidly than others. The mass-doubling time of Burkitt's lymphoma, is about one day whilst that of a typical breast cancer is about 100 days, (Zubrod, 1972).

This tumour mass doubling time is determined by the proportion of the dividing cells, called the "growth fraction". Thus a tumour with a high growth fraction will have a shorter doubling time. Dividing cells are more sensitive to cytotoxic agents and therefore tumours with a high growth fraction are more

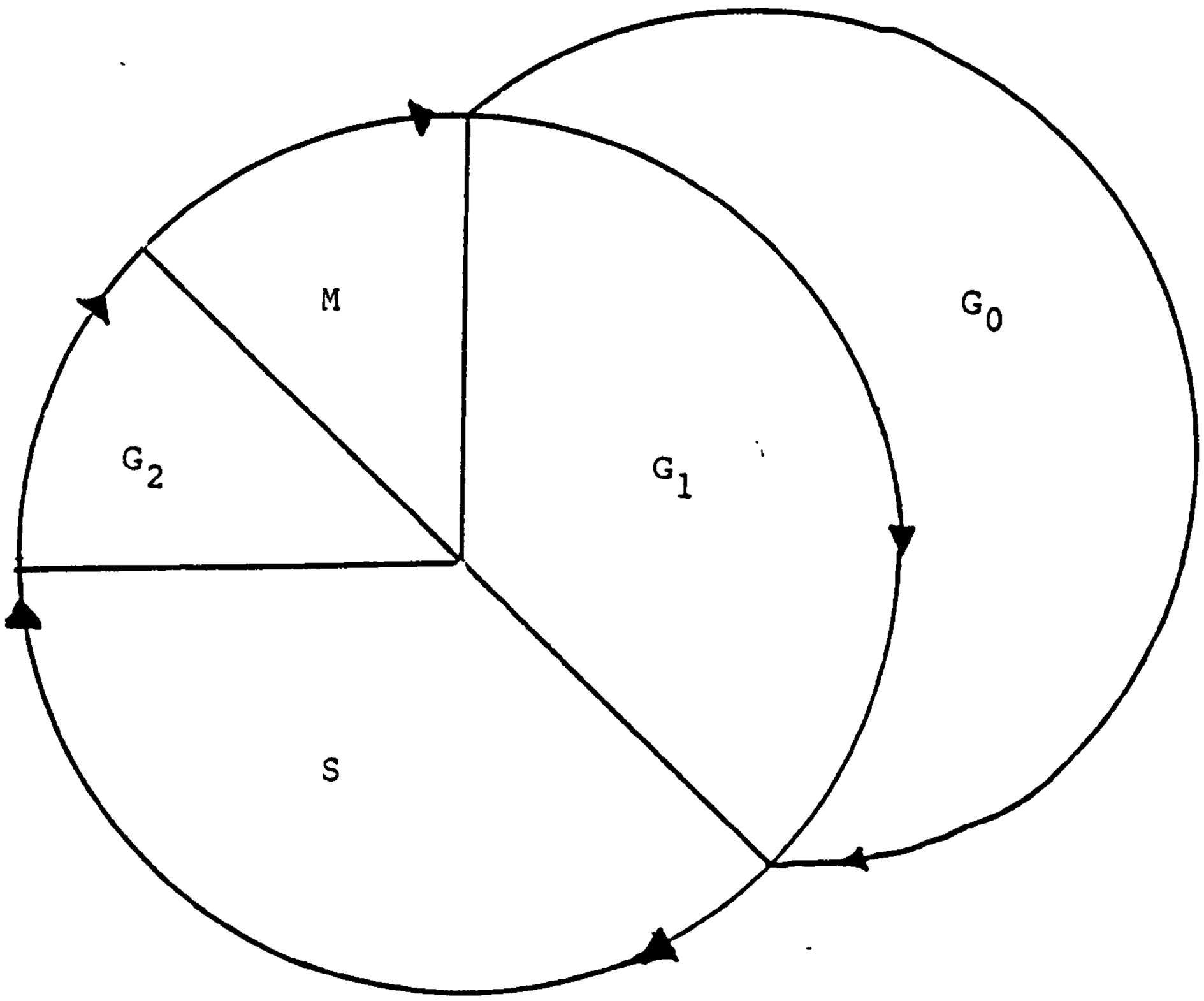
responsive to drug treatment. Normal tissues with a high growth fraction, for example bone marrow, are similarly susceptable to damage by cancer chemotherapeutic agents (Steel, 1973). Thus during treatment with anticancer drugs, bone marrow cells will be damaged by those drug levels which are necessary to kill cancer cells. Due to the type of cells that arise from the marrow, (eg. granulocytes, platelets), mylesuppression and leucopenia are two of the limiting complications of cancer chemotherapy.

1.3.1.2. Total Tumour Burden.

Tumour size is another factor which limits succesful chemotherapy in malignant diseases; large bulky tumours are not usually curable by chemotherapy. A high percentage of cells in a bulky tumour may be in a non-proliferative stage at the time of treatment and thus survive to re-establish the tumour mass. Drugs may not be able to penetrate into a solid tumour in sufficient concentration to effect a cell kill, due to poor vascularisation of tumour mass (Pratt and Ruddon, 1979). The killing of tumour cells by drugs appears to follow first-order kinetics, hence a given drug regimen will eliminate a constant proportion of cells rather than a constant number of cells.

1.3.1.3. The Cell Cycle.

To understand the observation that only certain cells in a tumour are susceptible to drugs, and also the mode of action of cytotoxic drugs, the phases of



G_1 : Begning of duplication of all the cell organelles, preparation for the replication of DNA within the nucleus.

S : Synthesis or replication of cellular DNA.

G_2 : Preparation for mitosis, end of duplication of the cell organelles.

M : Mitosis.

G_0 : "Resting" phase containing temporarly dormant cells.

Figure 2 : The cell cycle.

the cell cycle must be considered. It is generally considered that all proliferating cells which are synthesising DNA progress cyclically through a series of phases known as the cell cycle, as shown in figure 2, (Carter and Mathé, 1980).

The cell cycle is divided into a G_1 or intermitotic phase, an S or DNA synthesis phase, a G_2 or premitotic phase and an M or mitotic phase (Carter and Mathé, 1980). In all normal differentiated adult tissues and in most tumours, there is a population of viable cells that is not proliferating. These cells are sometimes said to be in the G_0 phase. If the majority of the tumour cells are in G_0 phase then these cells are less readily killed by cytotoxic agents and thus the tumour will not readily respond to chemotherapeutic agents. In the S phase, nucleic acid synthesis is taking place. This nucleic acid synthesis is inhibited by several drugs used in the treatment of cancer, including purine and pyrimidine antimetabolites, methotrexate, alkylating agents and drugs which bind to DNA such as doxorubicin. In the M, or mitotic phase the cells divide, and antitumour agents which are effective in this phase include vincristine and vinblastine.

1.3.1.4. Drug Resistance In Tumour Cell Populations.

A drug that initially is capable of killing cancer cells later becomes ineffective due to altered metabolic properties of the surviving cancer cells. Drug-resistant cells in a tumour cell population may re-establish the tumour mass after drug-sensitive cells

have been killed. Thus subsequent therapy with the initially effective drug may fail. Therefore it is common practice to use more than one chemotherapeutic agent, each having a different mode of action to reduce the problem of drug resistance.

1.3.1.5. General Condition Of The Patient.

A number of factors which determine the chemotherapeutic response are intrinsic to the host rather than the tumour cell. One important factor to consider is the general status of the patient and his/her ability to tolerate the cytotoxic effects of the drugs. Cancer chemotherapy is only worthwhile if the treatment will prolong the life of, or reduce the pain in a cancer sufferer.

All currently used cancer chemotherapeutic agents have side effects that must be considered when planning the treatment regimen. During drug treatment, the patient may become nauseated and with some drugs vomiting is common. With an aggressive, highly toxic regimen, the patient's mouth may become ulcerated, or alopecia, or infections may occur and tendency to bleed may develop. Although many side effects may be minimised with carefully conducted drug regimens, toxic effects like these clearly impose limitations on the use of some drugs, particularly in debilitated patients who tend to have a lower response rate and who tolerate such drugs poorly (Kennealey and Mitchell, 1977; Carter and Mathé, 1980; and Pratt and Ruddon, 1980).

In order to understand why a particular chemotherapeutic regimen is used in the clinic it is essential to appreciate the modes of action of these drugs.

1.4. THE STRUCTURE OF DNA.

This project is concerned with development of potential antitumour agents. Most of the currently available chemotherapeutic agents inhibit replication of the genetic material. Before discussing the mechanisms involved in the inhibition, it is necessary to review the composition and the structure of DNA.

1.4.1. Chemical composition of DNA.

DNA contains two types of heterocyclic bases, those containing a purine ring system and those containing a pyrimidine ring system. The purine bases in DNA are adenine and guanine and the pyrimidine bases are thymine and cytosine (Adams et al., 1976), although other bases may be found in small amounts, eg. N-methyladenine. The ratio of adenine to cytosine (and hence thymine to guanine) is dependent on the source of DNA but for most DNA the ratio of adenine to thymine and guanine to cytosine equals unity (Chargraff, 1950).

The bases are attached to the 1'-carbon atom of D-2-deoxyribose, (in the case of DNA), through an N-glycosidic bond. A purine or pyrimidine base attached to a sugar (ribose or deoxyribose) is termed a nucleoside, the addition of a phosphate group to the nucleoside results in a nucleotide. The construction of

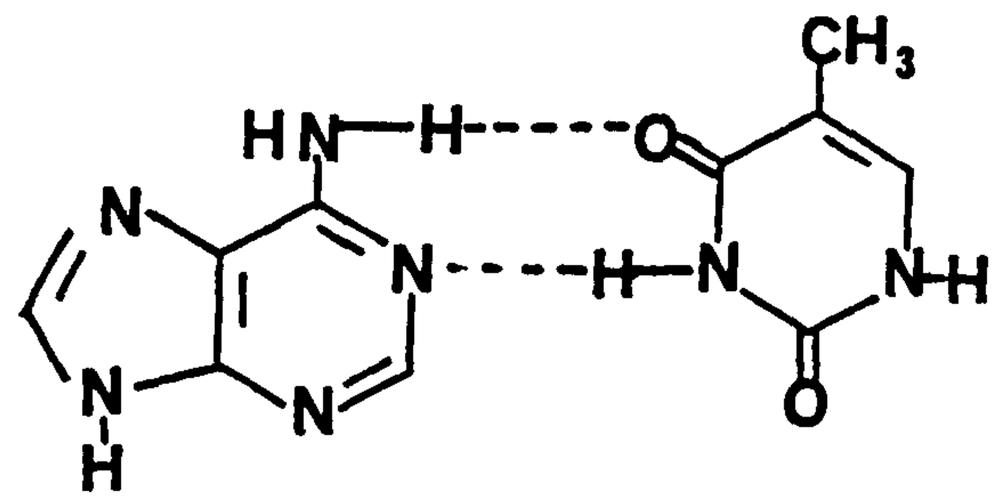
all nucleic acids is based on the nucleotide.

The primary structure of DNA consists of the sugar units of DNA linked through the 3'-phosphate group of one pentose and the 5'-hydroxyl group of a second molecule by a phosphodiester bond, producing a sugar phosphate chain.

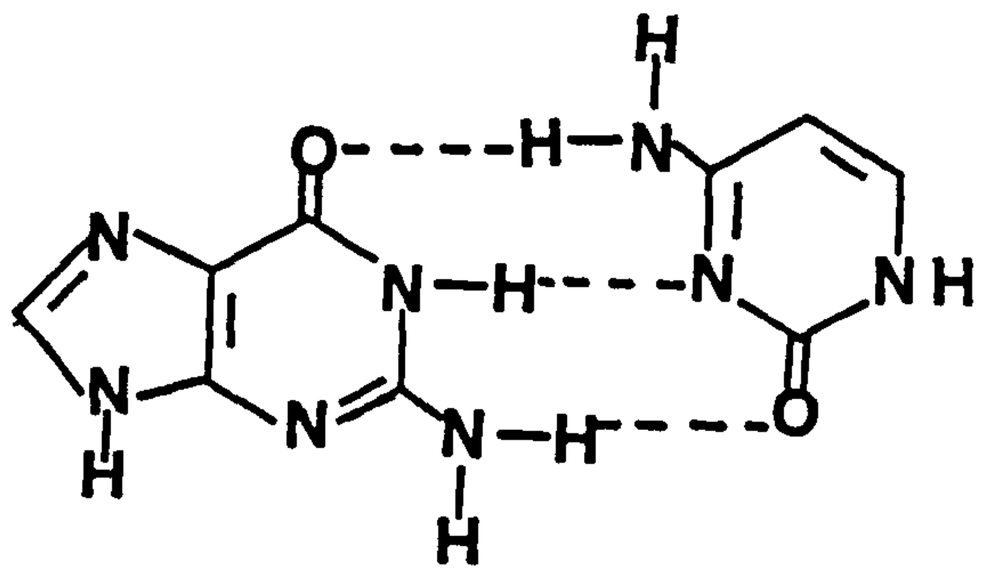
1.4.2. Secondary and tertiary structure of DNA.

The currently accepted structure of DNA was postulated by Watson and Crick (1953) using data from the X-ray diffraction studies of Wilkins et al. (1953). It was suggested that DNA is in the form of a right-handed double-stranded helix consisting of two antiparallel chains, the nucleotide linkage on one chain being 3'-5'; whilst the other chain is 5'-3'. The adenine residues of one strand are hydrogen bonded with thymine residues of the other strand and similarly guanine residues are hydrogen bonded to cytosine residues (figure 3). This base pairing is now generally accepted (Adams et al., 1976) since AT and GC base pairs are of equal dimensions and therefore allow uniform spacing between the two sugar-phosphate backbones of the double helix. The purine and pyrimidine bases are orientated towards the centre of the helix and are stacked on top of each other; their planes being perpendicular to the helix axis. It is this stacking which stabilises the DNA helix.

The exterior of DNA is polyanionic due to the presence of outwardly directed phosphate residues, and



Adenine Thymine



Guanine Cytosine

Figure 3 : Hydrogen bonding of base pairs.

the interior is hydrophobic due to the arrangement of bases. The major and minor grooves run down the outside of the helix and afford access to the bases. This Watson-Crick model of DNA has gained wide acceptance except for minor corrections in the postulated bond angles.

There have however been proposals for alternative structures for DNA in the recent years (Rodley et al., 1976; Sasisekharen et al., 1977; Pohl and Roberts, 1978). The most important of these proposed alternative structures for DNA, is the side-by-side (SBS) model, of Rodley et al. (1976). This model maintains the Watson-Crick base pairing and the antiparallel nature of the two chains but disputes the gross intertwining of the sugar-phosphate backbone. The right and left-handed segments of DNA are proposed to alternate, and bends in the sugar-phosphate backbone structure are proposed at every five base pairs. This model is consistent with X-ray diffraction patterns of DNA and also explains other properties of DNA such as the separation of the strands during replication. However the existence of the side-by-side model is extremely doubtful (Arnott, 1979) since studies using gel electrophoresis and supercoiled DNA provide evidence which strongly support the Watson-Crick model for DNA (Wang and Bauer, 1979; Crick et al., 1979).

1.4.3. Conformation of DNA.

Early studies on DNA showed that DNA can adopt three conformations, namely A, B, and C, which are

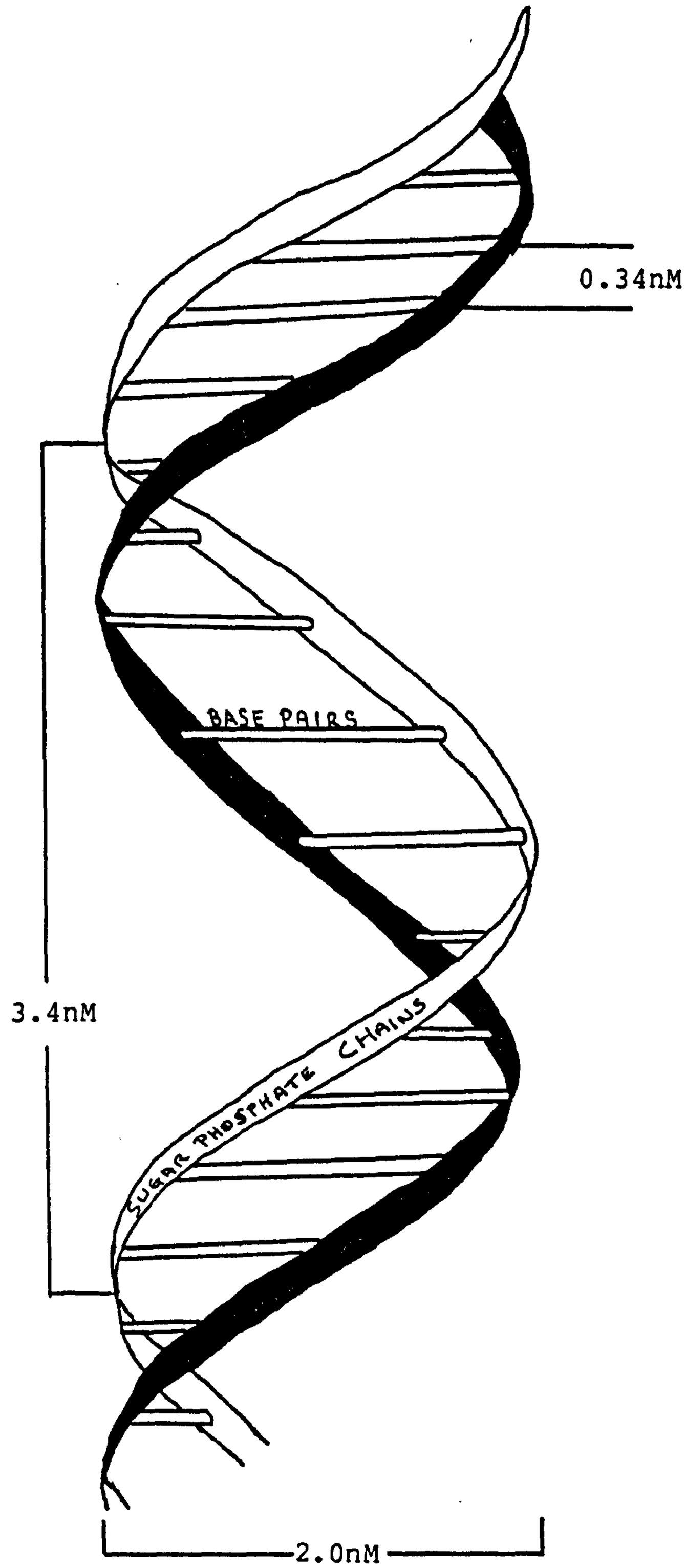


Figure 4 : Schematic representation of the Watson-Crick model of DNA.

dependent on the ionic strength of the solution (Arnott, 1970). The "native" form of DNA is the B-conformation which is that proposed by Watson and Crick. Figure 4 shows a schematic representation of the Watson-Crick model of DNA.

The B structure of DNA (Watson-Crick model) is characterised by an overall helix diameter of 20Å with base pairs stacked perpendicular to the helix axis and slightly twisted relative to one another. The bases overlap with the distance from the helix axis being 0.63Å, and are held together by hydrogen bonding (Watson and Crick, 1953). The helix is right-handed with ten bases per turn and a rise per base pair of 3.4Å. Two grooves of unequal width run around the outside of the helix, the larger is 22Å in diameter and is termed the major groove whilst the minor groove is 12Å in width. They are both 7Å in depth measured from the surface of the enveloping cylinder. The conformation of the deoxyribose is C2-endo (Arnott, 1970).

The A structure of DNA is found in fibres of lower relative humidity (75% compared to 92% for the B form) when the counterion for DNA is sodium, potassium or caesium. The structure is also in the form of a right handed helix, but differs from the B structure in that there are 11 base pairs per turn and a rise per base pair of 2.8Å. The bases are also tilted by 20°. The C form of DNA is found in fibres of lithium DNA at 66% relative humidity. The basic structure is similar to the B form, but has 9.3 base pairs per turn and a

rise per base pair of 3.1Å. The bases are tilted by 6° (Adams et al., 1976).

Previous work on the elucidation of DNA structure were based on fibre diffraction studies. However following the syntheses of dcuble stranded oligonucleotides, X-ray crystallography was used to define the structure. The B and A models were shown to be averaged structures and also novel DNA structures were found, the most significant being the Z form. This Z-DNA structure was proposed following studies on the 3-dimensional structure of crystalline $d(C-G)_3$ fragments (Wang et al., 1979; Davies and Zimmerman, 1980; Arnott et al., 1980). Two of the $d(C-G)_3$ molecules are combined to form a left-handed double helical segment with Watson-Crick base pairing. The model maintained the anti-parallel strands but had twelve base pairs per turn (compared to ten in the case of Watson-Crick model). The base pairs were tilted by 7° from the helix axis making a distance of 3.7Å between the base pairs along the axis, although they were stacked 3.4Å apart. In this model, the cytosine bases were in the anti-conformation, as in Watson-Crick model, while the guanine bases were in the syn-conformation. The puckering of the 2-deoxyribose rings also differed, being C3-endo for the two internal deoxycytidines. The conformation of the backbone about the C_4-C_5 bond was gauche-trans for the dG residues and gauche-gauche for the dC residues. The net result of these differences was to produce a staggered zig-zag course for the deoxyribose phosphate backbone and hence

the term "Z-DNA". In this case there was only one groove all the way along the axis of the molecule. The groove had a serrated edge of phosphate groups which are 8.5Å apart between the two edges and the groove itself is 9Å deep since it extends to the central axis of the 18Å diameter helix.

Similar structures have been proposed for poly(dG-dT) . poly(dA-dC) and poly(dG-dC) . poly(dG-dC) (Arnott et al., 1980). The Z-form can exist in physiological salt solutions when poly (dC-dG) is fully methylated at the cytosine 5 position (Behe and Felsenfeld, 1981). It can also coexist with B-DNA at low salt concentrations in closed circular plasmid DNA's (Peck et al., 1982). The demonstration that right- and left-handed segments can coexist in a single DNA chain may have profound implications for the role of DNA in regulatory processes. For example, Z-DNA maintained within a principally right-handed molecule, could be a store of negative windings. In appropriate conditions these could be used to compensate positive windings and produce a region of melted DNA available for polynucleotide synthesis. The left-handed segments could also be used to titrate out the negative supercoils.

Having first considered the structure of DNA, it is now necessary to examine the mechanisms by which currently available chemotherapeutic agents exert their effect in order to identify where rational design of antineoplastic drugs may be possible.

1.5. DRUGS USED IN THE TREATMENT OF CANCER.

Several classes of drugs have been introduced for the treatment of cancer during the past thirty years. The majority of these drugs depend for tumour cell selectivity on the fact that the tumour cells utilise amino acids, purine and pyrimidine bases and an energy source to a greater extent than normal cells.

Cancer chemotherapeutic agents may be divided into seven major classes, namely alkylating agents (Section 1.5.1), antimetabolites (Section 1.5.2), enzymes (Section 1.5.3), hormones (Section 1.5.4), mitotic inhibitors (Section 1.5.5), radio-sensitising agents (Section 1.5.6) and drugs which interact with DNA (Section 1.5.7, and 1.6). This classification is based upon our present understanding of the mode of acion of these drugs, (Bender et al., 1978; Chabner et al., 1975; Pratt and Ruddon, 1979). Figure 5 shows schematically the biochemical sites of action of the most common antitumour agents.

1.5.1. Alkylating agents.

Alkylating agents contain a substituted alkyl group which is capable of covalent reaction with cellular constituents (Price, 1975). The alkylating agents have a complex action: they alkylate DNA and various enzymes which are essential for cell division. Alkylating agents may cause cross linking of two strands of the DNA double helix, hence preventing

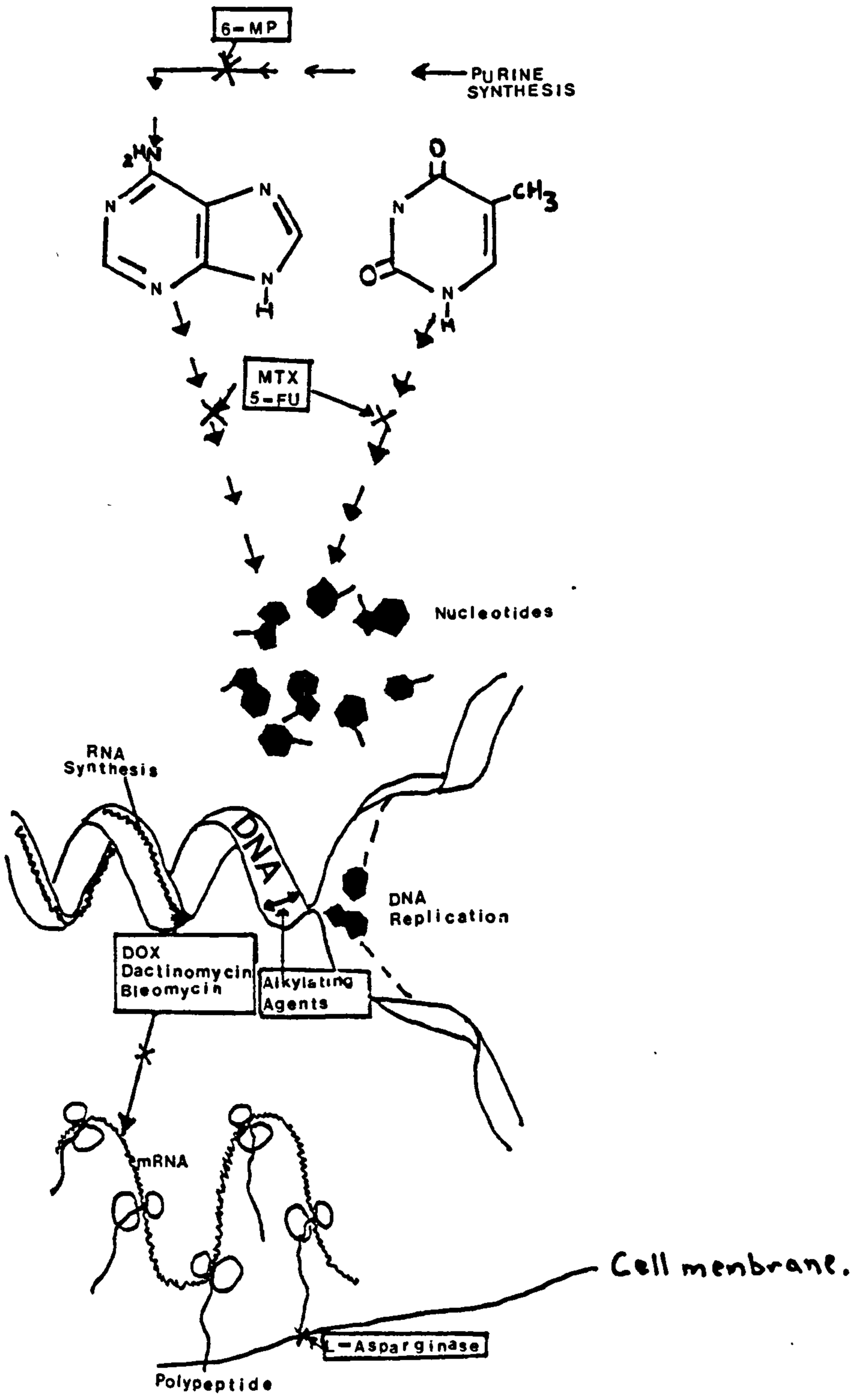


Figure 5 : Some biochemical sites where antitumour agents have been shown to interfere with nucleic acid synthesis and function.

replication.

Five major classes of alkylating agents are used in cancer chemotherapy: these are nitrogen mustards, nitrosoureas, triazenes, methane sulphonic acid esters, and ethylenimines (Pratt and Ruddon, 1979; Connors, 1975; Wilman and Connors 1983). The most commonly used alkylating agents include cyclophosphamide, melphalan, chlorambucil, dacarbazine (DTIC), busulphan and thiotepa.

1.5.2. The antimetabolites.

These agents substitute for normal metabolites in various biochemical pathways and their cytotoxicity is therefore greatly influenced by the level of natural metabolites with which they compete.

The clinically important antimetabolites are antagonists of either folic acid (for example methotrexate), or purines (for example 6-mercaptopurine), or pyrimidines (for example 5-fluorouracil).

1.5.3. Enzymes.

The only example in this group is L-asparaginase which causes depletion of endogenous plasma asparagine. This amino acid is non-essential for normal cells, but some malignant cells are unable to synthesise asparagine and require an exogenous source for protein synthesis (Horwitz et al., 1968).

1.5.4. Hormones.

Those hormones used in cancer chemotherapy include oestrogens, progestogens, and androgens. Synthetic antioestrogenic compounds and glucocorticoids are also used.

Cancers which originate in organs normally sensitive to the suppressant action or maturing effects of hormones may be inhibited by hormonal treatment. Oestrogens are extensively employed in the treatment of prostate carcinoma, whilst antioestrogenic drugs are used for the treatment of breast cancer, (Carter and Mathé, 1980).

1.5.5. Mitotic spindle inhibitors.

The two most clinically important drugs in this group are vincristine and vinblastine. They are mainly used in the treatment of the leukaemias in combination with drugs which inhibit DNA replication for example, alkylating agents, intercalating agents, and antimetabolites. The mitotic inhibitors act by interfering with the function of microtubules during mitosis. The clinical use of vincristine and vinblastine is limited by their neurotoxic side effects, (Weiss et al., 1974).

1.5.6 Radiosensitizing agents.

Ionising radiation has been extensively utilised in the treatment of cancer. The radiosensitivity of cells depends in part on the local concentration of

oxygen present at the time of irradiation. However, within a solid tumour mass a large number of tumour cells are hypoxic and thus not sensitive to radiotherapy. In an attempt to mimic the free radical forming ability of oxygen a number of nitrohetrocyclic compounds have been developed.

1.5.7. Drugs which interact with DNA.

A number of antitumour, antibacterial, and antiparasitic drugs inhibit nucleic acid synthesis; furthermore mutagens and carcinogens also exert their toxic effects in this manner. There are three levels at which nucleic acid inhibitors may exert their effects (Waring, 1981). Firstly they may inhibit nucleotide synthesis, (eg. the antimetabolites); secondly they may inhibit the polymerisation of nucleic acids by imparing the capacity of DNA to function as a template (eg. drugs which reversibly (or irreversibly) react with DNA). Thirdly they may inhibit polymerase or other enzymatic processes involved in the replication of DNA. Drugs which inhibit polymerisation of nucleic acids interact with DNA either irreversibly or reversibly. Replication of DNA may thus be inhibited by these interactions.

Drugs which bind irreversibly with DNA cause structural alterations within the DNA molecule, such as strand scission or removal of bases, or may crosslink the DNA strands. The alkylating agents may be classified in this group since bifunctional alkylating agents can covalently bind to DNA. Mitomycin C also

crosslinks the two complementary strands of DNA molecule; it is first reduced to the semiquinone form which then inserts into and becomes covalently attached to DNA (Lin et al., 1976).

Bleomycin has been extensively used in the treatment of neoplastic diseases. The interaction of bleomycin involves the formation of three strong hydrogen bonds between the drug molecule and DNA, insertion of bithiazole rings between the nucleic acid base pairs together with electrostatic binding (Povirk et al., 1979).

Drugs may interact reversibly with DNA either by binding to the exterior of the helix or by intercalation (drug insertion between successive base pairs) into the DNA molecule.

Netropsin and distamycin are antibiotics which have been shown to bind to DNA by reinforced electrostatic binding to the exterior of the helix. They bind to the minor groove of the B-form of DNA (Waring, 1981) and show A-T binding specificity. Distamycin has been shown to have antineoplastic activity in animals. The aromatic diamidines, berenil, and hydroxystilbamidine interact with DNA by a similar mechanism to that of distamycin.

The antibiotics chromomycin, mithramycin and olivomycin have been shown to bind externally to the DNA helix. The interaction of chromomycin with DNA is mediated by an antibiotic-Mg^{2+} complex (Waring, 1981).

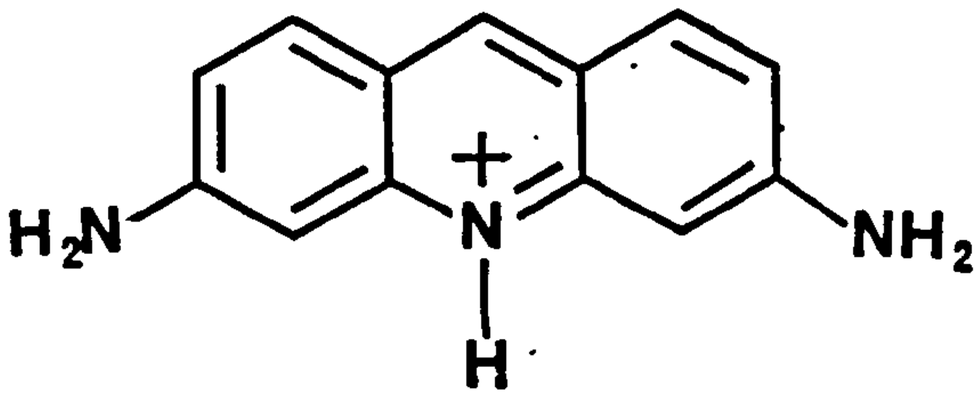
The most characteristic feature of the binding of these antibiotics to DNA is the requirement of a stoichiometrically equivalent amount of Mg^{2+} (Ward et al., 1965). Chromomycin appears to interact specifically with guanine residues of helical DNA.

In this work, compounds will be synthesised based on the mode of action of doxorubicin. One of the modes of antitumour activity of doxorubicin is by intercalation (see later) it is therefore necessary to examine the mechanisms underlying intercalation, and also the structural requirements for intercalating agents.

1.6. INTERCALATION.

The second mechanism by which drugs interact reversibly with DNA is by intercalation of the drug molecule between adjacent base pairs of DNA. Several anticancer drugs act by intercalation into DNA. These agents are uniquely suited for studies concerned with molecular basis of drug action since the precise structure and conformation of the receptor is well documented. Thus drugs which bind to DNA provide a good opportunity to examine in detail the nature of drug-receptor interactions. The advantage of designing drugs which interfere directly with DNA structure is that the binding to DNA can be quantified and the structural requirements for drug binding to DNA are known, thus allowing a considerable scope for structural variation.

Drug which interfere with the template role of



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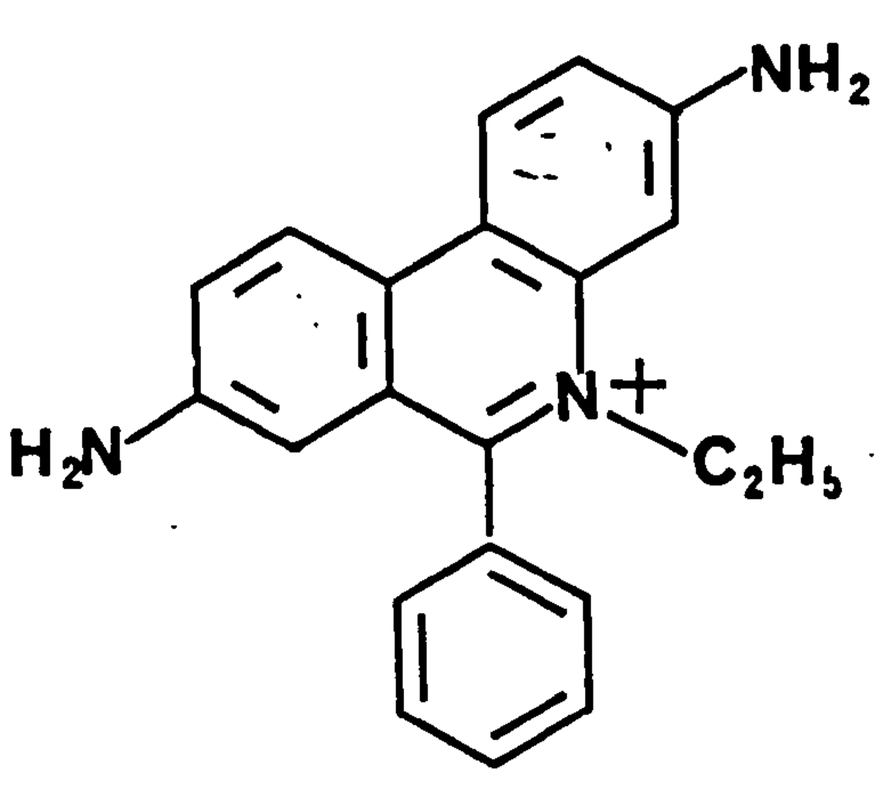


Br^- (4)

Figure 6 : Structures of two typical intercalating drugs: proflavine (3) and ethidium (4).

DNA either by causing structural changes such as strand scission (eg. bleomycin), removal of bases, or formation of covalent cross-links (eg. mitomycin) or by reacting with it to form a reversible DNA/drug complex. This reversible complex may be formed either by the drug binding to the exterior of the helix (for example, distamycin) or by a process termed intercalation, in which the drug molecule is inserted into the helix between succesive base pairs. Intercalation will be considered here since the structural requirements for this DNA/drug interaction can be readily identified. Proflavine (3) and ethidium bromide (4) (figure 6) are classic examples of intercalating compounds, and therefore the mode of interaction of these compounds will be considered first.

1.6.1. Lerman model for Intercalation.

The first detailed description of the model for drug intercalation into DNA was proposed by Lerman (1961). This model was based on the X-ray diffraction patterns of proflavine (3)-DNA complexes, and on the observation that there was an increase in viscosity with an associated decrease in sedimentation coeff-icient of DNA/drug complex. The hydrodynamic changes were attributed to a lengthening of DNA molecules caused by proflavine (3) binding, such that thay behaved as stiffer, more slender rods with a diminished mass per unit length. X-ray diffraction patterns of the DNA-proflavine (3) complex revealed the loss of

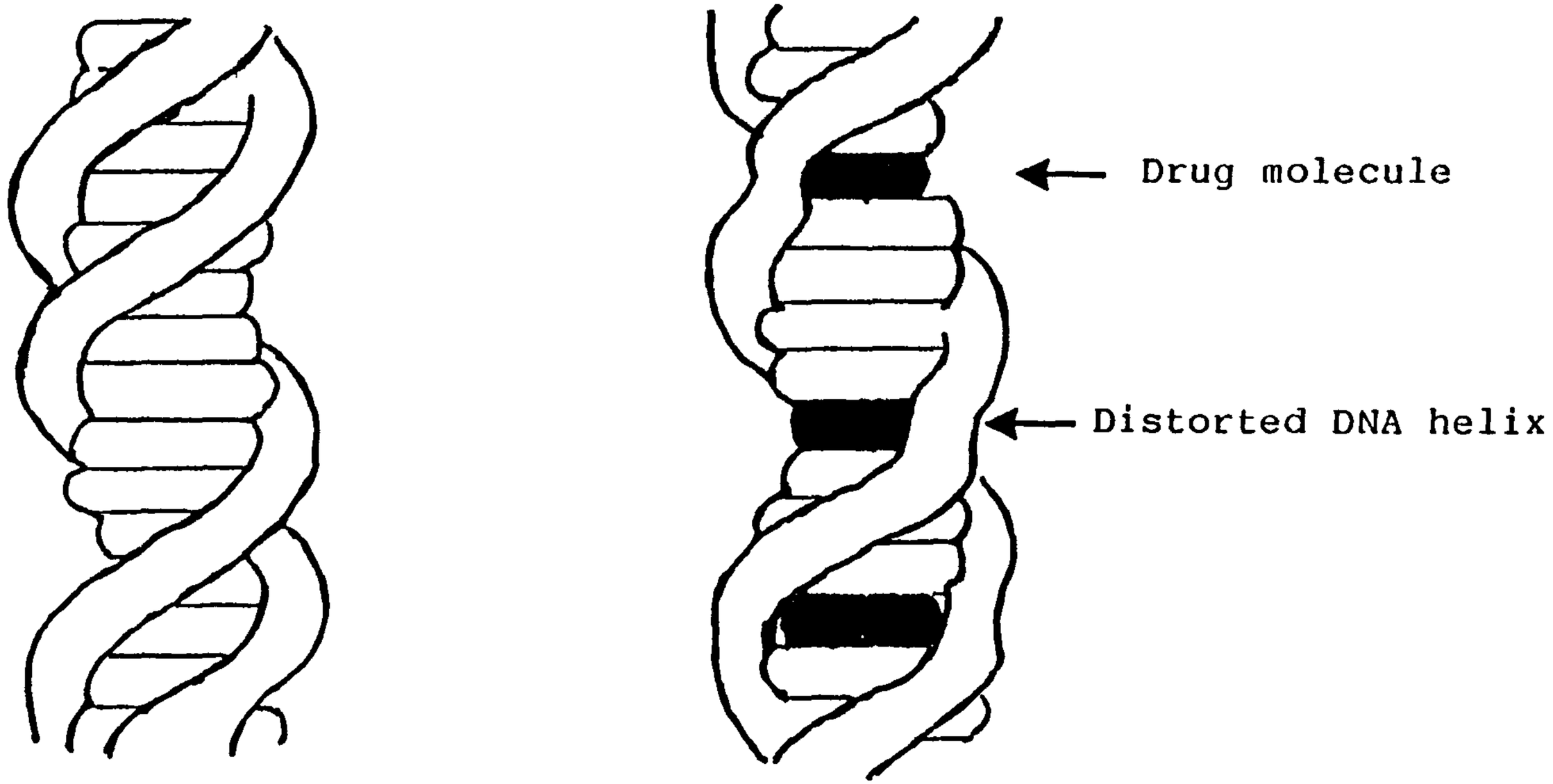


Figure 7 : Schematic representation of Lerman's (1961) model for intercalation of proflavine into DNA.

long-range regularity of the helical structure but retention of 3.4Å meridional reflections due to regular stacking of base pairs perpendicular to the helix axis.

Lerman (1961) proposed that the planar aromatic proflavine (3) molecule becomes inserted between adjacent base pairs of the double helix of DNA (Figure 7). The base pairs remain perpendicular to the helix axis, but they are moved apart, by 3.4Å, to accomodate the acridine molecule which lies in van der Waals contact between the base pairs. The intimate contact between the π-orbitals of the drug molecule and the base pairs would help to stabilize the complex via hydrophobic and charge-transfer forces. Local distortion of the helix occurs since it has to unwind in order to accomodate the drug molecule. Distortion of the helix due to local unwinding at intercalation sites would destroy the long-range regularity of the helix, as observed by X-ray diffraction studies. Lerman (1961) proposed an unwinding of angle 45° in his original model which he subsequently revised to 36°, (Lerman, 1964a).

Based on the results of studies with ethidium/DNA mixtures (4) Fuller and Waring (1964) further refined the intercalation model. This amended model was similar to that proposed by Lerman; but in addition suggested the formation of hydrogen bonds between the amino groups of the drug molecule and charged oxygen atoms of the phosphate groups of the DNA

backbone. They suggested an unwinding angle of 12°, the minimum necessary to permit intercalation of an aromatic ring system and the preferred angle since this would preserve maximal separation of the negatively charged phosphate groups. However, the currently accepted value for unwinding angle is 26°, (Wang, 1974, Waring, 1981). The model could readily accomodate proflavine (3), since the aromatic ring systems and the intramolecular distance between the amino groups of both proflavine (3) and ethidium (4) are very similar (Jones and Neidle, 1975). The phenyl and ethyl groups of these intercalating agents are thought to project into the minor groove of the DNA helix.

Further evidence has been obtained supporting the validity of the Lerman, Fuller-Waring model for intercalation. This further evidence came from fluorescence and dichroism studies (Lerman, 1963), X-ray diffraction studies (Neville and Davies, 1966; Luzati et al., 1961), auto-radiography (Cairns, 1972), amino group reactivity (Lerman, 1964b), electron microscopy (Freifeilder, 1971), and studies on the thermal denaturation of the DNA helix (Le Pecq and Paoletti, 1967).

In addition to intercalation, at relatively high drug concentrations, there is a weaker secondary binding which is a "stacking" process in which drug molecules bind to the exterior of the DNA helix and stack upon each other (Stone and Bradley, 1961; Blake and Peacock, 1968). This stacking is due to

electrostatic interaction of the positive centre of drug with negatively charged phosphate groups. However, this interaction is unlikely to be of significant importance *in vivo*, where relatively low drug concentrations are found. The intercalation process leads to the binding of one drug molecule per 2 to 2.5 nucleotide base pairs (Waring, 1981). The secondary external binding proceeds until one basic drug molecule is bound for every anionic phosphate group.

The factors which limit the intercalation to a maximal binding of one drug molecule per 2-2.5 base pairs is not fully explained by the Lerman model for intercalation. However the site excluded (or neighbour exclusion) model is now generally accepted. The model was first proposed by Cairns (1962) and assumes that drug molecules may intercalate anywhere along the length of DNA, subject only to the restriction that intercalation may not occur at adjacent base pairs. This restriction does not apparently result from any steric interference between bound drug molecules (Fuller and Waring, 1964), although it is possible that electronic interactions between the drug molecule and the base pairs in some way affects the charge distribution on base pairs rendering them less capable for interaction with drug molecule. Cairns (1962) showed, by theoretical calculations, that the maximum number of drug molecules which may occupy a potential binding site was 0.436 or one drug molecule per 2.3 base pairs; in agreement with the experimental values.

The use of synthetic self-complementary nucleotides such as dCpG has enabled subsequent workers to support and clarify the concept of intercalation. In addition to ethidium and proflavine, other DNA binding drugs such as daunorubicin have been shown to form intercalation complexes with dinucleotides (and tetranucleotides) in solution (Patel 1979, 1980). Detailed information of the nature of interaction was obtained by the use of high resolution NMR studies, and X-ray crystallography of the crystalline DNA/drug complexes (Sobell et al., 1978; Patel, 1980; Neidle, 1980). Based on these studies, a detailed model has been proposed to explain the interaction of ethidium with DNA (Sobell et al., 1978). This model suggested an unwinding angle of -26° (the currently accepted value) at the immediate site of drug intercalation, producing an effective angular unwinding of the helix by about -10°. This model also assumed that DNA "kinks" in order to accomodate ethidium molecules. DNA was proposed to bend towards the major groove, since the negatively charged phosphate groups do not approach each other significantly even after bending the DNA helix by up to 30°. In the kinked DNA structure, the base pairs are partially unstacked and the helical axis for the B-DNA sections above and below the kink are displaced by about -1.0Å. It is suggested that ethidium intercalates into the minor groove of this kinked DNA (Sobell et al., 1977). Three types of intercalation are possible; firstly, a drug (for example, ethidium) may intercalate exclusively from the minor groove of DNA,

binding first to the kink in DNA and then later intercalating into the interior of the helix; secondly, a drug (for example, daunorubicin) may intercalate from the major groove or thirdly, the drug (for example, 9-aminoacridines) may enter from either the major or the minor groove.

An important feature of this model is its requirement for strictly alternating C3'endo--(3',5')-C2'-endo sugar puckering. All the sugars in the Watson-Crick B-DNA model are puckered C2' endo, this necessitates a change of pucker in one deoxyribose ring of each strand at the intercalation site and therefore automatically imposes a condition of neighbour exclusion. However this may not be the **only** explanation for neighbour exclusion (Neidle, 1980) since alternating patterns of sugar puckering are not invariably seen with drug-dinucleotide complexes.

The classical intercalation models of Lerman (1961) and Fuller-Waring (1964) have been criticised in the literature. Both acridines and ethidium bind equally well at room temperature to heat-denatured DNA as well as to native DNA. A modified intercalation model has been proposed, based on this finding (Blake and Peacocke, 1968; Pritchard et al., 1966). In this model the positioning of the intercalated ring system with respect to DNA bases is shifted to lie between two adjacent bases on the same polynucleotide chain with the positively charged nitrogen in close proximity to the phosphate group. This type of model allows several

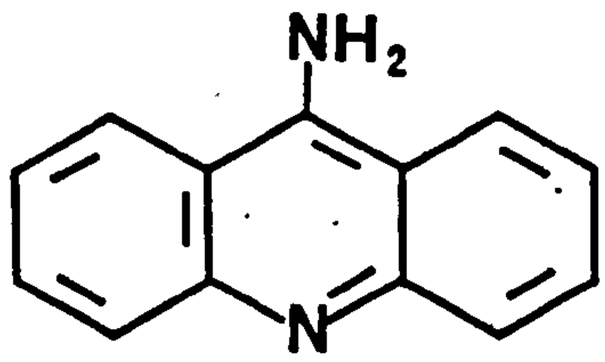
different modes of orientation of the aromatic chromophore intercalated between the stacked bases or base-pairs of DNA. Daunorubicin, is one of several compounds known to orientate perpendicular to the DNA helical axis (see 1.8.1).

An intensive effort has been made in the past two decades to synthesise drugs which intercalate into DNA. Daunorubicin and doxorubicin are two examples of drugs which have been isolated from microbiological sources, have proved to be useful as antitumour agents and subsequently shown to intercalate into DNA. The following section describes some of these intercalating compounds.

1.6.2. Other intercalating compounds.

1.6.2.1 Monofunctional intercalating agents.

Substitution of various groups at the C-9 position of the acridine nucleus results in compounds which are still capable of intercalation. For example 9-aminoacridine (5) (figure 8) binds strongly to DNA by intercalation (Dalgleish et al., 1971). The anti-malarial drug mepacrine (6) (figure 8) has been shown to intercalate into DNA (Ciak and Hahn, 1967) with an absolute preference for B-DNA (Plumbridge and Brown, 1977). Amsacrine (mAMSA, Figure 8), (7), was first reported in 1974 as an 9-anilincacridine derivative showing high activity against L1210 leukaemia (Cain and Atwell, 1974) and has subsequently been shown to be active in a number of experimental



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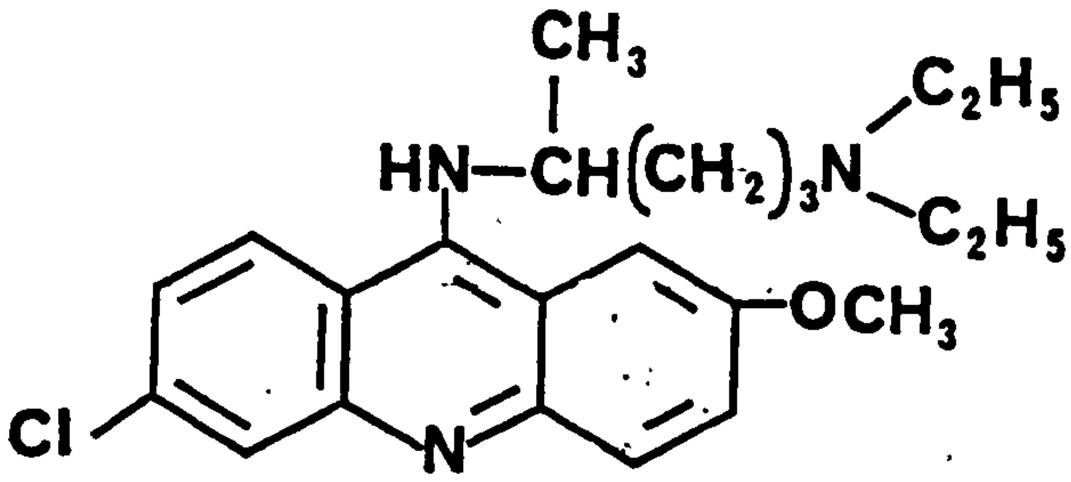

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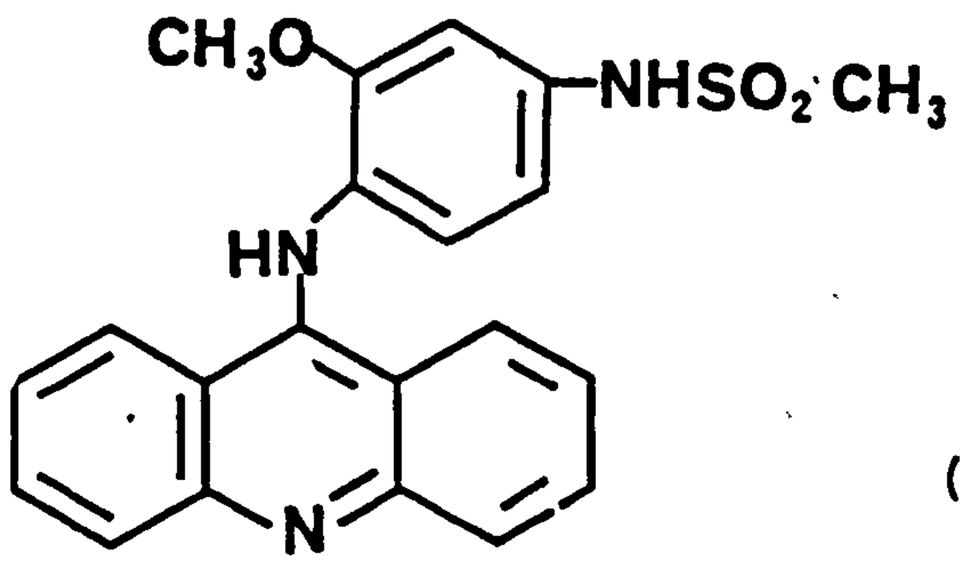

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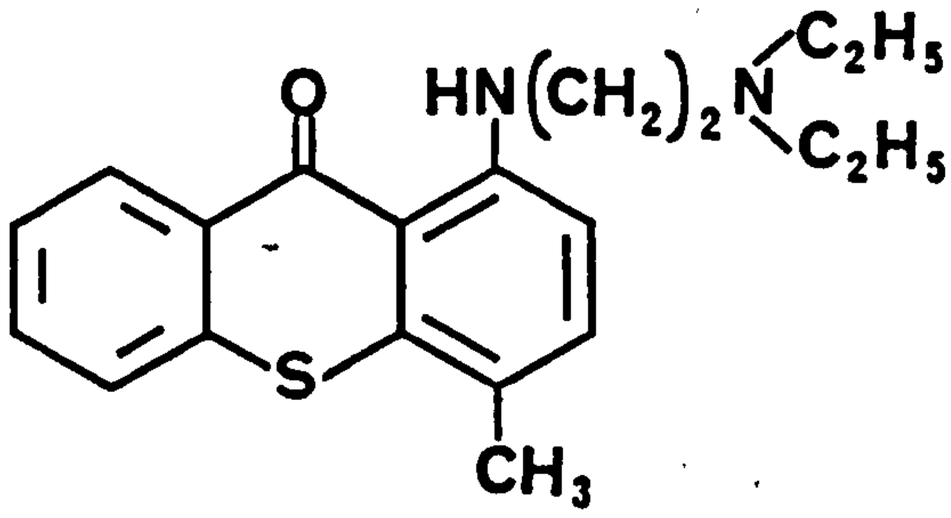


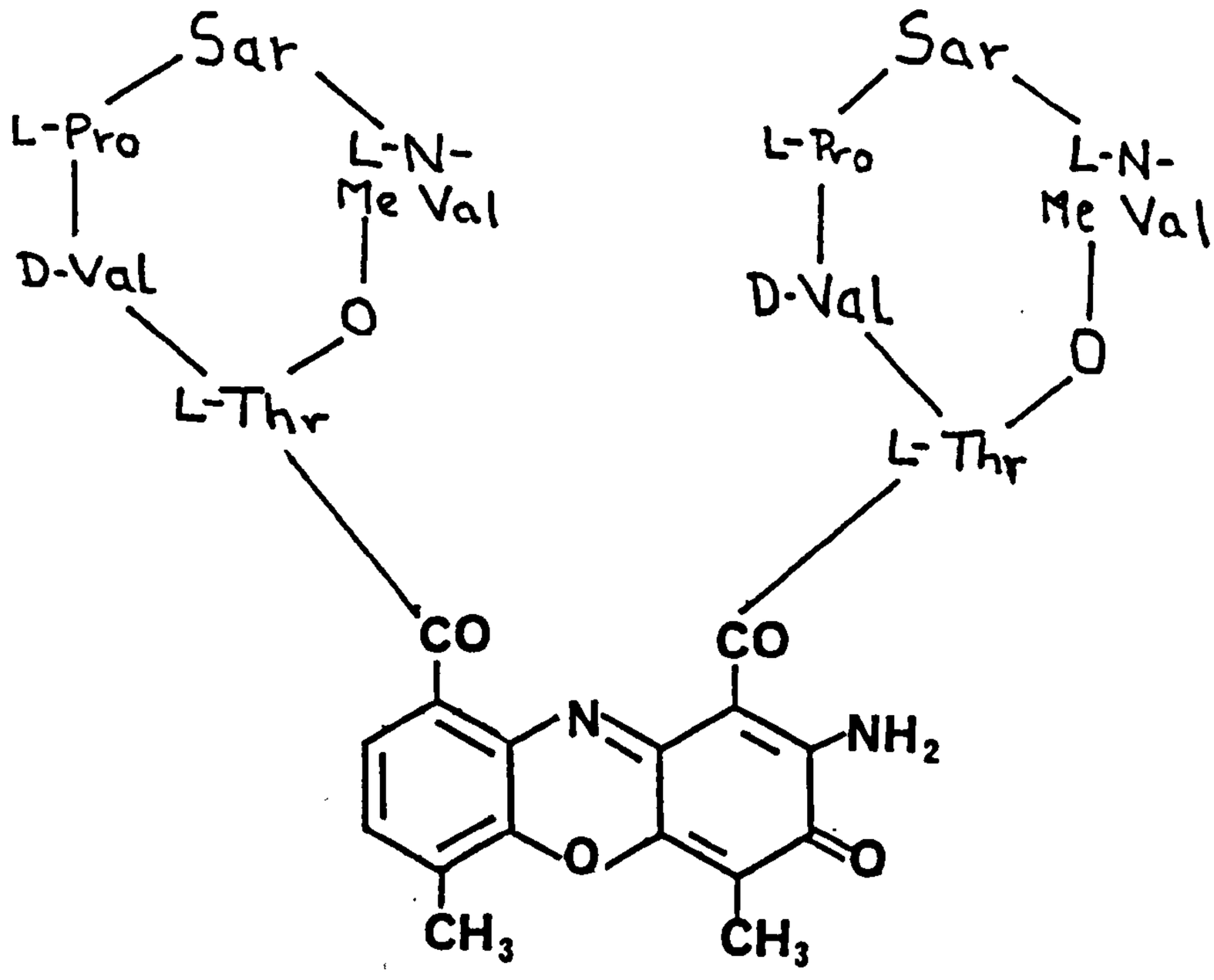
(8)

Figure 8 : Structure of the intercalating agents 9-aminoacridine (5), mepacrine (6), m-AMSA (7) and lucanthone (8).

tumours (Denny et al., 1983). Amsacrine has recently been marketed for treatment of neoplastic diseases. AMSA has been shown to preferentially bind to native DNA compared to heat denatured DNA or RNA (Wilson et al., 1981).

Lucanthone, (8) (figure 8), and its 4-hydroxymethyl analogue have been shown to be potent schistosomicidal drugs (Hirschberg, 1974). Both analogues have been shown to have a planar structure (Neidle, 1976) and are therefore able to intercalate into DNA. Lucanthone analogues with varying side chains have been prepared and a correlation between antibacterial activity and DNA binding ability established. It was shown that both a non-alkyl-substituted proximal nitrogen atom, and a correctly positioned terminal nitrogen atom were essential for DNA binding activity (Hirschberg et al., 1968).

Actinomycin D, (9) (figure 9) has been shown to be a potent antitumour antibiotic and contains a planar phenoxazine chromophore linked to two identical pentapeptides. This antibiotic has been shown to bind specifically to G-C base pairs with the requirement for the 2-amino group of guanine for interaction. The amino and quinoid groups on the chromophore are essential for intercalation. From these structural features and X-ray diffraction studies, Hamilton et al. (1963) proposed that the complex was stabilised by hydrogen bonds from the 2-amino group of the guanine base to the quinoidal



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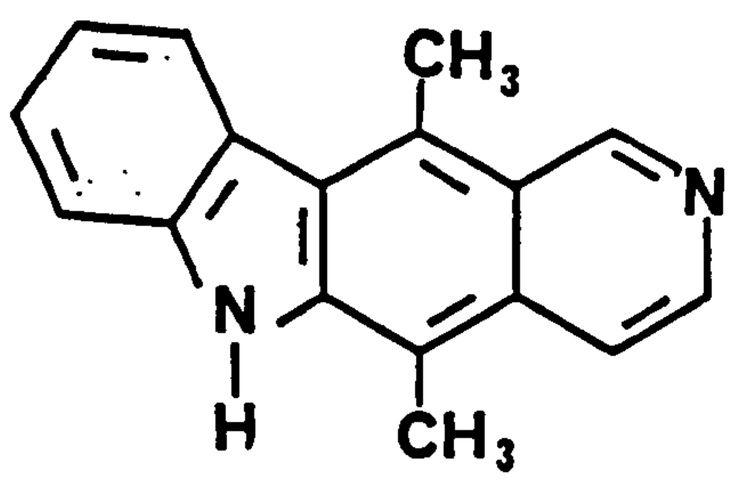


(10)

Figure 9 : Structure of actinomycin D (9) and ellipticine (10).

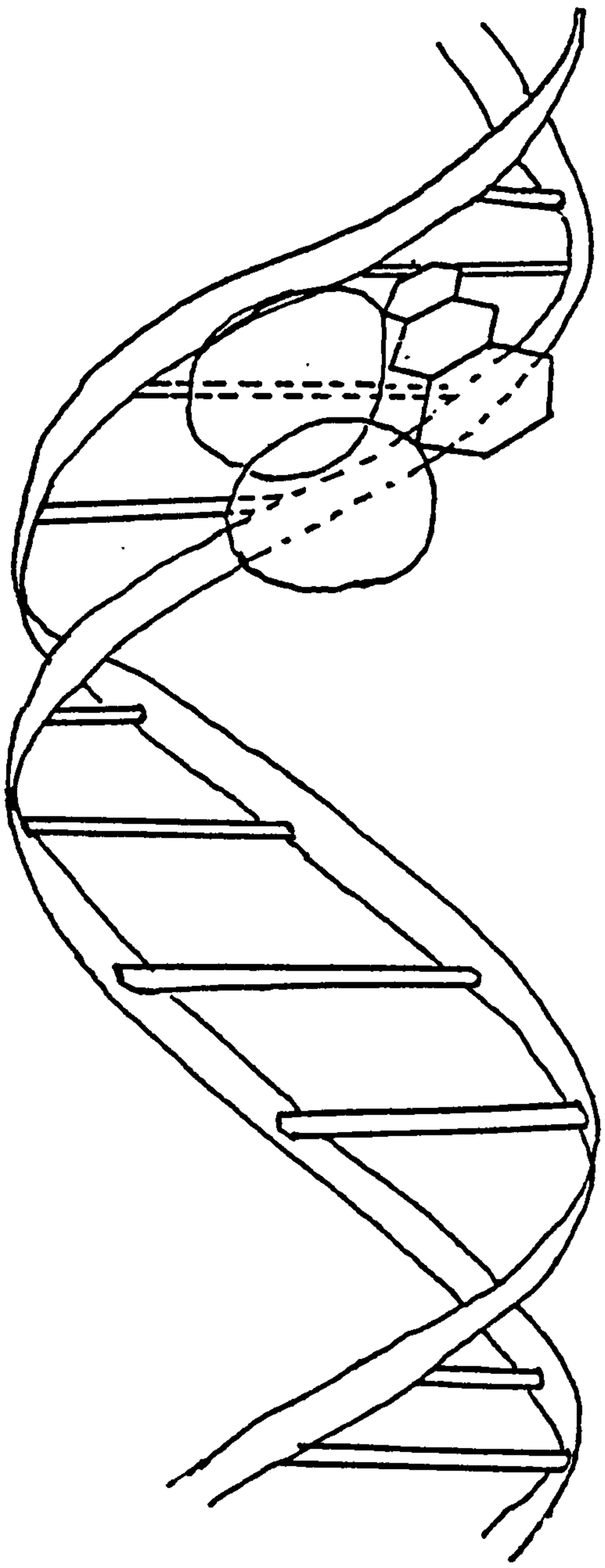


Figure 10 : Schematic representation of the interaction of actinomycin D (9) with DNA. The chromophore is represented as a simple anthracene ring system in perspective, the peptide lactones are shown as rings. (adapted from Waring, 1981).

oxygen of the actinomycin chromophore, and from the 2-amino group of the chromophore to N3 of the base and the sugar ring oxygen of deoxyguanosine. This would locate the chromophore in the minor groove of the DNA with its plane inclined at an angle of about 20° to the base pairs (figure 10). The peptide ring could then pack into the minor groove and stabilize the complex (Waring, 1968) further via four hydrogen bonds from the NH groups of the peptide to the phosphate oxygen atoms in the chain complementary to the the one containing the deoxyguanosine.

Ellipticine, (10) (figure 9) is an alkaloid which has pronounced antineoplastic activity in experimental tumours (Hartwell and Abbott, 1969). The compound is planar with approximately the same molecular dimensions as proflavine and is protonated in mildly acidic conditions. The compound has been shown to intercalate into DNA by both spectroscopic methods and by its ability to unwind closed circular DNA (Neidle, 1979).

1.6.2.2 Bifunctional intercalating agents.

There are many instances where increasing affinity of drugs for DNA improves therapeutic index (Neidle 1979). In the past decade attempts have been made to capitalise on this by using drugs which intercalate simultaneously at more than one base pair site. Such compounds, with two or more intercalating chromophores, were shown to have significantly higher

DNA-binding affinity than mono-functional intercalating agents. From the neighbour exclusion principle (see 1.6.1) the minimum theoretical distance between the two intercalating chromophores to achieve two-site binding is about 10.2Å. Therefore by appropriate choice of intercalating chromophores linked together by a "spacer" of an appropriate length it should be possible to design compounds which bind preferentially to a specific DNA sequence. For example, acridine chromophores have been utilised in a number of investigations for the synthesis of bifunctional intercalating molecules. A series of diacridines were synthesised with the spacer group $(CH_2)_n$ and their binding to DNA was investigated (Wakelin, et al., 1976, 1978; Chen et al., 1978). These studies showed that the minimum length of the spacer chain necessary for bis-intercalation to occur was 8.8Å. Later studies with diacridines substituted in the chromophores showed that the ability of these compounds to bis-intercalate was also dependent on the substitution pattern as well as on the nature of the spacer chain (Wright et al., 1980).

In addition to the acridines a number of other bis-intercalating agents are also known. These include derivatives of ethidium such as bis(methidium)spermines (Dervan and Becker, 1978), and quinoxaline antibiotics such as echinomycin and luzopeptin (Neidle, 1979; Waring and Fox, 1983). The nature of interaction of these bis-intercalating agents has been extensively studied (Waring, 1981; Waring and Fox, 1983). A common

feature of all bisintercalating agents is the relatively high affinity constants (10^9 to 10^{14} M^{-1} compared to 10^5 to 10^7 M^{-1} for monofunctional intercalating agents) and large unwinding angles (Waring, 1981) (35-55° for bis-intercalating agents compared to 8-26° for the monofunctional intercalating agents).

Recently a triacridine derivative has also been described and shown to bind to DNA (Atwell et al., 1983). The tris-intercalating agent unwinds closed circular DNA by angle of 45°, which is significantly larger than those produced by monofunctional and bifunctional intercalating agents.

It is apparent from the preceding discussion that certain minimum structural requirements must be satisfied before a drug can intercalate into DNA. These requirements consist of a planar, tri or tetracyclic ring system, containing a substituent amino group which can initially bind electrostatically to the phosphate groups on the DNA. Further stabilisation would be afforded by groups, such as hydroxyl groups, which could hydrogen bond to other groups in the DNA.

Anthracycline antibiotics have been widely studied in the recent years, since some possess potent antitumour activity. Doxorubicin in particular has been shown to possess a broad spectrum of activity. However all anthracyclines have severe dose-limiting toxicity. The principal aim of this project is to design potential antitumour agents with at least equivalent

cytotoxicity, but without the dose limiting toxicity of the anthracyclines. Many of the anthracyclines have been shown to intercalate into DNA (Brown, 1978). Intercalation is only one of several biochemical mechanisms by which the anthracyclines are thought to exert their cytotoxic action. It is therefore necessary to review the clinical activity and the biochemical mechanisms by which the anthracyclines are thought to exert their antitumour action.

1.7. THE ANTHRACYCLINES.

1.7.1 Introduction.

The anthracycline group of antitumour antibiotics were isolated from various species of Streptomyces; their discovery, isolation and structure elucidation have been extensively reviewed (Arcamone, 1981; Brown, 1978, 1983; Remers, 1979). The anthracyclines are planar naphthacenediones and usually occur as glycosides. Doxorubicin and daunorubicin are glycosides of the amino sugar daunosamine, and the aglycones adriamycinone and daunomycinone respectively (figure 1).

Following the isolation of these anthracyclines in the late 1960's there have been considerable efforts made to produce structural analogues of daunorubicin and doxorubicin with improved antitumour activity. Previous work on the development of anthracyclines has been of an empirical nature (reviewed by Brown, 1978). The earliest approach has been to search for novel

anthracyclines produced by newly isolated microorganisms or by strains of anthracycline-producing organisms derived by exposure to some form of mutagenic treatment. Doxorubicin was isolated as a product of a mutant strain of the daunorubicin-producing organism (Arcamone, 1981). A second approach has been to chemically modify known anthracyclines, or to couple a synthesised aglycone to daunosamine or a synthesised alternative sugar to a natural aglycone. A final approach has been to attempt to mimic the biological actions of doxorubicin (and daunorubicin) in simplified, totally synthetic analogues. This latter approach has been used in this project. Each of these approaches has led to the introduction of new analogues which have a higher therapeutic index than the parent anthracyclines. This work has been extensively reviewed, (Brown and Imam, 1984; Brown, 1978, 1983; Du Vernay, 1981; Di Marco, 1981; Crook et al., 1981; Henry, 1979; Arcamone, 1981), and therefore only developments which have had a significant impact on the understanding of the modes of antitumour action and toxicity of anthracyclines will be considered here. It is necessary to discuss the clinical activity of anthracyclines before considering the mode of action of these drugs.

1.7.2. Clinical activity of anthracyclines.

The two most prominent anthracyclines used in the clinic are doxorubicin and daunorubicin. They are important drugs in cancer chemotherapy, despite their

inhibitory effects on rapidly dividing tissues (which leads to myelosuppression, alopecia, and stomatitis) and their specific toxicity to the heart (Carter, 1980, Young et al.,1981). Daunorubicin has been used in the induction of remission in acute lymphocytic leukaemia, however its use in maintenance of remission is limited by the dose-related cardiotoxicity. Doxorubicin in contrast is used as a single agent or in combination therapy for the treatment of a wide spectrum of solid tumours (Wiernik, 1980). It should be noted that there is only a small structural difference between daunorubicin and doxorubicin. However as a result of the replacement of one of the hydrogens at C_{14} of daunorubicin by a hydroxyl group the spectrum of antitumour activity of doxorubicin is profoundly altered.

When daunorubicin is used, as a single agent, for the treatment of acute nonlymphoblastic leukaemia (ANLL) it produces a complete remission in 40-50% of patients (Wiernik and Serpik, 1972). It also has significant activity against childhood and adult acute lymphoblastic leukaemia (ALL); although it is rarely necessary to add this drug to vincristine and prednisone induction therapy, however, the drug is of use in refractory or relapsed patients (Aur et al., 1971). Daunorubicin has not been fully evaluated for maintenance therapy in acute leukaemia. This is primarily due to the fact that induction therapy usually requires most of the allowable dose (ie before the cumulative cardiotoxic effects become prominent).

However by the use of combination chemotherapy employing a relatively low dose of daunorubicin it is possible to employ daunorubicin for reinduction and maintainence therapy, (Wiernik, 1980).

Doxorubicin has the broadest spectrum of anticancer activity of any drug in use today (Carter, 1975; Weirnik, 1980). It is active in haematological malignancies, metastatic or inoperable sarcomas, hepatoma and a variety of childhood solid tumours (O'Brian et al., 1973, Carter, 1982). Following the use of doxorubicin in combination with cyclophosphamide, methotrexate and 5-fluorouracil for the treatment of metastatic breast cancer, a 50% response rate was obtained (Kennealy et al., 1978). Doxorubicin has been used either alone or in combination with other chemotherapeutic agents in the treatment of a variety of sarcomas and also the following solid tumours: pancreatic cancer, hepatocellular carcinoma, metastatic hepatoblastoma, ovarian adenocarcinoma, advanced endometrial cancer, metastatic prostate carcinoma, metastatic testicular cancer and most forms of broncogenic carcinoma (Cortes et al., 1974; Blum and Carter, 1974; Horton et al., 1978; Wiernik, 1980). Doxorubicin is also useful against metastatic disease and as an adjuvant to surgery for the primary treatment of some sarcomas (Benjamin et al., 1975).

The anthracyclines represent a significant advance in the treatment of ANLL, doxorubicin has the broadest spectrum of clinical activity of all known

anticancer drugs. However the anthracyclines have limiting toxicities in man which prevents exploitation of their antitumour activity to the full extent. These toxicities are summarised in the following section.

1.7.3. Toxicity of anthracyclines.

Both daunorubicin and doxorubicin produce toxic effects typical of drugs which inhibit nucleic acid synthesis (Bonadonna et al., 1970). All patients treated with these agents experience alopecia, stomatitis occurs in about 75% of patients. Nausea and vomiting are also common side effects (Brown, 1978). Unusual pigmentation of nails or tongue may result following administration of daunorubicin or doxorubicin (Weirnik, 1980). Other side effects of these drugs include sublingual haemorrhage, nephrotoxicity and severe local necrosis during administration due to extravasation at the site of injection (Reilly, et al., 1977). These drugs are also carcinogenic (Brown, 1978).

However the most serious dose limiting toxicity of these drugs is cardiotoxicity. This can be subdivided into types; firstly reversible and transient electrocardiographic changes which occur within a few hours of administration of the drug (Gilladoga et al., 1976); secondly an irreversible cardiomyopathy which develops one to six months after therapy; which may lead to congestive cardiac failure. The incidence of this fatal cardiotoxicity increases with a cumulative dose in excess of $550mgM^{-2}$ (Blum and Carter, 1974;

Young et al., 1981). Although the monitoring of cardiac function can reduce the risk of cardiotoxicity, (Minow et al., 1977), the ideal solution would be to identify and negate the cause of the toxic effect.

In the last 15 years an intensive effort has been made to differentiate between the biochemical mechanisms involved in the cardiotoxic effects and antitumour action of anthracyclines. The following sections describe these mechanisms.

1.8. MECHANISM OF ACTION OF DOXORUBICIN AND DAUNORUBICIN.

The anthracyclines have many cellular actions and these can broadly be divided into three mechanisms; namely interaction with DNA, free radical generation and interaction with membranes.

1.8.1. Interaction of anthracyclines with DNA.

The most recognised property of doxorubicin and daunorubicin is their effect on the nucleus; they can intercalate into DNA (Arcamone, 1981; Brown, 1978, 1983; Neidle and Sanderson, 1983; Patel et al., 1981; Plumbridge and Brown, 1977; Quigley et al., 1980), and cause sister chromatid exchange and chromosome aberrations (Nersted, 1978). The DNA shows protein associated single strand and double strand breaks possibly due to binding of topoisomerase which ineffectually attempts to relieve the strain in the helix induced by intercalative binding of the drug (Ross et al., 1978; Ross and Smith, 1982). For both

drugs the maximum binding that can occur is about two drug molecules per five base pairs (Phillips et al., 1978). On saturation of the intercalation sites, further drug molecules can bind to the exterior of the helix, presumably by electrostatic interaction with the phosphate groups (Zunino et al., 1977). It has recently been shown that at low levels of drug binding, doxorubicin (or daunorubicin) bind in a cooperative manner to DNA (Graves, and Krugh, 1983); the binding of the first drug molecules induce further molecules to bind more readily to DNA. The cooperative binding is dependent upon the ionic strength, which suggests DNA flexibility is necessary in this cooperative binding process. Data on the interaction of doxorubicin and daunorubicin with chromatin, in general parallel results obtained with calf thymus DNA, with only a slight reduction in the apparent binding constants (Zunino et al., 1980; Sabeur et al., 1979)

Two models have been proposed for the intercalation of daunorubicin into DNA. The first model, based on X-ray diffraction data, was proposed by Pigram et al, (1972) and has been refined by Neidle (1979). In this model the planar aromatic ring system is inserted into the DNA helix, with the plane of the chromophore being parallel to the planes of the DNA base pairs, accompanied by a small degree of untwisting of the DNA sugar-phosphate backbone. The daunosamine sugar lies in the major groove of the DNA with its amino group interacting electrostatically with the second phosphate residue away from the intercalation

site. This model has received a widespread acceptance since it satisfactorily explains much of the DNA binding data for daunorubicin and doxorubicin (Neidle, 1979). The total binding site spans three base pairs, in good agreement with the experimental data of approximately 0.17 binding sites per nucleotide. When daunosamine is altered by acetylation of the amino group, or the configuration of the sugar is altered the biological activity and DNA-binding is diminished (DiMarco et al., 1976, 1977).

This model for the interaction of daunorubicin with DNA has however been superceded by a new model in which the drug "skewers" the DNA. This model is based on evidence from X-ray crystallographic data of daunorubicin-oligonucleotide complexes (Quigley et al., 1980; Rich et al., 1981) and high resolution NMR studies (Patel et al., 1981). This second model is consistent with theoretical model building (Henry, 1976; Neidle and Taylor, 1979; Nakata and Hopfinger, 1980). It was shown from X-ray crystallography that two drug molecules are bound per self-complementary oligonucleotide (d(CpGpTpApCpG)) duplex. In spite of the choice of four possible sites (CG, GT, TA, or AC), the drug is preferentially intercalated between the terminal CG base pairs. The drug molecules show minor groove intercalation, in contrast to the earlier molecular models proposed. The amino sugar fits into the minor groove; however this position excludes any interaction between the charged amino group and backbone phosphates. The chromophore lies skew to the

GC base pairs, and rings A and D protude from them, in complete agreement with NMR observations (Patel et al., 1981). The B and C ring overlap with base pairs is small, although the hydroxyquinone oxygen atoms on each side of the drug molecule play a stabilising role, by being stacked with G2 and G6 guanine bases. The least understandable feature of this model is its inability to account for the role of the charged amino group in DNA binding. It is possible that the amino group acts in the initial electrostatic recognition of the exterior of the DNA molecule, and thus brings the intercalative grouping into the appropriate orientation for its binding (Quigley et al., 1980).

Doxorubicin and daunorubicin both inhibit the synthesis of DNA and RNA in vitro, in support of the observation that template DNA is the site of binding (Arcamone, 1981). It is not known whether they inhibit DNA and RNA synthesis to the same extent (Wang, et al., 1972; Zunino et al., 1974). However there is evidence to suggest that DNA replicative enzymes are inhibited to a greater extent than DNA repair enzyms (Sartiano, et al., 1979); This enzymic inhibition can only be reversed by the addition of excess DNA and not by additional enzyme (Zunino et al., 1974), consistent with a direct drug template (DNA) interaction.

1.8.2. Free radical generation.

The anthracyclines, daunorubicin and doxorubicin, contain a quinone moiety which can be enzymatically reduced to semiquinone and hydroquinone,

and may then reoxidise, generating reactive oxygen species including the superoxide anion, hydrogen peroxide and hydroxyl radicals. This redox cycling of anthracyclines may occur under aerobic or anaerobic conditions.

1.8.2.1. Aerobic redox cycling of Anthracyclines.

One electron reduction of the anthraquinone moiety of anthracyclines generates a semiquinone free radical. Bachur et al., (1977) suggested that the anthracycline antibiotics enter the single electron transfer chain at a point between NADPH and cytochrome P-450, forming semiquinones which reduce molecular oxygen to the superoxide anion (Figure 11); an analogous mechanism was proposed by Thayer (1977). It is now known that NADPH-dependent cytochrome P-450 reductase is one of several enzymes which can provide the electron necessary for the reduction (Bachur et al., 1979; Doroshow and Reeves, 1981). Xanthine oxidase is also capable of transferring electrons to the anthracycline chromophore. Superoxide anions formed via redox cycling of the anthracycline are further converted to hydroxyl radicals and peroxides (Winterbourne, 1981). It is the hydroxyl radicals which are thought to be involved in cellular damage. The general mechanism for the formation of $OH^{\bullet}$ radicals may be represented by the following equations:

$$HO_2^{\bullet} + HO_2^{\bullet} \xrightarrow{SOD} H_2O_2 + O_2$$

$$CMX.Fe(II) + H_2O_2 \longrightarrow CMX.Fe(III) + OH^{\bullet} + OH^{-}$$

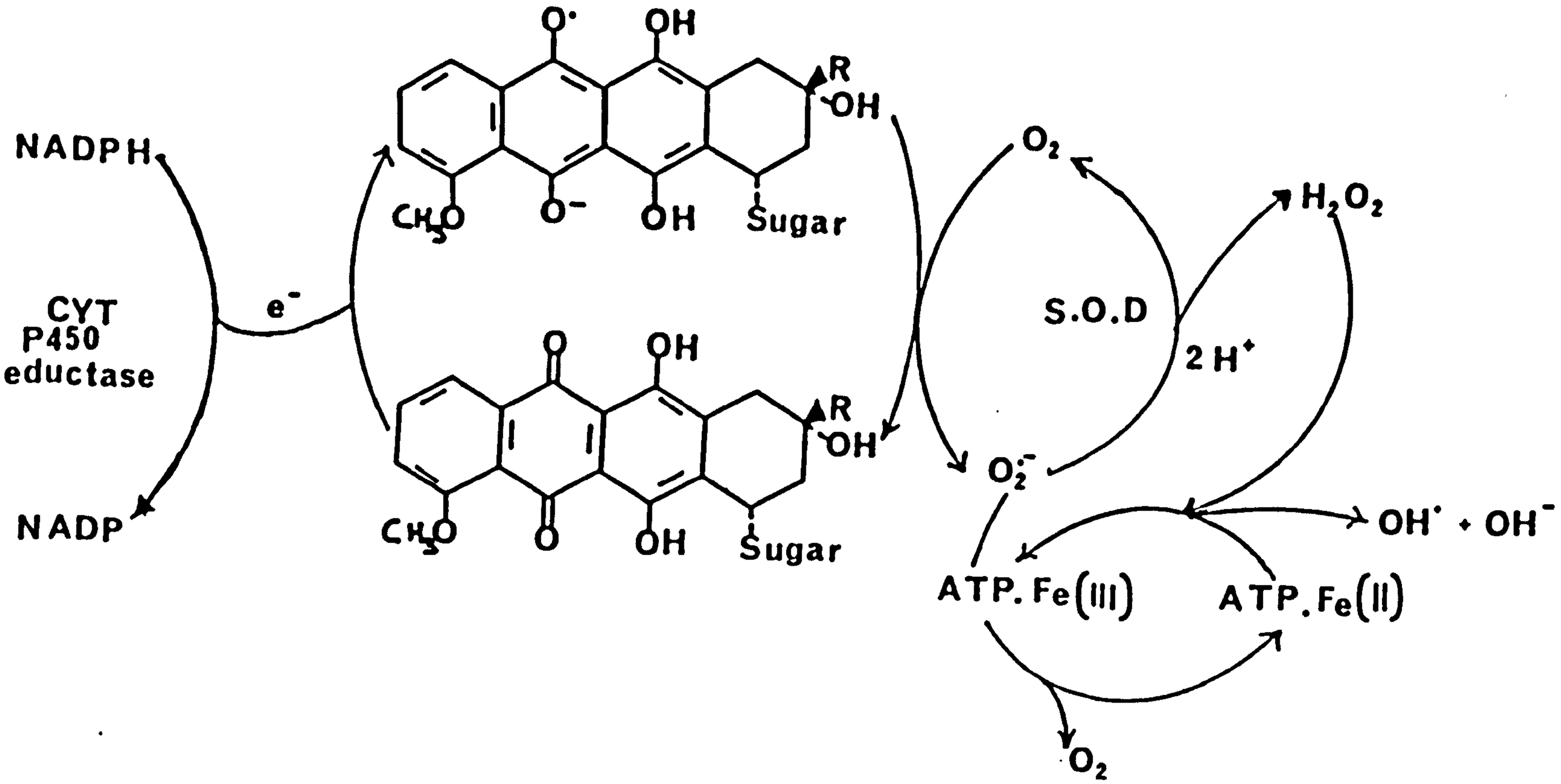


Figure 11 : Aerobic redox cycling of anthracycline antibiotics.

where SOD = superoxide dismutase and CMX.Fe(II) = iron complexed with ATP or protein. The $OH^{\bullet}$ radical is the most reactive radical formed in biological systems and reacts indiscriminately to degrade any molecule within diffusion distance (Fridovich, 1977). It may for example, react with lipid membranes (hence initiating lipid peroxidation), or with DNA, causing lesions including single strand breaks (Gutteridge and Togg, 1982, Berlin and Haseltine, 1981). Hydroxyl radicals may also be produced by reaction of semiquinone free radicals with hydrogen peroxide, this is not dependent on the presence of an iron complex (Winterbourn, 1981).

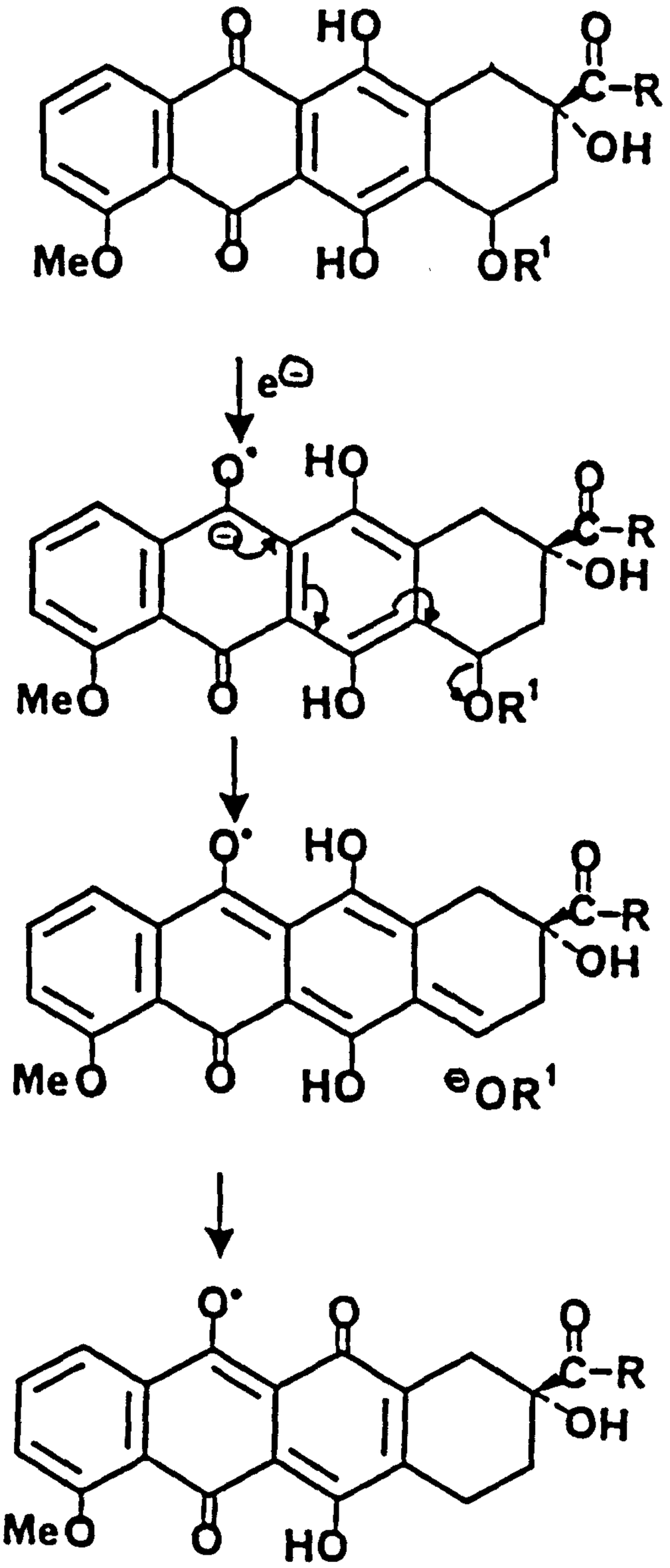
Goodman and Hochstein (1977) suggested that cardiotoxicity resulting from anthracycline chemotherapy is caused by this superoxide production leading to the initiation of lipid peroxidation. Doxorubicin is known stimulate lipid peroxidation in rat heart tissue (Mimnaugh et al., 1981). Several enzymes are known to catalyse the removal of these reactive oxygen species. For example, superoxide dismutase catalyses the dismutation of superoxide anions to hydrogen peroxide which in turn may be converted to water by catalase or by glutathione peroxidase using reduced glutathione (GSH) as a source of reducing equivalents. The concentrations of these protective enzymes is much lower in the cardiac tissue (Herzog and Fahimi, 1974) compared to other tissues such as liver and kidney. Nohl and Jordan (1983) showed

that in the cardiac tissue the anthracycline semiquinone radical shuttels electrons to hydrogen peroxide rather than molecular oxygen. This results in the generation of highly reactive hydroxyl radicals, instead of superoxide anion radicals.

The deleterious effects of reactive oxygen species in biological systems is well documented (Fridovich, 1979; Bachur, 1982a, 1982b, 1982c; Morehouse et al., 1983). The most important consequence of free radical generation is the damage to membranes as a result of lipid peroxdation, resulting in cellular damage. Furthermore these reactive oxygen species are capable of reacting with proteins and nucleic acids. One of the mechanisms involved in the cytotoxic action of doxorubicn is suggested to be the reaction of free radicals with deoxyribose (Bates and Winterbourn, 1982) resulting in DNA strand scission (Someya and Tanaka, 1979). Bachur et al., (1982c) showed that isolated nuclei were capable of reducing doxorubicin to the corresponding semiquinone with concurrent formation of reactive oxygen metabolites.

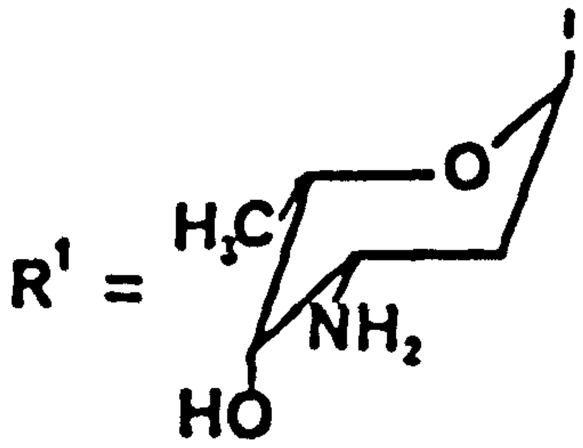
1.8.2.2. Anaerobic reduction of Anthracyclines.

Under anaerobic conditions the sugar moiety, (daunosamine in the case of daunorubicin and doxorubicin), of anthracycline glycosides may be eliminated by enzymatic reduction (Komiyama et al., 1979). A one electron reduction of the quinone, mediated by NADPH-dependent cytochrome P-450, is



R= CH_3 (Daunorubicin)

R= CH_2OH (Doxorubicin)

R^1 = 

(Daunosamine)

Figure 12 : Anaerobic reduction of anthracyclines.

followed by the elimination of the C-7-substituted sugar residue (figure 12). However it is possible that a free radical at C-7 may also be formed (Sinha and Gergory, 1981). A two electron reduction of anthracycline is also possible, which may give rise to an alkylating C-7 quinone methide metabolite (Moore, 1977). The reactive metabolites thus formed may bind covalently to cellular constituents, for example DNA. Sinha and Chignell (1979) showed that the covalent binding of anthracyclines to DNA correlates with chromosomal damage and with increased frequency of sister chromatid exchange.

1.8.2.3. Interaction of Anthracyclines with membranes.

Both doxorubicin and daunorubicin interact with components of cell membranes. Doxorubicin has been shown to have a high affinity for phospholipids such as cardiolipin; the complex formed is stabilised by electrostatic interaction between the amino group of daunosamine and the phosphate residues of the lipid (cf. DNA) (Goormaghtigh et al., 1980). The interaction between doxorubicin (or daunorubicin) and phospholipids results in the alteration of membrane fluidity (Kraczmar and Tritton, 1979; Goldman et al., 1978).

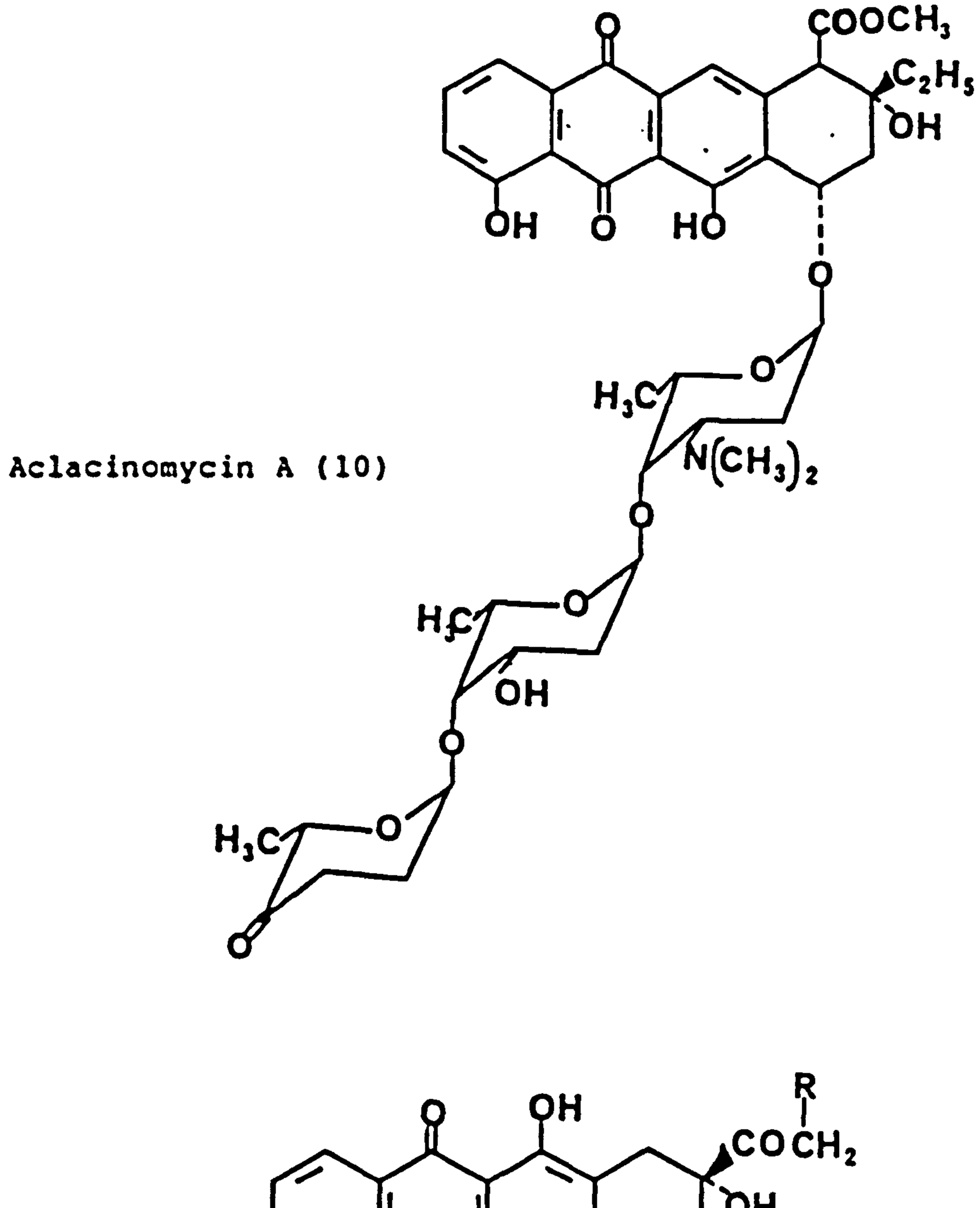
The formation of complexes between anthracyclines and phospholipids excludes cytochrome c oxidase from its essential cardiolipin environment, thereby inhibiting the electron transport chain of mitochondrial respiration. Since cardiac muscle cells are densely populated with respiring mitochondria, the

normal functioning of the cardiac muscle may be hindered.

It has been shown that doxorubicin can be cytotoxic without entering cells; presumably this is mediated via a membrane effect (Tritton and Yee, 1982; Tokes et al., 1982; Tritton et al., 1983). The inactive anthracycline analogue 4-demethoxy-7,9-epi-daunorubicin when covalently coupled to polyglutaraldehyde microspheres, acquired a significant cytostatic activity when evaluated with doxorubicin resistant and sensitive L1210 leukaemia cells (Rogers and Tokes, 1984).

The dose-limiting cardiomyopathy associated with doxorubicin (and daunorubicin) treatment may be as result of reductive metabolism of the drugs. This reductive metabolism leading to formation of highly reactive oxygen metabolites (eg. hydroxyl radicals), with subsequent lipid peroxidation. The damage to cardiac tissue may partially be exacerbated due to the presence of low levels of protective reducing agents (eg. GSH); further damage would also occur as a result of association of the anthracyclines with essential phospholipids.

In the past two decades several anthracyclines have been isolated or prepared, in an attempt to improve the therapeutic indices of these drugs. These attempts have been reviewed elsewhere (Brown, 1983; Arcamone, 1981). Aclacinomycin A (10) (figure 13) has



Aclacinomycin A (10)

R= OH 5-iminodoxorubicin (12)

R= H 5-iminodaunorubicin (11)

Figure 13 : Some anthracyclines with high therapeutic index.

been shown to be almost as active as doxorubicin, but is less cardiotoxic (Hori et al., 1977). Aclacinomycin A preferentially inhibits the synthesis of RNA, both in vitro and in vivo (Oki, 1977; Yamaki et al., 1978). It is the quinone moiety of anthracycline which undergoes redox cycling, and hence replacement of the quinone group by another group would be likely to yield a compound which is not able to redox cycle. For example 5-imino- derivatives of doxorubicin and daunorubicin (11, 12) (figure 13) have been synthesised (Acton and Tong, 1981; Tong et al., 1979) in an attempt to reduce the cardiotoxic effects of the parent compounds. Both analogues are as active as the parent compounds but 5-iminodoxorubicin (12) is less potent than doxorubicin. Both compounds are more difficult to reduce polarographically and to reoxidise, with a resultant decrease in nicking of DNA (Lown et al., 1979). Recently morpholinyl derivatives of anthracyclines have also been synthesised, these compounds have been found to be 100 to 1000 fold more potent than doxorubicin against P388, and L1210 leukaemias, and B16 melanoma (Acton et al., 1984).

Anthracyclines can be viewed as substituted anthraquinones, so an analysis of the antitumour effects of anthraquinones would be worthwhile particularly since some anthraquinones and their glycosides have antibacterial and antipsoriatic activity (Friedman, 1980; Anton and Haag-Berrurier, 1980). A number of amino-, hydroxy-, and nitro-anthrquinones are known to be mutagenic (Brown

and Brown, 1976; Brown and Dietrich, 1979) and the related anthrone dithranol has been widely used in the treatment of psoriasis because of its cytotoxic effects (Brown, 1983).

In this work substituted anthraquinones will be designed and synthesised with structural similarities to the anthracyclines. It is therefore necessary to review the synthesis and biological activity of antitumour anthraquinones.

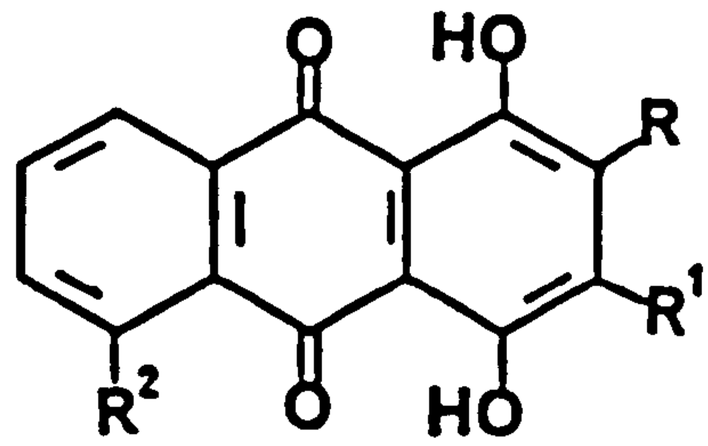
1.9. SUBSTITUTED ANTHRAQUINONES.

The essential structural requirements for an intercalating agent were described earlier (see 1.6). An intercalating agent should have a planar, π electron rich chromophore, the optimal size of which is equivalent of 3 or 4 annelated benzene rings, and hence is of about the same dimensions as the purine-pyrimidine hydrogen-bonded base pairs of DNA (Cain, 1974). Intercalation into DNA is found to be enhanced when an amino group is also present in the drug. However since it is the anthraquinone moiety of the anthracyclines which intercalates into DNA (see 1.9.1), suggests that it should be possible to design anthraquinones which mimic anthracyclines. In 1966 it was shown that some anthraquinones undergo spectral shifts in the presence of DNA (Swanbeck, 1966). Subsequently Müller et al., (1971), showed that it is possible to delete the cyclohexene ring of the anthracyclines, leaving the simpler anthraquinone

chromophore, and still retain the ability to bind to DNA. Since 1971 many anthraquinones have been synthesised and of these, two compounds (ametantrone and mitoxantrone) have been introduced in the clinic. Mitoxantrone (Novantrone) has recently been marketed for the treatment of breast cancer.

1.9.1 Substitution at the 2 and / or 3 positions.

Doxorubicin can be regarded as a 1,4-dihydroxy-2,3-disubstituted anthraquinone (figure 1); the first anthraquinones modelled on doxorubicin were of this type. Müller et al., (1971) studied such compounds, 13-15 (figure 14) and found they bind to DNA; however no details were given. Subsequently it was shown that the 2-substituted-1,4-dihydroxy-anthraquinones (16) and (17) (figure 14) inhibited DNA and RNA synthesis (Henry, 1976). It was shown that anthraquinones such as (18) did not bind to DNA (Double and Brown, 1976). It was suggested that this was due to the side chain being insufficiently basic to be ionised at physiological pH. Henry (1979) also reported the syntheses of daunosamine substituted anthraquinones for example compound (19), and showed that the compound inhibited DNA and RNA synthesis in vitro; however compound (19) was inactive against P388 tumours. Compounds such as (20) were shown to intercalate into DNA (Bennett et al., 1982), however no in vivo antitumour activity was shown. Bis-substituted sulphonamides, ethers and esters of anthraquinones (eg. compounds 21-23, figure 15) have been shown to have



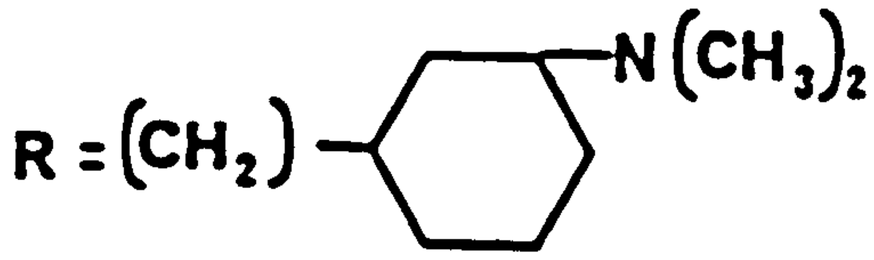
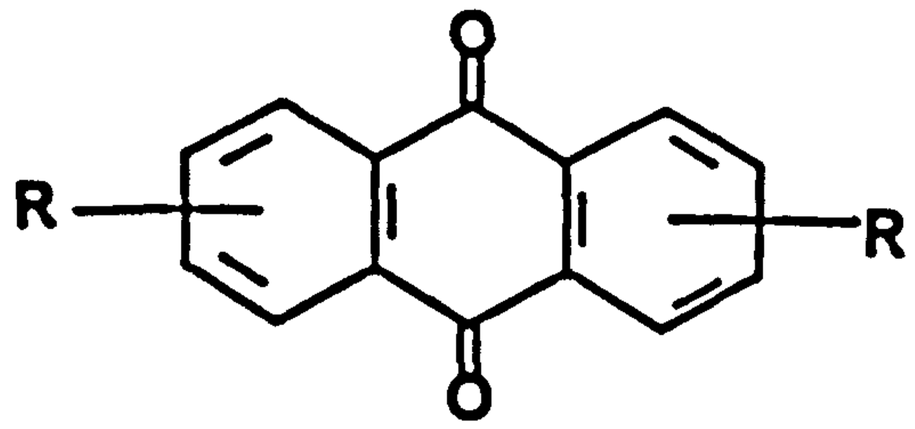
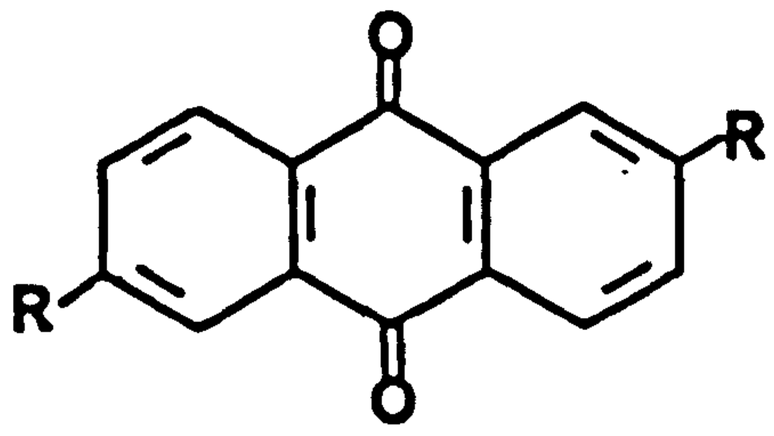
13		R^1 = H	R^2 = OH
14	$R=CH_2N(CH_3)_2$	R^1=H	R^2=OH
15	$R=R^1=(CH_2)_2N(CH_3)_2$		R^2=H
16	$R=NH(CH_2)_3N(CH_3)_2$	$R^1=R^2=H$	
17	$R=S(CH_2)_2N(CH_3)_2$	$R^1=R^2=H$	
18	$R=CH_2-C_6H_5-(pN(C_2H_5)_2)$	$R^1=R^2=H$	
19	$R=CH_2$-O-daunosamine	R^1= H	$R^2=OCH_3$
20	$R=CH_2CONH(CH_2)_2NCH_3$	$R^1=R^2=H$	

Figure 14 : Some 2-substituted anthraquinones shown to intercalate into DNA.



21 R= $SO_2NH(CH_2)_3N(n\text{-}C_4H_9)_2$



22 R= $O(CH_2)_2N(C_2H_5)_2$

23 R= $CO_2(CH_2)_3N(C_2H_5)_2$

24 R= $-N{=}CO\ CH\text{-}N(CH_3)_2$

25 R= $-NH\text{-}CO(CH_2)_n\text{-}N(CH_3)_2$ n= 1 or 2

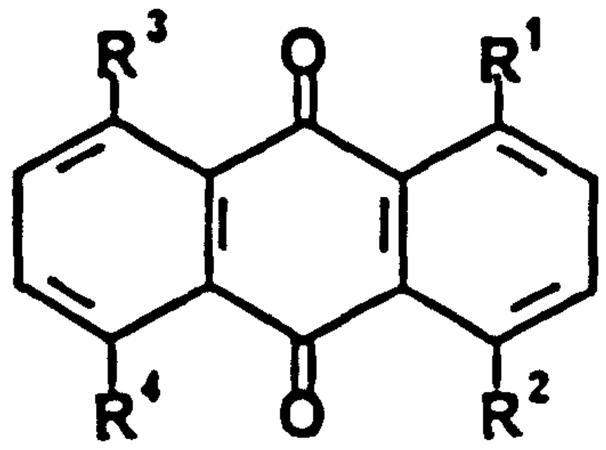
Figure 15 : Chemotherapeautically active bis-substituted anthraquinones.

antiviral activity (Grisar et al., 1974; Sill, et al., 1974). Some basic substituted 2,6-diamidino and 2,6-diacetamino anthraquinones (24,25) have been shown to be active against Entamoeba histolytica and Trichomonas vaginalis (Winkelman and Raeter, 1979).

There is thus evidence that 2-substituted anthraquinones may have a potential as chemotherapeutic agents, and can intercalate into DNA (Bennett et al., 1982; Brown, 1983).

1.9.2. Substitution at 1, 4, 5, and 8 positions.

In 1975, Double and Brown reported the synthesis of 1-, 1,4-, 1,5-, and 1,8- aminoalkylamino-substituted anthraquinones (compounds 26-30, figure 16), which were designed as intercalating agents incorporating those features of the anthracyclines which were essential for intercalation. These compounds were found to intercalate into DNA, with affinity constants of 0.5-4.2 $X10^{6}M^{-1}$; however they were devoid of in vivo antitumour activity (Brown, 1983). Further work by Lederle Research, Inc., showed that similar anthraquinones were active in vivo against a number of experimental tumours, with ametantrone (31) showing optimum activity (Murdock, et al., 1979). It was also shown that activity and potency were further enhanced by 5,8-hydroxylation of the anthraquinone chromophore; with mitoxantrone (32) showing optimum activity. Some of these compounds were also reported by Zee-Cheng and Cheng (1978); notably compound (31). From these earlier studies on 1,4-substituted anthraquinones, it was shown



26	R^1= $NHCH(CH_3)(CH_2)_3N(C_2H_5)_2$	R^1=R^3=R^4=H
27	R^1=R^2= $NHCH(CH_3)(CH_2)_3N(C_2H_5)_2$	R^3=R^4=H
28	R^1=R^3= $NHCH(CH_3)(CH_2)_3N(C_2H_5)_2$	R^2=R^4=H
29	R^1=R^4= $NHCH(CH_3)(CH_2)_3N(C_2H_5)_2$	R^2=R^3=H
30	R^1= $NHCH(CH_3)(CH_2)_3N(C_2H_5)_2$	R^2=OH R^3=R^4=H
31	R^1=R^2= $NHCH_2CH_2NHCH_2CH_2OH$	R^3=R^4= H
32	R^1=R^2= $NHCH_2CH_2NHCH_2CH_2OH$	R^3=R^4= OH
33	R^1=R^2=$NH(CH_2)_2NHCH_2CH_3$	R^3=R^4= H
34	R^1=R^2= $(CH_2)_2NH(CH_2)_2OH$	R^3=R^4= H
35	R^1=R^2= $(CH_2)_2$—N⟨⟩—NC_5H_{10}	R^3=R^4= H
36	R^1=R^2= $(CH_2)_5OH$	R^3=R^4= H
37	R^1=R^2= $(CH_2)_2S(CH_2)_2OH$	R^3=R^4= H

Figure 16 : 1-, 1,4- 1,5-, and 1,8- Substituted anthraquinones

that cytotoxic activity is greatest where (a) there is disubstitution on the anthraquinone chromophore; (b) where there is a dimethylene spacer between the two nitrogens; (c) where the aliphatic amine is secondary; and (d) the substituent is an hydroxyethyl group (Murdock et al., 1979; Zee-Cheng and Cheng, 1978, Zee-Cheng et al., 1979). Mitoxantrone (32) showed activity which was either equivalent, or superior to that of doxorubicin against certain experimental tumours (Johnson et al., 1979). Since these earlier studies, mitoxantrone (32) and ametantrone (31) have undergone extensive trials in animals as well as in man, this work will now be reviewed.

1.9.2.1. Antitumour activity of Mitoxantrone (32) and Ametantrone (31).

Mitoxantrone has a wide spectrum of activity against experimental murine tumours including L1210 and P388 leukaemias, B16 melanoma and mouse colon 26 tumour (Cheng et al., 1979; Fujimoto and Ogawa, 1982; Johnson et al., 1979; Wallace et al., 1979; Zee-Cheng and Cheng, 1978; Zee-Cheng et al., 1979). It was also shown to be active against mouse colonic adenocarcinomas, and mouse mammary carcinomas (Smith, 1983). Mitoxantrone is more effective than doxorubicin in extending survival times of animals bearing tumours (Murray and Wallace, 1980) and it has a wide spectrum of activity in a human tumour-cloning system (Von Hoff et al., 1981). However the only tumour type to show complete resistance to mitoxantrone was colon carcinoma. In another study, using the same clonogenic assay system, it was shown

that mitoxantrone was more effective as a cytotoxic agent than doxorubicin (Cowan et al, 1983) and there was a lack of cross resistance 27-34% of the time, with mitoxantrone being more active than doxorubicin. Ametantrone has been shown to be less active than mitoxantrone and doxorubicin (Drewinko et al., 1983; Smith, 1983; Johnson et al., 1979).

Synergism has been demonstrated between mitoxantrone and cis-platin, 5-fluorouracil, vincristine, and DTIC against murine colonic and mammary adenocarcinoma (Corbett et al., 1982), and with irradiation against L1210 leukaemia (Kimler and Hacker, 1981). Following these encouraging results, mitoxantrone rapidly entered phase I clinical trials (Von Hoff et al., 1980; Valdivieso et al., 1981; Alberts, et al., 1980). Mitoxantrone was generally well tolerated, with leucopaenia being the dose limiting toxic effect. Mitoxantrone treatment induced remission in some patients (Alberts et al., 1980) and showed little evidence of cardiotoxicity. Phase I trials were also started for ametantrone (Van Echo et al., 1981; Loesch et al., 1983).

In phase II clinical trials it was shown that mitoxantrone induced a partial tumour regression in patients with metastatic breast cancer (Yap et al., 1981; Stuart-Harris and Smith, 1982; Stuart-Harris, et al. 1984). A complete response was obtained in some patients with refractory acute leukaemia (Estey et al., 1983). On the basis of these clinical trials

mitoxantrone (Mitozanthrone, Novantrone®) has recently been marketed in the United Kingdom for the treatment of breast cancer.

In the last three years there have been some reports of cardiotoxicity induced by mitoxantrone, however in most cases patients had received prior chemotherapy (including doxorubicin) or irradiation and therefore at this early stage it is uncertain if mitoxantrone is less cardiotoxic than doxorubicin. Earlier trials in animals showed that mitoxantrone was less cardiotoxic than doxorubicin (Henderson et al., 1982; Cheng et al., 1979). The biochemical mechanisms involved in the cytotoxicity of mitoxantrone, including intercalation are described in the following sections.

1.9.2.2. Mecanism of action of mitoxantrone.

The principal mode of cytotoxic action of mitoxantrone is thought to be due to interaction of the drug with DNA. Mitoxantrone has been shown to inhibit nucleic acid synthesis in vitro (Johnson et al., 1981). It gives nuclear aberrations similar to those produced by doxorubicin. It is not cell-cycle specific and although it is more effective against cells in the growth phase, it is also cytotoxic against non-dividing cells (Murray and Wallace, 1980). Treatment with either mitoxantrone or ametantrone produces an accumulation of cells in the G2 phase (Evenson et al., 1979, 1980; Kimler, 1980; Kapuscinski et al., 1981). Mitoxantrone has been shown to be localised in the cell nucleus following exposure to drug, although some is found in

the cytoplasm (Kapuscinski et al., 1981). It is associated with DNA and RNA, and cells treated with DNAase and RNAase fail to bind the drug. Both mitoxantrone and ametantrone cause sister-chromatid exchange and chromosome breaks (Au et al., 1981). As with other intercalating agents mitoxantrone has been found to give protein-associated single strand breaks in DNA (Cohen et al., 1980) and to unwind covalently closed circular DNA (Kapuscinski et al., 1981; Lown et al., 1984). Mitoxantrone thus appears to exert its cytotoxic action by a similar mechanism to that of doxorubicin.

Mitoxantrone and other related compounds were shown to stabilise DNA to thermal denaturation (Johnson et al., 1979); however this property could not be related to the antitumour activity of these drugs. For example, the 1,4-bis-ethylaminoethylamino analogue (33) gave a greater stabilisation of DNA than did mitoxantrone but was shown to be much less active against all tumour systems evaluated (Johnson et al., 1979). Comparison of a 1,8-bis-substituted compound with its 1,4-bis-substituted analogue showed the latter to be a more effective DNA-binding agent. However it was shown that the 1,5-bis-substituted analogue stabilised DNA to a greater extent than did its 1,4-bis-substituted counterpart and required much lower concentrations to inhibit nucleic acid synthesis. However, 1,5-substituted anthraquinones were found to be totally inactive in vivo (Johnson et al., 1979). However when interpreting the data of Johnson et al.

(1979), an explanation for these observations unrelated to DNA binding should not be ruled out. One explanation of these results is that analogues inactive *in vivo* may have a poor access to tumour cells due to differences in lipid solubility, or may be differentially bound to proteins, or may be inactivated.

Although the above evidence indicates that intercalation is the most important mechanism of DNA binding of mitoxantrone (and related compounds), the precise nature of the interaction of drug with DNA is not known. Mitoxantrone has been implicated as cardiotoxic in several patients, and recent work elsewhere has concentrated on the biochemical mechanisms involved in its possible cardiotoxicity and these will now be described.

1.9.2.3. Potential cardiotoxicity of mitoxantrone and related compounds.

In the last 3-4 years there have been a number of reports in the literature which indicate that mitoxantrone may be cardiotoxic (Pratt *et al.*, 1983; Schell *et al.*, 1982; Sparano *et al.*, 1982; Henderson *et al.*, 1982; Unverferth *et al.*, 1983); in the majority of these clinical studies patients had received prior chemotherapy and hence cardiotoxicity may have resulted from exposure to other agents. Both mitoxantrone and ametantrone were found to be less cardiotoxic than doxorubicin in animal studies (Cheng *et al.*, 1979).

Kharasch and Novak (1981, 1983), showed that mitoxantrone and ametantrone are not as readily

activated to semiquinone free radicals as are the anthracyclines. These drugs stimulate NADPH oxidation and superoxide generation to a lesser extent than the anthracyclines. Furthermore mitoxantrone was found to be a poor substrate for cytochrome P-450 reductase as well as being an inhibitor of this enzyme. Both mitoxantrone and ametantrone inhibited lipid peroxidation *in vitro* (Mimnaugh *et al.*, 1982); indeed mitoxantrone (and ametantrone) actually inhibited doxorubicin-stimulated lipid peroxidation (Kharasch and Novak, 1982a). Mitoxantrone has also been shown to inhibit the formation of doxorubicin semiquinone free radicals *in vitro*, (Basra, J., personal communication). Ametantrone has been shown to inhibit microsomal oxidative drug metabolism, in addition to inhibiting lipid peroxidation, (Kharasch and Novak, 1982b).

These studies show that mitoxantrone (and related compounds) probably exert their antitumour action by interacting with DNA and are probably less cardiotoxic than doxorubicin. It remains to be seen whether mitoxantrone is as useful a cancer chemotherapeutic agent as doxorubicin. Whatever the outcome, synthetic anthraquinones have provided new ammunition in the arsenal of drugs used for the treatment of cancer.

To date little information is available on the nature of interaction of anthraquinones with DNA. In this work an attempt will been made to rationalise the precise nature of the anticipated interaction of

substituted anthraquinones with DNA in order to allow the design of more selective antitumour agents.

1.10. AIMS

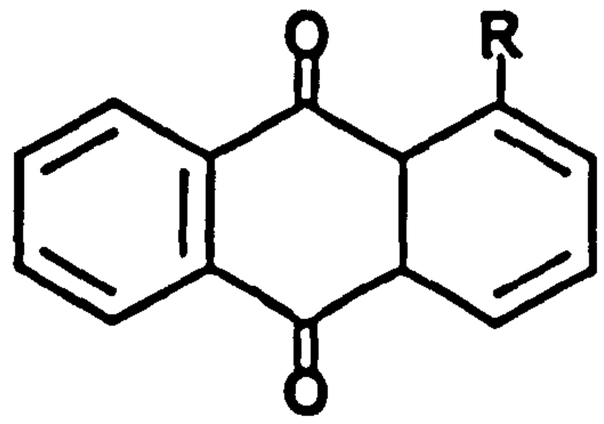
The anthracycline antibiotics daunorubicin and doxorubicin, have been used extensively in the treatment of neoplastic diseases (see 1.7.2). They have a multiplicity of cellular effects including metabolic reduction (see 1.8.2), and interaction with phospholipids and cellular membranes (see 1.8.3). However the most obvious effect of these drugs is their effect on the nucleus. As well as their ability to intercalate into DNA (see 1.8.1), they cause protein associated double and single strand breaks in DNA, they give rise to sister chromatid exchange and chromosome aberrations. Whatever the mode of cytotoxic action it is clear that intercalating anthracyclines are cytotoxic.

The minimal structural requirements for intercalation are a planar, tricyclic chromophore (such as the anthraquinone chromophore of doxorubicin), with a positively charged group, such as an amino group, capable of interacting with phosphate residues in the DNA (see 1.6.1). Based on these structural requirements several anthraquinones have previously been synthesised (see 1.9), and shown to intercalate into DNA, in accordance with their design; with the 1,5-disubstitution pattern having the highest affinity for DNA (Plumbridge et al., 1980). The majority of these anthraquinones were shown not to posses antitumour activity in vivo. It was later shown that

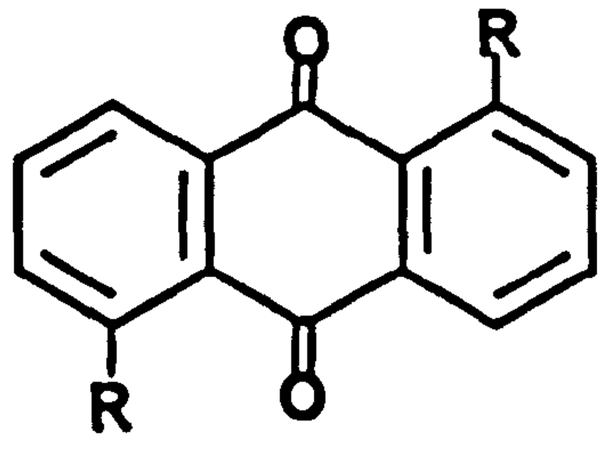
some 1,4-bis-(2'hydroxyethylamino)ethylamino substituted anthraquinones (notably ametantrone and mitoxantrone, see 1.9.2) possesed *in vivo* antitumour activity (Zee Cheng and Cheng, 1978; Murdock *et al.*, 1979). However these are 1,4-disubstituted derivatives and as indicated above it had previously been shown that the 1,5-disubstituted anthraquinone had a higher affinity to DNA.

The effect of substitution pattern on the binding of anthraquinones to DNA, in two series of 1-, 1,4-, 1,5-, and 1,8-substituted anthraquinones (Figures 17a and 17b), will be examined in this project. It has previously been shown (Johnson *et al*, 1979) that the 1,4-disubstituted anthraquinones containing 2-(hydroxyethylamino)ethylamino side chains were cytotoxic against tumour cells *in vivo*. In order to exploit this cytotoxic property, anthraquinones substituted with this side chain will be synthesised. The two nitrogen atoms, in this case, are separated by two methylene units, and the 2-(diethylamino)ethylamino side chain will be used for the synthesis of the second series of aminoalkylamino- anthraquinones.

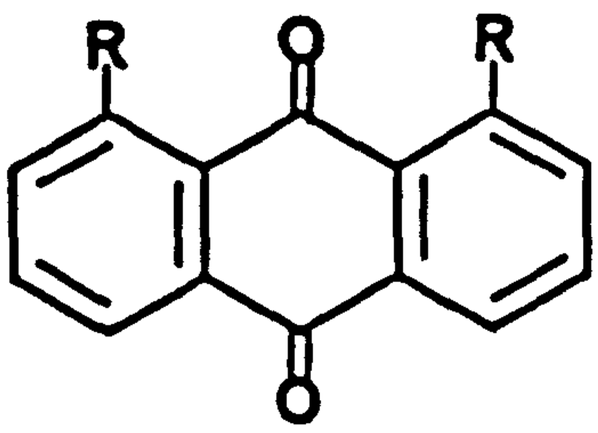
The ability of these proposed anthraquinones, to bind to DNA will be investigated. Suitable methods will be selected to distinguish qualitatively between external binding and intercalation of anthraquinones into DNA including the effect of DNA on spectral properties of drug, and the effect of drug on thermal denaturation of DNA. Further methods will be chosen to



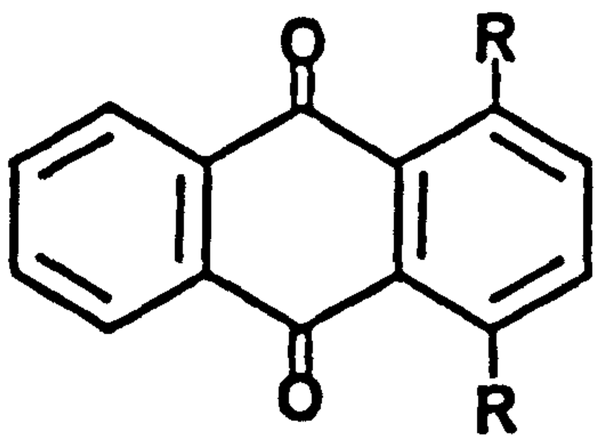
1-[2'-(Diethylamino)ethylamino]anthracene-9,10-dione (compound 1A).



1,5-bis[2'-(Diethylamino)ethylamino]anthracene-9,10-dione (compound 2A).



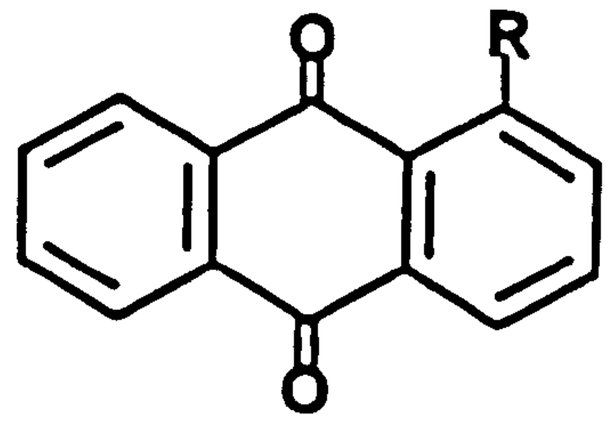
1,8-bis[2'-(Diethylamino)ethylamino]anthracene-9,10-dione (compound 3A).



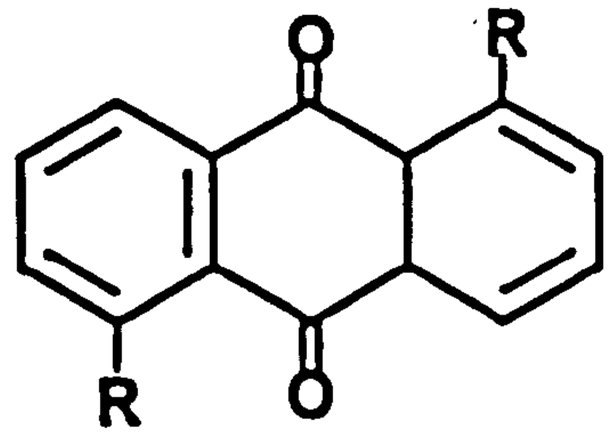
1,4-bis[2'-(Diethylamino)ethylamino]anthracene-9,10-dione (compound 4A).

$$R = -NHCH_2CH_2N(C_2H_5)_2$$

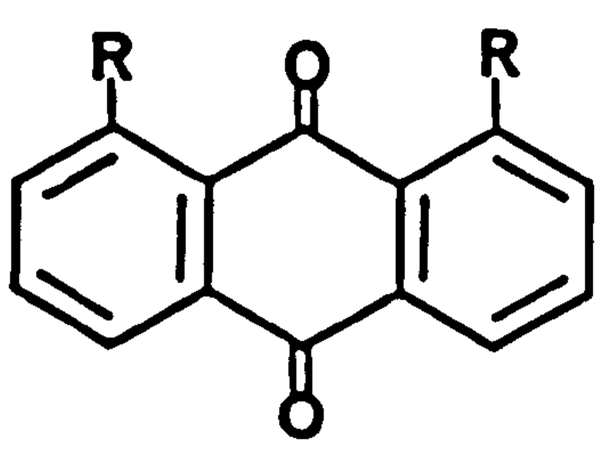
Figure 17a : Proposed 2'-Diethylaminoethylamino substituted anthraquinones (A series).



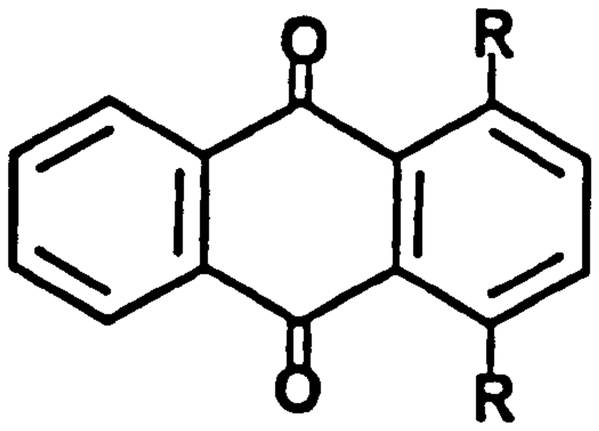
1-[2'-(Hydroxyethylamino)ethylamino]anthracene-9,10-dione (compound 1B).



1,5-bis[2'-(Hydroxyethylamino)ethylamino]anthracene-9,10-dione (compound 2B).



1,8-bis[2'-(Hydroxyethylamino)ethylamino]anthracene-9,10-dione (compound 3B).



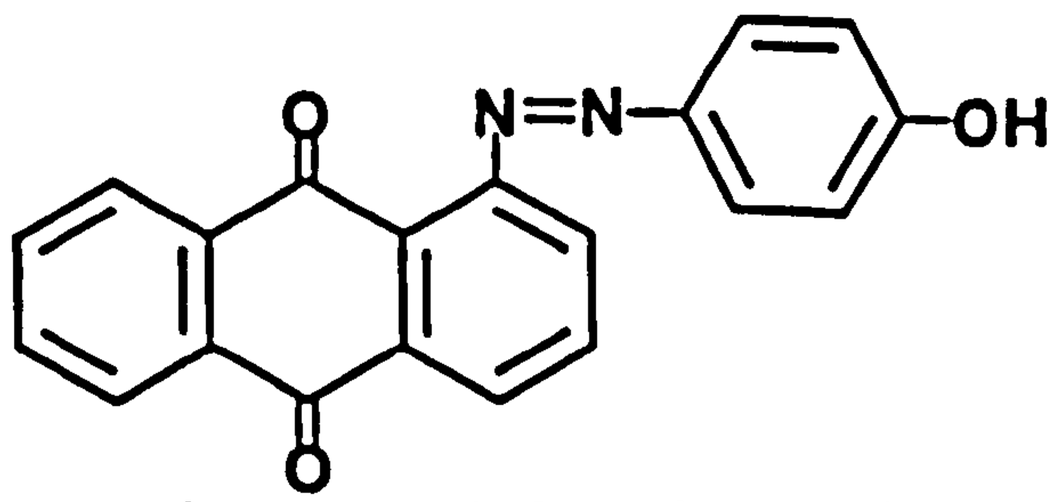
1,4-bis[2'-(Hydroxyethylamino)ethylamino]anthracene-9,10-dione (compound 4B).

$R = -NHCH_2CH_2NHCH_2CH_2OH$

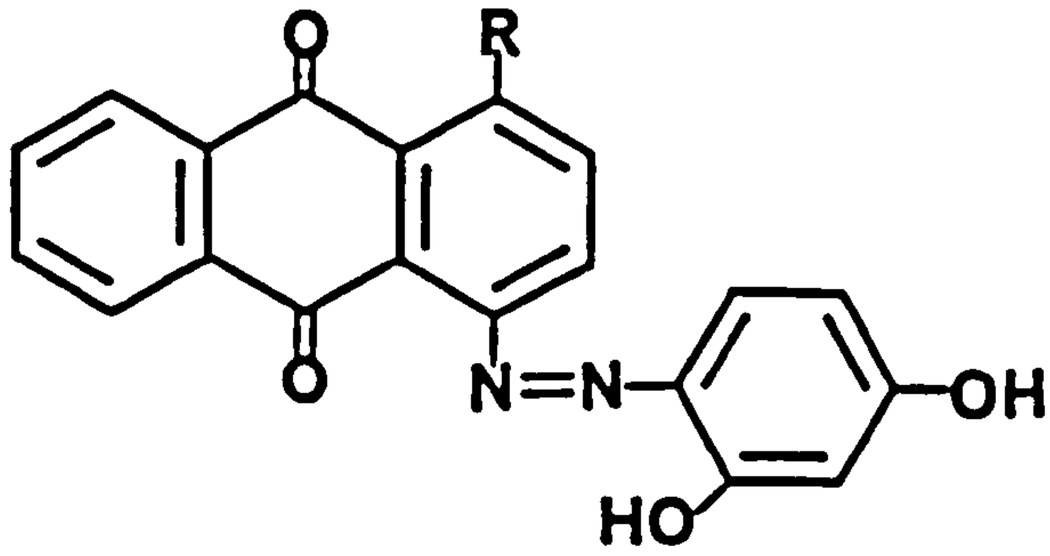
Figure 17b : Proposed 2'-Hydroxyethylaminoethylamino-substituted anthraquinones (B series).

quantitate differences between the binding of anthraquinones to DNA; these include determination of affinity constants (by spectrophotometric titration), determination of dissociation constants by stopped-flow spectrophotometry and the effect of the compounds on the unwinding of closed circular DNA. The results from solution studies on DNA-binding of substituted anthraquinones will be correlated with the results from computer graphics modelling (performed by S.A.Islam and S.Neidle at King's College, London), of their fit into a DNA intercalation site.

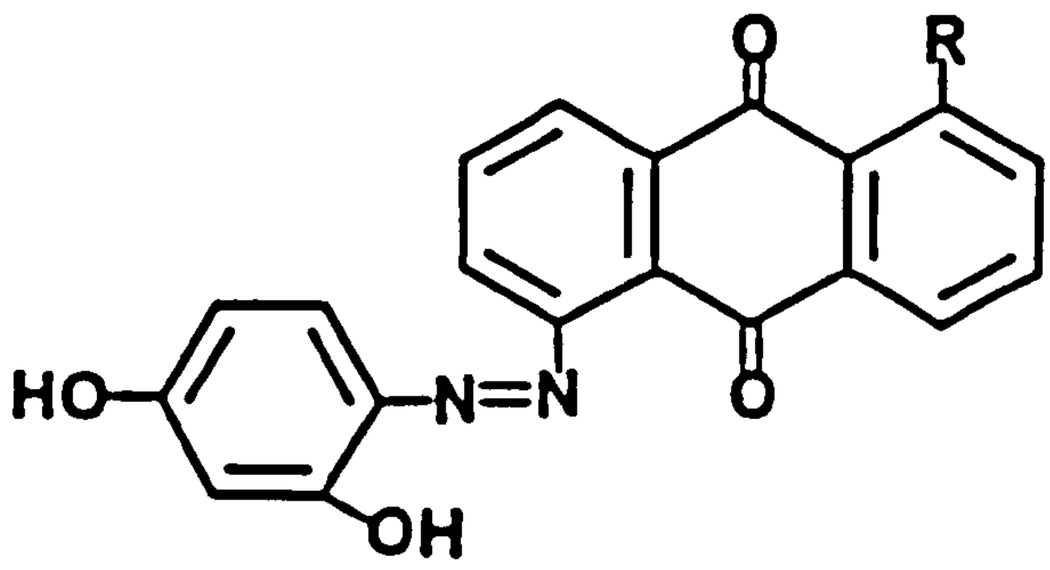
The use of chemotherapeutic agents in the treatment of neoplastic disease is limited by the severe toxicity of these agents to the patient and a rapid development of resistance to the drugs. Furthermore certain tumours, eg. liver tumours, are refractory to most currently available drugs. As seen previously, doxorubicin is one of the drugs which has been extensively used in the treatment of solid tumours including the treatment of hepatocellular carcinoma. Pro-drugs will be designed, based on doxorubicin, which can be selectively activated by the tumour cells. The pro-drugs proposed (figure 17c) have been designed so that they should not intercalate into DNA but should release the intercalating anthraquinone (Fig. 17c) when metabolised by intracellular enzymes.



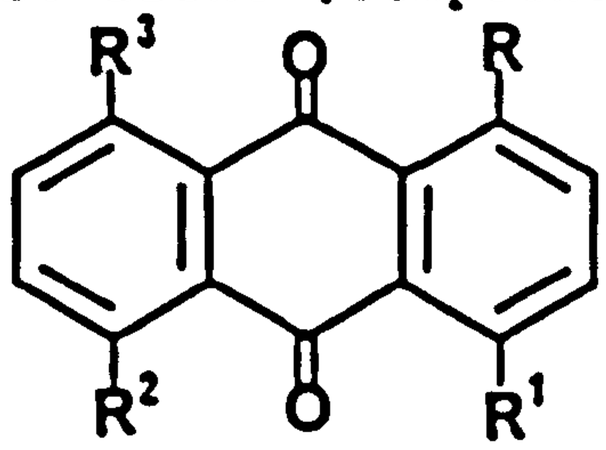
1-(4'-Hydroxyphenyl)azoanthracene-9,10-dione (compound 1C).



1-[2'-(Diethylamino)ethylamino]-4-(2",4"-dihydroxyphenyl)azoanthracene-9,10-dione (compound 2C).



1-[2'-(Diethylamino)ethylamino]-5-(2",4"-dihydroxyphenyl)azoanthracene-9,10-dione (compound 3C).



R= $NHCH_2CH_2N(C_2H_5)_2$

1-Amino-4-[2'-(diethylamino)ethylamino]anthracene-9,10-dione (compound 2Ci; R1=NH2, R2=R3=H).

1-Amino-5-[2'-(diethylamino)ethylamino]anthracene-9,10-dione (compound 3Ci; R1=R3=H, R2=NH2).

1-Amino-5,8-bis-[2'-(diethylamino)ethylamino]-4-hydroxyanthracene-9,10-dione (Compound 4Ci; R1 = R, R2=NH2, R3=OH).

Figure 17c: Proposed Anthraquinone pro-drugs and their potential metabolites.

2. RESULTS AND DISCUSSION.

In this chapter, the synthesis of the compounds will be discussed first. Then their binding to DNA will be considered, and the data compared with that obtained from computer graphics molecular modelling. Finally the *in vitro* metabolism of the synthesised pro-drugs will be discussed. The *in vivo* and *in vitro* antiproliferative testing of compounds, synthesised in this work, will also be discussed.

2.1. SYNTHESES.

The compounds synthesised in this work will be divided into three groups:

A. 2'-(Diethylamino)ethylaminoanthracene-9,10-diones (Compounds 1A-4A)

B. 2'-(Hydroxyethylamino)ethylaminoanthracene-9,10-diones (Compounds 1B-4B)

C. Pro-drugs and their potential metabolites (Compounds 1C-3C and 2Ci-3Ci).

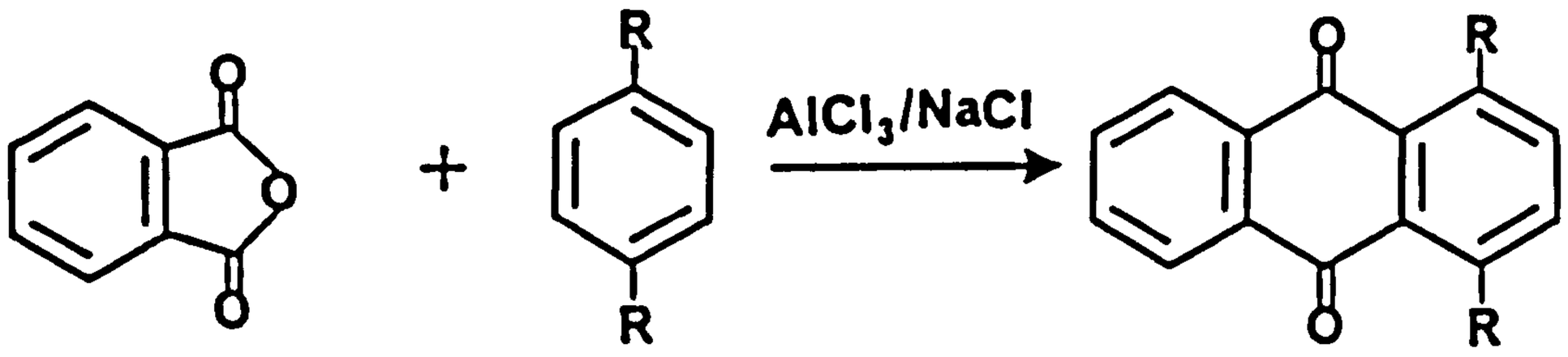
The term anthracene-9,10-dione follows the IUPAC nomenclature and is used throughout the experimental chapter. However, in this chapter the more usual (but trivial) name anthraquinone will be used.

The final compounds synthesised in this work are shown in figures 17a-17c, together with their codes.

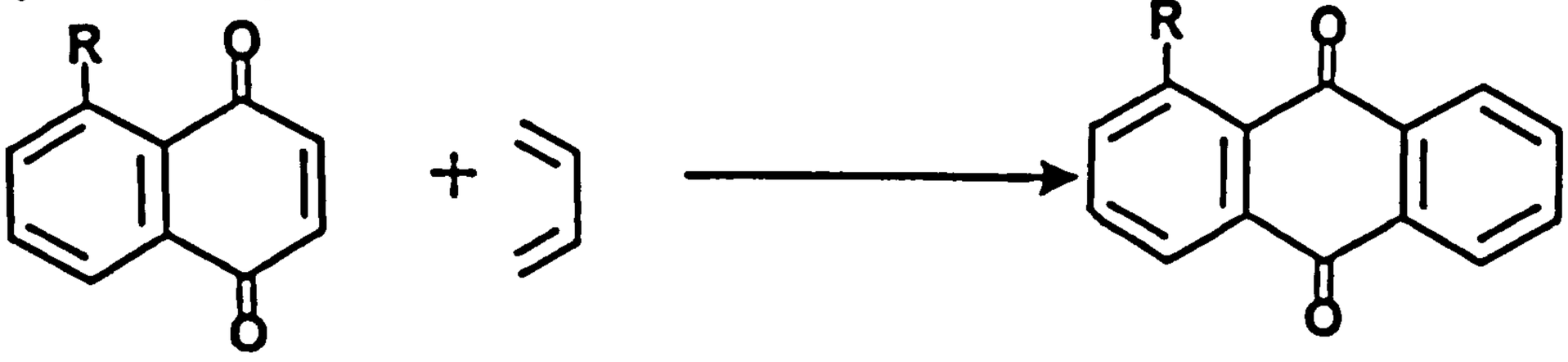
2.1.1. Synthesis of 2'-(diethylamino)ethylaminoanthraquinones (A series).

There are at least four possible methods for synthesis of aminoalkylaminoanthraquinones, these include Friedel Craft's acylation, Diels-Alder reaction, alkylation of aminoanthraquinones, and substitution of appropriate haloanthraquinone.

Friedel-Crafts acylation is the classical method for anthraquinone synthesis. Substituted phthalic anhydrides have been utilised in either a double concurrent ($AlCl_3$/NaCl melt), or consecutive, process. The reaction lacks regioselectivity, and furthermore in the case of aminoalkylamino substituted anthraquinones, protection of amino groups is required.

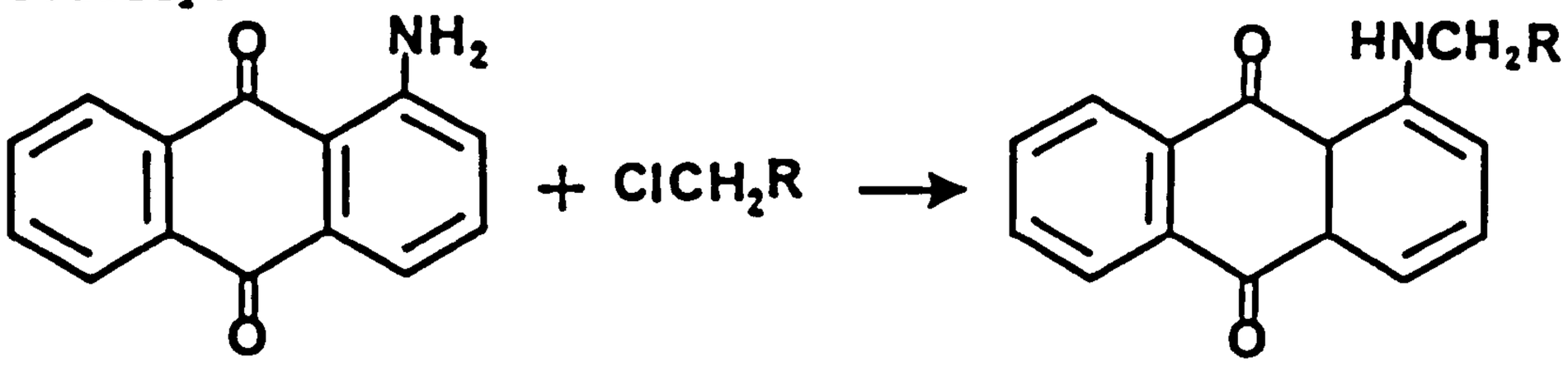


The Diels-Alder reaction may be used in the synthesis of anthraquinones. This method usually involves the reaction of a suitable dienophile with a quinone eg.



This method has been used extensively in the synthesis of anthracyclinones (reviewed by Brown and Imam, 1984.).

As an alternative to synthesising the anthraquinone chromophore as a last step, commercially available anthraquinones can be utilised. One possible method is to use aminoanthraquinones and alkylate the amino groups, this is possible where the appropriate alkylating agent is available or can be synthesised readily.



A more versatile route is by substitution of a haloanthraquinone with an appropriate amine, and this route has previously been used in the synthesis of aminoalkylaminoanthraquinones (Double and Brown, 1975). The reaction would be expected to proceed readily due to a reduced electron density at Cl (figure 18). In this work 1-, 1,5- and 1,8- aminoalkylaminosubstituted anthraquinones were prepared by this method; the 1,4-bis substituted analogue could not be readily prepared by this method since the corresponding dihaloanthraquinone in not available commercially. This compound was therefore prepared by an alternative route.

A two electron reduction of 1,4-dihydroxy-anthraquinone (quinizarin), yields leucoquinizarin (1,4,9,10-tetrahydroxyanthracene). The latter compound has previously been used in the synthesis of 1,4-disubstituted anthraquinones (Greenhalgh and Hughes, 1968). A major advantage of this method is that

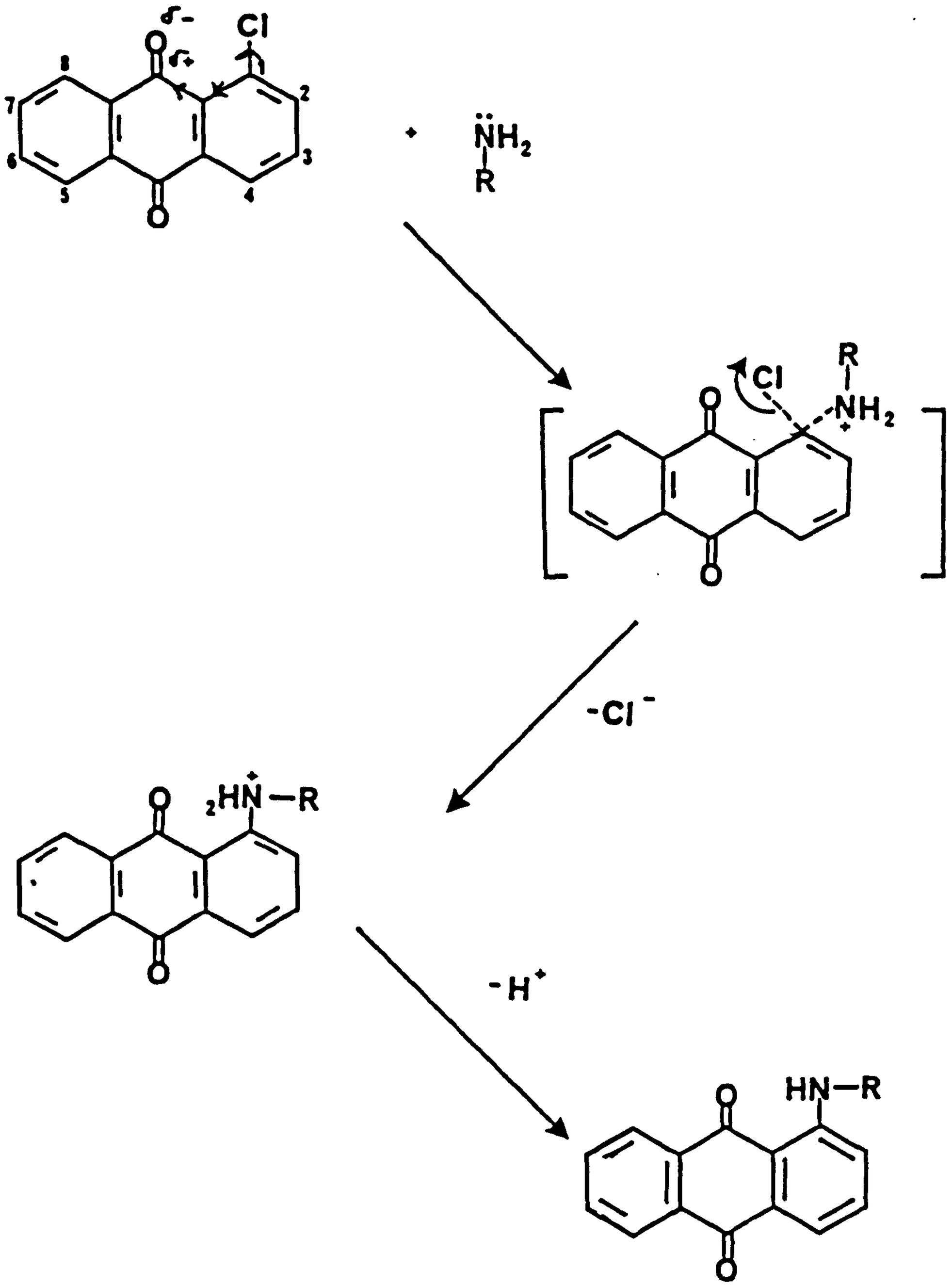


Figure 18: Mechanism of reaction of chloroanthraquinones with primary amines.

much milder conditions may be used (see later).

2.1.1.1. Synthesis of 1-[2'-(diethylamino)ethylamino]-anthraquinone (1A).

This compound was synthesised (see 3.1.1.1) by heating 1-chloroanthraquinone under reflux in excess (10 fold) of 2-(diethylamino)ethylamine until all the chloroanthraquinone had reacted. The reaction was monitored by the colour change of the mixture from pale orange (λ max 380nm) to deep red (λ max 490nm). Throughout this work it was found that the shift in λ max, of the reaction mixture was a good indication that the reaction had taken place. Furthermore, since the final product is much more polar (due to the basicity of the tertiary amine side chain), the reaction was also monitored by TLC.

After completion of the reaction the mixture was cooled, excess acid added and the mixture extracted with ether, to remove any non-polar products. The desired product and unreacted amine would be expected to remain dissolved in the aqueous layer as the hydrochloride salts. It was found that the anthraquinone hydrochloride could be extracted from the aqueous layer with chloroform. The chloroform was dried, evaporated <u>in vacuo</u> and the residue redissolved in deionised water. The solution was then made alkaline and extracted with chloroform. The free base of the product was obtained by evaporation of chloroform followed by recrystallisation.

The structure of the final product was

elucidated by spectroscopic methods, elemental analysis and also by X-ray crystallography, the latter was performed by Dr. S.Neidle and Dr. R. Kuroda (King's College, London). The λ max of the product was 495 nm compared to 380nm of the starting material. This bathochromic shift is expected due to the contribution of the lone pair of electrons of the nitrogen to the chromophore. This change in λ max is similar to that reported by other workers (Double and Brown, 1975). The final product was basic also indicates that there is an aliphatic as well as an aromatic amino group present. The infrared spectrum confirmed the presence of an NH (bonded) group and quinone carbonyl at 1660 cm^{-1}. The NMR spectrum of the product was in agreement with the structure except that the amino proton (NH) was found not to be washed by D_2O shake, this is undoubtably due to the fact that the NH proton forms a strong intramolecular hydrogen bond with the quinone carbonyl. Mass spectral data were also as expected. The fragmentation pattern is shown in figure 19. The purity of the free base was confirmed by elemental analysis.

Further evidence for the structure was obtained by X-ray crystallography, (Almond et al,1983), the anthraquinone chromophore was found to be highly planar with a dihedral angle of 2.7° between the two terminal aromatic rings. The amino proton forms a strong intramolecular hydrogen bond with the carbonyl oxygen (hence the lack of exchange with D_2O seen in the NMR spectrum) with an overall effect to somewhat increase the effective planar area of the molecule.

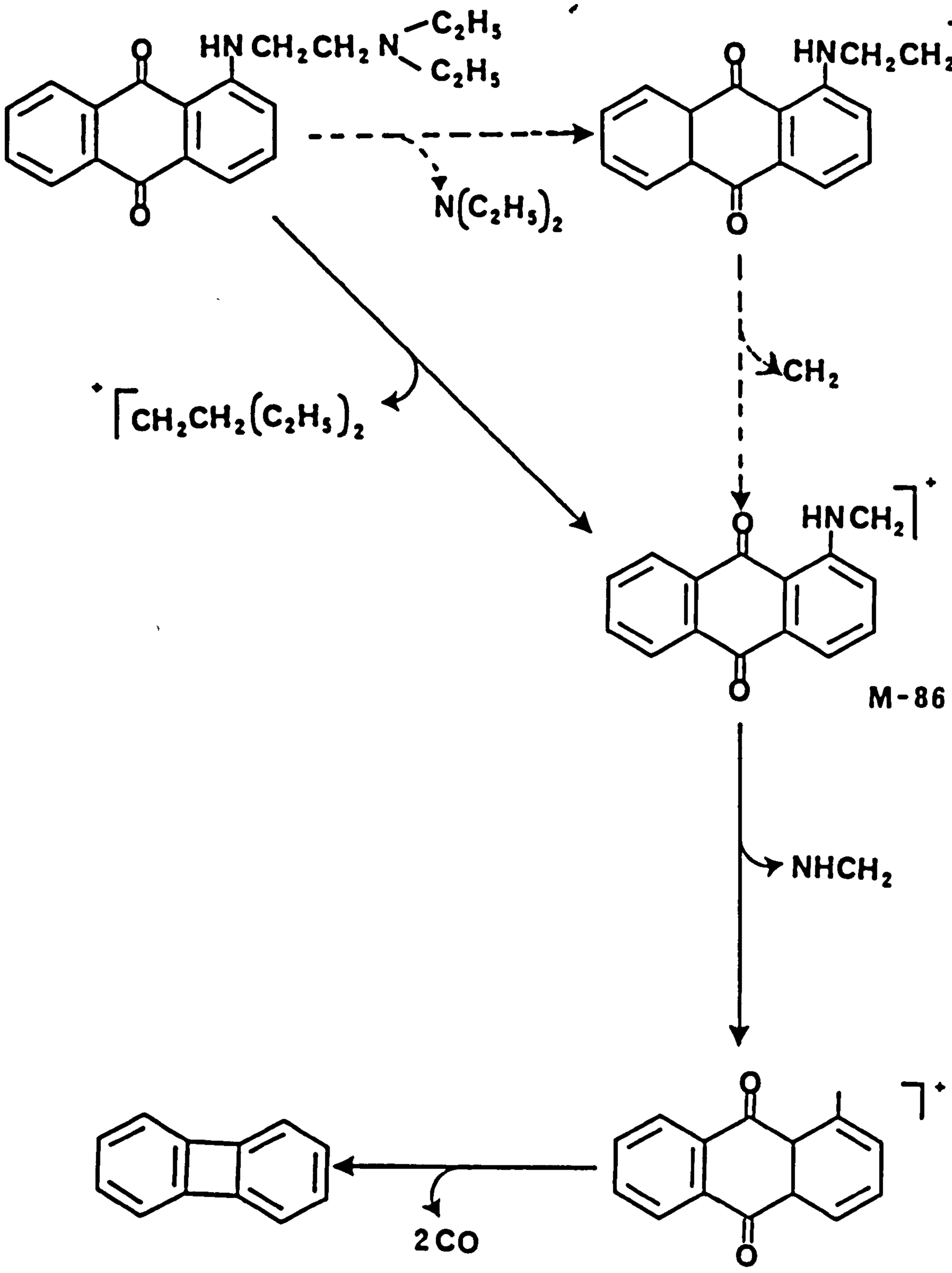


Figure 19: A common fragmentation pathway of 2'-(diethylamino)ethylaminoanthraquinones.

2.1.1.2. Synthesis of 1,5-bis and 1,8-bis-[2'-(diethylamino)ethylamino]anthraquinones (2A, 3A).

These compounds were synthesised by a method which was similar to the synthesis of 1-[2'-(diethylamino)ethylamino]anthraquinone (see 3.1.1.2 & 3.1.1.3). The appropriate dichloroanthraquinone, in ten fold excess of 2-(diethylamino)ethylamine, was heated under reflux until all the chloroanthraquinone had reacted. The reaction was monitored by the change in colour from pale orange (λ max 380nm) to deep red (λ max 515nm) in the case of the 1,5-, and mauve (λ max 536nm) in the case of the 1,8-disubstituted analogue. The reaction was also monitored by TLC, the compounds would be anticipated to be more polar than the starting chloroanthraquinones and monosubstituted anthraquinone (compound 1A). The Rfs in MeOH/$CHCl_3$ (1/1) were 0.95, 0.70, 0.50, and 0.55 for dichloro-, monosubstituted-, 1,5-, and 1,8-disubstituted anthraquinones respectively.

After completion of each reaction, the mixture was cooled and excess acid added; the mixture was then extracted with ether, to remove any non-polar products. The hydrochloride salts of unreacted amine and substituted anthraquinones would be expected to remain in the aqueous phase. The hydrochloride salts of the disubstituted anthraquinones are less lipophilic than the monosubstituted compounds, therefore the latter were readily removed by extraction of the aqueous phase with chloroform. The monosubstituted anthraquinones would be expected to have a λ max similar to compound

(1), whilst the disubstituted anthraquinones should have a longer λ max due to the presence of two amino groups on the chromophore. Basification of the aqueous phase followed by extraction with chloroform and evaporation in vacuo yielded a mixture of the disubstituted anthraquinone and the starting amine; the latter could be readily detected by its distinctive smell. The amine was removed by washing the residue several times with water until further amine could not be detected. The last traces of water were removed by suspending the residue in absolute ethanol and evaporating in vacuo. Recrystallisation of the products from absolute ethanol yielded the free bases.

The structures of the final products were elucidated by spectroscopic methods, elemental analyses and also by X-ray crystallography. The λ max in the visible region were 515nm and 536nm for 1,5- and 1,8-disubstituted anthraquinones respectively compared to 380nm for dichloro- and 495nm for monosubstituted anthraquinones. The final products were basic and polar, as expected. Both compounds were shown to be more polar than monosubstituted anthraquinone. Infrared spectra were similar to that of the monosubstituted anthraquinone and confirmed the presence of NH (bonded) group (3420 and 3280 cm^{-1} respectively for 1,5- and 1,8- disubstituted compounds), and quinone carbonyls at 1660, and 1670 cm^{-1}. The ^{1}Hnmr of the products was in agreement with the assigned structure. Mass spectral data were also as expected with a fragmentation pattern similar to that of monosubstituted anthraquinone

(figure 19). The purity of the free bases was confirmed by elemental analysis.

X-ray crystallography revealed that the anthraquinone chromophores are highly planar with dihedral angles of 2.4°, (Almond et al., 1983), and 2.0° (Islam et al., 1983), between the two terminal aromatic rings, for the 1,5- and 1,8-disubstituted anthraquinones respectively. The amino proton forms a strong intramolecular hydrogen bond with the carbonyl oxygen with an overall effect to somewhat increase the effective planar area of the molecule.

2.1.1.3. Synthesis of 1,4-bis-[2'-(diethylamino)ethyl-amino]anthraquinone (4A).

This compound could not be readily synthesised from the appropriate dihalo anthraquinone as the latter is not available commercially, however the compound was readily synthesised by the method of Greenhalgh and Hughes (1968). This method involves the reaction of leucoquinizarin (1,4,9,10-tetrahydroxyanthracene) with an appropriate primary amine (figure 20). Leucoquinizarin exists as the keto form in solution (Bloom and Hutton, 1963). The reaction proceeds by nucleophilic attack by the amine at the carbonyl group with subsequent elimination of H_2O to yield the imine. Oxidation of the imine with tautomerism to the enamine yields the free base, and this mechanism is shown in figure 20.

A major advantage of this method of synthesis is that the reaction can proceed at much lower

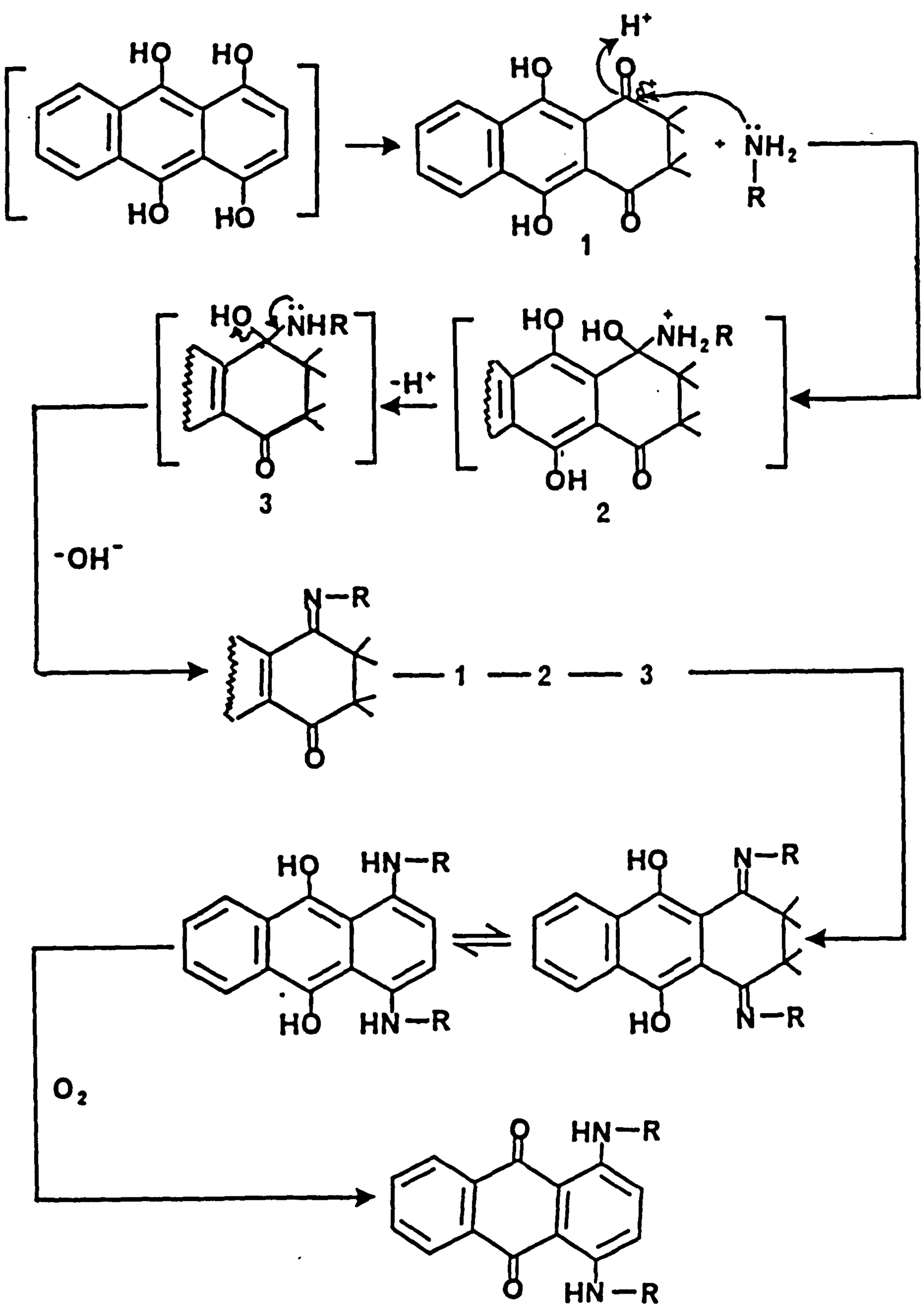


Figure 20: Mechanism of reaction of leucoquinizarin with primary amines.

temperatures, 50-60°C compared to 100-140°C in the case of substitution of chloroanthraquinones. As will be seen later, cyclisation of a side chain containing primary or secondary amino groups, can occur at the higher temperatures.

Compound (4A) was synthesised by heating leucoquinizarin and excess amine (10 fold excess) in butan-1-ol at 50-60°C under nitrogen, until all the leucoquinizarin had reacted (as determined by TLC). During this time the colour of the reaction mixture changed from dark brown to deep green. TLC of the reaction mixture indicated that the green compound was polar and basic (Rf 0.5; $CHCl_3$/MeOH, 1/1), furthermore the colour of the spot changed over a period of 2 hours to blue. At the completion of the reaction the mixture was aerated at 50°C to oxidise the substituted leuco-anthraquinone to the substituted anthraquinone. This product was purified essentially as for 1,5-disubstituted anthraquinone.

Visible spectroscopy revealed that the compound has two peaks at 626nm (λ max) and 582nm, compared to the λ max of 440nm of quinizarin. The compound was polar as would be expected. The spectral data were similar to that of 1,5-disubstituted anthraquinone, the infrared spectrum confirmed the presence of NH (bonded), and quinone carbonyl. ^{1}Hnmr and mass spectra were consistent with the assigned structure and elemental analysis confirmed that the compound was pure.

2.1.2. Synthesis of 2'-(hydroxyethylamino)ethylamino-anthraquinones (B series).

These compounds may be synthesised by analogous methods to those described for the synthesis of 2'-(diethylamino)ethylamino substituted anthraquinones, however since a secondary amino group is present in the side chain the products may cyclise to yield 1,2,3,4-tetrahydronaptho-2,3-quinoxaline-7,12-dione derivatives (figure 21). This occurs more readily at the higher temperatures involved in substitution of chloroanthraquinones. The mechanism for this reaction is shown in figure 21.

As seen previously leucoquinizarin may be utilised in the synthesis of 1,4-disubstituted anthraquinones and this route has been used by other workers for the synthesis of 1,4-bis[2'(hydroxyethyl-amino)ethylamino]anthraquinone (Zee Cheng and Cheng, 1978; Murdock et al. 1979). Since the hydroxyanthraquinones can be readily prepared (by Friedel Craft's acylation), and are also available commercially, the possibility of utilising 1-, 1,5-, and 1,8-dihydroxy anthraquinones for the synthesis of substituted anthraquinones was investigated.

The dihydroxy anthraquinones could be reduced to their leuco forms by dithionite, under nitrogen, in sodium hydroxide. However, unlike leucoquinizarin, these anthraquinones could not be isolated and were found to readily reoxidise to the dihydroxy anthraquinones. An alternative to isolating the leuco

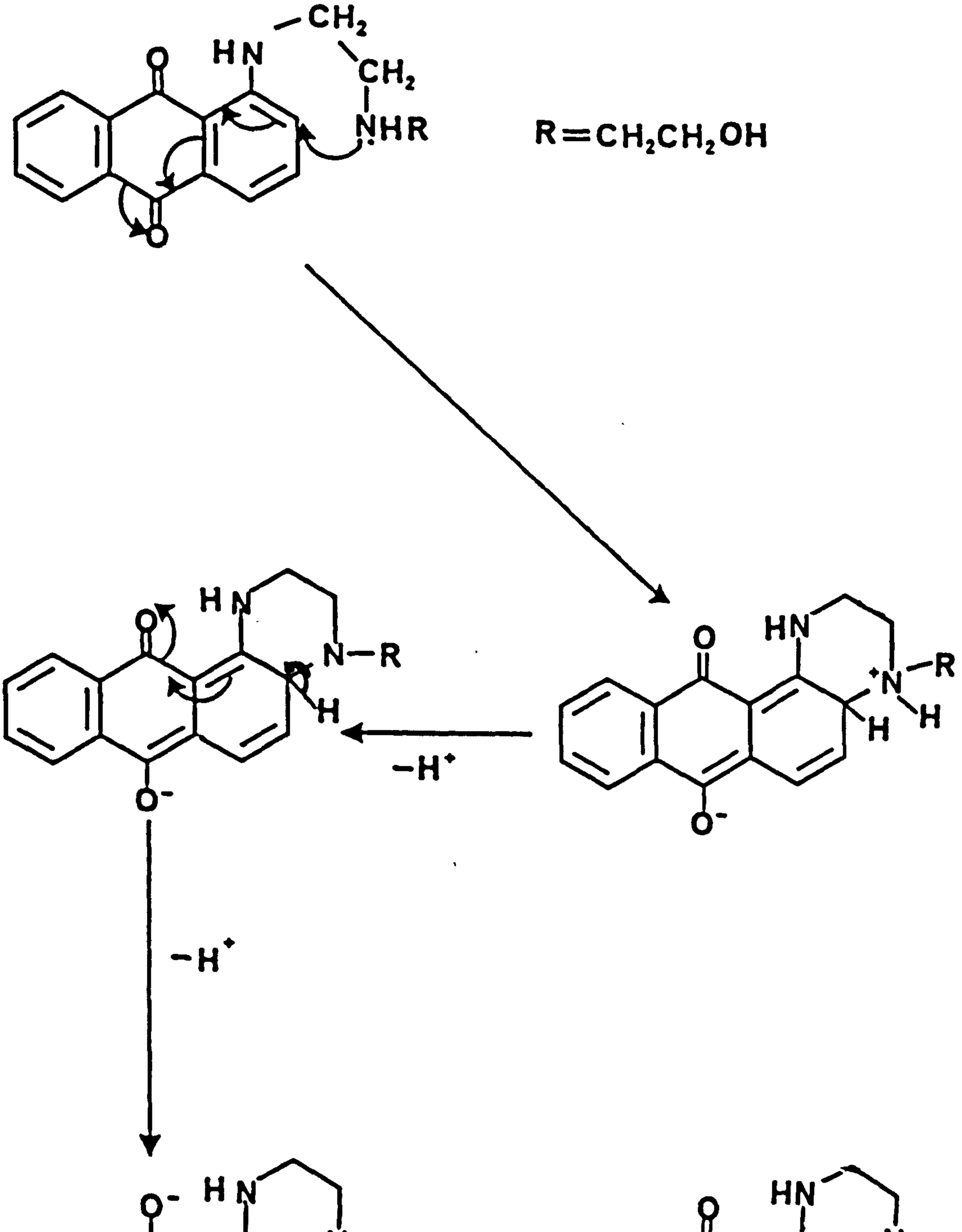


Figure 21: Mechanism of cyclisation of 2'-(hydroxy-ethylamino)ethylamino- anthraquinones.

anthraquinones would be to synthesise and react them in situ. After reduction of the dihydroxy anthraquinone the appropriate amine was added and the reaction allowed to proceed for a period of 72 hours, at 60-65°C. During this time there was no change in overall colour; TLC of the reaction mixture indicated the presence of only the starting dihydroxyanthraquinone.

The reaction conditions as described above were unsuitable for the synthesis of 1-, .1,5- and 1,8-bis-[2'-(hydroxyethylamino)ethylamino]anthraquinones, and therefore the substitution of chloro anthraquinones at lower temperatures was investigated. In a preliminary experiment 1-chloroanthraquinone was suspended in 2-(hydroxyethylamino)ethylamine by mechanical stirring and the temperature raised to 40°C. The mixture was maintained at this temperature for a period of 8 hours and the reaction monitored by TLC. The temperature of the reaction mixture was then raised by steps of 5°C. After each increment, the reaction was maintained at that temperature for 4 hours, whilst monitoring the reaction by TLC. It was shown that the minimum temperature necessary for the reaction to proceed was 90°C and therefore this temperature was utilised in the synthesis of 1-, 1,5-, and 1,8-bis-[2'-(hydroxyethylamino)ethylamino]antharquinones.

2.1.2.1. Synthesis of 1-[2'-(hydroxyethylamino)ethyl-amino]-anthraquinone (1B).

Compound (1B) was synthesised (see 3.1.2.1) by heating 1-chloroanthraquinone in excess, (20 fold), of 2-(hydroxyethylamino)ethylamine at 90°C with mechanical stirring until the majority of the chloroanthraquinone had reacted. During this time the colour of the reaction mixture changed from pale orange (λ max 380nm) to deep red (λ max 495nm). The reaction was also monitored by TLC. The compound was purified as for 1-[2'(diethylaminoethylamino]anthraquinone (1A) except that it was found that the impure product could be isolated by pouring the reaction mixture into a large volume of water. Further purification was achieved by solvent extraction and recrystallisation as for compound 1A.

The λ max of the product, in the visible region of the spectrum, was 498 nm compared to 380nm of the starting material. The bathochromic shift in λ max is similar to that of 1-[2'-(diethylamino)ethylamino]-anthraquinone (compound 1, λ max 495nm). The final product was found to be basic and polar. The infrared spectrum confirmed the presence of OH (3400 cm^{-1}), NH (3260 cm^{-1}, bonded) groups and quinone carbonyl at 1670 cm^{-1}. The NMR spectrum of the product was in agreement with the assigned structure. The presence of NH (2.4 δ) proton (D_2O) washable, as well as the presence of NH (9.9 δ) (not replaced by D_2O) protons indicate that the side chain had not cyclised. Mass spectral data

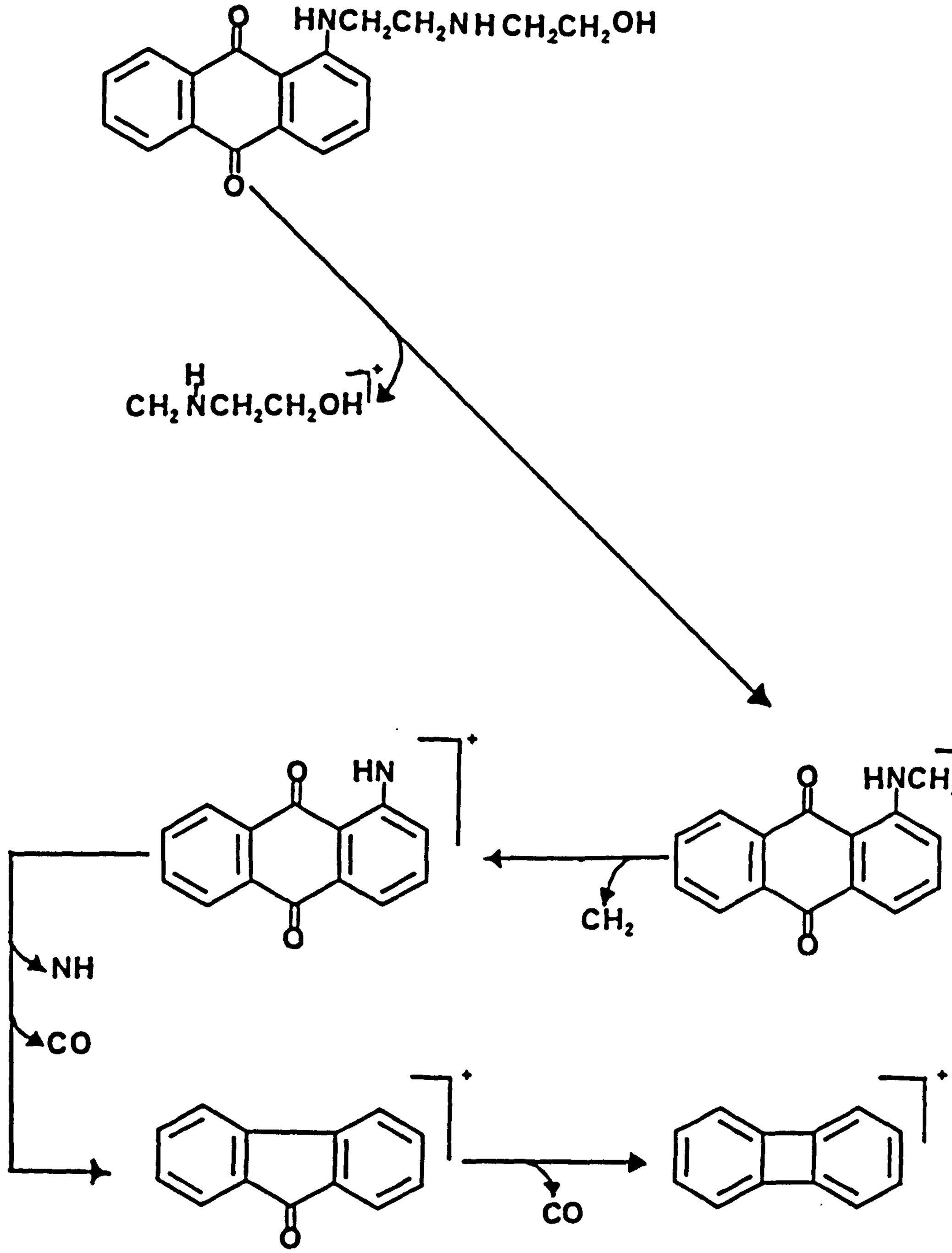


Figure 22 : A common fragmentation pathway of 2'-(hydroxyethylamino)ethylamino-anthraquinones.

were as expected and the fragmentation pattern is shown in figure 22. Elemental analysis confirmed that the product was pure.

2.1.2.2. Synthesis of 1,5-bis-[2'-(hydroxyethylamino)-ethylamino]-anthraquinone (2B).

Compound (2B) was synthesised (see 3.1.2.2) and purified by the same method as for compound (2A) except that the reaction was carried out at 90°C and the mixture was stirred mechanically. There was a similar change in colour, from pale orange to deep red (λ max 518nm) as the reaction progressed. After removal of unreacted anthraquinone and monosubstituted anthraquinone the compound was precipitated by the addition of excess sodium hydroxide, and filtered. Recrystallisation of the product yielded the pure 1,5-bis-[2'-(hydroxyethylamino)ethylamino]anthraquinone.

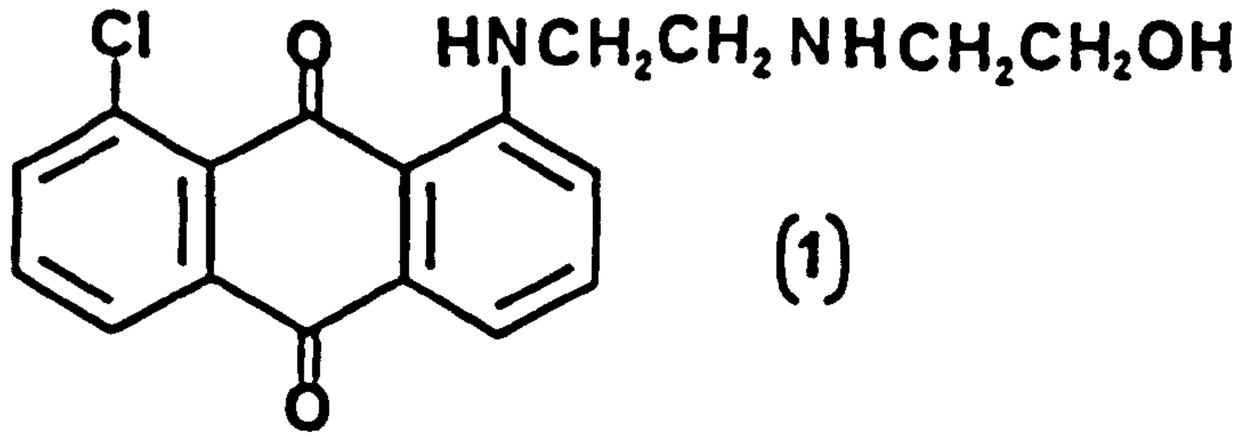
The λ max of the product, in the visible region, was 518 nm compared to 380nm of the starting material. The λ max of the product was similar to that of compound (2A), (515nm). The infra red spectrum was similar to that of 1-[2'-(hydroxyethylamino)ethylamino]-anthraquinone (1B), confirming the presence of OH, NH, and quinone carbonyl. The free base was insufficiently soluble in most of the commonly used solvents for ^{1}Hnmr, including chloroform, dimethylsulphoxide, and methanol therefore the ^{1}Hnmr spectrum of the hydrochloride salt was obtained, in D_2O. The ^{1}Hnmr spectrum was consistent with the assigned structure except the resonances corresponding to NH protons were

absent, as would be expected. Mass spectral data were also consistent with the assigned structure. Elemental analysis confirmed that the compound was pure.

2.1.2.3. Synthesis of 1,8-bis-[2'-(hydroxyethylamino)-ethylamino]-anthraquinone (3B).

Compound (3B) was synthesised (see 3.1.2.3) by the same method as for compound (2B), however it was found that during the reaction several products were formed with similar polarities (difference in Rf <0.3 when using MeOH/EtAc 1/1), and therefore the final product could not be purified by the simple solvent extraction methods used thus far. Possible structures of these compounds are shown in figure 23. The monosubstituted products would be expected to be less polar than the disubstituted analogues whilst the cyclised disubstituted product would be expected to have a longer λ max than the desired product, due to contribution of a lone pair of electrons by the third nitrogen to the chromophore

"Flash" column chromatography, with "sequential elution" is a more selective way of separating the components with similar polarities than solvent extraction. The term "sequential elution" is used here to describe gradual <u>stepwise</u> change in the polarity of the eluent rather than by a continous gradient. It was first necessary to remove any unreacted amine before column chromatography, since the amine is not very soluble in organic solvents and could therefore block the column. The amine was removed by washing the



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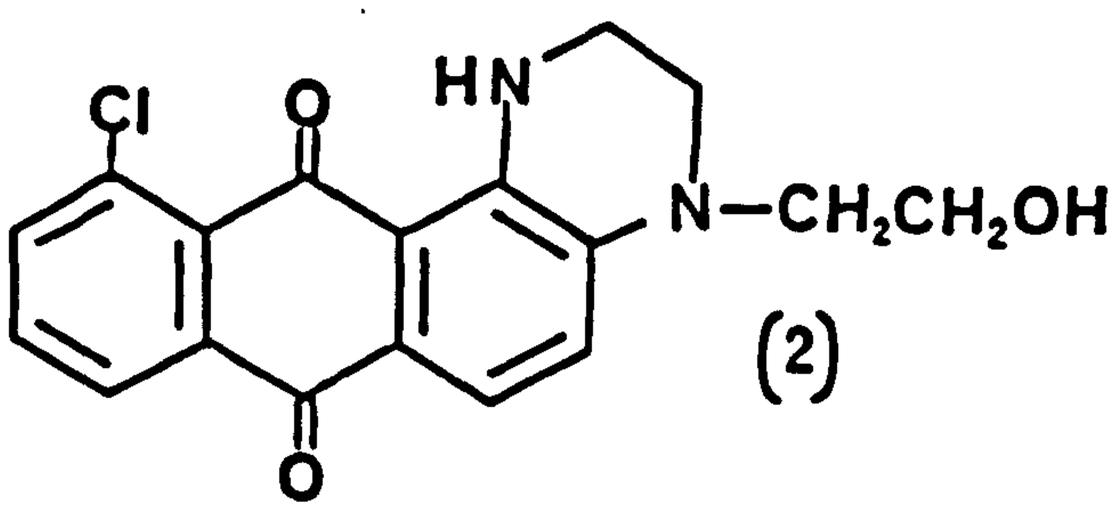

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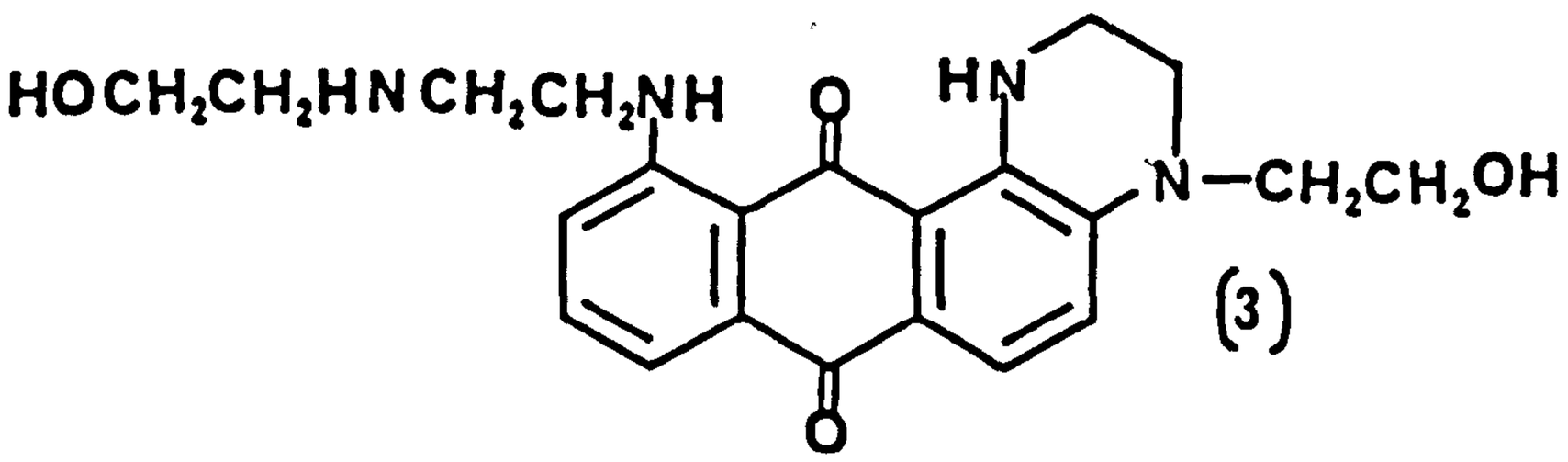

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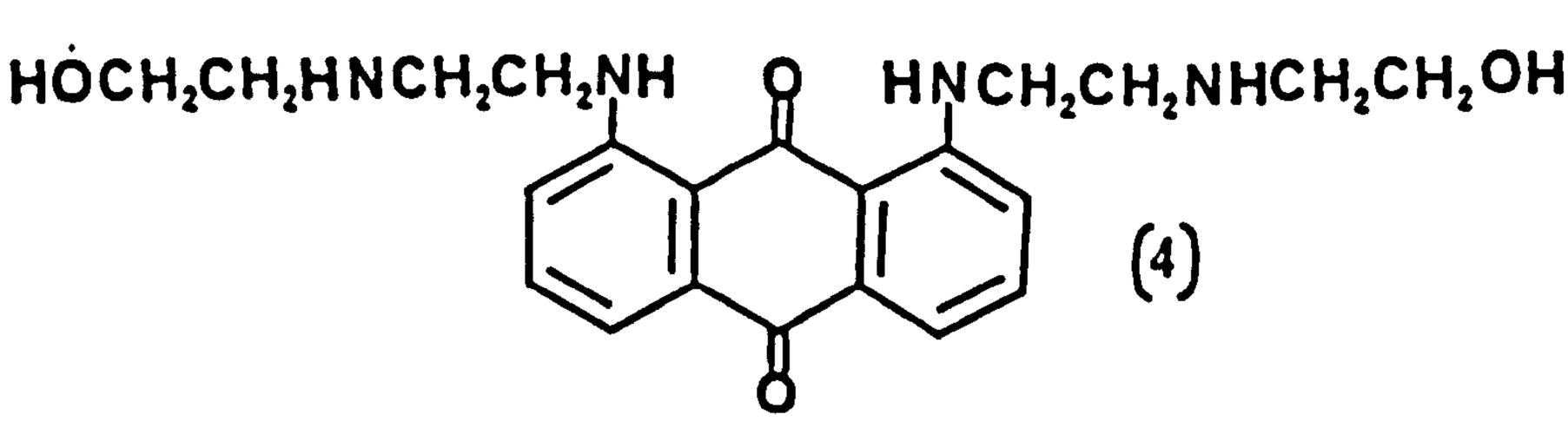


(4)

Figure 23 : Possible products following the reaction of 1,8-dichloroanthraquinone with 2-(hydroxyethylamino)ethylamine.

reaction mixture several times with water, the last traces of water were removed by addition of absolute ethanol followed by evaporation *in vacuo*.

Having removed the amine, the residue was dissolved in ethyl acetate/ methanol (2/1) and chromatographed (for details of eluent used and the technique see chapter 3). Fractions containing a particular single component (confirmed by TLC) were pooled and evaporated *in vacuo*. The most polar component was characterised first, since it was found to be the major component and also for the reasons mentioned above it was anticipated to be the desired product. The λ max of the product was found to be 540nm compared to 536nm for compound (3A). This gave an initial indication that the most polar fraction was most probably compound (3B). This component also had very similar Rf to compound (2B) (0.20 and 0.15 respectively for compounds (2B) and (3B)).

Further evidence for the structure was obtained by IR, ^{1}Hnmr, MS, and elemental analysis. The infrared spectrum confirmed the presence of OH (3420cm^{-1}), NH (3260cm^{-1}), and quinone carbonyl (1660cm^{-1}). NMR of the product was in agreement with the assigned structure. The presence of NH (2.4 δ , 2H) protons (D_2O) washable, as well as the presence of NH (9.9 δ, 2H) (not replaced by D_2O) protons indicate that the side chain had not cyclised. The fact that the λ max is in the same region as for 1,8-bis[2'-(diethylamino)ethylamino-

anthraquinone (3A), and that two D_2 replaceable protons are present indicates that the product is 1,8-bis-[2'-(hydroxyethyl)aminoethylamino]anthraquinone. Mass spectral data were in agreement with the assigned structure and elemental analysis confirmed that the compound was pure.

2.1.2.4. Synthesis of 1,4-bis-[2'-(hydroxyethylamino)-ethylamino]-anthraquinone (4B).

Compound (4B) was synthesised (see 3.1.2.4) by the same method as compound (4A) using leucoquinizarin and 2-(hydroxyethylamino)ethylamine. After oxidation the reaction mixture was filtered to yield the free base. Recrystallisation from ethanol/water mixture yielded the pure base. As with previous reactions the reaction was monitored by change in colour and also by TLC. The colour of the reaction changed from dark brown to green and, when aerated, to blue. There were two peaks in the spectrum of the final product in the visible region, at 628nm (λ max) and 583nm, the spectrum was similar to that obtained for compound (4A) (626 and 584nm respectively).

Further evidence for the structure was obtained by IR, ^{1}Hnmr, MS, and elemental analysis. The infrared spectrum indicated the presence of OH (3420cm^{-1}), NH (3280cm^{-1}), and quinone carbonyl (1610cm^{-1}). ^{1}Hnmr and mass spectral data were in agreement with the assigned structure and elemental analysis confirmed that the compound was pure.

In all cases hydrochloride salts were prepared by dissolving the base in a minimum quantity of an appropriate dry organic solvent (eg. ether, 2-methoxyethanol) and bubbling dry hydrogen chloride through the solution.

2.1.3. Synthesis of anthraquinone pro-drugs.

Most currently available antitumour agents are not selectively toxic to neoplastic cells. As seen earlier (Chapter 1), the most common toxicity of these agents to the host is depression of bone marrow function. In some cases the use of antitumour agents is limited by other ireversible toxicity, for example dose limiting cardiotoxicity of the anthracycline antibiotics. Furthermore the neoplastic cells become resistant to these drugs by a number of mechanisms. One method of reducing the toxicity, hence increasing the therapeutic index, of these drugs is to design drugs which are selectively activated by neoplastic cells.

In this work anthraquinone pro-drugs were designed such that these drugs could be selectively activated by target cells. The liver was chosen as the target organ because to-date only a limited number of chemotherapeutic agents are available for treatment of primary hepatocellular carcinoma. The use of fluoropyrimidines, nitrosoureas, and folate antagonists has usually yielded a poor response rate (Falkson et al., 1978). The single most effective drug is doxorubicin, which caused tumour regression in 35-50%

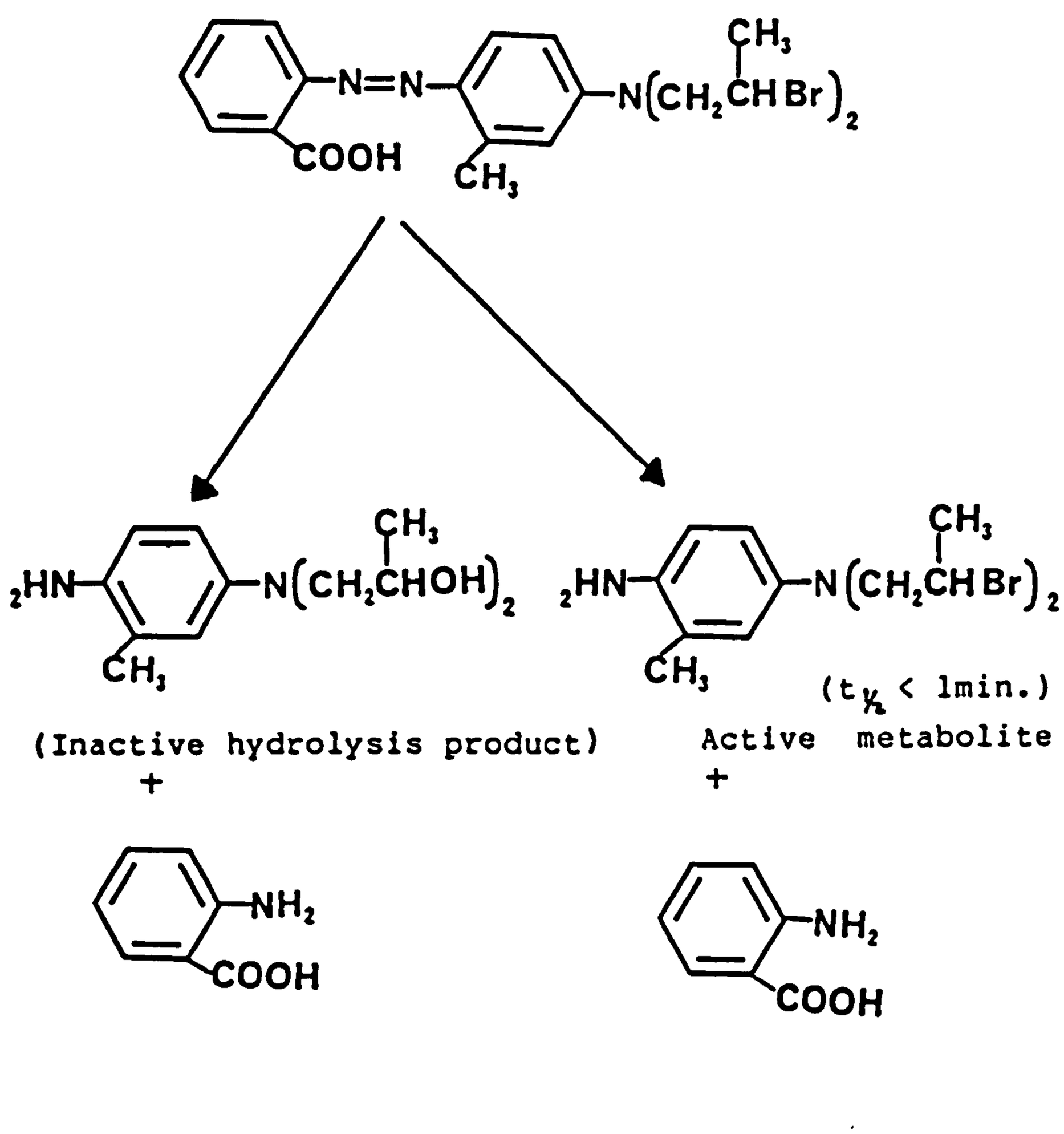


Figure 24 : Activation of azo-mustard by reduction in vivo by azo reductase. (From Connors, 1980).

of hepatocellular carcinoma cases and an improved survival time (Oon and Friedman, 1982). As described earlier (Chapter 1), one of the possible modes of action of doxorubicin is by interaction with DNA to inhibit replication of genetic material. It was anticipated that drugs which interact with DNA could be linked to inactive molecules to form pro-drugs. Furthermore with appropriate choice of linkages the active drug could be released by intracellular enzymes, and hence a selective targetting of drug to the tissue would be achieved. For example, Bukhari et al. (1973), synthesised azomustards (figure 24) and showed that these compounds are converted to the highly reactive phenylenediamine derivatives, by hepatic azo-reductase. It was therefore desirable to synthesise azo anthraquinones as pro-drugs, examine their metabolism by liver fractions in vitro, and study the interaction of these compounds with DNA. The following section discusses the syntheses of azoanthraquinones. Metabolism studies and DNA interactions will be discussed later in this chapter (Section 2 and 3).

In order to investigate whether azoanthraquinones can be metabolised to the corresponding aminoanthraquinones, by liver azo reductase enzymes 1-(4'-hydroxyphenyl)azoanthraquinone was synthesised from anthraquinone-1-diazonium chloride. The latter compound is available commercially as a stabilised zinc complex or can be readily synthesised from 1-aminoanthraquinone (Saunders, 1949).

2.1.3.1. Synthesis of 1-(4'-hydroxyphenyl)azo—anthraquinone (12).

The zinc complex of anthraquinone-1-diazonium chloride is stable at room temperature, the coupling to phenol was carried out at room temperature. The mechanism of coupling of diazonium cations to phenols is shown in figure 25. The diazonium cation can undergo electrophilic aromatic substitution. This however, is a weak electrophile compared to species such as $^{+}NO_2$ and will normally only attack highly reactive aromatic compounds such as phenols (Sykes, 1975). The introduction of electron withdrawing groups into the o- or p-positions of the diazonium cation enhances its electrophilic character. Diazonium cations exist in acid and slightly alkaline solution. In more strongly alkaline solutions they are converted to diazohydroxides, PhN=N-OH and further into diazotate anions, $PhN=N-O^{-}$ and coupling reactions are therefore carried out under acidic or more usually (in the case of phenols) under slightly alkaline conditions. With phenols a slightly alkaline pH is used since it is the phenoxide (PhO^{-}), and not the phenol (PhOH), that undergoes attack by the diazonium cation. As with other cases of electrophilic attack on phenols, the C-substituted product is obtained rather than the O-substituted product (Sykes, 1975). Removal of a proton from the intermediate is facilitated by one or other of the basic species present in solution. Coupling usually takes place in the p-, rather than the o-, position because of the excess of attacking

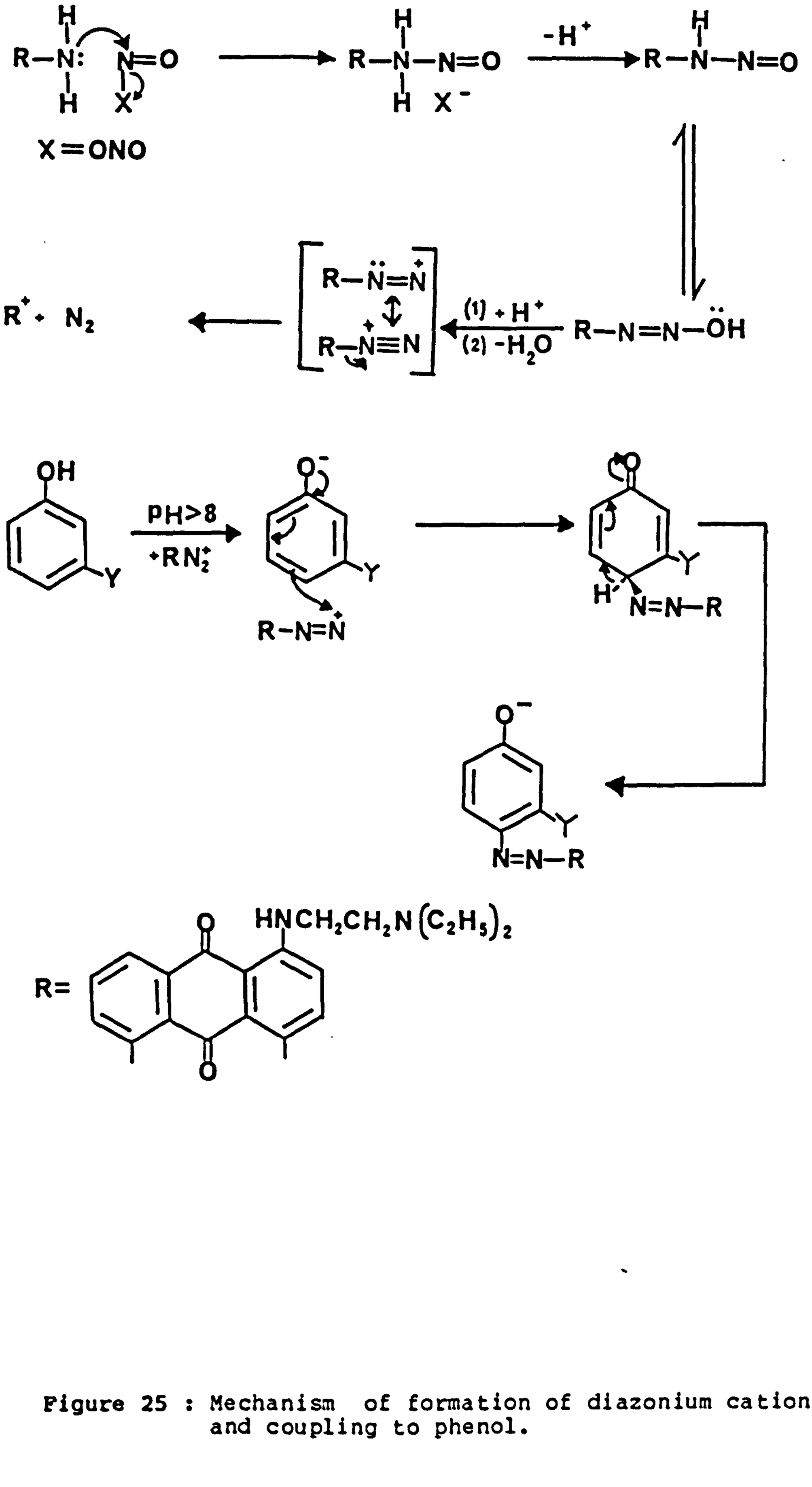


Figure 25 : Mechanism of formation of diazonium cation and coupling to phenol.

electrophile ArN_2^+.

Anthraquinone-1-diazonium chloride (zinc complex) was added to a solution of phenol in sodium hydroxide and the mixture stirred. The colour of the mixture changed from slight yellow to deep orange. The coupling reaction was monitored by TLC since the azoanthraquinone would be expected to be more polar than aminoanthraquinone due to the presence of phenolic OH. The mixture was stirred until completion of the reaction (as monitored by TLC). The resulting precipitate was filtered and washed first with sodium bicarbonate solution to remove any unreacted phenol, and finally with water. The solid was dried and recrystallised from ethanol.

The azoanthraquinone was characterised by spectroscopy and elemental analysis. The infra red spectrum showed the presence of phenolic OH (3400 cm^{-1}), and quinone carbonyl (1620 cm^{-1}). The observation that there was not a peak for NH_2 is consistant with coupling having taken place. The ^{1}Hnmr spectrum showed the presence of aromatic protons (7.1-7.6m δ), and OH (D_2O replaceable, 5.2s δ). Mass spectral data were in agreement with the assigned structure and elemental analysis confirmed that the compound was pure.

From DNA binding studies it was shown that 2'-(diethylamino)ethylamino substituted anthraquinones do bind to DNA by intercalation (see later sections)

with the 1,4-, and 1,5-disubstituted anthraquinones having a higher affinity than the 1- and 1,8-disubstituted anthraquinones for DNA. The compounds also have antiproliferative activity *in vitro* against HeLa cells; however the compounds were inactive *in vivo* against P-388 leukaemia. Therefore pro-drugs incorporating these anthraquinones would be of particular value if they could be delivered to the target organ. Subsequent activation, of the pro-drug, by the target organ could be used to release the cytotoxic compound.

In order to synthesise aminoalkylamino-anthraquinone pro-drugs it is necessary to first synthesise aromatic aminoanthraquinones, substituted with aminoalkylamino side chains. The synthesis of 1-amino-2'-(diethylaminoethylamino) substituted anthraquinones will be described first, and azo coupling of these compounds to phenols will then be discussed.

2.1.3.2. Synthesis of 1-amino-4-[2'-(diethylamino)-ethylamino]anthraquinone (2Ci).

Compound (2Ci) was synthesised (see 3.1.3.1) by the same method as compound (1A) using 1-amino-4-bromo anthraquinone and 2-(diethylamino)ethylamine. As the reaction progressed the colour of the mixture changed from deep red (λ max 490nm) to blue (λ max 600nm). The reaction was also monitored by TLC, in this case two blue spots were present one had the same Rf as 1,4-disubstituted anthraquinone (4A), whilst the other

was less polar than compound (4A). It was found that the two compounds could not be separated by solvent extraction and were therefore separated by "flash" column chromatography after removing unreacted amine. The more polar compound was shown to be compound (4A), since the U.V. and IR spectra were identical. The less polar of the two components was further purified by recrystallisation, and characterised as (2Ci) by spectroscopy and elemental analysis.

A solution of the purified product produced two peaks in the visible region of the spectrum, at 606nm (λ max), and 565nm. These two peaks are characteristic of 1,4-diaminoanthraquinones. The infra red spectrum indicated the presence of NH_2 (3400 cm^{-1}), NH (bonded, 3260 cm^{-1}), and quinone carbonyl (1645 cm^{-1}). ^{1}Hnmr and mass spectral data were consistent with the assigned structure. Elemental analysis confirmed that the product was pure.

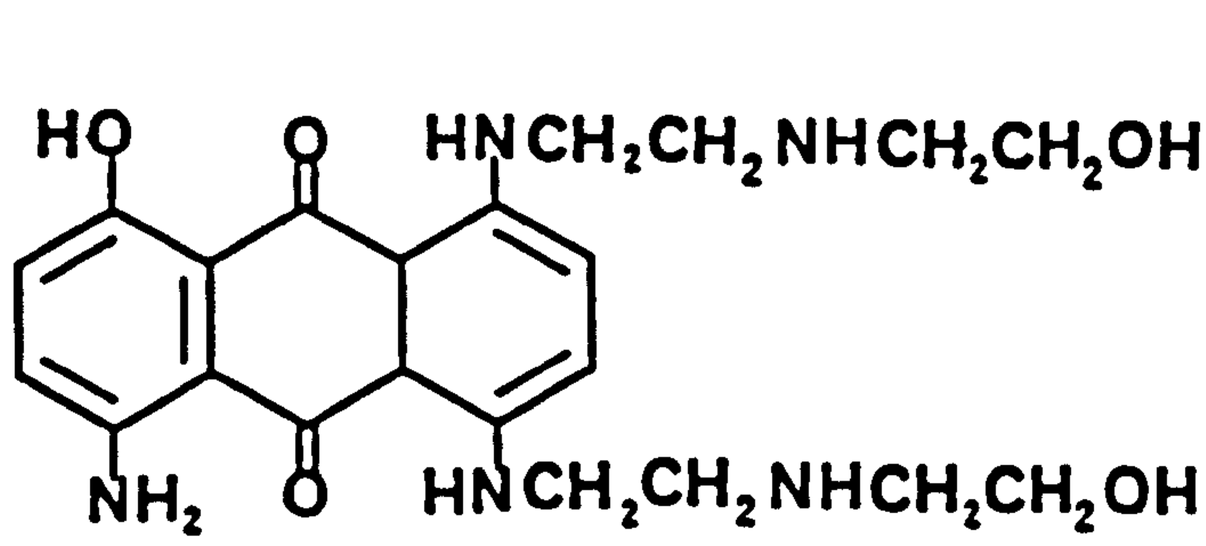
2.1.3.3. Synthesis of 1-amino-5-[2'-(diethylamino)-ethylamino]anthraquinone (3Ci).

Compound (3Ci) was prepared and purified (see 3.1.3.2) by the method described for compound (2Ci) using 1-amino-5-chloroanthraquinone and 2-(diethyl-amino)ethylamine. In this reaction the colour of the mixture changed from orange (λ max 450) to deep red (λ max 507).

Compound (3Ci) was characterised by spectroscopy and elemental analysis. The λ max, of the pure compound, was 507nm and this is in the same region as

compound (2A). The infrared spectrum was comparable to that of compound (2Ci), ^{1}Hnmr and mass spectral data were consistent with the assigned structure. Elemental analysis confirmed that the compound was pure.

Having synthesised compounds (2Ci) and (3Ci) as model compounds the next step was to synthesise an amino-substituted anthraquinone with a higher affinity for DNA. On the basis of DNA binding work, on the first two series of anthraquinones (see 2.2), it was decided to synthesise a 1,4-bis-substituted- anthraquinone with a primary aromatic amino group in the 5 (or 8) position of the chromophore. Previous workers had synthesised compounds of this type also containing a hydroxy group in the 8-position (Nippon Kayaku Co., Japan, 1982).



This compound is a structural analogue of mitoxantrone, and posseses antitumour properties.

2.1.3.4. Synthesis of 1-amino-5,8-bis-[2'-(diethyl-amino)ethylamino]-4-hydroxy-anthraquinone (4Ci).

Compound (4Ci) was synthesised (see 3.1.3.3) essentially by the method described in a patent (Nippon Kayaku Co., 1982). 1,5-Diamino-4,8-dihydroxy-anthraquinone and 2-(diethylamino)ethylamine (ten fold excess) were suspended in aqueous methanol (50%), under

nitrogen, and sodium dithionite added. The mixture was heated, under nitrogen, in an oil bath at 60-65°C, and the reaction monitored by TLC. The product would be expected to be more polar than the starting anthraquinone due to the presence of an aliphatic amino group, Heating was continued until no further product was formed, during which time the colour of the reaction mixture changed from blue to dark green. This change in colour was as expected, due to the formation of reduced aminoanthraquinones (starting material and the substituted product). The mixture was then aerated, at 50-60°C, to oxidise the leucoanthraquinones; this was assumed to be completed when the colour of the reaction mixture had changed to blue. The mixture was evaporated in vacuo and inorganic impurites removed by suspending the residue in water and extracting with ethyl acetate. The ethyl acetate solution was then evaporated in vacuo and the compound purified by "flash" column chromatography. Fractions containing a single component were pooled and evaporated in vacuo. Only two components were present, starting anthraquinone and the more polar 2'-(diethylamino)-ethylamino substituted anthraquinone. The latter component was further purified by recrystallisation, and characterised by spectroscopy and elemental analysis.

The compound exhibited two peaks in the visible region of the spectrum, at 630nm (λ max) and 585nm. These two peaks are characteristic for 1,4-bis-amino-alkylamino-substituted anthraquinones. The infra-red

spectrum showed the presence of NH and OH (bonded) and quinone carbonyl. ^{1}Hnmr and mass spectral data were consistent with the assigned structure. Elemental analysis confirmed that the compound was pure.

Having synthesised aminoanthraquinones (compounds 2Ci-4Ci), they were azotised and coupled to resorcinol. Resorcinol was chosen since the presence of hydroxyl or amino groups in close proximity to the azo bond enhance the enzymatic reduction (Shargel et al., 1984). Resorcinol was also chosen because it couples more readily than does phenol (Saunders, 1949).

2.1.3.5. Synthesis of 1-[2'-(diethylamino)ethylamino]-4-(2",4"-dihydroxyphenyl)azoanthraquinone (2C).

1-Amino-4-[2'-(diethylamino)ethylamino]anthraquinone (see 3.1.3.5) was dissolved in hydrochloric acid and cooled in a NaCl/ice bath (0-5°). Sodium nitrite was then added and the reaction allowed to proceed until all the aminoanthraquinone had reacted. During this time the colour of the solution changed from deep blue to orange/green. The reaction was also monitored by adding a small aliquot of the reaction mixture into excess sodium bicarbonate solution; where any unreacted amine precipitated out.

The mixture containing azotized anthraquinone was added to a solution of resorcinol in sodium hydroxide, also at 0-5°. The coupling reaction was allowed to proceed until all the diazonium salt had reacted; this was indicated by the colour of the

mixture which changed from orange/green to dark green. The reaction was also monitored by TLC, since the azo compound was expected to be more polar than the amine, due to the presence of two phenolic groups.

After completion of reaction any unreacted amine was extracted with dichloromethane and this was discarded. The aqueous layer was evaporated *in vacuo* and the last traces of water were removed by adding absolute ethanol followed by further evaporation *in vacuo*. Inorganic material was removed by suspending the residue in chloroform and filtering. The solution was then evaporated *in vacuo* and the resultant green solid purified by "flash" column chromatography. Further purification was attained by recrystallisation.

The azo product was characterised by spectroscopy and elemental analysis. There were two peaks in the visible region of the spectrum, 626nm (λ max) and 480nm, compared to 606nm and 565nm for the starting amine. This bathochromic shift is as expected, due to extention of conjugation by the azo linkage. The infrared spectrum indicated the presence of OH, NH, and quinone carbonyl. ^{1}Hnmr and mass spectral data were consistent with the assigned structure. The purity of the azo-anthraquinone was confirmed by elemental analysis.

2.1.3.6. Synthesis of 1-[2'-(diethylamino)ethylamino]-5-(2",4"-dihydroxyphenyl)azoanthraquinone (3C).

Compound (3C) was prepared essentially by the same method as compound (2C) using 1-amino-5-[2'-(diethylamino)ethylamino]anthraquinone. It was found that in this case the product precipitated out at the completion of reaction. The precipitate was collected and washed several times with water, to remove inorganic material. The product was then washed with cold ethanol to remove traces of water and dried overnight in vacuo at 60°. Compound (3C) was further purified by "flash" column chromatography.

Compound (3C) was characterised by spectroscopy and elemental analysis. The λ max of the purified product was 520nm compared to 507nm for the starting amine. The infrared spectrum indicated the presence of OH, NH, and quinone carbonyl. ^{1}Hnmr and mass spectral data were consistent with the assigned structure. The final product was confirmed to be pure by elemental analysis.

The hydrochloride salts of compounds (2C) and (3C) could not be prepared as it was found that the azo compounds degraded to the parent amine in the presence of HCl.

2.1.3.7. Attempted synthesis of 1,4-bis-[2'-(diethylamino)ethylamino]-5-(2",4"-dihydroxyphenyl)-azo-8-hydroxyanthraquinone (4C).

Attempts to prepare compound (4C) from 1-amino-5,8-bis-[2'(diethylamino)ethylamino]-4-hydroxy-

anthraquinone (4Ci) were unsuccesful. The aminoanthraquinone could readily be diazotised and coupled to resorcinol using the same conditions as for the synthesis of compound (2C). The diazotisation and coupling were monitored by TLC. However the azo-anthraquinone could not be isolated since it was rapidly degraded to the parent aminoanthraquinone (4Ci). The conversion to the aminoanthraquinone was completed in less than 10 minutes, as monitored by TLC. Attempts to couple compound (4Ci) to phenol were similarly unsuccesful.

Having synthesised the substituted anthraquinones (1A-4A, 1B-4B), the anthraquinone pro-drugs (1C-3C) and their corresponding intermediates (2Ci-4Ci); the interaction of these compounds with DNA was examined.

2.2. DNA BINDING STUDIES.

The compounds prepared in this work were designed to intercalate into DNA, with or without activation, in accordance with the principal mode of cytotoxic action of doxorubicin (Brown, 1983; see 1.8). Determination of the interaction of these compounds (and doxorubicin) with DNA was therefore of fundamental importance in the assessment of potential antitumour activity of these compounds. Furthermore pro-drugs (compounds 2C & 3C) of anthraquinones were designed with the intention that they should not intercalate into DNA, but that when metabolised, they should yield the corresponding anthraquinones (compounds 2Ci & 3Ci) which would then intercalate into DNA; therefore the interaction of all compounds prepared in this work with DNA was examined.

In order to fully evaluate the DNA-binding of the compounds prepared, methods of studying their binding to DNA must be selected which will allow both the nature of binding and the affinity of the drug for DNA to be determined. Drugs can interact with DNA in several ways; for example external binding (see 1.5.7). However the compounds prepared in this work were designed to intercalate into DNA. It is therefore important here to use methods which will distinguish the intercalative mode of binding from other modes and allow determination of the affinity of the drug for DNA when intercalation is shown. Consequently, two types of

tests have been used; firstly a series of qualitative tests, the results of which can collectively show whether the compounds intercalate. Secondly, tests to quantitate the affinity of the compounds for DNA. The methods used in this work will be discussed in detail together with the results obtained and then alternative methods, not employed here, will be discussed briefly. These alternative methods were not used in this work since sufficient data were obtained (from the methods used in this work) to draw conclusions on the nature of interaction and affinity of drug for DNA.

The methods employed in this work can be classified into two groups. Firstly those that monitor changes, usually spectroscopic, in the properties of drug and secondly those that monitor changes in the properties of DNA.

2.2.1. Changes in the spectral properties of drug.

Changes in the fluorescence and UV visible absorbance properties of drug can occur on its binding to DNA (Neidle, 1978; Waring, 1981). Methods which monitor such changes were used here to examine the interaction of doxorubicin and substituted anthraquinones, (compounds 1A-4A, 1B-4B, 2C-3C and 2Ci-3Ci), with DNA. Firstly methods which monitor changes in the absorbance properties of the drug will be considered then methods which utilise fluorescence techniques will be discussed.

The effect of DNA on the spectral properties of

drug can be studied provided the absorbance spectrum of the DNA does not significantly interfere with the absorbance spectrum of the drug. Since the λ max of DNA is 260nm and the λ max of the compounds prepared in this work is >400nm these methods can be used. The methods monitoring changes in the absorbance properties of the drug can further be divided into three groups:

1. Effect of DNA on the absorbance spectrum of the drug.
2. Spectrophotometric titration - determination of the apparent affinity constant. (K)
3. Stopped-flow analysis of drug/DNA kinetics - determination of the dissociation rate constant.

The first method is a qualitative, and indicates the nature of binding of the drug to DNA. The second and third methods are quantitative and indicate the differences in the binding of drugs to DNA.

2.2.1.1. Effect of DNA on the absorbance spectrum of drug.

When drug molecule intercalates into DNA, the λ max of the drug typically shifts to a longer wavelength (bathochromic shift) and the molar extinction at the λ max of the free drug decreases (hypochromic shift) (Waring, 1981). If the spectrum of a solution of free drug and the spectra of solutions containing drug and increasing concentrations of DNA are superimposed, then all the spectra will pass through a single point, which is termed the isosbestic point (Waring, 1981). However this will only occur if

there is a single spectroscopically distinct bound form of the drug molecule in addition to the free drug. The appearance of a clear isosbestic point is mandatory if such spectra are to be used quantitatively in the determination of the affinity of drug for DNA (see later). The intercalation of a drug into DNA gives certain spectral characteristics which are not shown by drugs which undergo external binding to DNA. However some drugs, such as proflavine, which are known to intercalate into DNA (Neidle, 1978), do not exhibit a clear isosbestic point (Waring, 1981). Thus absence of an isosbestic point does not necessarily eliminate intercalation as the mode of binding.

All compounds studied here were shown to obey Beer-Lambert's law over the concentration range used in this work ($1 \times 10^{-7} - 5 \times 10^{-4}$M). All compounds were next examined to see if their spectra show an isosbestic point when the DNA concentration was varied. All compounds (except the pro-drugs compounds 2C and 3C) were shown to give an isosbestic point in the visible region of the spectrum. Figure 26 shows the effect of increasing DNA concentration on the visible spectrum of compound 1A; similar spectra were also obtained for compounds 2A-4A, 1B-4B and 2Ci-3Ci. The data obtained are summarised in table 1. All compounds studied showed bathochromic and hypochromic shifts on binding to DNA. The pro-drugs (compounds 2C-3C) however did not, show any significant changes in their spectra (figure 27). The shifts in λ max and decrease in extinction of all compounds were of the same order as

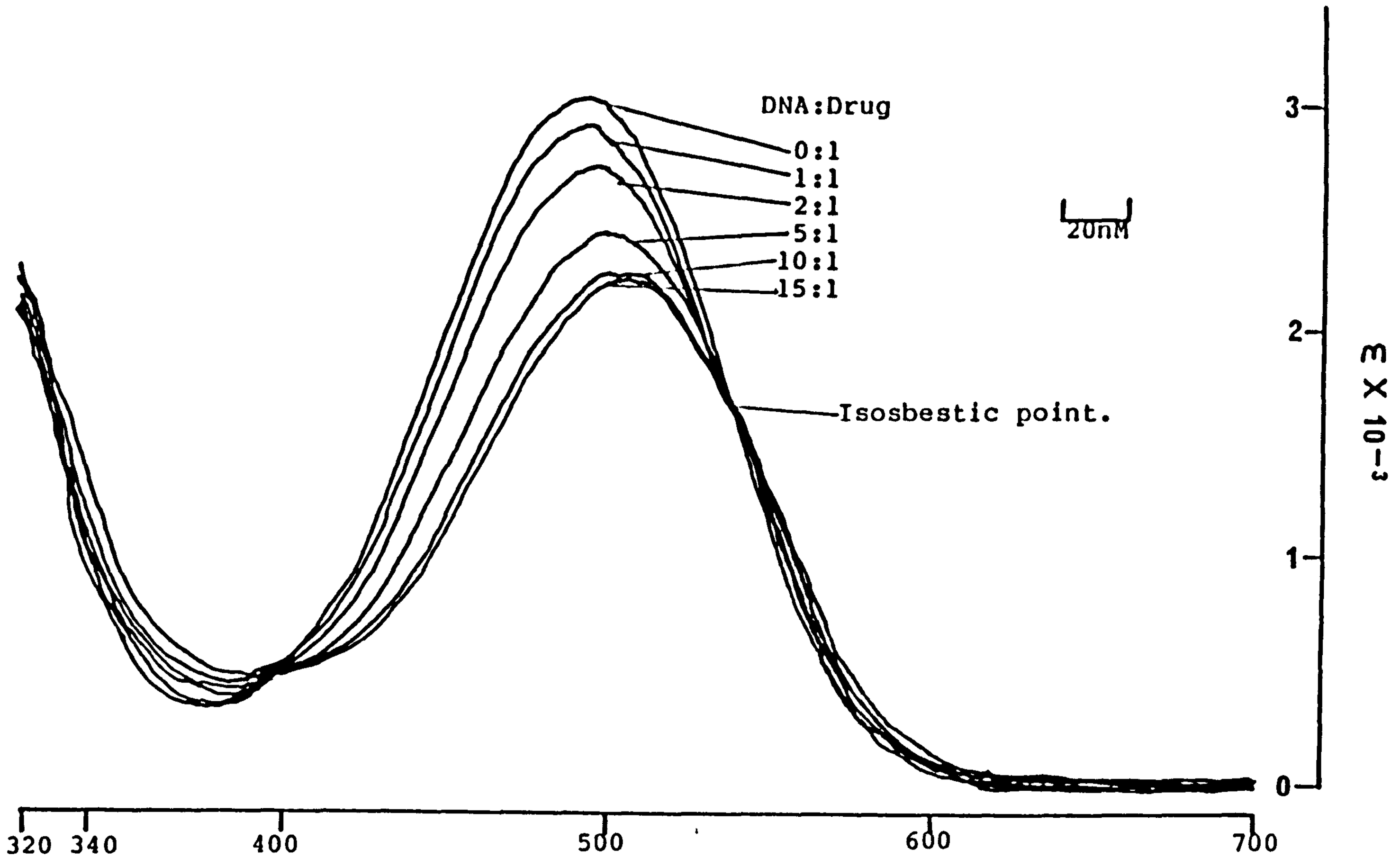


Figure 26 : Effect of DNA on the spectral properties of anthraquinones (compound 1A).

DNA:drug ratios - same as for compound 1A (Figure 26).

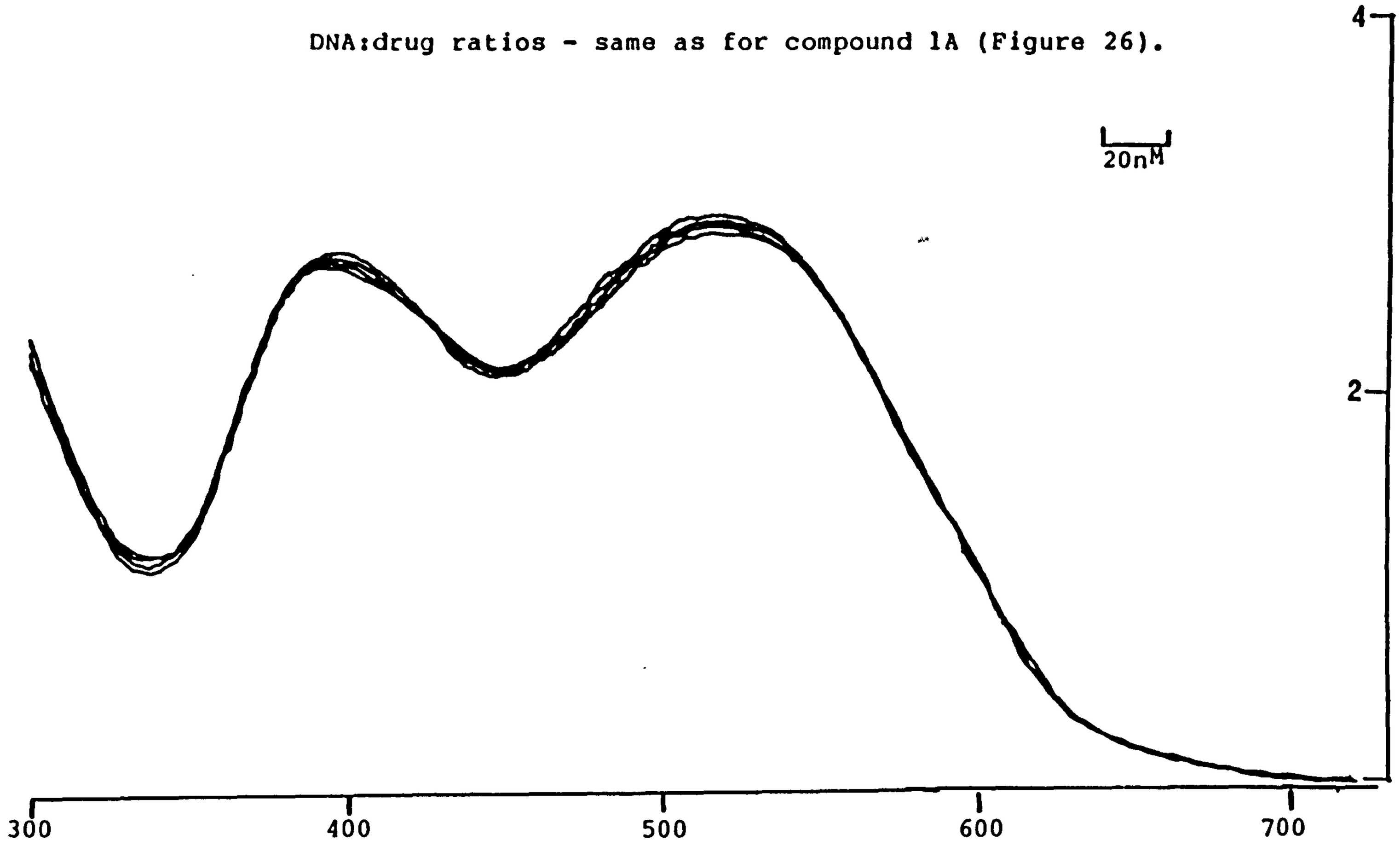


Figure 27 : Effect of DNA on the spectral properties of anthraquinone pro-drug (compound 3C).

Compound	Isosbestic points (nm)	Shift in λ max[1,2] (nm)	% decrease in extinction (εfree, εbound)
Doxo--rubicin	545,410	10	42.0 (9680,5540)
2'-(Diethylaminoethylamino)anthraquinones			
1A	538,400	10	37.0 (3200,2010)
2A	562,360	12	27.0 (9500,6930)
3A	585,340	11	32.0 (7900,5370)
4A	637,550	20	42.0 (14900,8640)
2'-(Hydroxyethylamino)ethylaminoanthraquinones			
1B	572,344	10	32.0 (6290,4280)
2B	574,332	15	32.0 (11900,8095)
3B	590,340	11	33.0 (8450,5660)
4B	637,430	15	38.0 (10120,6275)
Pro-drugs and their "metabolites"			
2Ci	615,382	10	25.0 (9590,7190)
2C	none	-	no dec. (3220,3220)
3Ci	560,340	10	33.0 (11200,7500)
3C	none	-	no dec. (3200,3200)

1. Shift in λ max at DNA:drug ratio of 10:1, drug concentrations in all cases were 25 μM in tris (0.008M), NaCl (0.05M) buffer (pH 7.40).

2. Difference between λ max of fully bound drug and free drug.

Table 1 : Data from the determination of effect of DNA on the absorbance spectrum of drug.

for doxorubicin. From these preliminary results it appears that the anthraquinones (1A-4A, 1B-4B and 2Ci-3Ci) do indeed interact with DNA by the same mode as doxorubicin; ie. intercalation. Furthermore this evidence suggests that the prodrugs appear not to intercalate into DNA.

On binding to DNA, compounds 1A-4A, 1B-4B and 2Ci-3Ci show a 10-20nm bathochromic shift in the λ max and a decrease in extinction of between 27 and 42%. These values are comparable with those for doxorubicin which shows a 10nm bathochromic shift in the λmax and a 35% decrease in extinction. The pro-drugs (compounds 2C-3C) did not show any changes in the spectrum as the DNA concentration was increased, whilst the corresponding potential aminoanthraquinone metabolites (compounds 2Ci-3Ci) exhibited isosbestic points, and showed bathochromic and hypochromic shifts of the same order as compound (1A).

These results are consistant with an intercalative mode of action of the anthraquinones (1A-4A, 1B-4B and 2Ci-3Ci). In order to quantitate the differences in the binding of these compounds to DNA, affinity constants were determined by spectrophotometric titration.

2.2.1.2. Spectrophotometric titration studies.

When DNA is added to a solution of a drug which intercalates into DNA, for example doxorubicin, there is a progressive decrease in the molar extinction of the drug at the λ max of the unbound drug as the proportion of DNA is increased. This occurs up to a limiting concentration of DNA, after which there is no further decrease in the extinction; at this point the drug is fully bound to DNA. Therefore if the absorbance is recorded at this λ max at various DNA:drug ratios, extinctions obtained at these ratios may be calculated. The fraction of drug bound can thus be calculated, at any particular concentrations of DNA and drug as shown later. From this it is possible to determine the affinity of the drug for DNA. This method is quantitative, giving information on the affinity rather than on the nature of the interaction.

For the spectrophotometric titration method to be valid it is essential that both the free drug and the bound drug obey Beer-Lambert's Law over the drug concentrations used for the assay. A clear isosbestic point must also be present, since this indicates that there are only two spectroscopically distinct forms of the drug; the bound and the free drug. Hence the fraction of the bound drug is proportional to the fractional decrease in extinction. All compounds (except pro-drugs) showed distinct isosbestic points in this work and therefore the use of spectrophotometric

titration method is valid for this study. Before discussing the results obtained by this method it is necessary to discuss how the experimental data were interpreted.

The interaction of a drug with DNA may be represented as follows:

$$\underset{C}{\text{Drug}} + \underset{n-r[DNAp]}{\text{DNA}} \rightleftharpoons \underset{r[DNAp]}{\text{Drug-DNA complex}}$$

Where C is the concentration of drug, r is the number of sites occupied by the drug per [DNAp], n-r is the number of unoccupied sites per [DNAp]. The concentration of the bound drug may be related to the concentration of the unbound drug by the Law of mass action:

$$K = \frac{r}{c(n-r)} \qquad (1)$$

where K is the affinity constant and is the ratio of association and dissociation constants k_1 and k_{-1}; r is the number of drug molecules bound per DNAp (concentration of DNA is expressed in terms of phosphorus); c is the concentration of unbound drug and n is the number of binding sites for drug per DNAp.

Equation (1) is usually rearranged to a linear form, invariably by the method of Scatchard (1949). This enables calculation of K and n.

$$r/c = -Kr + Kn \qquad (2)$$

A plot of r/c vs r gives a line of slope -K and an intercept Kn on the ordinate axis. In order to calculate r and r/c, the fraction of bound drug (α) is determined. In order to estimate α it was necassary to determine the extinction due to free drug (ε_f), the extinction due to bound drug (ε_b) and the extinction of the sample after each addition of DNA (ε_{obs}). Extinction of free drug was obtained from the absorbance of the free drug, extinction of the sample under test is obtained from the absorbance of the drug/DNA mixture. The extinction of the bound drug was estimated from a plot of ε_{obs} vs DNAp/c_t, where c_t is the total drug concentration (Plumbridge et al., 1978). The fraction of drug bound after each addition of DNA was calculated by method of Peacocke and Skerret (1956), equation 3:

$$\alpha = \frac{\varepsilon_f - \varepsilon_{obs}}{\varepsilon_f - \varepsilon_b} \qquad (3)$$

r and r/c can now be calculated from α since:

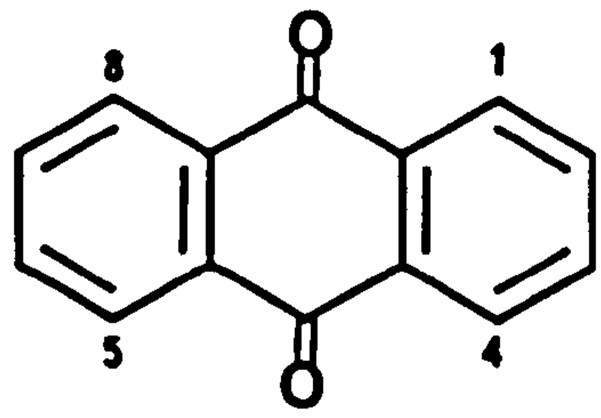
$$c = (1 - \alpha)c_t \quad \text{and} \quad r = \alpha\, c_t/\text{DNAp}.$$

A plot of r/c vs r was then used to calculate K and n by means of regression analysis. However, curvature was often encountered since the Scatchard plot will only be linear if there is a single type of non-interacting site. In the case of interaction of drugs with DNA there are at least two radically different binding sites available. The drugs can either bind externally to the phosphate residues or can intercalate into DNA

(Waring, 1981). The affinity constants (K) for interaction at the two sites usually vary considerably; K for external binding usually about 10^4 compared to 10^6 for intercalation (Blake and Peacock, 1968). The two sites may be treated separately on the binding curve. Furthermore not all intercalating sites are available for intercalation (see chapter 1, 1.6.1.), due to "nearest neighbour exclusion".

In order to use this method for the determination of affinity constant, it is first necessary to ensure that both the free and bound forms of the drug obey Beer-Lambert's Law. It was shown that dilute solutions of all compounds prepared in this work obeyed Beer-Lambert's law in the concentration range from 1×10^{-4} to 2×10^{-6}M. Furthermore it was shown that the bound form of drug obeyed Beer-Lambert's law by dilution of the final solution, containing excess DNA, at the end of the spectrophotometric titration. The absorbance at the λ max of the drug was monitored after addition of each aliquot of DNA solution.

In all cases the drug was fully bound at a DNA:drug ratio of >10:1, this enabled reliable estimates for ε_b to be determined. Scatchard plots were produced for each drug (figures 28-29) and were of comparable shape to that obtained for doxorubicin under identical conditions. The estimated K and n values were of the same order as those for doxorubicin (ie $K \approx 10^6$, $n \approx 0.2$). The values of K and n for the substituted anthraquinones, obtained, by Scatchard plots are shown



Compound	K_M x I_a 10^{-6}	n[b]	substitution pattern[c]
Doxo--rubicin	4.42 (0.43)[d]	0.19 (0.002)[d]	
2'-(Diethylaminoethylamino)anthraquinones			
1A	1.48 (0.14)	0.20 (0.004)	1-
2A	3.97 (0.18)	0.21 (0.002)	1,5-
3A	1.71 (0.07)	0.20 (0.001)	1,8-
4A	3.17 (0.20)	0.22 (0.006)	1,4-
2'-(Hydroxyethylamino)ethylaminoanthraquinones			
1B	1.03 (0.09)	0.19 (0.001)	1-
2B	3.20 (0.08)	0.22 (0.003)	1,5-
3B	2.35 (0.11)	0.22 (0.002)	1,8-
4B	2.83 (0.10)	0.19 (0.004)	1,4-

a. Affinity constant determined by Scatchard plots.

b. Number of binding sites per DNA phosphate.

c. Substitution pattern of anthraquinones.

d. Standard deviation (n=3).

Table 2 : DNA binding properties of substituted anthraquinones: determination of affinity constants.

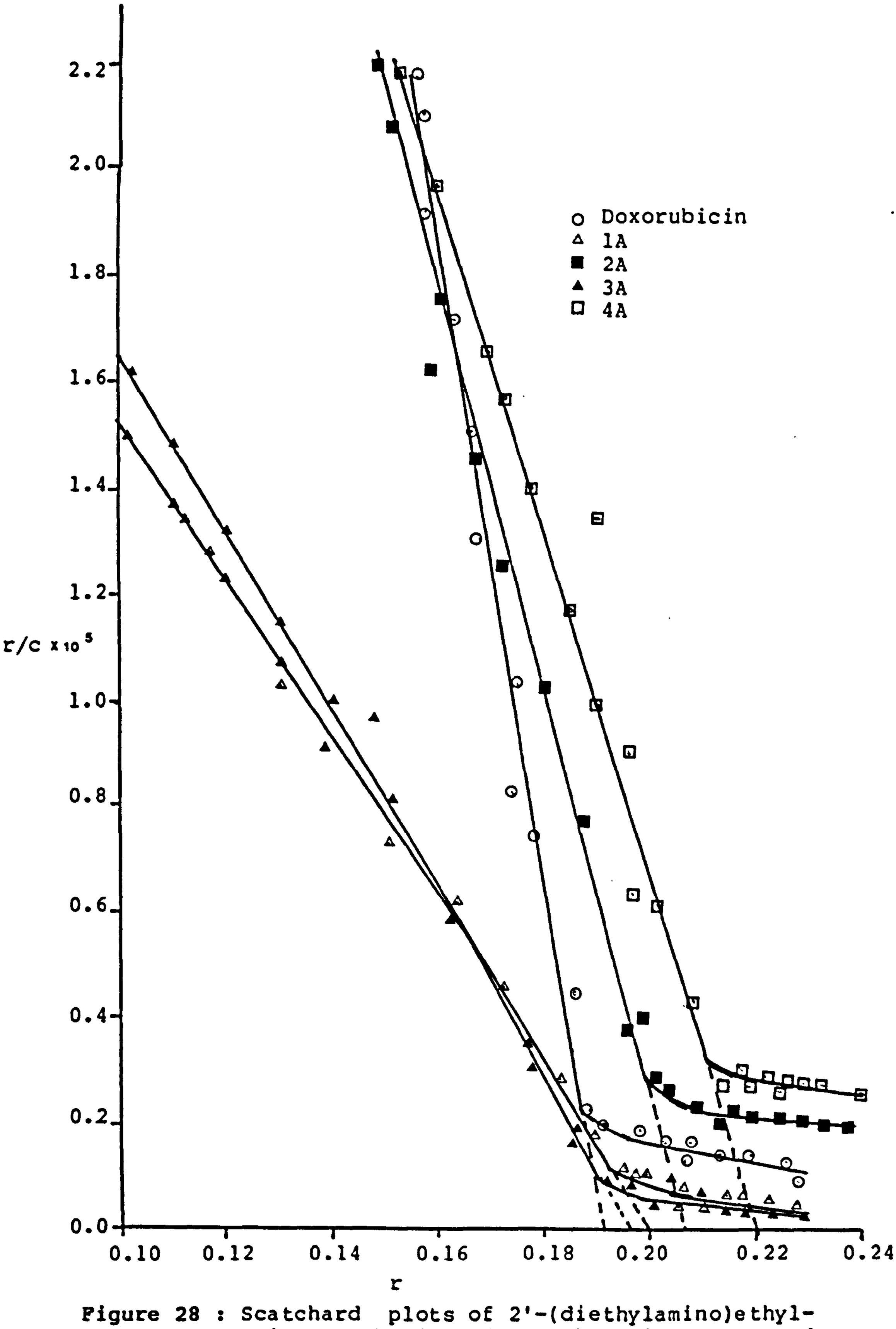


Figure 28 : Scatchard plots of 2'-(diethylamino)ethylamino substituted anthraquinones and doxorubicin.

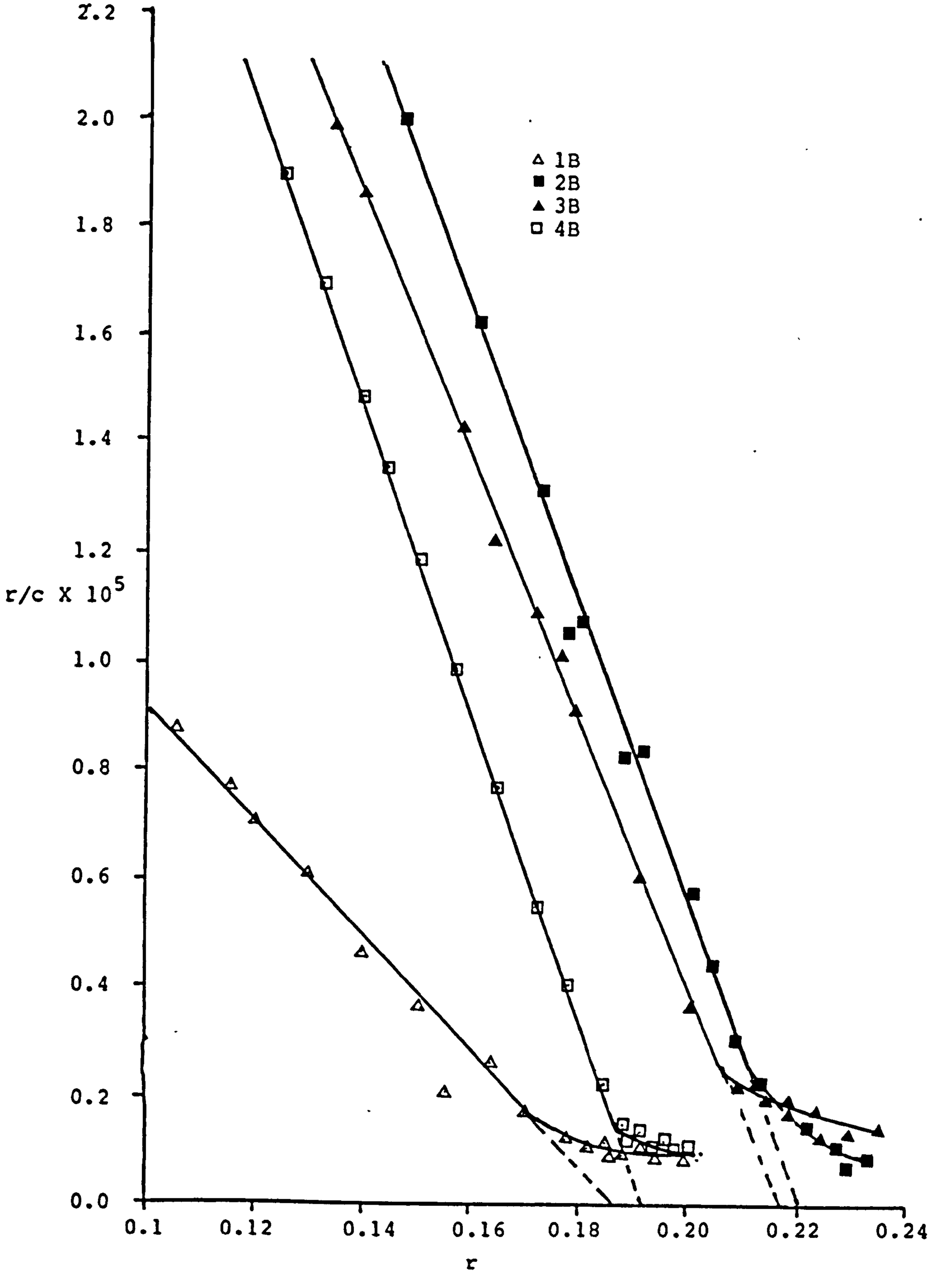


Figure 29 : Scatchard plots of 2'-(hydroxyethyl-amino)ethylamino substituted anthraquinones.

in table 2. These results are consistent with intercalation as a mode interaction of these anthraquinones with DNA. The pro-drugs did not show any significant binding to DNA, as evidenced by the lack of change in the spectral properties of these drugs. Therefore the affinity constants for these compounds could not be determined. From the results obtained it can clearly be seen that all the compounds (1A-4A, 1B-4B), except the prodrugs, bind to DNA with the 1-substituted anthraquinones (compounds 1A and 1B) having the least affinity for DNA, whilst the 1,5-bis-substituted anthraquinones (compounds 2A and 2B) have the highest affinity in each of the series. The order of affinity, for DNA, in both series of compounds is 1,5- > 1,4- > 1,8- > 1-substituted anthraquinones. The 2'-(diethylamino)ethylamino-anthraquinones have consistently higher affinities for DNA than the 2'-(hydroxyethylamino)ethylamino-anthraquinones, except in the case of 1,8-bis-substituted anthraquinones.

Computer graphics modelling studies (performed by S.Islam and S.Neidle, King's College, London.) of the interaction of 2'-(diethylamino)ethylamino-substituted anthraquinones with DNA, showed that these anthraquinones could intercalate into d(CpG) in one of two orientations, either parallel or perpendicular to the long axes of the base pairs. The 1-substituted compound (1A) can intercalate into d(CpG) from either the major or the minor groove (figure 30-32). The more

stable major-groove complex shows binding in the parallel orientation. The 1,8-substituted compound (3A) was shown to only intercalate from the major groove, due to steric hindrance in the minor groove. In this case the compound binds parallel to the long axes of the base pairs (figure 33).

The 1,5-substituted anthraquinone (2A), was shown to bind to DNA by "straddling" across the intercalation site (figure 34). The disubstituted anthraquinone cannot simply be inserted into the intercalation site of d(CpG), since its thickness dimension is about 5.5Å. This would require an initial base pair separation of at least 9Å (5.5 + 3.4A). In order for compound (2A) to intercalate, DNA-breathing (transient base pair unstacking) has to occur to allow the docking of drug molecule into the receptor site. The anthraquinone must first interact with non-base-paired DNA residues, which subsequently hydrogen-bond together. Hence once the anthraquinone has intercalated into DNA, DNA-breathing is required before dissociation can take place. It was shown that prior disruption of base pairs is required not only immediately at, but also adjacent to, the binding site.

The 1,4-substituted anthraquinone (4A) was shown to bind to DNA in a perpendicular orientation with the side chains in the major groove (figure 35). However the compound can also bind to DNA by "straddling" across the intercalation site (figure 36); in this case this is only possible after full geometric minimisation

of the dinucleoside geometry.

To date, computer graphics modelling studies by Neidle and Islam, have been completed for the first series of anthraquinones (compounds (1A)-(4A)). Modelling studies for the interaction of the second series of anthraquinones (compounds (1B)-(4B)) are currently in progress.

Figures 30-36: Computer drawn views of the intercalation model of compounds 1A-4A. In all cases the top view is perpendicular to the base-pair plane, and the other two are perpendicular to this plane. The compounds are shown in dashed lines. The bottom plot is a space-filling representation.

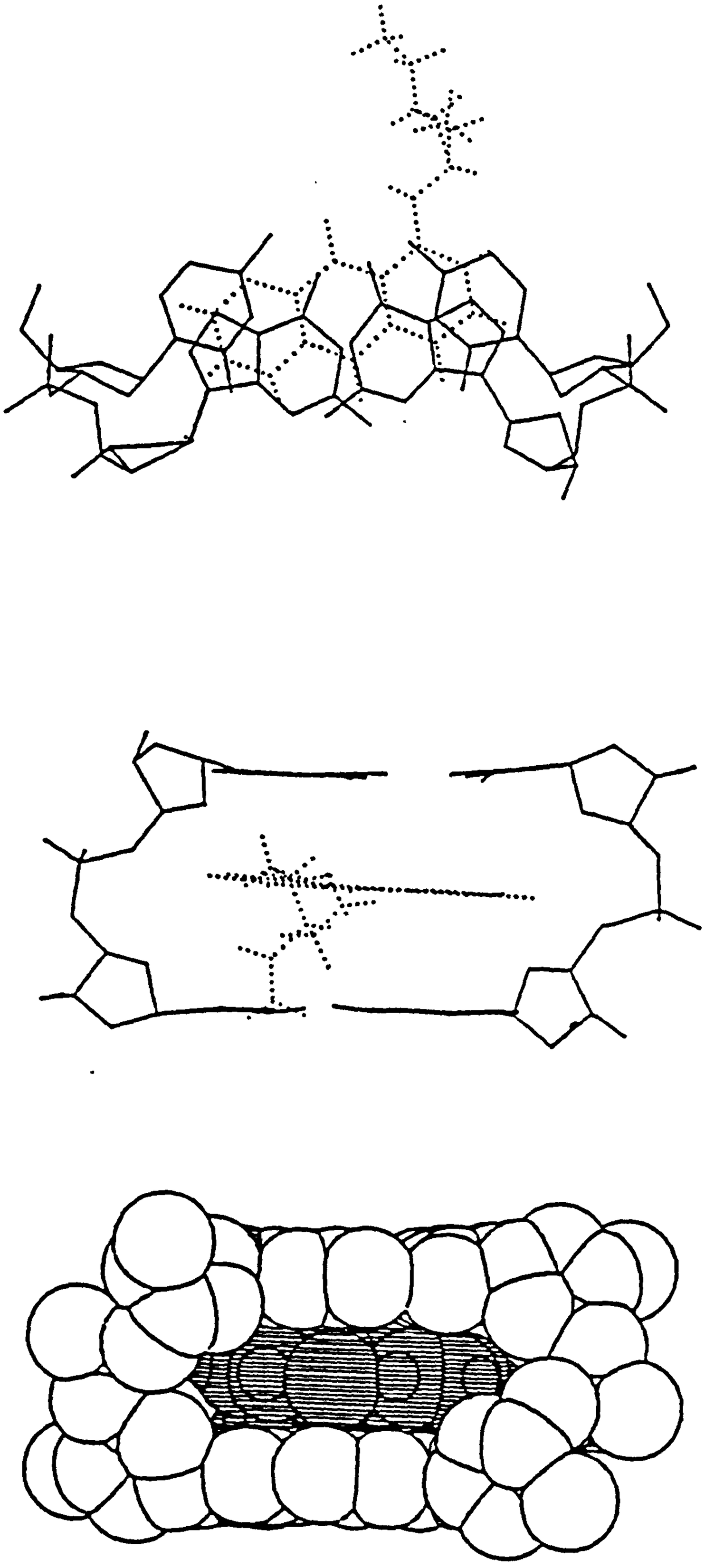


Figure 30 : Intercalation of compound 1A via the major groove of DNA.

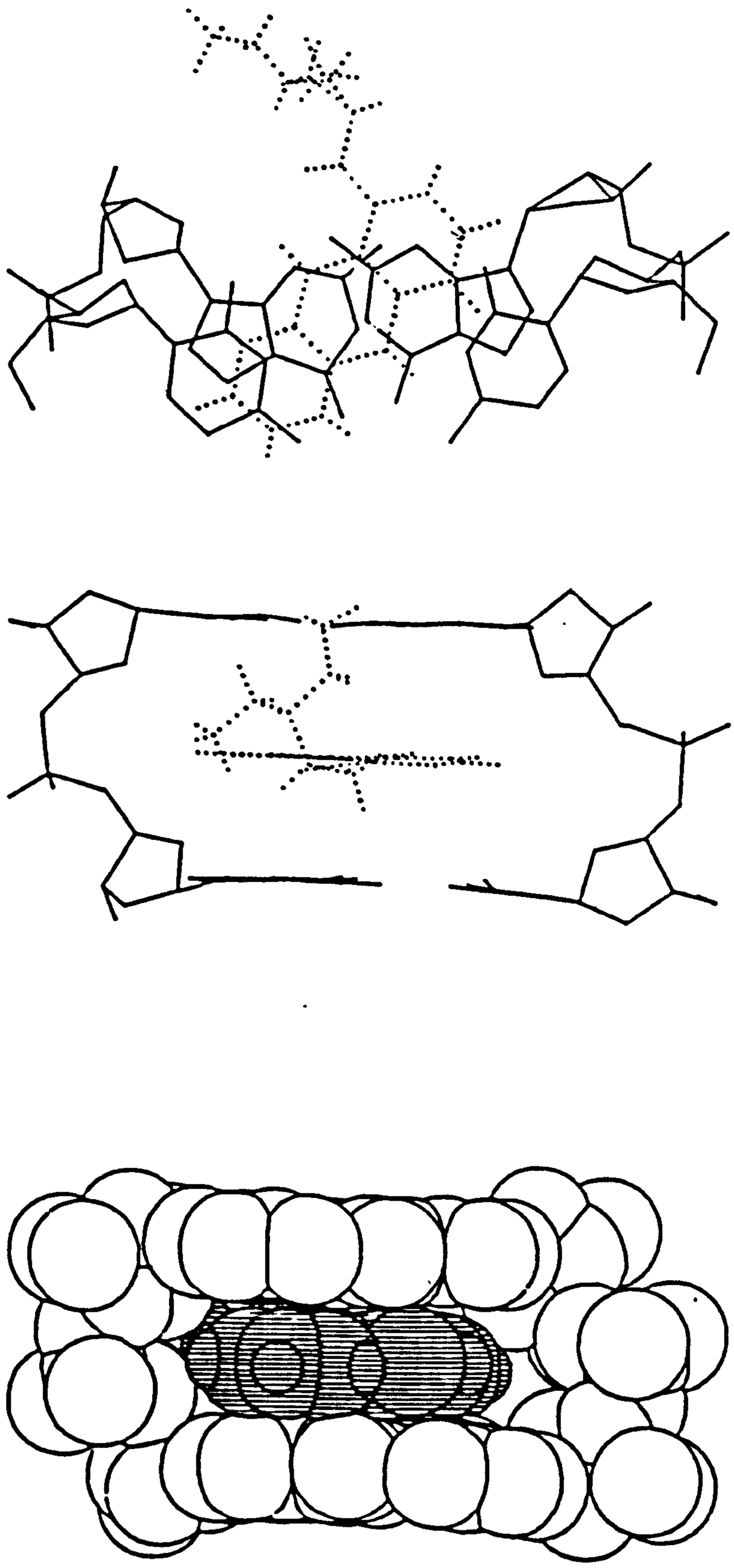


Figure 31 : Intercalation of compound 1A via the minor groove of DNA.

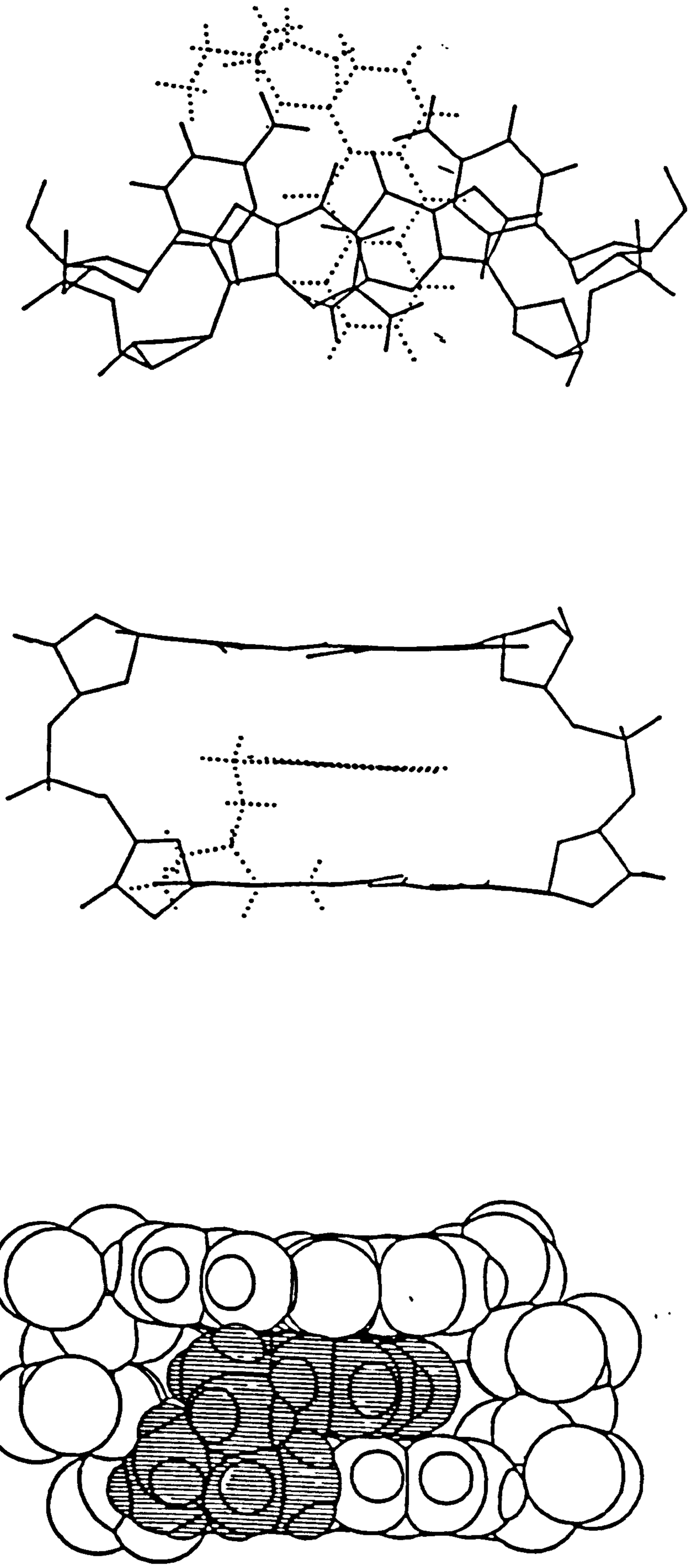


Figure 32 : Intercalation of compound 1A perpendicular to the base pairs.

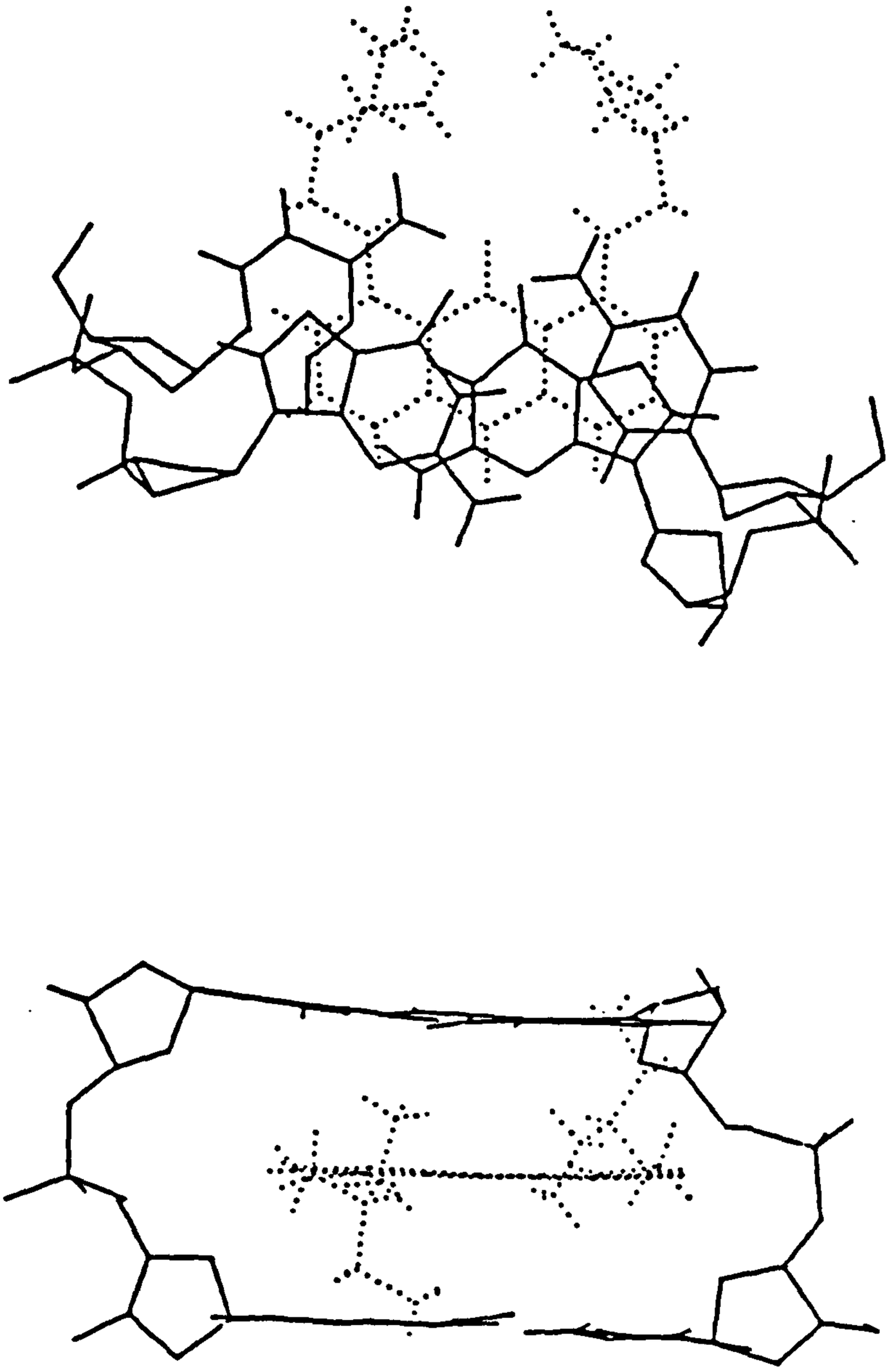
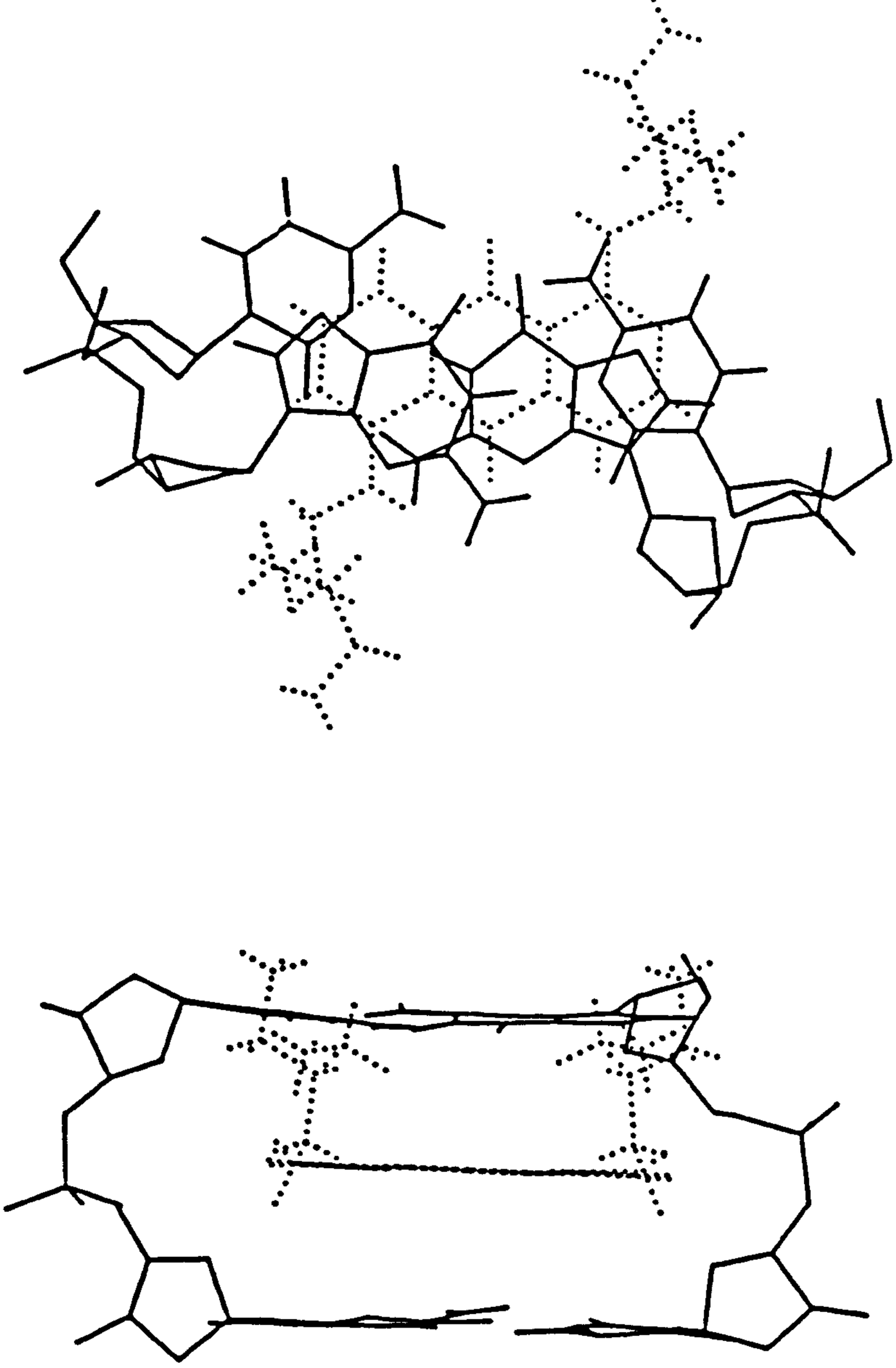


Figure 33 : Intercalation model for compound 3A.



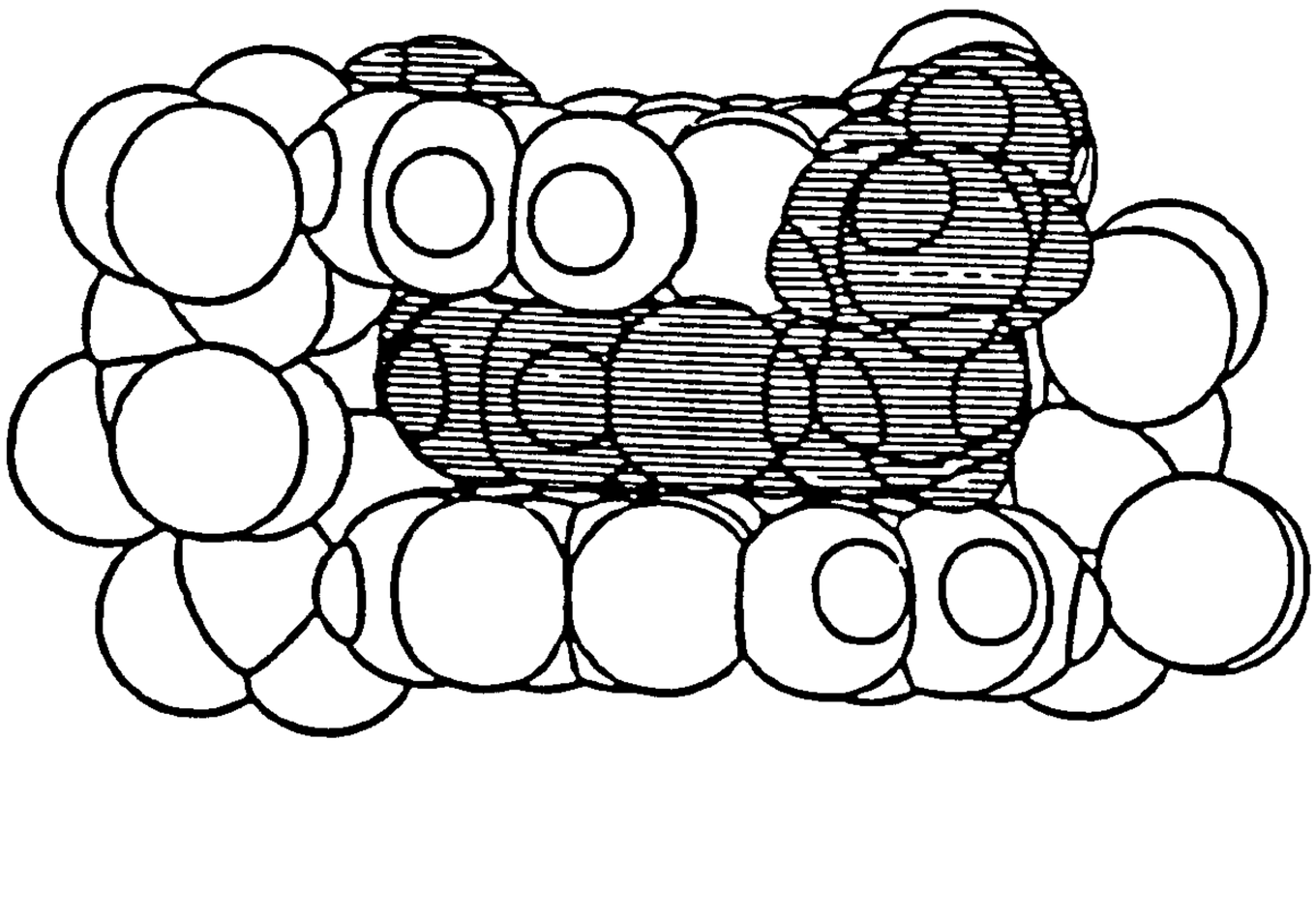
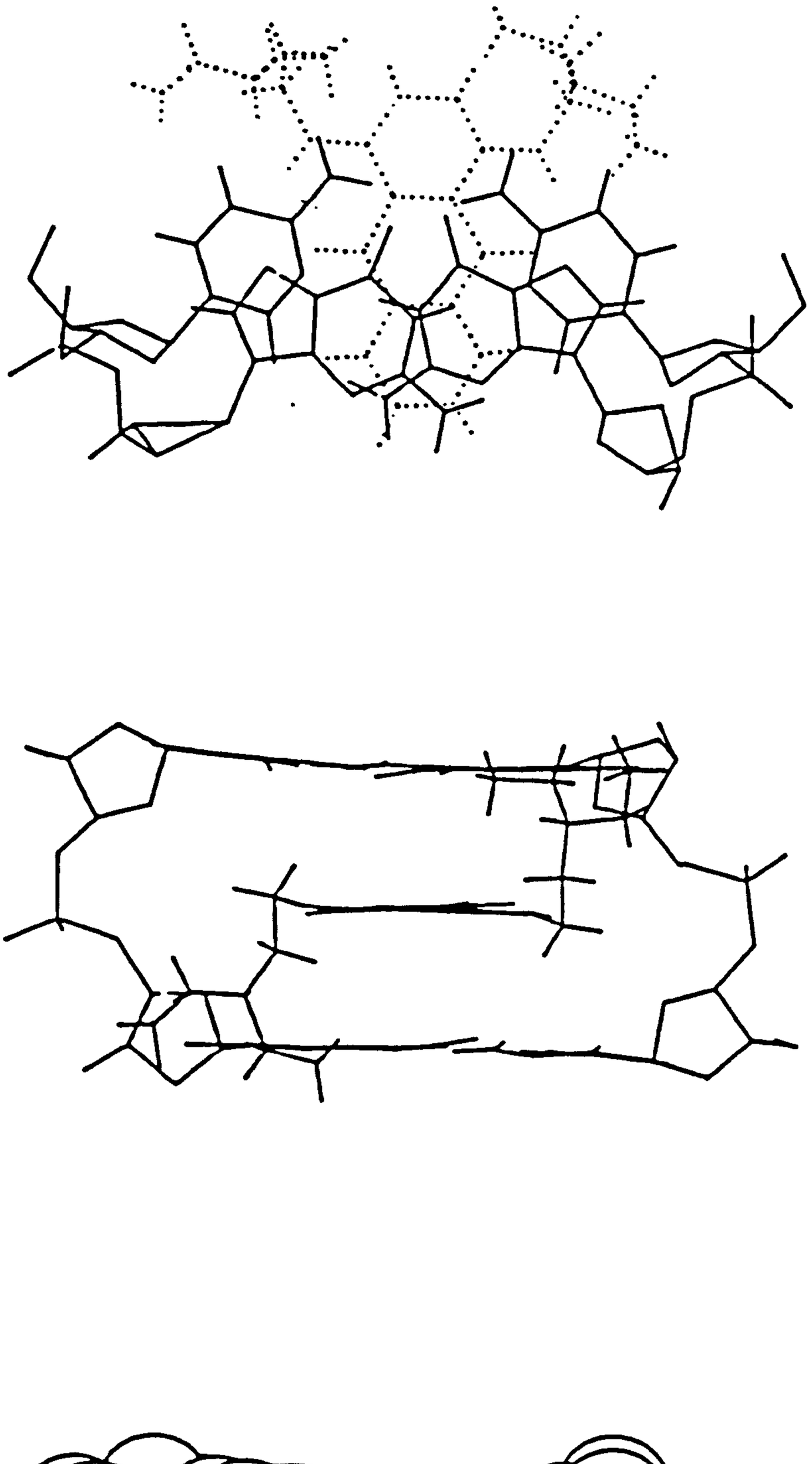


Figure 34 : Intercalation model for compound 2A.



Figures 35 : Major grove intercalation for compound 4A.

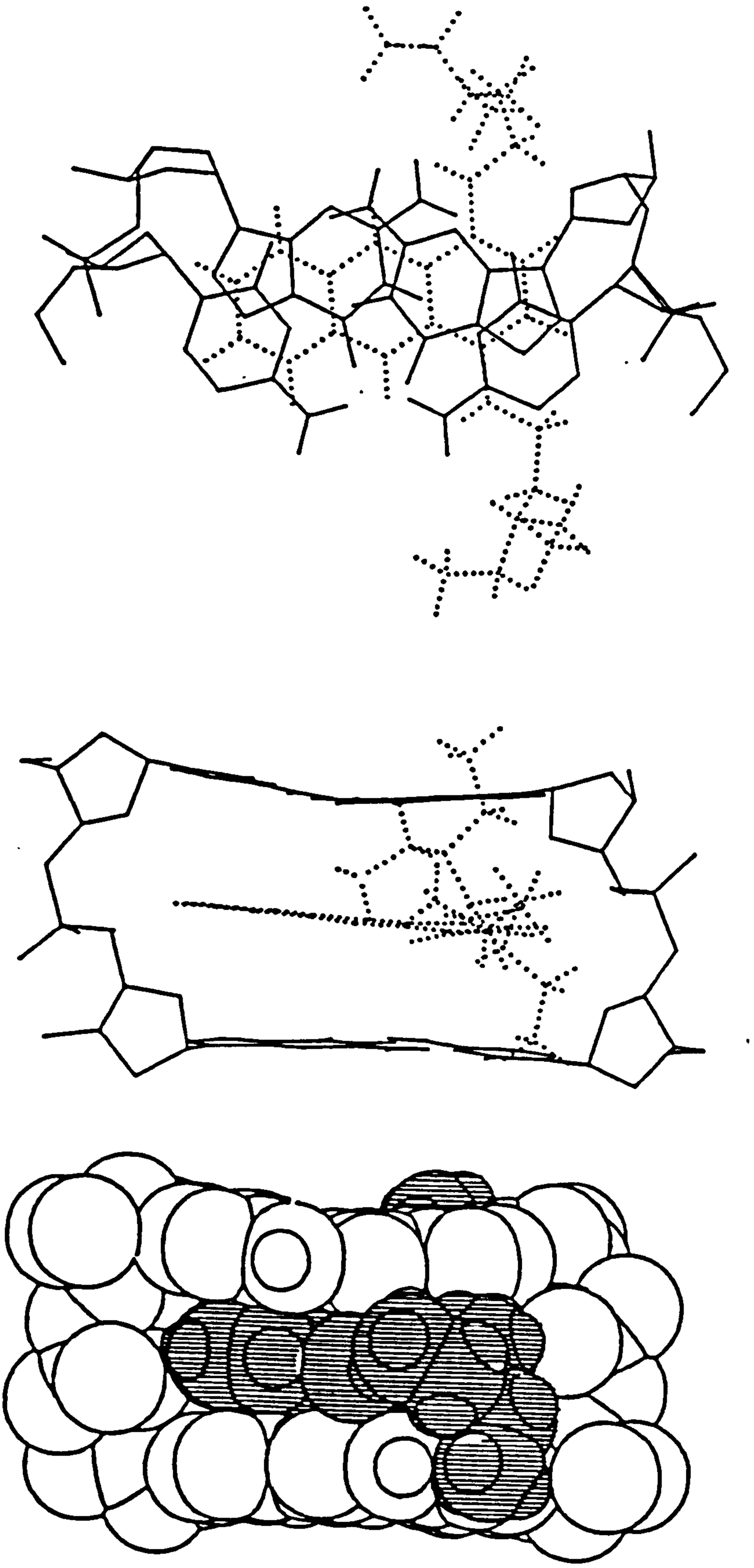


Figure 36 : "Straddle" intercalation model for compound 4A.

These computer graphics modelling studies have shown that the 1,5- and 1,4-disubstituted anthraquinones, (2A) and (4A), would be expected to dissociate less readily from DNA than the 1- or 1,8-disubstituted anthraquinones. In order for compound (2A) (and (4A)) to dissociate from DNA, DNA breathing is required.

The spectrophotometric titration method only gives the affinity constant (K) (the ratio of association (k_1) and dissociation (k_{-1}) constants), for the binding reaction. It would therefore be informative to use a method which gives information on the rate of dissociation of drug from (or association of drug with) the receptor, since this would further validate computer graphics modelling studies. In this work the dissociation of the anthraquinones, 1A-4A and 1B-4B, and the anthracyclines doxorubicin and daunorubicin from DNA were examined.

2.2.1.3. Determination of dissociation rate constants of DNA-anthracyclines and DNA-anthraquinones by stopped-flow spectrophotometry.

Both the anthracyclines and the substituted anthraquinones are ionised at physiological pH so that in addition to the intercalative mode of binding there will be a secondary mode of external binding to the helix, as occurs with other basic intercalating agents, (see 1.6). The equilibrium can thus be represented as:

$$\text{DNA} + \text{Drug} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{extern.}}{\text{DNA--boundDRUG}} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{intercal.}}{\text{DNA-Drug.}}$$

In support of this, T-jump and stopped flow studies with daunorubicin and polynucleotides and nucleic acids show a biexponential decay pattern (Grant and Phillips, 1979; Forster et al.,1980). In this study all solutions were prepared in tris (0.015M) NaCl (0.2M) buffer pH 7.4, since at this ionic strength only the intercalation mode of binding should be present.

The dissociation rate constant for a particular DNA/drug complex with was determined at 20°, 25° and 37°C using sodium dodecyl sulphate (SDS) to disrupt the DNA-drug complex. This agent has been used previously to disrupt DNA/drug intercalated systems, (Grant & Phillips, 1979; Muller & Crothers, 1968; and Wilson et al., 1976) including daunorubicin/DNA complexes (Grant & Phillips, 1979 and Wilson et al., 1976). In one study it was confirmed that alteration of the SDS concentration had minimal effect ($\pm$3%) on dissociation rate constant (Wilson et al., 1976). The reaction can be represented as:

$$\text{DNA-Drug} \xrightarrow{k_d} \text{DNA + Drug}$$

$$\text{SDS+DRUG} \xrightarrow{k_s} \text{SDS-Drug}$$

where k_d is the first order dissociation rate constant. The DNA-drug dissociation is the rate controlling step since the sequestering of drug with SDS is a diffusion controlled process. It is not possible, by analogy with the rate constants for other intercalating systems, to unambiguously determine whether the measured rate constant, k_d, is identical to k_{-2} of the two-step mechanism.

Drug dissociation from DNA was monitored by recording the increase in extinction at the λmax of free drug, over a period of about 6 half lives. In each case at least eight data points were collected and fitted to both a single exponential, and a biexponential equation using a microcomputer programme. In all cases the data conformed to a single exponential curve with a correlation coefficient >0.99; the data did conform to a biexponential model (R< 0.8). Hence this confirms the suitability of the ionic strength of the solution.

The dissociation of anthracyclines and anthraquinones from DNA was shown to conform to first order kinetics for more than four half-lives for all complexes under these conditions. The first-order rate plots for the dissociation of DNA-daunorubicin, DNA-doxorubicin and DNA-anthraquinone (1A-4A and 1B-4B) complexes at 20°C, are shown in figures 37 and 38. The results have also been summarised for this and other temperatures (25 and 37°C) in table 3. In all cases there was an increase in the rate constant with temperature. The ranking order for the dissociation rate constants, of the substituted anthraquinone-DNA complexes was the same at 20, 25, and 37°C. Measurements were taken at 37°C to ensure that the order is not changed at physiological temperatures. All reactions were studied at 20°C because this enabled a better reproducibility of the data than at higher temperatures. Dissociation rate constants were also

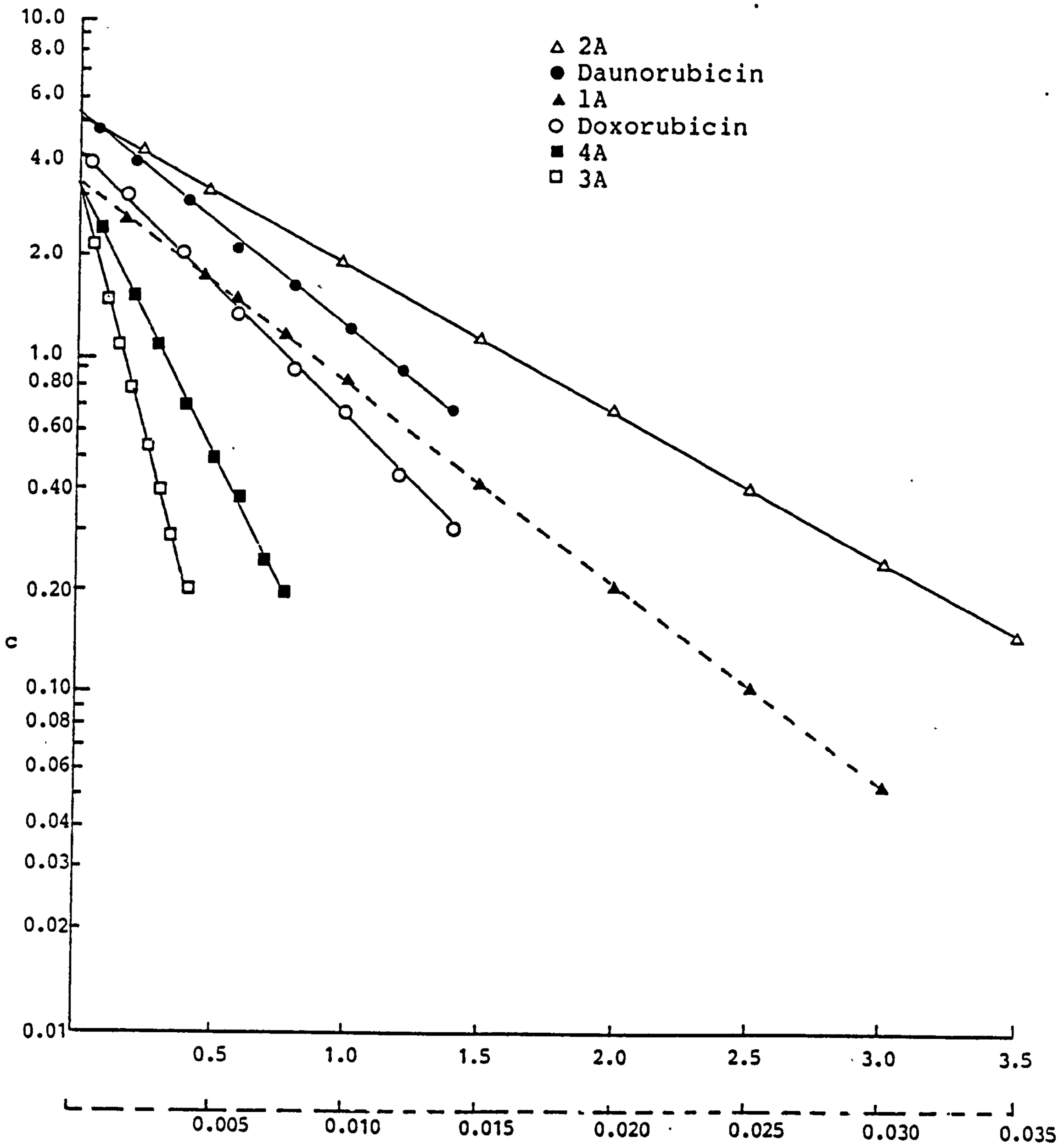


Figure 37 : First order dissociation plots of 2'-(diethylamino)ethylamino anthraquinones and anthracyclines from calf thymus DNA.

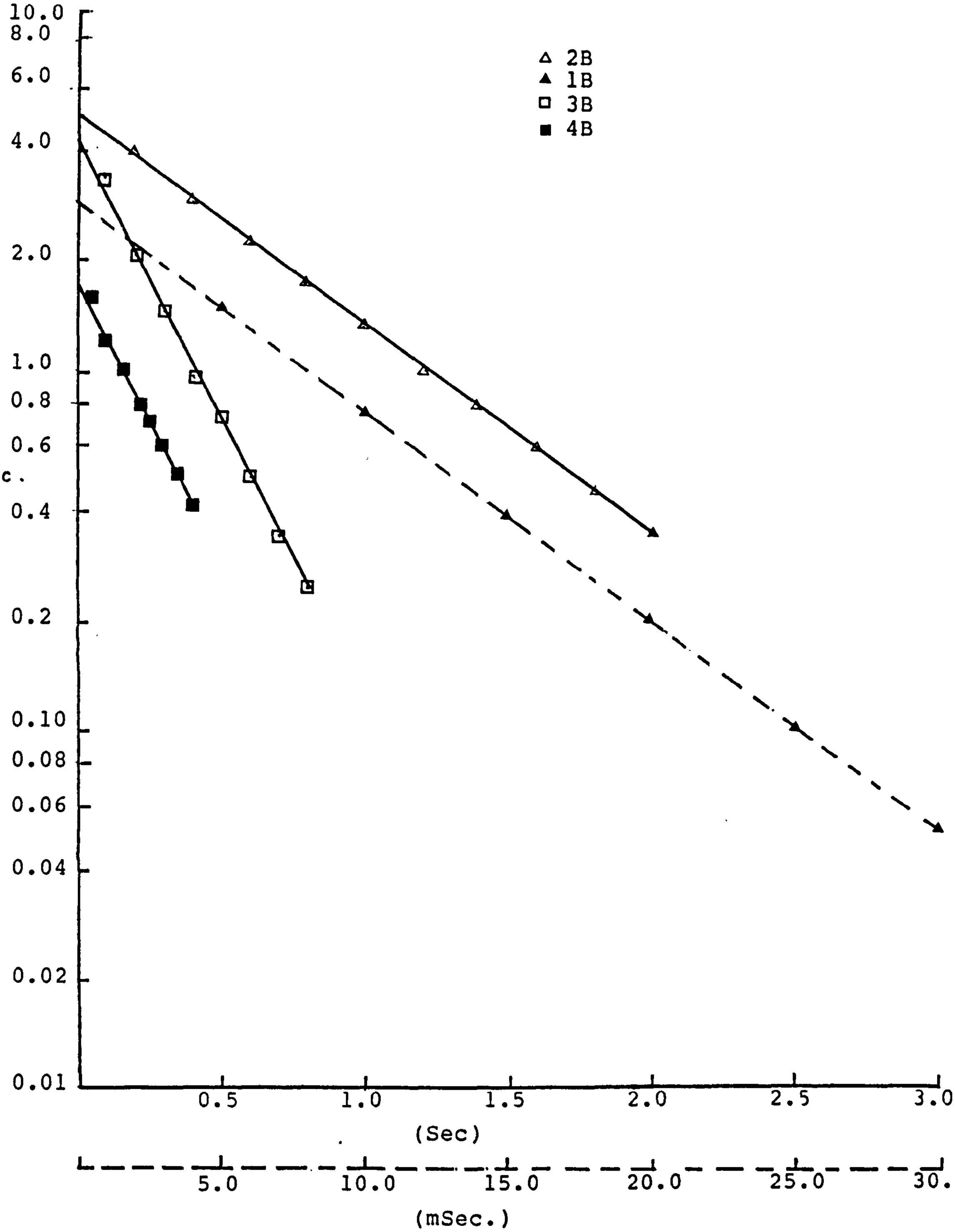


Figure 38 : First order dissociation plots of 2'-(hydroxyethylamino)ethylamino anthraquinones from calf thymus DNA.

DRUG	Dissociation rate constant S^{-1} 20°C	25°C	37°C	DRUG CONCN.[1] Mx 10^5
Daun.[2]	1.43 (0.17)[3]	2.17 (0.2)	10.93 (0.76)	3.43
Doxo.[2]	1.84 (0.19)	2.07 (0.87)	9.87 (1.02)	3.45
1A	>150	-	-	3.42
2A	0.91 (0.12)	1.8 (0.05)	7.50 (0.3)	3.05
3A	6.99 (0.52)	17.26 (1.2)	47.97 (5.6)	3.741
4A	3.73 (0.14)	7.5 (0.38)	35.66 (3.30)	3.198
1B	>127	-	-	3.332
2B	1.39 (0.13)	1.93 (0.7)	19.61 (2.74)	3.874
3B	3.69 (0.42)	7.08 (0.46)	24.28 (1.41)	3.663
4B	3.82 (0.34)	6.02 (0.7)	19.61 (2.74)	3.874

[1] Final drug concn. after mixing with SDS =0.5 X stated concn. Final SDS concn in all cases 0.1%.

[2] The values for daunorubicin (I) and doxorubicin (II) are not significantly different ($p > 0.99$, $n=5$).

[3] Standard deviation ($n=5$)

Table 3: Dissociation rate constants for DNA complexes of anthracyclines and substituted anthraquinones.

measured at 25°C as this is standard temperature for physicochemical studies.

The dissociation rate constants for daunorubicin/DNA complexes are of the same order as those obtained by previous workers for polydeoxynucleotide-daunorubicin (3.3 S^{-1} at 20 and 27.0 S^{-1} at 37°C) and for salmon sperm DNA-daunorubicin complexes (2.88 S^{-1} at 15°C) (Grant & Phillips, 1979; and Wilson et al., 1976).

The dissociation rate constants for DNA complexes with disubstituted anthraquinones were of the same order as for anthracycline complexes. In the case of the monosubstituted anthraquinones, (1A) and (1B) the dissociation rate constants were greater than 120 S^{-1} at 20°C and could not be measured accurately at higher temperatures. The dissociation rate constants for the 1,8-disubstituted compounds (3A & 3B) were significantly lower than for the 1- substituted derivatives (1A & 1B) but were greater than for the 1,4-bis-substituted anthraquinones (4A & 4B). The 1,5-bis-substituted anthraquinones had the lowest dissociation rate constants, 0.91 and 1.39 S^{-1} respectively for 2A and 2B compared to 3.73 and 3.82 S^{-1} for the 1,4-disubstituted anthraquinones (compounds 4A and 4B respectively) even though the affinity constants for these compounds were shown to be similar. It is not possible to calculate the association rate constants (k_2) from the determined affinity constants as the buffer used here was of a higher ionic strength

than that used in the determination of the affinity constants (0.2M NaCl compared to 0.05M NaCl).

As seen earlier it was shown from computer graphics modelling, (performed by S.A.Islam and S.Neidle), of the interaction of anthraquinones with the self-complementary d(CpG) dinucleotide, that in order to accomodate the 1,5-disubstituted anthraquinone (2A), DNA-breathing (transient base pair unstacking) has to occur to allow the docking of drug molecule into the receptor site. Hence once the anthraquinone has intercalated into DNA, DNA-breathing is required before dissociation can take place. This is not necessary with the other compounds, though compound 4A can also bind in this manner as well, so explaining the very slow dissociation of the DNA/(2A) complex compared to the DNA complexes with 1-; 1,8-; or 1,4-bis-substituted anthraquinones and the anthracyclines.

As mentioned earlier the effect of DNA on the fluorescence properties of the drug can be examined to show that a particular drug intercalates into DNA. The compounds synthesised in this work were found to exhibit insufficient fluorescence for DNA-drug binding studies. However by using a compound which is known to intercalate into DNA and which has a large change in quantum yield on release from DNA (such as ethidium bromide), it was possible to examine the DNA binding of the compounds synthesised in this work, by fluorescence spectroscopy by competitive displacement of ethidium from DNA. This type of competitive study gives

information on both the nature of interaction of drugs with DNA and the relative affinity of the drugs to DNA. The effect of compounds, 1A-4A, 1B-4B, 2C-3C and 2Ci-3Ci, on the binding of ethidium to DNA will now be discussed.

2.2.2. Effect of drug on the binding of ethidium to DNA.

The interaction of ethidium bromide with DNA has been extensively reviewed, (Waring, 1981, Yielding et al., 1983). Ethidium bromide has been shown to bind to DNA by intercalation. When binding to DNA the quantum yield of fluorescence of ethidium is enhanced (Paoletti,and Le Pecq, 1971). Hence if a compound binds to the same site in the DNA helix as ethidium the drug will compete with ethidium for that site. Providing the compound, in the absence of DNA, has no effect on the fluorescence of ethidium it is possible to monitor the effect of that compound on fluorescence enhancement.

If ethidium (or another intercalating agent) bound to DNA is irradiatiated with polarised light, the fluorescence emitted will be polarised. This fluorescence polarisation is due to a reduced rotational movement of the drug, however in the absence of DNA the fluorescence will be depolarised. If another drug also binds to the same intercalation site, it will displace ethidium and therefore the polarisation of fluorescence will be reduced. This property can be used to quantify the relative differences in the affinities

of drugs for DNA, and also to show that an intercalative mode of binding occurs.

The effect of compounds (1A-4A, 1B-4B, 2C-3C and 2Ci-3Ci) on the fluorescence enhancement of ethidium bound to DNA will be discussed first. Then the effect of these compounds on the polarisation of fluorescence of ethidium bound to DNA will be examined.

2.2.2.1. The effect of drug on the fluorescence enhancement of ethidium bromide due to binding to DNA.

The ability of the substituted anthraquinones (compounds 1A-4A, 1B-4B, 2C-3C and 2Ci-3Ci) to displace intercalated ethidium bromide was investigated. Aliquots of ethidium bromide solution were added sequentially to a 2.0ml volume of, each of the following solutions; calf thymus DNA in buffer; calf thymus DNA and drug in buffer (10:1 DNAp:drug ratio); drug in buffer; and buffer (for details of experimental conditions see 3.2.2.1). The fluorescence of each solution was recorded after each addition, at 596nm (excitation 476nm) at 25°C.

For each compound investigated fluorescence reading vs ethidium:DNA ratio was plotted (figures 39-41). These plots show that fluorescence enhancement, and hence DNA-binding, of ethidium is reduced when compounds (1A-4A, 1B-4B and 2Ci-3Ci) are also bound to DNA. However in the case of pro-drugs (compounds 2C-3C) there was no significant change in the fluorescence enhancement due to ethidium binding to DNA, indicating

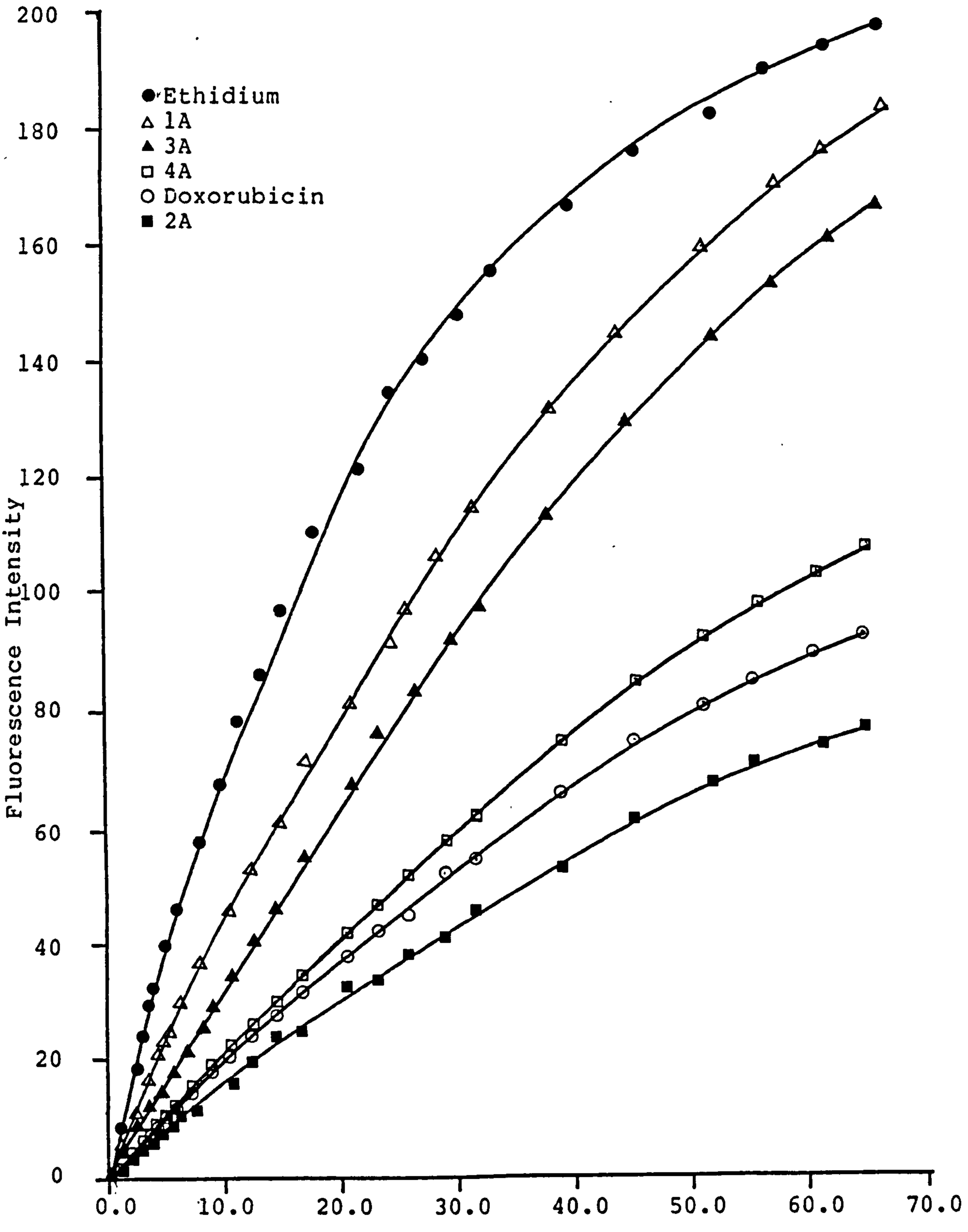


Figure 39 : Effect of doxorubicin and 2'-(diethylamino)ethylamino-substituted anthraquinones (1A-4A) on the fluorescence enhancement of ethidium binding to DNA.

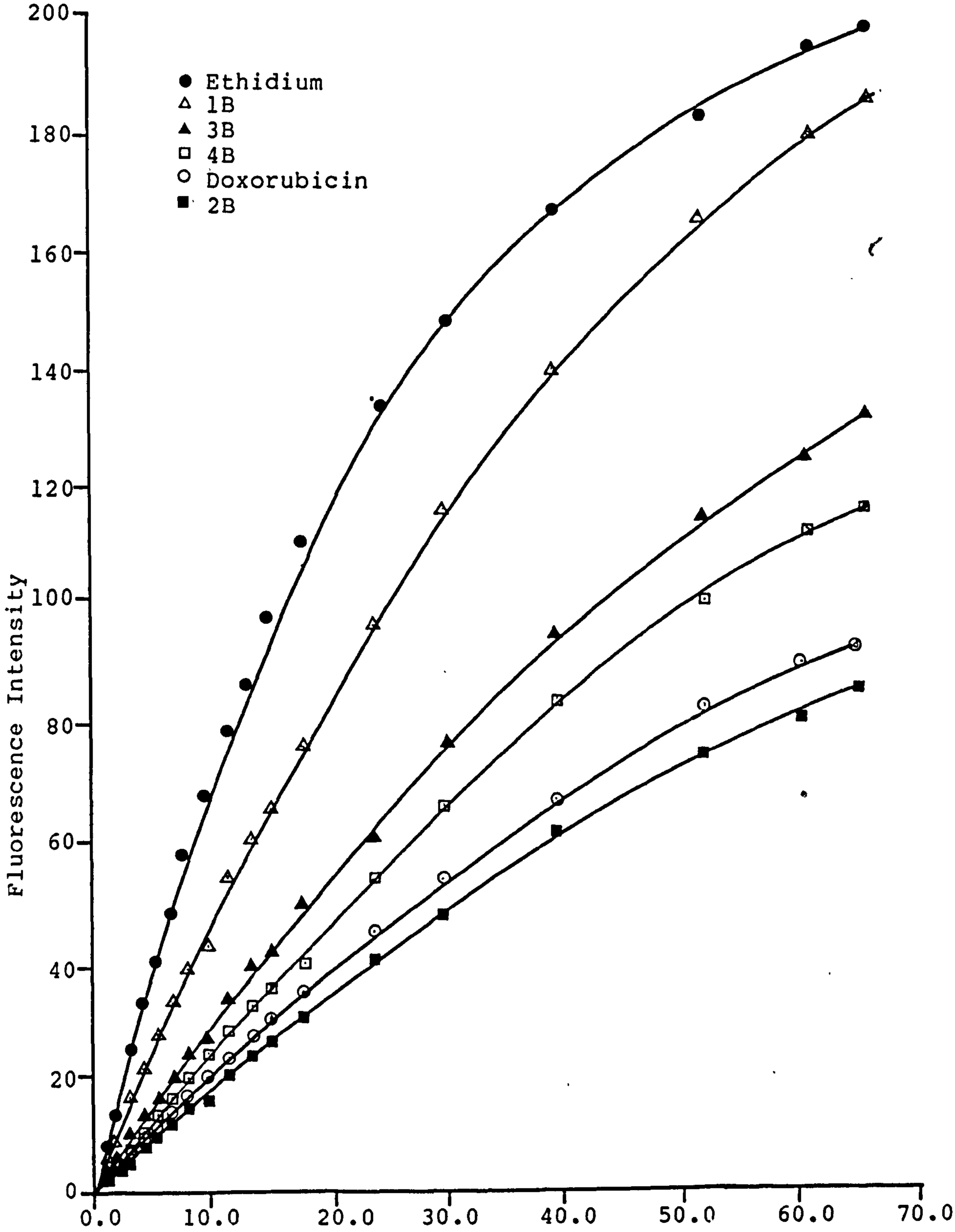


Figure 40 : Effect of doxorubicin and 2'-(hydroxyethylamino)ethylamino-substituted anthraquinones (1B-4B) on the fluorescence enhancement of ethidium binding to DNA.

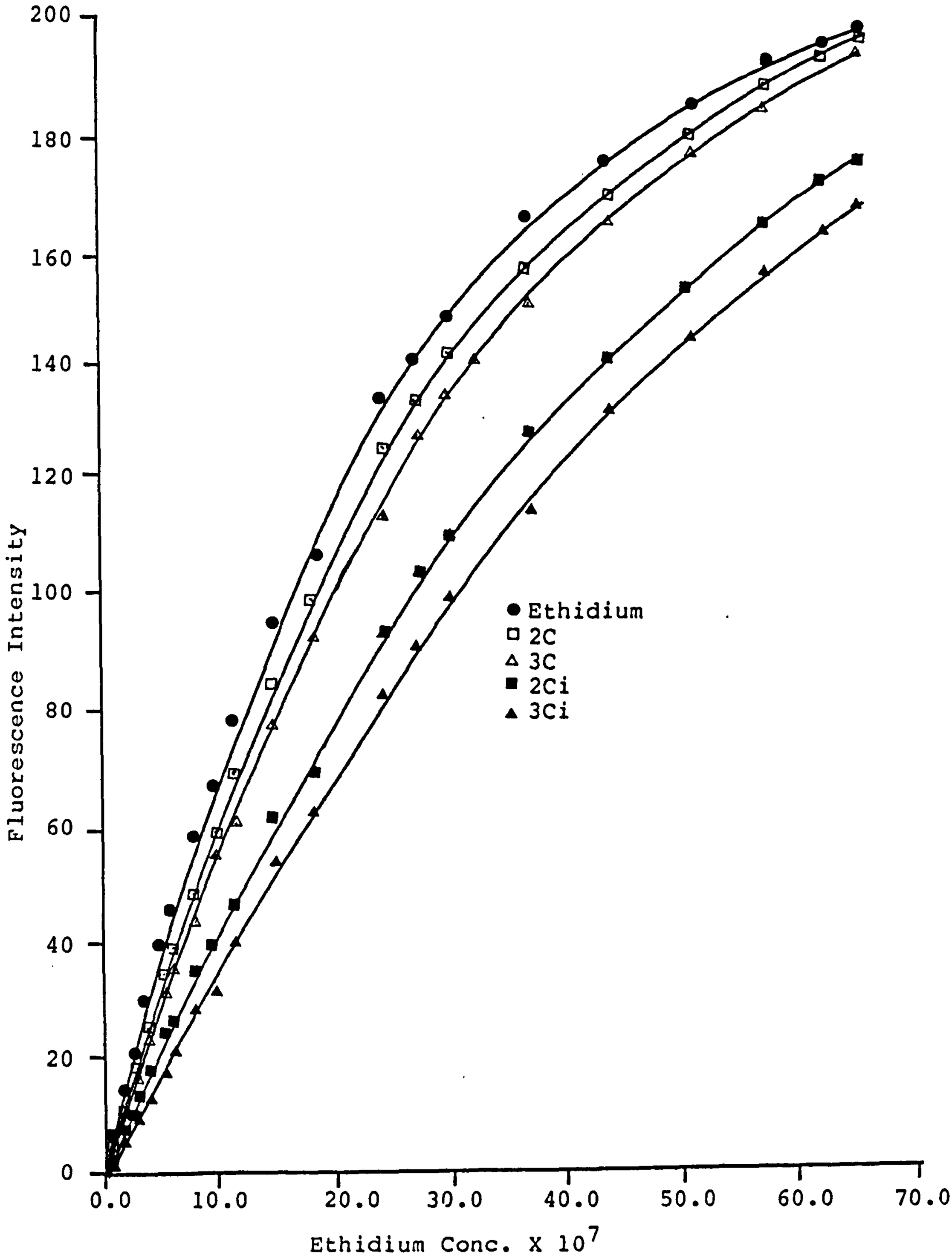


Figure 41 : Effect of prodrugs (2C & 3C) and their "metabolites" (2Ci & 3Ci) on the fluorescence enhancement of ethidium binding to DNA.

that the pro-drugs do not bind to DNA.

The monosubstituted anthraquinones (1A & 1B) had the least effect on the fluorescence enhancement, whilst the 1,5-substituted anthraquinones (2A & 2B) caused a maximal change. These results show that substituted anthraquinones are able to displace ethidium from its binding (intercalation) site.

It has recently been shown that certain intercalating agents (for example m-AMSA), reduce the fluorescence of ethidium bound to DNA without physically displacing it (Baguley & Le Bret, 1984). This reduction in fluorescence intensity may be as a result of reversible formation of electron-transfer complexes between the intercalating drug and the excited state of ethidium. Therefore in order to distinguish between ethidium displacement and formation of electron-transfer complexes a more selective method was also used in this work.

When a molecule binds to DNA, by intercalation, the molecule is constrained in its orientation in the time between exitation and fluorescence. If a solution of DNA-drug complex is irradiated with plane polarised light, fluorescence emmitted by the drug molecule will also be polarised. Therefore the effect of varying DNA concentrations on the polarisation of ethidium was investigated.

Aliquots of DNA solution were sequentially added to each of three separate cuvettes containing ethidium

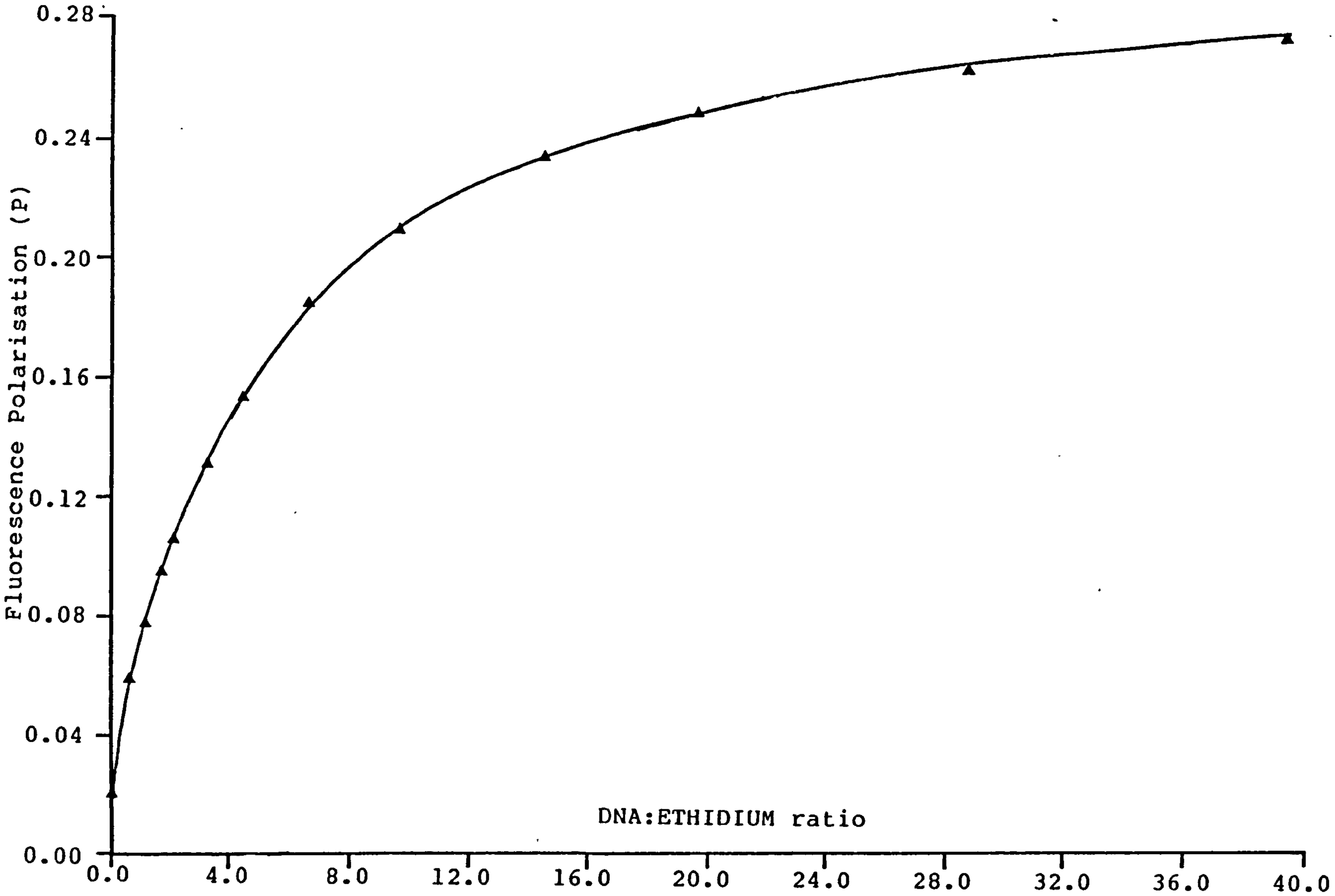


Figure 42 : Effect of DNA on the fluorescence polarisation of ethidium.

bromide and also to one cuvette containing buffer. After each addition the polarisation, P, of fluorescence was measured, (see 3.2.2.2 for details). A graph of polarisation (P) vs DNA:ethidium ratio was plotted (figure 42). From this graph it can be seen that the polarisation increases with increasing DNA:ethidium ratio up to a maximal value (P=0.26), at a DNA:ethidium ratio of 6:1. At DNA:ethidium ratios of greater than 6:1 all the ethidium will be bound to DNA and the DNA will therefore have some unoccupied sites. However, at low DNA:ethidium ratio (less than 4.5:1) all the binding sites are occupied by ethidium, therefore if another intercalating agent is added some of the ethidium will be displaced by the drug. This property may be utilised in quantifying the relative differences in the affinity of drugs to DNA.

2.2.2.2. The effect of drug on the fluorescence polarisation of ethidium bound to DNA.

Aliquots of drug solution were added, to each of eleven flasks containing DNA:ethidium (4:1) solution, to give a range of concentrations. Aliquots of drug solution were similarly added to controls containing ethidium only. Fluorescence polarisation of solution in each of the flasks was determined and the % of ethidium bound to DNA calculated from:

$$\%\text{binding} = \frac{P_{sample} - P_{control}}{P_{max}} \times 100$$

where P_{sample} is the polarisation of DNA+ethidium+drug, $P_{control}$ is the polarisation of drug+ethidium and P_{max}

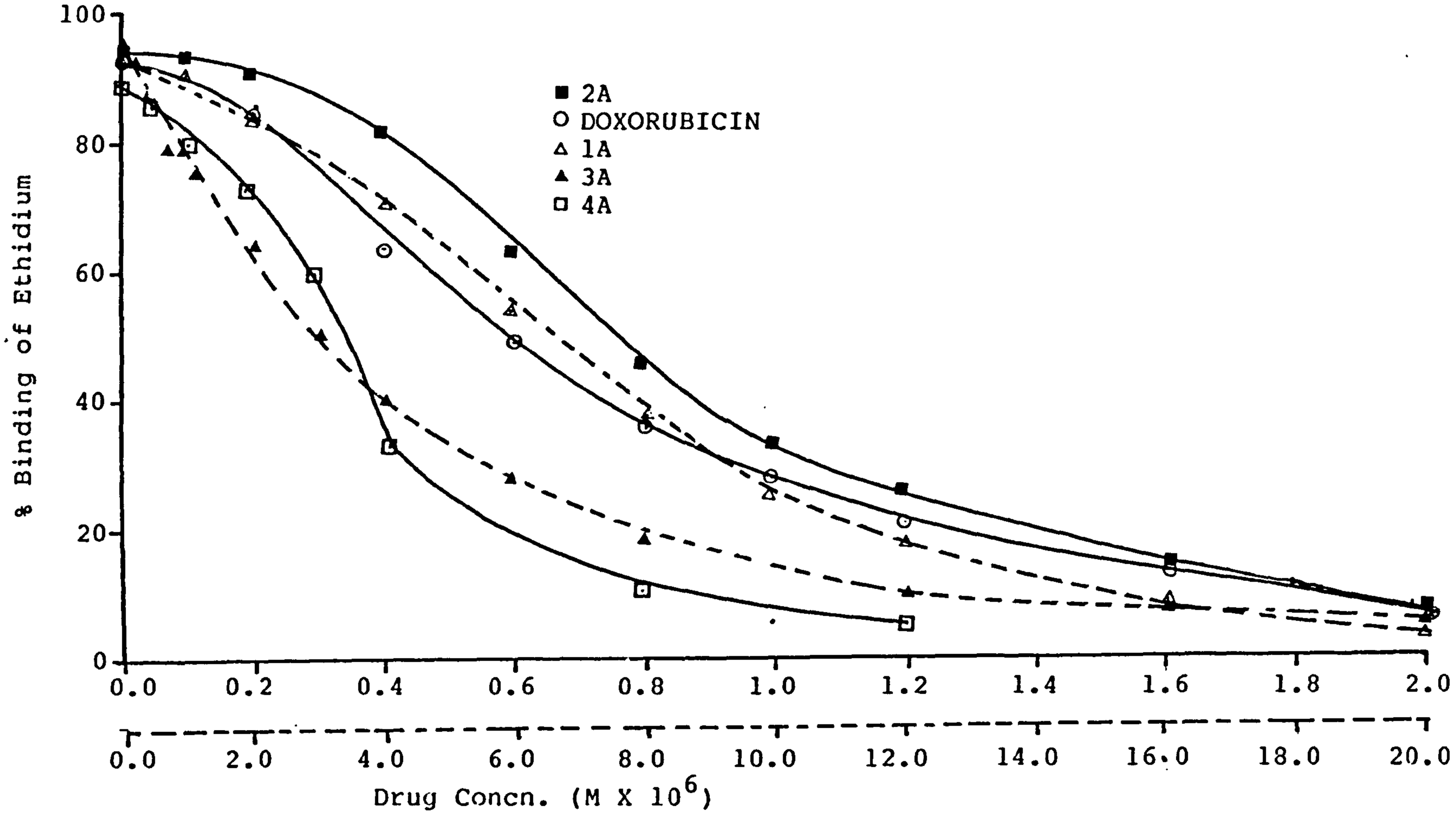


Figure 43 : Displacement of ethidium bromide by doxorubicin and 2'-(diethylamino)ethylamino- substituted anthraquinones (1A-4A).

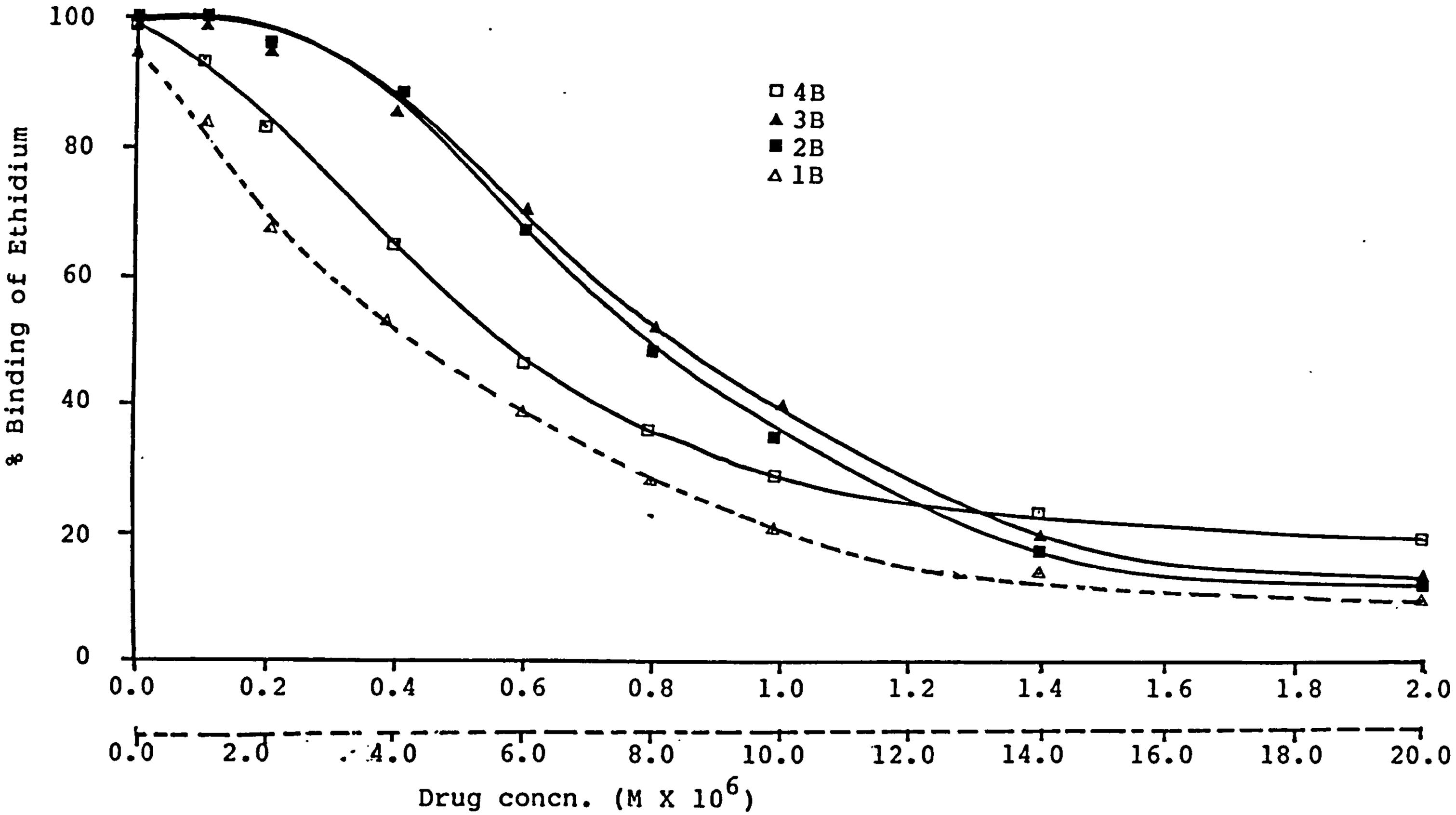


Figure 44 : Displacement of ethidium bromide by 2'-(hydroxyethylamino)ethylamino-substituted anthraquinones (1B-4B).

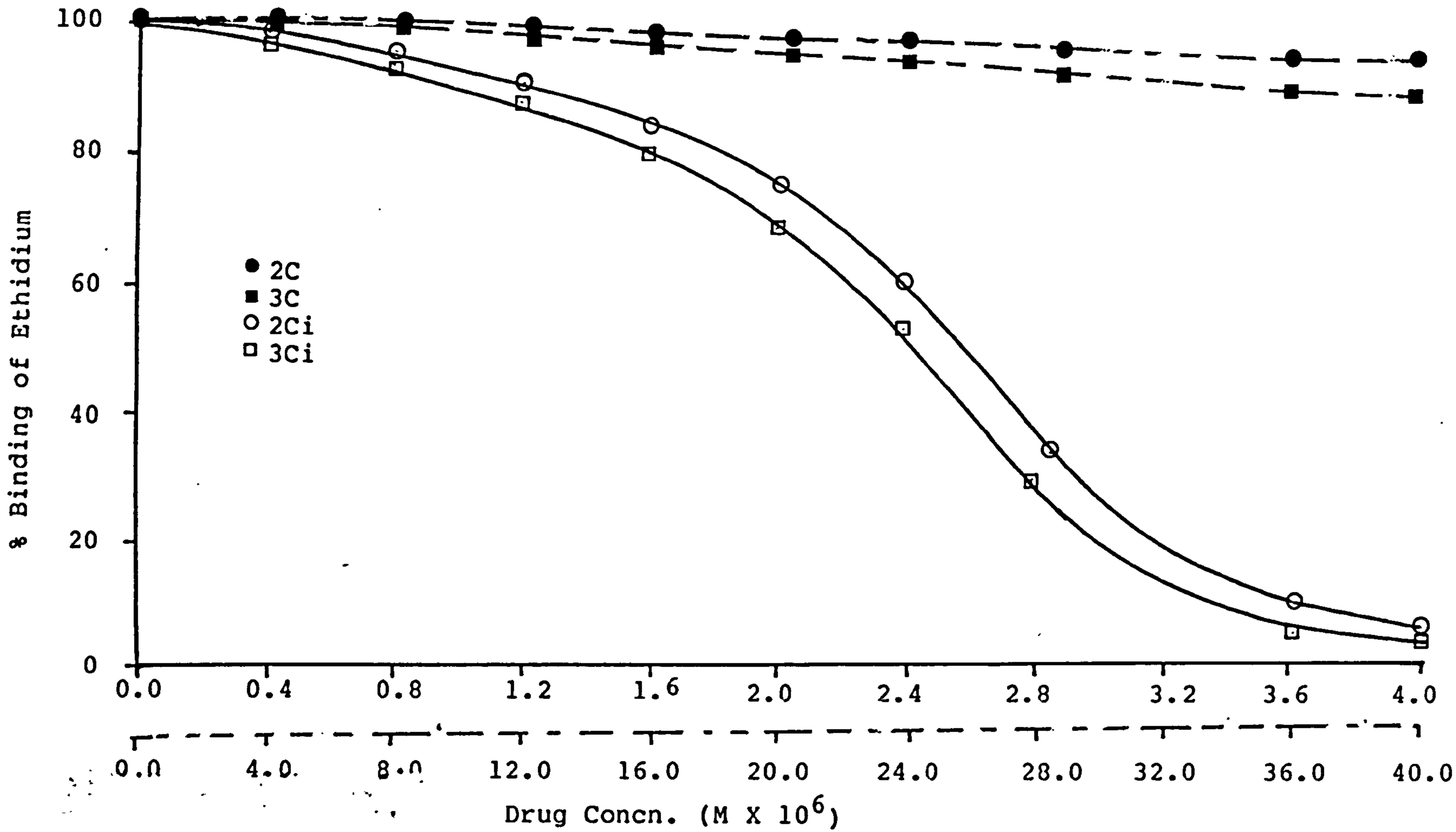
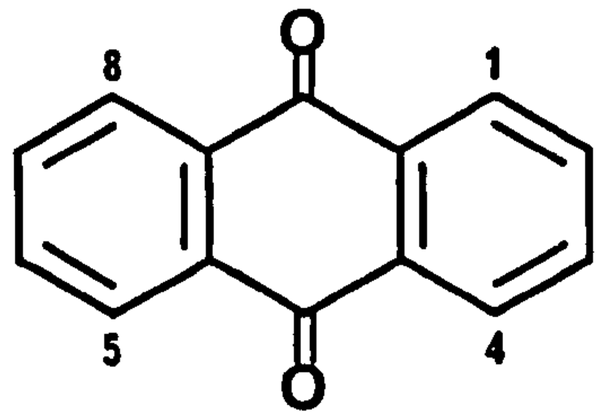


Figure 45 : Comparision of displacement of ethidium bromide by prodrugs (2C-3C) and their "metabolites" (2Ci-3Ci).



Compound	substitution pattern	Concentration required to displace 50% of ethidium ($M \times 10^6$)
Doxorubicin	-	0.70 (0.02)[1]
2'-(Diethylamino)ethylaminoanthraquinones		
1A	1-	7.50 (0.05)
2A	1,5-	0.85 (0.01)
3A	1,8-	3.50 (0.04)
4A	1,4-	0.35 (0.02)
2'-(Hydroxyethylamino)ethylaminoanthraquinones		
1B	1-	5.00 (0.08)
2B	1,5-	0.90 (0.02)
3B	1,8-	0.90 (0.03)
4B	1,4-	0.50 (0.01)
Prodrugs and "metabolites"		
2Ci	"metabolite"	2.60 (0.05)
2C	prodrug	>40.0 (-)
3Ci	"metabolite"	2.50 (0.04)
3C	prodrug	>40.0 (-)

1 Standard deviation (n = 9).

Table 4 : Displacement of ethidium bromide by doxorubicin, substituted anthraquinones, and prodrugs.

is the polarisation of DNA+ethidium (4:1 ratio).

The experiment was repeated at least nine times for each compound in order to ensure that the results were statistically reliable. A graph of %Binding of ethidium vs drug concentration was plotted for each of the compounds prepared in this work (figures 43-45). From these binding isotherms it can be seen that the displacement of ethidium from its binding site, by doxorubicin and the substituted anthraquinones (1A-4A, 1B-4B, and 2Ci-3Ci), is qualitatively similar. However the prodrugs (2C & 3C) do not have any significant effect on the displacement of ethidium. The concentrations of drug required to displace 50% of ethidium are given in table 4.

It was shown that significantly ($P < 0.02$, $n=9$) lower concentrations of 1,4-substituted anthraquinones (4A & 4B, 0.35 and 0.50 x 10^{-6}M respectively) are required to displace ethidium from DNA than is required for doxorubicin (0.70 X 10^{-6}M) or 1,5-substituted anthraquinones (2A & 2B, 0.85 and 0.90 x 10^{-6}M respectively). However doxorubicin and the 1,5-disubstituted anthraquinones have higher affinity constants and, as shall be seen later, higher Δ Tms. This may be because the 1,5-substituted anthraquinones can only bind to DNA by "straddling" (see 2.2.1.3), whilst the 1,4-substituted anthraquinones may bind to DNA without the requirement for DNA breathing. In the previous experiment, when ethidium was added to a DNA-drug complex, it was shown that 1,5-substituted

anthraquinones had a maximal effect on the inhibition of fluorescence enhancement of ethidium binding to DNA. This apparent anomaly may be due to the 1,5-substituted anthraquinones dissociating less readily from DNA than do the 1,4- derivatives.

Higher concentrations of monosubstituted anthraquinones, (7.5 and 5.0 x 10^{-6}M for 1A & 1B respectively), and 1-8-substituted anthraquinones, (3.5 and 0.8 x 10^{-6}M for 3A & 3B respectively), are required to displace ethidium from its binding site. These values correlate with the relative affinity constants for each compound tested.

In the case of the prodrugs these compounds did not displace ethidium to any significant extent at concentrations of 4 x 10^{-5}M, this concentration is twenty fold greater than that at which the metabolites give displacement of ethidium.

It has so far been shown both qualitatively and quantitatively that the anthraquinones synthesised in this work do indeed bind to DNA by intercalation, whilst their azo derivatives (prodrugs) do not. In order to further investigate the interaction of these anthraquinones with DNA, further studies were designed to show whether the DNA helix is stabilised to heat denaturation by these compounds and also whether on binding, these compounds cause unwinding of the DNA in order to accomodate the drug.

2.2.3. Effect of drug on the physical properties of DNA.

2.2.3.1. Effect of drug on thermal denaturation properties of DNA.

When double-stranded DNA molecules are subjected to increase in temperature or extremes of pH, the DNA denatures to give two single-stranded molecules as a result of rupturing of the hydrogen bonds in the double helix. On denaturation there is an increase in absorbance of the DNA solution, so the change in absorbance can be used to monitor denaturation (Marmur and Doty, 1962). The temperature at which half the total increase in absorbance has occurred is known as the melting (or transition) temperature (Tm).

The intercalation of a drug molecule into the DNA helix stabilises the macromolecule such that more energy is required to separate the two strands. Thus the Tm of DNA is increased due to a change in enthalpy (Zunino et al., 1972). However if the drug binds externally to the helix then there is generally no significant change in the Tm. By measuring the change in the absorbance with temperature of the DNA solution at the DNA λ max (260nm), in the presence and absence of the drug, the Δ Tm (difference inTm's) can be determined. This is a measure of the stability of the drug/DNA complex. The increased stability imparted to the DNA helix by the drug is a measure of the affinity of binding. It should be noted however, that thermal stability of DNA is also influenced by changes in ionic

strength. As the ionic strength is increased, so is the stability of the helix, and hence the Tm. Furthermore the stability of the DNA helix is also affected by changes in the base composition; DNAs with high guanine-cytosine content have higher Tms than DNAs with high adenine-thymine content (Marmur and Doty, 1962).

The Tm of calf thymus DNA in absence of drug was shown to be 71.2 $\pm$ 0.1°C in pH7.4, Tris (2.88mM), NaCl (18mM), buffer. The Tm values obtained for DNA, DNA and either substituted anthraquinones or doxorubicin (at 10:1 DNA/drug ratio) are shown in table 5. Typical melting curves for DNA and DNA+drug (compound 1A) are shown in figure 46.

However in the case of DNA/anthraquinone complexes of (2A) and (4A) it was shown that the change in absorbance at 260nm was not an accurate measure of DNA melting. The increase in absorbance was significantly higher (80%) than that of DNA alone (50%). This was due to the bound drug having a lower extinction coefficient than the free drug at 260nm; both compound (2A) and (4A) have λ max, in the uv region, close to the λ max of DNA. At the isosbestic point there will be little (or no) change in the absorbance due to the release of drug as DNA denatures. In this way it was possible to monitor change in the absorbance of DNA at the isosbestic points, in the UV region (figure 47), of DNA+compound 2A (245nm) and DNA+compound 4A (268nm). The isosbestic points in the UV region of the spectrum were determined as follows.

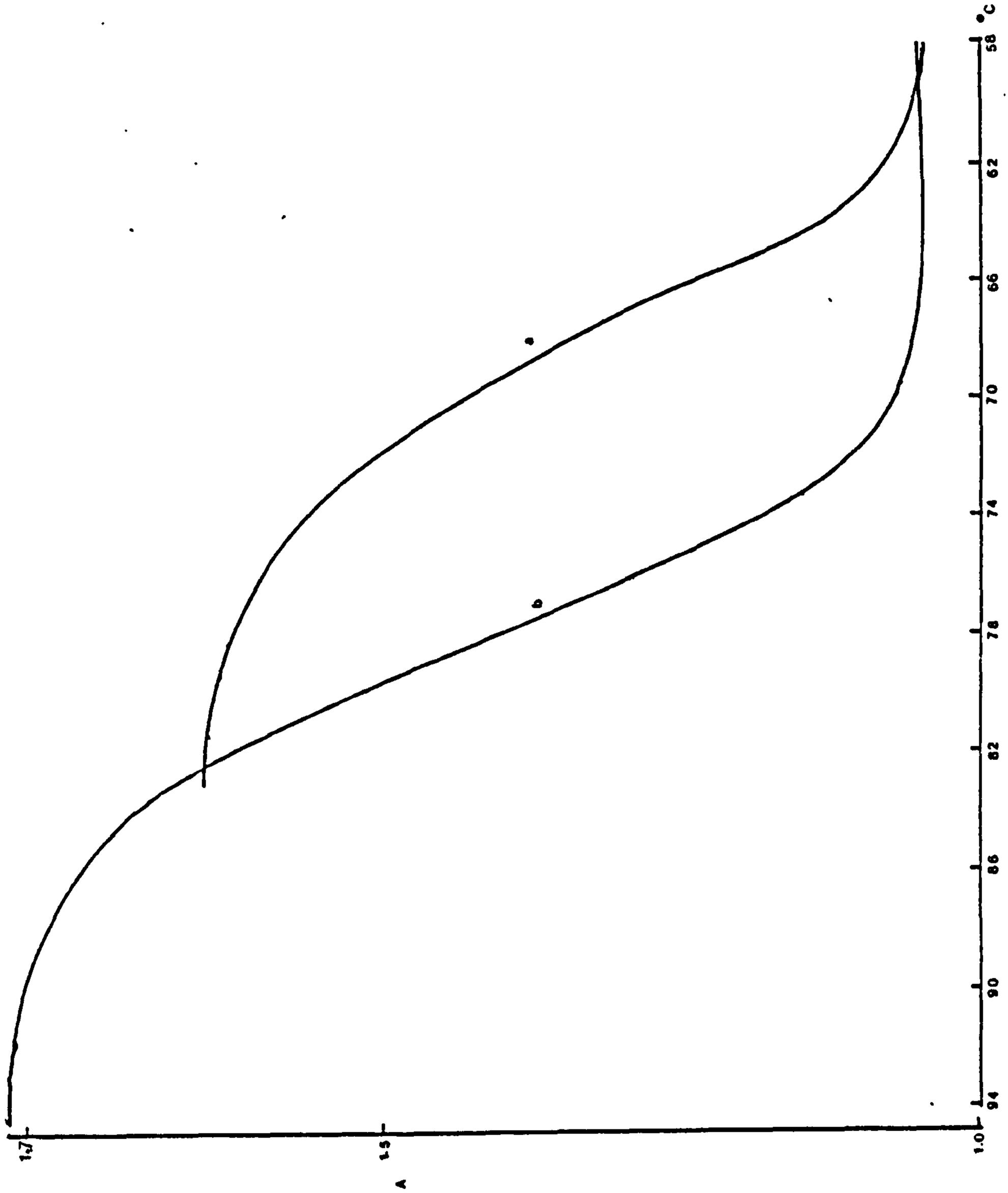


Figure 46 : Melting curves of DNA (curve a) and DNA+anthraquinone (1A) (curve b), in the temperature range 58° - 94°C.

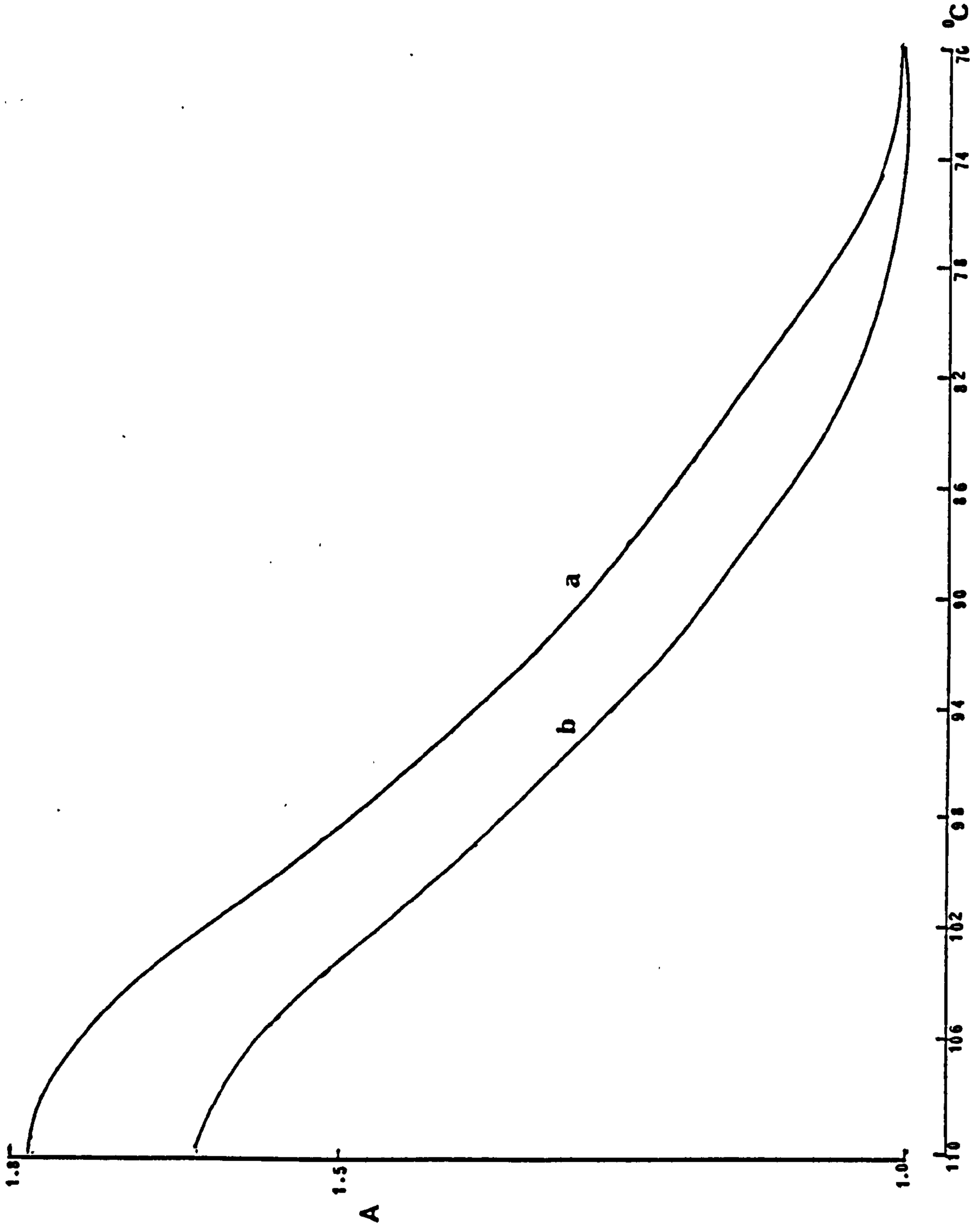
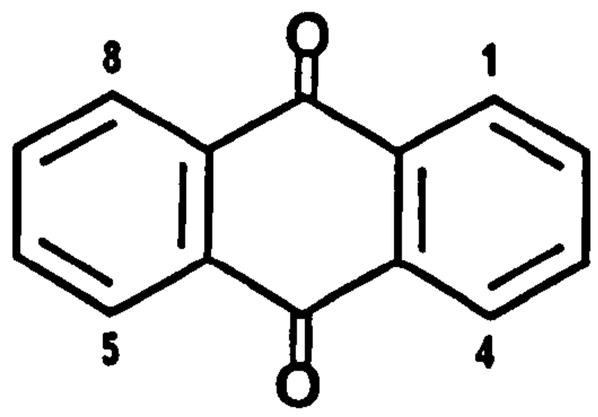


Figure 47 : Melting curves of DNA+anthraquinone (2A) at 260 (curve a) and at isosbestic point, 245nm, (curve b) in the temperature range 70° - 110°.



Compound	Tm(DNA)[2]	Tm(DNA+drug)[2]	ΔTm[2]
Doxorubicin	71.2°C	87.5°C	16.3°C
2'-(Diethylamino)ethylamino anthraquinones			
1A (1-)[1]	71.2	80.0	8.8
2A (1,5-)	71.2	98.4	27.2
2[3]	71.2	96.3	25.1
3A (1,8-)	71.2	80.7	9.5
4A (1,4-)	71.2	92.5	21.3
4[3]	71.2	90.2	20.0
2'-(Hydroxyethylamino)ethylamino anthraquinones			
1B (1-)	71.2	79.2	8.0
2B (1,5-)	71.2	94.8	23.6
3B (1,8-)	71.2	89.4	18.2
4B (1,4-)	71.2	90.7	19.5
Prodrugs and their potential metabolites			
2C	71.2	71.3	0.1
2Ci	71.2	80.1	8.9
3C	71.2	71.4	0.2
3Ci	71.2	78.4	7.2

[1]The numbers in brackets refer to substitution patern.

[2]Mean Tm values ± 0.1°C in all cases, (n=60, for DNA; n=4 for DNA+drug).

[3]Tm at isosbestic point in the UV region.

Table 5: ΔTm values for calf thymus DNA incubated with substituted anthraquinones (pH7.4, 0.018M NaCl, 0.00288M Tris buffer).

The spectra of DNA+drug were recorded against DNA blanks (see 3.2.1.2); the concentrations of DNA in both reference and sample cuvette were identical and therefore only the effect of DNA on the absorbance spectrum of drug was recorded. Using this method, compounds (2A) and (4A) showed isosbestic points at 245 and 268nm respectively. These wavelengths were used to determine the ΔTms of these anthraquinones.

From these data it was shown that all aminoalkylamino substituted anthraquinones (1A-4A, 1B-4B, and 2Ci-3Ci) stabilise DNA to thermal denaturation; however the azoanthraquinones (2C,3C) were not shown stabilise DNA to any siginificant extent (Δ Tm < 0.2°C). The monosubstituted anthraquinones (1A and 1B) have ΔTm values (8.8 and 8.0°C respectively) less than that obtained with doxorubicin (16.3°C), and this is consistent with the lower affinity of these anthraquinones as shown by data already discussed (see 2.2.1 and 2.2.2). The 1,5-substituted anthraquinones (2A and 2B) and the 1,4-substituted anthraquinones (4A and 4B) give significantly higher ΔTms than doxorubicin. This may be due to the 1,5- and 1,4-substituted anthraquinones dissociating less readily, from DNA, than does doxorubicin as shown previously (2.2.1.3).

The ΔTm values obtained in this work gives some indication of binding ability of drug to DNA. Further evidence of the nature of the interaction of these anthraquinones with DNA can be obtained by examining

the effect of drug on covalently closed circular DNA (cccDNA).

2.2.3.2. Effect of drug on covalently closed circular DNA (PM-2 DNA).

The effect of substituted anthraquinones on the unwinding of bacteriophage PM-2 DNA was examined by gel electrophoresis. All cccDNAs share the following properties: they contain a covalently closed double stranded polydeoxynucleotide and, as shown elsewhere by examination under an electron microscope, they are supercoiled (Adams et al., 1976). The supercoiling makes this type of DNA unusually compact and it therefore has an abnormally high sedimentation coefficient. When a drug intercalates into DNA, to accomodate the drug, local unwinding of the helix must occur, resulting in an increase in p, the average number of base-pairs per turn. Consequently, the number of supercoils must change as p is varied. It has previously been shown that during the initial stages of intercalation of drug, the number of right-handed supercoils decrease (figure 48) and the sedimentation coefficient of closed circular DNA falls (Crawford and Waring, 1967). At a critical level of binding the initial deficiency of turns in the closed circular molecules is just balanced by the increase in p due to accumulated drug-induced unwinding and the molecules behave as relaxed open circles. As further drug binding occurs, the additional unwinding forces the open circles to supercoil in the opposite sense

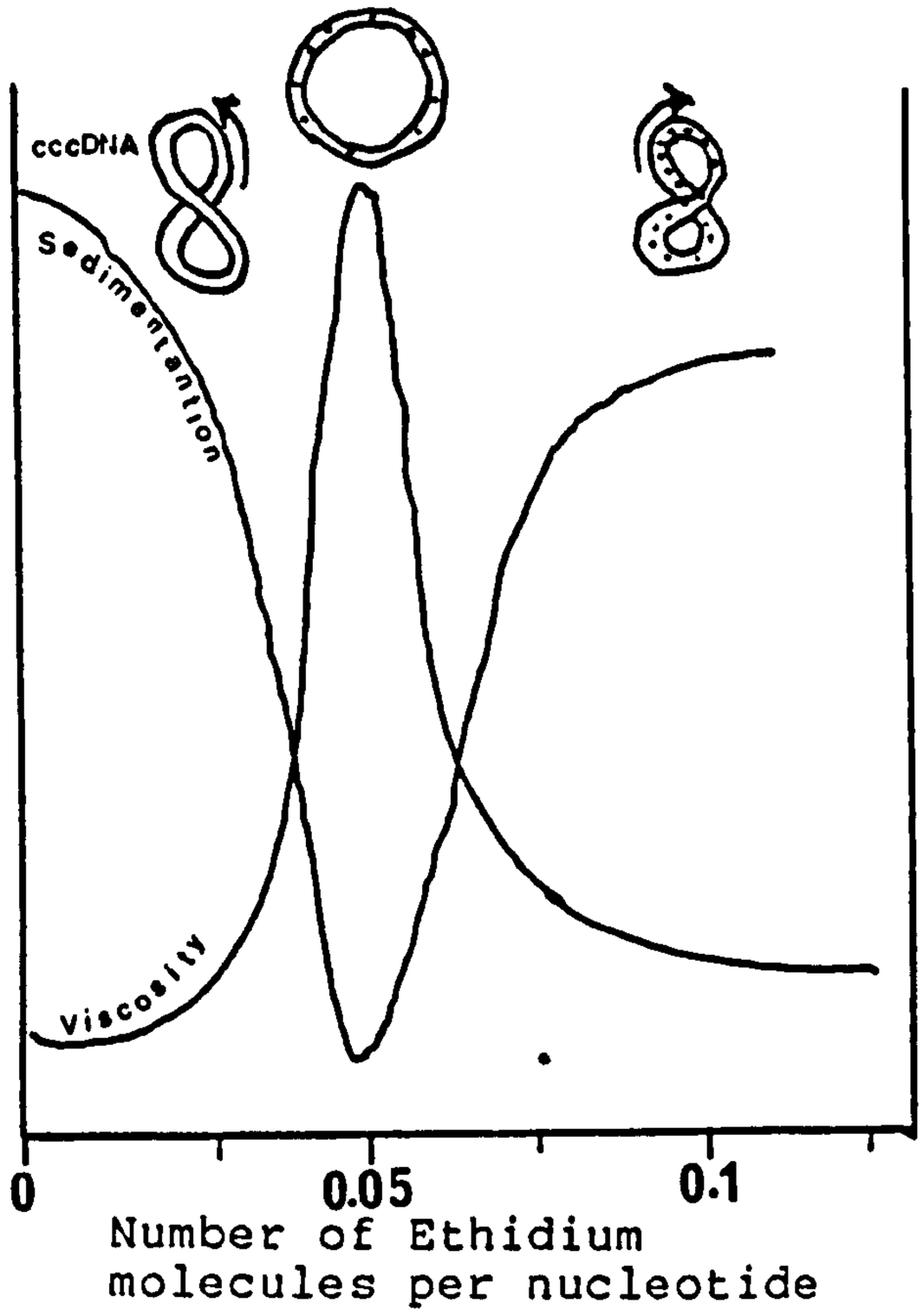


Figure 48: Effect of ethidium on the superhelical density of cccDNA (from Waring, 1981).

resulting in an increase in the sedimentation coefficient.

A major disadvantage of employing sedimentation velocity drug titrations is that the procedure requires large amounts of DNA and drug, furthermore only one concentration of drug may be use per centrifugation and therefore this method is also relatively time consuming. Alternatively, agarose gel electrophoresis can be used to separate covalently closed circular DNA from the relaxed covalently open circular DNA molecules of the same molecular weight (Keller et al., 1974). If the superhelical density of DNA is known then it is possible to determine the degree of DNA unwinding by the drug providing the affinity constant (K) and the number of drug molecules bound per base pair (n) is known. For the anthraquinones used in this work the K and n values were determined by means of spectrophotometric titration.

Agarose gels containing differing concentrations of drug were prepared and the DNA+drug complex layered on top together with bromophenol blue. Bromophenol blue and cccDNA are negatively charged at pH 7.4 and therefore migrate towards the anode. The anthraquinones, on the other hand, are positively charged at pH7.4 and migrate towards the cathode. It is possible to perform the titration with dye solely in agarose gels if the electrophoresis is stopped before the DNA band reaches the ascending boundary of the drug. In this way it is possible to run many gels

Figure 49: Gel electrophoresis of ccc-DNA (PM-2) in the presence of a range of concentrations of compound (3A), from left to right 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.8 and 1.0 µg/ml.

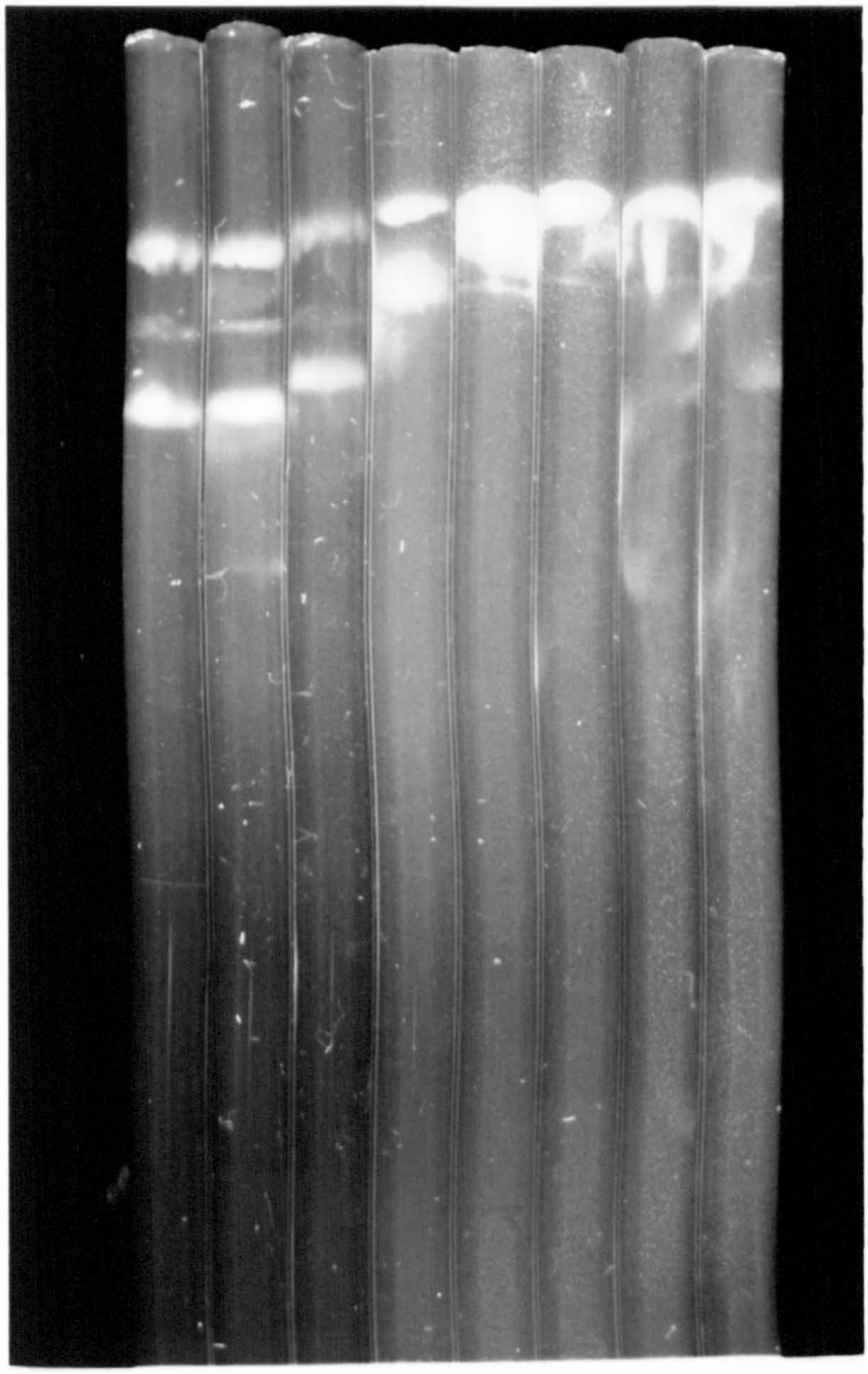
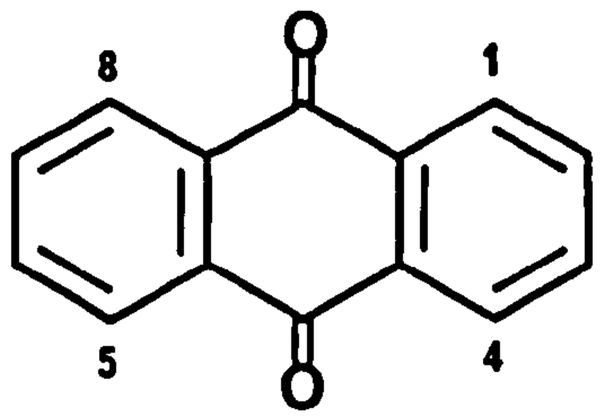


Figure 50: Gel electrophoresis of ccc-DNA (PM-2) in the presence of a range of concentrations of compound (1A), 0, 1.0, 2.0, 3.0, 4.0, 5.0, 8.0, and 10.0 µg/ml.



Compound	Critical Concentration[1] ($M \times 10^{7}$)	Unwinding angle[2] (Ø)
Ethidium	2.759	26.0
Doxorubicin	2.759	14.3
2'-(Diethylamino)ethylamino anthraquinones.		
1A (1-)[4]	22.30	10.6
2A (1,5-)	3.15	14.2
3A (1,8-)	7.874	14.3
4A (1,4-)	3.15	14.2
2'-(Hydroxyethyl)aminoethylamino anthraquinones		
1B (1-)	23.09	12.46
2B (1,5-)	3.93	13.51
3B (1,8-)	7.86	11.65
4B (1,4-)	3.93	18.02
Prodrugs and their "metabolites"		
2C[3]	>>1000	-
2Ci	22.30	10.7
3C[3]	>>1000	-
3Ci	22.30	10.7

1 Concentration of drug at which the PM-2 DNA almost co-migrated with nicked DNA.

2 The degree of local unwinding of the DNA helix due to each molecule of drug bound to DNA.

3 These azo anthraquinones did not have any effect on the migration of PM-2 DNA in the concentration range used (1×10^{-8} M to 1×10^{-4} M).

4 The numbers in brackets refer to the substitution pattern.

Table 6: "Critical concentrations" and calculated unwinding angles of cccDNA obtained with ethidium bromide doxorubicin, and substituted anthraquinones.

containing differing concentrations of drug at the same time. This method has previously been used to determine the superhelical densities of a number of cccDNAs including PM-2 DNA (Espejo and Lebowitz 1976). Hence knowing the superhelical density it is possible to calculate the degree of DNA unwinding caused by each molecule of drug. In this work a method based on that described by Espejo and Lebowitz (1976) was used.

Electrophoresis of PM-2 DNA in the presence of aminoalkylamino anthraquinones showed that, as with ethidium and doxorubicin, the rate of migration of PM-2 DNA decreased as drug concentration increased (i.e. the DNA was unwound); this reached a minimal value when the DNA almost co-migrated with the nicked PM-2 present in the PM2 DNA sample. As the drug concentration was increased further, the rate of migration increased progressively (i.e. DNA was supercoiled in the opposite sense). Figures 49 and 50 show typical electrophoretic runs. The concentration of drugs at which the DNA almost co-migrated with nicked DNA, the critical concentrations, are shown in table 6.

To calculate the degree of unwinding due to each bound molecule of drug, it is necessary to know the superhelical density of the PM-2 DNA and the number of drug molecules bound per DNA phosphate when the supercoils in the PM-2 DNA are totally unwound. The latter was calculated from the Scatchard equation:

$$r = \frac{Kcn}{1+Kc} \qquad (1)$$

K and n were determined for each drug (see 2.2.1.2.), and substituting c' (for c), the critical concentration of drug, into equation (1) yields the value of r', the number of drug molecules/DNA phosphate which gives full relaxation of the supercoiled DNA. The method of calculating unwinding angle was that used by Espejo and Lebowitz (1976) and DeLeys and Jackson (1976), and is as follows:

$$\sigma_0 = \frac{-10\emptyset r'}{180} \qquad (2)$$

where σ_0 is the superhelical density of PM-2 DNA and $\emptyset$ is the unwinding angle. The superhelical density for PM-2 DNA quoted by Espejo and Lebowitz is 0.042 based on an unwinding angle of 12° for ethidium; this has now been corrected and the currently accepted value for the unwinding angle for ethidium is 26° (Waring, 1981): the amended value for the unwinding angle produces a recalculated value of 0.0911 as the superhelical density for PM-2 DNA. Equation (2) thus becomes:

$$-\emptyset = \frac{1.638}{r'} \qquad (3)$$

The critical concentrations of drug required to relax cccDNA are shown in table 6. Using the K and n values from the Scatchard plots (table 2), the unwinding angles were calculated (table 6). From these results it can be seen that all substituted anthraquinones unwind cccDNA whilst the prodrugs have no significant effect on the migration of cccDNA. The local unwinding of the helix will only occur if the drug intercalates. These

results again show that the substituted anthraquinones interact with DNA by intercalation.

To summarise the results so far; it has been shown that the substituted anthraquinones (1A-4A, 1B-4B, and 2Ci-3Ci) a) unwind cccDNA, b) stabilise DNA to thermal denaturation, c) displace ethidium bromide from it intercalation site, d) have dissociation and affinity constants of the same order as doxorubicin, and e) exhibit isosbestic points on binding to DNA. These observations prove the hypothesis that these compounds interact with DNA by intercalation. Furthermore the prodrugs which were designed not to intercalate into DNA do not exhibit any of the above mentioned properties, and therefore do not intercalate into DNA.

2.2.4. Additional methods used previously in DNA-drug interaction studies.

Several literature methods have previously been described in the study of DNA-drug interactions (Waring, 1981; Wilson & Jones, 1981; Reinert, 1983; Patel, 1979). In recent years extensive use has been made of high resolution NMR to study the interaction of drugs with DNA (Patel, 1979), including studies of the interaction of daunorubicin with synthetic polynucleotides (Patel and Canuel, 1978; see 1.8.1). It has been recently shown elsewhere by NMR studies that 1,5-bis[2'-(diethylamino)ethylamino]anthraquinone, compound (2A), has some preference for A-T sites on

DNA, (Feigon, et al., 1984); this is consistent with the "straddle" mode of binding of this compound to DNA, proposed from this work, since A-T regions have a higher population of transient base-pair disruptions. Circular dichroism studies have shown that the interaction of daunorubicin with DNA produces characteristic alteration in the CD spectrum of the drug, which vary with DNA-drug ratios (Krug & Young, 1977).

In order to monitor changes in the properties of drug on binding to DNA, electrochemical and physical methods have been utilised in addition to spectroscopic methods. Furthermore polarography was used to determine whether quinone-containing drugs intercalate into DNA, since there is a reduction in the wave height at the half-wave potential on binding of the drug to DNA (Calendi et al., 1965; Berg et al., 1981).

When a drug intercalates into DNA there is a stiffening of the DNA molecule together with an increase in length. This results in an increase in viscosity of the DNA solution (Lerman, 1961). Therefore by monitoring this change in viscosity of a DNA solution on addition of drug, it is possible to determine if the drug intercalates (Berg & Echart, 1970). In addition to changes in viscosity, there is a characteristic change in the sedimentation coefficient of DNA (Zunino et al., 1972, Waring, 1981).

Equilibrium dialysis has been used previously to determine binding constants for DNA-drug interactions

(Zunino et al., 1972). In this method, the unbound drug molecules are allowed to equilibrate with DNA across a semipermeable membrane. After equilibration, the drug concentration is measured on both sides of the membrane and, knowing the total drug and macromolecule concentrations, the concentration of bound and free drug may be determined. Other methods where the free drug is physically separated from DNA-drug complex include ultracentrifugation, ultrafiltration, and partition methods.

None of the above methods were used in this work since the use of spectrophotometric and electrophoretic methods have shown conclusively that the aminoalkylamino-substituted anthraquinones intercalate into DNA and allowed determination of the affinity constants and dissociation rate constants.

It was shown in this work (see 2.2.1 - 2.2.3) that prodrugs of anthraquinones do not interact with DNA. However these prodrugs were designed such that active anthraquinones would be liberated in the target tissue (in this case liver). It was therefore important to determine whether the target tissue was capable of metabolising these prodrugs to release the corresponding aminoanthraquinones.

2.3. PRELIMINARY STUDIES OF AZOANTHRAQUINONE METABOLISM BY LIVER FRACTIONS in vitro.

One of the functions of the liver is to eliminate lipophylic compounds via metabolism to more polar metabolites. Therefore liver cells contain

several hundred types of enzymes capable of a number of biochemical reactions including conjugation, hydroxylation, oxidation and reduction of foreign compounds. Azo dyes have been widely used as colour additives in food and cosmetics. Reductive clevage of azo compounds by liver yields the corresponding amines (Miller and Miller, 1948, Muellier and Miller, 1950).

It has previously been shown that mammalian azoreductase activity is mainly localised in the liver microsomes (Miller and Miller, 1948; Walker, 1976; Fujita and Peisach, 1982). It has also been shown that azobenzenes may be reduced to the corresponding aminobenzenes by rat liver microsomes *in vitro* under anaerobic conditions in the presence of NADPH (Mueller and Miller, 1950). Subsequently it has been shown that purified NADPH-cytochrome c reductase catalyses the reduction of azo dyes to primary amines (Hernandez *et al.*, 1967; Fujita and Peisach, 1982). However, not all of the liver microsomal azoreductase activity could be attributed to this flavoprotein; some of the azoreductase activity was inhibited by carbonmonoxide, suggesting the possible involvement of cytochrome P-450 (Fujita and Peisach, 1976). Furtheremore cytochrome b_5 was also reported to function as an electron carrier in the metabolism of azo compounds (Fuijita and Peisach, 1977). Thus microsomal azoreductase system may consist of several different enzymes. Azoreductase activity has also been reported in the hepatic cytosol (Huang *et al.*, 1979) in this case either NADH or NADPH may function as electron donors (Daniel, 1969).

The azoanthraquinones (compounds 1C-3C) were designed to be metabolised by liver to the corresponding aminoanthraquinones (1-aminoanthraquinone, compounds 2Ci & 3Ci). Therefore the metabolism of these compounds was examined *in vitro* using microsomal and cytosolic fractions from rat liver. The methods for preparing these subcellular fractions are described in chapter 3.

The azoanthraquinones were not very soluble in aqueous media and therefore concentrated solutions (1mg/ml) of each drug in ethanol were prepared. A solution of the appropriate drug (0.1ml) was added to each of five tubes containing either microsomal or cytosolic fraction, buffer, and NADPH regenerating system, or NADH in the case of cytosolic fraction (see chapter 3) at 0°C. Controls were set up so that any non-specific azo reduction could also be determined. The following controls were used:

Drug +

1. Buffer
2. NADPH regenerating system (or NADH) + buffer.
3. Micromosomal (or cytosolic) fraction + buffer
4. Heat denatured microsomal (or cytosolic) fraction + NADPH regenerating system (or NADH) + buffer.

Nitrogen (O_2 free) was bubbled through all the mixtures to attain near anaerobic conditions and the tubes sealed, since the azoreductase activity of microsomal preparation is reduced in the presence of oxygen.

Preliminary work showed that the metabolism of these prodrugs was essentially complete after four hours under the conditions described. The tubes were incubated at 37°C for 0, 0.5, 1.0, 2.0 and 4.0 hours and the mixture extracted with dichloromethane after addition of sodium hydroxide. The organic layer was carefully removed and an aliquot (20µl) spotted onto a TLC sheet, using the appropriate aminoanthraquinone and azoanthraquinone as standards.

Since there is a difference in the Rfs of azoanthraquinone and aminoanthraquinones, TLC was chosen as a method to show if metabolism of azoanthraquinones had taken place. This method has the advantage over spectrophotometric assay, in that other metabolites which may be formed can also be detected.

It was shown that all azoanthraquinones could be reduced by both microsomal and cytosolic fractions. In the case of cytosolic reduction, the azoreduction proceeded without the addition of NADH, this could possibly be due to the presence of some NADH in the cytosolic fraction. However the amount of aminoanthraquinone formed was considerably less than in the presence of NADH. None of the other controls showed any metabolism of the azoanthraquinones.

During the incubation period two spots were formed for each of the pro-drugs, the first spot had the same colour and Rf as the pro-drug (0.5, 0.3, and 0.3 respectively for compounds 1C, 2C and 3C). The intensity of this spot decreased as the incubation

progressed. The second spot had the same colour and Rf as the corresponding amino anthraquinones (0.8, 0.5, 0.55 respectively for 1-aminoanthraquinone, compounds 2Ci and 3Ci), the intensity of this spot increased in parallel to the decreasing intensity of the spot corresponding to the pro-drug. In all cases the prodrug spot could not be detected, (neither by visible inspection nor under UV light) within one hour, in the microsome incubates, and one and half hours in cytosol incubates.

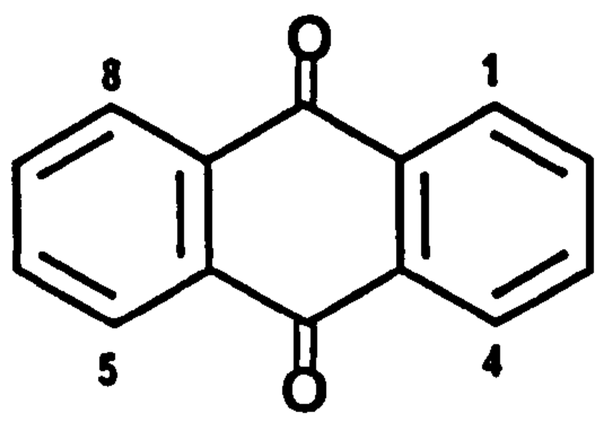
These preliminary studies showed that it is possible to design prodrugs of anthraquinones, which yield anthraquinones capable of interacting with DNA when metabolised by liver.

All anthraquinones prepared in this work were screened for antitumour activity *in vitro* and *in vivo*; the results obtained will be described briefly in the following section.

2.4. ANTITUMOUR ACTIVITY

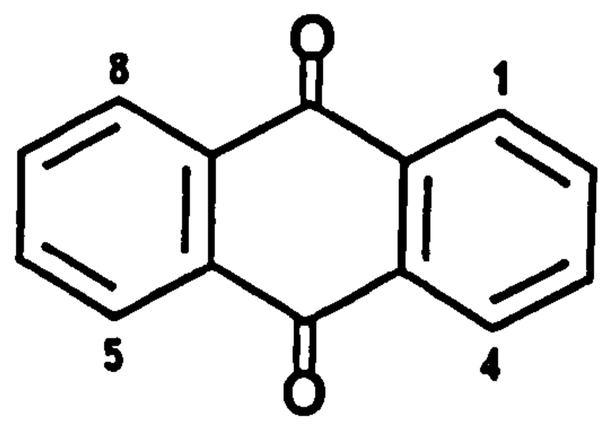
The compounds prepared in this work were designed to be potential antitumour agents and therefore the antitumour properties of these drugs in vitro and in vivo were investigated. The substituted anthraquinones (1A-4A and 1B-4B), anthraquinone prodrugs (2C & 3C) and their metabolites (2Ci & 3Ci) have been evaluated for their antiproliferative activity in vitro using CCRF-CEM human leukaemia cells by Lilly Research Centre, Windlesham, Surrey. The substituted anthraquinones (1A-4A and 1B-4B) have also been screened for their antiproliferative activity in vitro against HeLa cells, essentially by a similar method to that described by Uyeki et al., (1981), (performed by M.Partridge, Leicester Polytechnic, Leicester). The first series of anthraquinones (1A-4A) have been evaluated for their activity against P-388 leukaemia in vivo by the Cancer Research Campaign Experimental Cancer Chemotherapy Unit, University of Aston, Birmingham. The testing procedures have not yet been completed but the results obtained to date are shown in Tables 7-9.

From the results obtained to date, compounds 1A-4A do not show any significant activity (table 9) against mice bearing P-388 leukaemia in vivo. However all compounds showed antiproliferative activity in vitro: compound (4B) was shown to be the most potent (IC_{50}0.2μM) against CCRF-CEM human leukaemia. Compound



Compound	Substitution pattern	IC_{50}(μm)	IC_{50}(μg/ml)
(2'-Diethylamino)ethylamino anthraquinones			
1A	1-	5.02	1.8
2A	1,5-	1.77	0.9
3A	1,8-	2.55	1.3
4A	1,4-	3.34	1.7
(2'-Hydroxyethylamino)ethylamino anthraquinones			
1B	1-	5.48	1.9
2B	1,5-	1.44	0.7
3B	1,8-	1.23	0.6
4B	1,4-	0.21	0.1
"Pro drugs" and their metabolites			
2Ci	-	4.82	1.8
2C	-	10.48	4.8
3Ci	-	4.82	1.8
3C	-	23.58	10.8

Table 7 : Antiproliferative activity of anthraquinones in vitro (CCRF-CEM human leukaemia assay). Performed by Lilly Research Centre, Windlesham, Surrey.



Compound	Substitution pattern	$IC_{50}(\mu M)$
Mitoxantrone	1,4-[1]	1.0
(2'-Diethylamino)ethylamino anthraquinones		
1A	1-	51.0
2A	1,5-	9.0
3A	1,8-	27.0
4A	1,4-	25.0
(2'-Hydroxyethylamino)ethylamino anthraquinones		
1B	1-	17.0
2B	1,5-	2.6
3B	1,8-	5.7
4B	1,4-	1.0

[1]Mitoxantrone is a 5,8-dihydroxy substituted analogue of compound 4B.

Table 8 : Antiproleferative activity of anthraquinones in vitro against HeLa cells. Performed by M.Partridge, Leicester Polytechnic, Leicester.

Compound	Dose $mg.kg^{-1}$	Schedule	T/C
1A	16	QO1DXO1	95
	8		100
	4		100
	2		98
	1		105
2A	128	QO1DXO1	92
	64		105
	32		100
	16		105
	8		98
	4		107
	2		98
	1		102
3A	8	QO1DXO1	107
	4		105
	2		104
	16	QO4DXO3	102
	8		98
	4		91
4A	128	QO1DXO1	111
	64		97
	32		103
	16		112
	8		107
	4		100
	2		95
	1		117

T/C= % survial of drug treated mice/controls. T/C values <120 are not significant.

Daunorubicin at 16mg.kg on a QO1DXO1 schedule gave T/C 143 and at 4mg.kg on a QO4DXO3 schedule gave T/C 128.

Table 9 : In vivo activity of 2'(diethylamino)ethyl-amino-substituted anthraquinones (1A-4A) against leukaemia P-388. Performed by by the Cancer Research Campaign Experimental Cancer Chemotherapy Unit, University of Aston, Birmingham

(4B), ametantrone, was originally synthesised by Murdock et al., (1979) and Zee Cheng and Cheng (1978) and is currently in Phase II clinical trials.

The (2'-hydroxyethyl)aminoethyl anthraquinones show a greater antiproliferative activity in vitro than do the corresponding (2'-diethylamino)ethylamino analogues against HeLa and CCRF leukaemia cells. The monosubstituted anthraquinones (1A & 1B) are significantly less active than the disubstituted anthraquinones.

The 1,5-bis-substituted anthraquinone (2A) was the most potent of the (2'diethylamino)ethylamino-substituted anthraquinones (A series) (IC_{50} 1.77μM and 8.2μM vs CCRF human leukaemia and HeLa cells respectively) in vitro. The 1,8-bis-substituted anthraquinone (3A) showed higher antitumour activity than the 1,4-bis-substituted anthraquinone (4A), against CCRF human leukaemia (IC_{50} 2.55 and 3.34μM respectively for compounds 3A and 4A). However compounds (3A) and (4A) are almost equipotent (IC_{50} 27.0 and 25.0μM respectively) against HeLa cells.

The 1,4-bis- substituted anthraquinone (4B) was the most potent of the (2'-hydroxyethylamino)ethyl-amino-substituted anthraquinones (B series), (IC_{50}, 0.21 and 0.1μM respectively against CCRF human leukaemia and and HeLa cells) in vitro. The 1,8-bis-substituted anthraquinone (3B) (IC_{50} 1.23μM) was more effective than the 1,5-bissubstituted anthraquinone (2B) (IC_{50} 1.44μM) against CCRF human leukaemia.

However compound (2B) was more potent than compound (3B) (IC_{50} 2.6 and 5.7μM respectively) against HeLa cells.

These data are not exactly as predicted from the DNA binding studies and this could be due to several factors. For example, the compounds may bind differentially to components, such as proteins, present in the growth medium. However it can clearly be seen from the results presented in this work that the disubstituted anthraquinones have a greater antiproliferative activity *in vitro* than the monosubstituted analogues. Furthermore the presence of the hydroxyethylaminoethylamino side chain further enhances the potency of these compounds. This is supported by recent reports on the clinical effectiveness of mitoxantrone and ametantrone in the treatment of cancer (Estay *et al.*, 1983; Loesch *et al.*, 1983)

The prodrugs (compounds 2C and 3C) and their metabolites have been tested only against CCRF human leukaemia *in vitro*. The metabolites were found to be significantly more active than the prodrugs, as predicted by the DNA binding studies. Metabolites of these prodrugs (2Ci and 3Ci) were equipotent (IC 50 4.82μM) and only marginally more active than compound (1A; IC 50 5.02μM), showing that the presence of an aromatic amino group at position 4 or 5 of the anthraquinone chromophore does not significantly alter the activity of monosubstituted anthraquinones.

Compound 2C (IC 50 10.48μM) was more potent than compound 3C (IC 50 23.58μM). This reduction in potency of the pro-drugs may be due to the fact that the compounds need to be metabolised <u>in vivo</u> by the liver to the parent amino anthraquinone before they can interact with DNA; in accordance with their design.

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3. EXPERIMENTAL

3.1. SYNTHESIS OF SUBSTITUTED ANTHRACENE-9,10-DIONES.

All chemicals were purchased from the Aldrich Chemical Company, Gillingham. Kodak Chromagram sheets (No. 13181) were used for all TLC work. Unless otherwise stated, potassium bromide (KBr) discs were used for all infra-red spectroscopy, $CDCl_3$ was used as a solvent for ^1H NMR, and phosphate buffer (0.2M, pH 7.0) was used for U.V./Vis spectroscopy. Elemental analyses were carried out by CHN Analysis Ltd., Wigston, Leics. The following instruments were used in this work,

Perkin-Elmer 552-S UV/Vis spectrophotometer,

Perkin-Elmer 298 Infra-Red spectrophotometer,

Hitachi Perkin-Elmer R 600 (60MHz) NMR spectrometer,

V.C. Micromass 16F mass spectrometer.

3.1.1. Synthesis of 2'-(diethylamino)ethylamino-anthracene-9,10-diones (A series).

3.1.1.1. Synthesis of 1-[2'-(diethylamino)ethylamino]-anthracene-9,10-dione (1A).

1-Chloroanthracene-9,10-dione was purified by recrystallisation from nitrobenzene. 2-(Diethylamino)-ethylamine (98%+) was used without further purification.

1-Chloroanthracene-9,10-dione (12.1g, 0.05mol) was dissolved in 2-(diethylamino)ethylamine (58g, 0.5mol) and the solution heated under reflux for 2

hours. The reaction mixture was cooled in an ice bath and excess hydrochloric acid (10M, 100ml) added slowly whilst maintaining the temperature below 15°C.

The acidic mixture was extracted sequentially with ether (3 X 200ml) and chloroform (3 X 200ml). The ethereal layers were discarded and the bulked chloroform extracts were dried and evaporated *in vacuo*. The residue was dissolved in deionised water, made alkaline (pH13), with sodium hydroxide and extracted with chloroform (3 X 200ml). The bulked extracts were evaporated *in vacuo* and washed several times with water. Finally the solid was suspended in absolute ethanol and the solvent evaporated *in vacuo* . The solid was dried over P_2O_5 *in vacuo* and recrystallised from absolute ethanol to yield the free base (9.67g, 60%), Mp 156-158°C.

TLC Rf 0.7 ($MeOH/CHCl_3$ 1/1).

Elemental analysis: found, C 74.77, H 7.11, N 8.46%.

Calc. for $C_{20}H_{22}N_2O_2$, C 74.51, H 6.83, N 8.69%.

IR cm^{-1} ; 3285 (NH), 3000-2800 (CH_2 CH_3), 1660 (quinone carbonyl), 1585 (aromatic C=C).

^{1}Hnmr: 9.7 (NH, 1H); 7.2-8.3(m) (aromatic, 7H); 3.3(q) (CH_2, 2H); 2.6(m) (CH_2, 6H); 1.1(t) (CH_3, 6H).

m/e (EI) 322 (M^+), other major fragments; 250 (M-72), 236 (M-86), 151, (M-171), 86, (M-236).

UV λ max. (ε X 10^{-3}) (HCl salt) 495 (3.22), 315 (2.99), 279 (5.64), 248 (16.16).

The hydrochloride salt was prepared by dissolving the base in ether (sodium dried) and

bubbling dry HCl gas for 2 minutes whilst stirring the solution. The resultant precipitate was filtered and dried over P_2O_5 in vacuo.

3.1.1.2. Synthesis of 1,5-bis-[2'-(diethylamino)ethyl-amino]-anthracene-9,10-dione (2A).

1,5-Dichloroanthracene-9,10-dione was purified by recrystallisation from toluene. 2-(Diethylamino)-ethylamine (98%+) was used without further purification.

1,5-Dichloroanthracene-9,10-dione (5.52g, 0.02mol) was dissolved in 2-(diethylamino)ethylamine (23,2g, 0.2mol) and refluxed for 4 hours. The reaction mixture was cooled in an ice bath and excess hydrochloric acid (10M, 100ml) slowly added whilst maintaining the temperature below 15°C using an ice bath.

The acidic mixture was extracted sequentially with ether (3 X 200ml) and chloroform (3 X 200ml). The aqueous layer was made alkaline (pH 13-14) with sodium hydroxide, extracted with chloroform (3 X 200ml), and the bulked extracts dried and evaporated in vacuo. The solid residue was washed several times with deionised water, the last traces of water removed by adding absolute ethanol and evaporation in vacuo. The solid was dried over P_2O_5 in vacuo and recrystallised from absolute ethanol to yield the free base (4.19g, 48%), Mp >300°C.

TLC Rf 0.50 ($MeOH/CHCl_3$ 1/1).

Elemental analysis: found, C 71.31, H 8.31, N 12.60%.
Calc. for C_{26} H_{36} N_4 O_2, C 71.52, H 8.31, N 12.83%.
IR cm^{-1} ; 3420 (NH), 3000-2800 (CH_2 CH_3), 1660 (quinone carbonyl), 1585 (aromatic C=C).
^{1}Hnmr: 9.7 (NH, 2H); 6.8-7.6(m), (aromatic, 6H); 3.4(t), (CH_2, 4H); 2.7(m), (CH_2, 12H); 1.1(t), (CH_3, 12H).
m/e (EI) 435 (M^{-1}), other major fragments; 363 (M-73), 349, (M-87), 264, (M-172), 235, (M-201), 86, (M-350), 58, (M-378).
UV λ max. (ε X 10^{-3}) (HCl salt) 515 (9.50), 282 (10.72), 231 (31.46), 201 (20.17).

The hydrochloride salt was prepared as described for (1A).

3.1.1.3. Synthesis of 1,8-bis-[2'-(diethylamino)ethyl-amino]-anthracene-9,10-dione (3A).

This was prepared by the same method as compound (2A) using 1,8-dichloroanthracene-9,10-dione (recrystallised from toluene, 5.52g, 0.02mol) and diethylaminoethylamine (23.2g, 0.2mol), with heating under reflux for 3 hours.
Yield 2.62g, (30%) of free base Mpt. >300°C.
TLC Rf 0.55 (MeOH/$CHCl_3$ 1/1).
Elemental analysis: found, C 71.31, H 8.32, N 12.50%.
Calc. for C_{26} H_{36} N_4 O_2, C 71.52, H 8.31, N 12.83%.
IR cm^{-1} ; 3280 (NH), 2790-2980 (CH_2 CH_3), 1670 (quinone carbonyl), 1585 (aromatic C=C).
^{1}Hnmr: 9.7 (NH, 2H); 7.1-7.5(m), (aromatic, 6H);

3.25(q), (CH_2, 4H); 2.5(m), (CH_2, 12H); 1.2(t), (CH_3, 12H).

m/e (EI) 436 (M^+) other major fragments; 363 (M-73), 350, (M-86), 235, (M-201), 100, (M-336), 86, (M-350), 58, (M-378).

UV λ max. (ε X 10^{-3}) (HCl salt) 536 (7.90), 316 (5.37), 250 (9.07), 236 (33.05), 201 (23.55)

3.1.1.4. Synthesis of 1,4-bis-[2-(diethylamino)ethyl-amino]-anthracene-9,10-dione (4A).

1,4,9,10-Tetrahydroxyanthracene (leucoquinizarin), was purified by repeated recrystallisation from acetone:water mixture (9:1). 2-(Diethylamino)ethylamine (98%+), was used without further purification.

1,4,9,10-Tetrahydroxyanthracene (4.84g, 0.02mol) was suspended in butan-1-ol (100ml) then 2-(diethylamino)ethylamine (23.2g, 0.2mol) added dropwise with stirring under nitrogen (oxygen free, and dried). The suspension was then heated to 50-55°C for 2 hours, using an oil bath, and allowed to stand overnight at room temperature. The mixture was then aerated for a period of 8 hours at 50-55°C and the resultant mixture evaporated in vacuo. The residue was dissolved in hydrochloric acid (2N, 200ml). The solution was then extracted sequentially with ether (3 X 200ml) and chloroform (3 X 200ml).

The aqueous layer was then made alkaline (pH 13-14), extracted with chloroform (3 X 200ml), and the bulked extracts dried and evaporated in vacuo. The solid

residue was washed several times with deionised water and the last traces of water removed by adding absolute ethanol followed by evaporation in vacuo. The solid was dried over P_2O_5 in vacuo and recrystallised from absolute ethanol to yield the free base (4.88g, 56%), Mp 175-177°C.

TLC Rf 0.5 ($MeOH/CHCl_3$ 1/1).

Elemental analysis: found, C 71.51, H 8.33, N 12.82%.

Calc. for $C_{26}\ H_{36}\ N_4\ O_2$, C 71.52, H 8.31, N 12.83%.

IR cm^{-1} ; 3420 (NH), 3000-2800 (CH_2 CH_3), 1660 (quinone carbonyl), 1585 (aromatic C=C).

^{1}Hnmr: 9.7 (NH, 2H); 7.2-8.4(m), (aromatic, 6H); 3.5(t), (CH_2, 4H); 2.7(m), (CH_2, 12H); 1.1(t), (CH_3, 12H).

m/e (EI) 436 (M^+), other major fragments; 236 (M-173), 151, (M-285), 86, (M-350), 69, (M-367), 58, (M-378).

UV λmax. (ε X 10^{-3}) (HCl salt) 626 (10.12), 582 (9.55), 256 (24.72), 207 (17.99).

The hydrochloride salt was prepared as described for (1A).

3.1.2. Synthesis of 2'-(hydroxyethylamino)ethylamino-anthraquinones (B series).

3.1.2.1. Synthesis of 1-[2'-(2-hydroxyethyl)aminoethyl-amino]-anthracene-9,10-dione (1B).

1-Chloroanthraquinone was purified by recrystallisation from nitrobenzene. 2-Aminoethylamino-ethanol (97%) was used without further purification.

1-Chloroanthraquinone (9.7g, 0.04mol) was suspended in 2-aminoethylaminoethanol (83.2g, 0.8mol) by mechanical stirring and heated to 90°C in an oil bath for two hours.

The reaction mixture was cooled in an ice bath, and poured into deionised water (500ml). The resulting solid was filtered and dried in vacuo at room temperature for 48 hours. The solid was suspended in hydrochloric acid (10N, 200ml) and stirred for 24 hours. The mixture was centrifuged, the supernatant decanted, diluted with deionised water (300ml) and sequentially extracted with ether (4x200ml) and chloroform (4x200ml). The aqueous layer was made alkaline (pH 13-14) with sodium hydroxide and extracted with chloroform. The bulked organic extracts were dried and evaporated to dryness in vacuo and the solid recrystallised from chloroform: hexane (1:1), to yield the free base (5.5g, 44%), Mp 106-108°C.

TLC Rf 0.7 (MeOH/EtAc 1/1).

Elemental analysis: found, C 67.95 H 5.96, N 8.78%.

Calc. for $C_{18}H_{18}N_2O_3 . 0.5H_2O$: C 67.96, H 5.96, N

8.77%.

IR Cm^{-1} (KBr Disc); 3400 (OH), 3260 (NH), 3000-2800 (CH_2 CH_3), 1670 (quinone carbonyl), 1580 (aromatic C=C).

^{1}Hnmr: 9.9 (NH, 1H); 7.2-8.4(m), (aromatic, 7H); 4.1(s), (OH); 3.5(m), (CH_2, 8H); 2.4(s), (NH-aliphatic side chain).

m/e (EI) 311 (M^{+1}), other major fragments; 237 (M-73), 222, (M-87), 74, (M-236).

UV λ max. (ε X $10E^{-3}$) in phosphate buffer (0.2M, pH7.0) (HCl salt) 498 (6.23), 313 (5.82), 278 (10.6), 247 (32.29).

The hydrochloride salt was prepared by dissolving the base in 2-methoxyethanol and bubbling dry HCl gas for 2 minutes whilst stirring the solution. Diethylether was then added to the solution, the resultant precipitate filtered and dried over P_2O_5 in vacuo.

3.1.2.2. Synthesis of 1,5-bis-[2'-(2-hydroxyethyl)amino-ethylamino]anthracene-9,10-dione (2B).

1,5-Dichloroanthraquinone, was purified by recrystallisation from toluene. 2-Aminoethylamino-ethanol (97%) was used without further purification.

1,5-Dichloroanthraquinone (11.04g, 0.04mol) was suspended in 2-aminoethylaminoethanol (83.2g, 0.8mol) by mechanical stirring and heated to 90°C in an oil bath for four hours. The reaction mixture was cooled in an ice bath and excess hydrochloric acid (10M, 100ml) was added slowly whilst maintaining the temperature

below 15°C, using an ice bath.

The acidic mixture was extracted sequentially with ether (3 X 200ml) and chloroform (3 X 200ml). The aqueous layer was made alkaline (pH 13-14) and the resulting suspension filtered. The solid was washed several times with cold deionised water and recrystallised twice from 2-methoxyethanol/ethyl acetate (2/1), to yield the free base (5.2g, 31.5%), Mp 125-127°C.

TLC Rf 0.2 (MeOH/EtAc 1/1).

Elemental analysis: found, C 61.32 H 7.01, N 13.00%.

Calc. for $C_{22}H_{28}N_4O_4 . 1.0H_2O$: C 61.40, H 6.98, N 13.02%.

IR Cm^{-1} ; 3410 (OH), 3260 (NH), 2920-2830 (CH_2 CH_3), 1660 (quinone carbonyl), 1590 (aromatic C=C).

^{1}Hnmr: (HCl salt) (D_2O) 6.8-7.5(m) (aromatic, 6H); 4.0(m) (CH_2, 12H); 3.5(m) (CH_2, 4H).

m/e (EI) 412 (M^+), major fragments; 264 (M-148), 236, (M-176), 74, (M-338).

UV λ max. (ε X $10E^{-4}$) in phosphate buffer (0.2M, pH7.0) (HCl salt) 518 (1.15), 281 (1.24), 230 (4.00).

The hydrochloride salt was prepared as described for (1B).

3.1.2.3. Synthesis of 1,8-bis-[2'-(2-hydroxyethyl)-aminoethylamino]anthracene-9,10-dione (3B).

1,8-Dichloroanthraquinone, was purified by recrystallisation from toluene. 2-Aminoethylamino-ethanol (97%) was used without further purification.

1,8-Dichloroanthraquinone (5.52g, 0.02mol) was suspended in 2-aminoethylaminoethanol (41.6g, 0.4mol) by mechanical stirring and heated to 90°C in an oil bath for three hours. The reaction mixture was cooled to room temperature and a mixture of ethanol (200ml) and deionised water (400ml) added. The solution was evaporated *in vacuo* and the residue washed several times with dionised water. The last traces of water were removed by the addition of absolute ethanol (100ml) followed by evaporation *in vacuo*. The solid was then dried *in vacuo* at room temperature overnight.

The compound was purified by "flash" column chromatography using the following solvent mixtures:

EtAc/MeOH	10/0	300ml
EtAc/MeOH	9/1	300ml
EtAc/MeOH	8/2	300ml
EtAc/MeOH	6/4	300ml
EtAc/MeOH	4/6	300ml
EtAc/MeOH	2/8	300ml
EtAc/MeOH	0/10	300ml

50ml fractions were collected and chromatographed on TLC plates. Fractions containing a single component were pooled and evaporated *in vacuo*. Only one of the components (most polar) was fully characterised: and it

was shown to be the desired compound. This component was recrystallised from ethanol to yield the free base (0.6g, 7.3%), Mp 130-132°C.

TLC Rf 0.15 (MeOH/EtAc 1/1).

Elemental analysis: found, C 63.30, H 6.46, N 13.56%.

Calc. for $C_{22}H_{28}N_4O_4$: C 64.08, H 6.80, N 13.59%.

IR Cm^{-1} ; 3420 (OH), 3260 (NH), 2920-2830 (CH_2 CH_3), 1660 (quinone carbonyl), 1590 (aromatic C=C).

^{1}Hnmr (DMSOd^6), 9.7 (NH, 1H); 7.1-7.6(m), (aromatic, 6H); 4.0(m), (CH_2, 12H); 3.5(m), (CH_2,4H).

m/e (EI) 411 (M^{-1}), other major fragments; 322 (M-90), 266, (M-146), 74, (M-338), 44, (M-368).

UV λmax. (ε X $10E^{-3}$) in phosphate buffer (0.2M, pH7.0) (HCl salt) 540 (8.45), 318 (5.12), 280 (10.87), 236 (35.27).

3.1.2.4. Synthesis of 1,4-bis-[2'-(2-hydroxyethyl)amino-ethylamino]anthracene-9,10-dione (4B).

1,4,9,10-Tetrahydroxyanthracene (leucoquinizarin), was purified by repeated recrystallisation from acetone:water mixture (9:1). 2-Aminoethylaminoethanol (97%) from Aldrich Chemical Company, was used without further purification.

Compound (4B) was prepared by the same method as compound (4A) using 1,4,9,10-tetrahydroxyanthracene (4.84g, 0.02mol) and 2-aminoethylaminoethanol (20.8g, 0.2mol). After aeration the mixture was filtered through a sintered glass funnel and the solid washed with hexane/ethanol (1/1, 2x 200ml) mixture. The compound was recrystallised from ethanol/water mixture

(1/4), to yield the free base (5.3g, 64.3%), Mp 156-158°C.

TLC Rf 0.2 (MeOH/EtAc 1/1).

Elemental analysis: found, C 63.96, H 6.91, N 13.56%.

Calc. for $C_{22}H_{28}N_4O_4$: C 64.08, H 6.80, N 13.59%.

IR Cm^{-1} ; 3420.(OH), 3280 (NH), 2920-2830 (CH_2 CH_3), 1610 (quinone carbonyl), 1580 (aromatic C=C).

^{1}Hnmr (CD_3COOD): 7.2-8.1(m) (aromatic, 6H); 4.0(m) (CH_2, 12H); 3.5(M) (CH_2, 4H).

m/e (EI) 411 (M-1), other major fragments; 264 (M-148), 236, (M-176), 74, (M-338).

UV λ max. (ε X $10E^{-4}$) in phosphate buffer (0.2M, pH7.0) (HCl salt) 628 (1.49), 583 (1.50), 256 (3.35), 204 (2.62).

The hydrochloride salt was prepared by dissolving the base in absolute ethanol and bubbling dry HCl gas for 2 minutes whilst stirring the solution. The resultant precipitate was filtered and dried over P_2O_5 in vacuo.

3.1.3. Synthesis of Azoanthraquinones.

These azoanthraquinones were synthesised from their corresponding amino anthraquinones and therefore the synthesis of aminoanthraquinone intermediates will be described here first.

3.1.3.1. Synthesis of 1-amino-4-[2'(diethylamino)ethyl-amino]-anthracene-9,10-dione (2Ci).

1-Amino-4-bromoanthracene-9,10-dione (a gift from I.C.I) was purified by recrystallisation from glacial acetic acid. 2-(Diethylamino)ethylamine (98%+) was used without further purification.

1-Amino-4-bromoanthracene-9,10-dione (2.92g, 0.01 mol) was dissolved in 2-(diethylamino)ethylamine (11.6g, 0.1mol) and the solution heated under reflux for 30 minutes. The reaction mixture was cooled in an ice bath, and deionised water (50ml) added. The solution was evaporated in vacuo and the residue washed several times with deionised water. The last traces of water were removed by the addition of absolute ethanol (50ml) followed by evaporation in vacuo. The solid was then dried in vacuo at room temperature overnight.

The solid was redissolved in hexane/ethyl acetate (1/1) mixture and purified by "flash" column chromatography using the following solvent mixtures:

Hexane/EtAc	1/1	150ml
Hexane/EtAc	0/1	150ml
EtAc/MeOH	9/1	150ml
EtAc/MeOH	8/2	150ml
EtAc/MeOH	6/4	150ml

25ml fractions were collected and chromatographed on TLC sheets (solvent EtAc/MeOH, 8/2). Fractions containing a single component were pooled and evaporated in vacuo. Only one of the components (Rf 0.5) was fully characterised, and found to be the

desired product. This component was recrystallised from a mixture of hexane and ethyl acetate (2/1) to yield the free base (1.5g, 44.5%), Mp 112-113°C.

TLC Rf 0.5 (EtAc/MeOH 1/1)

Elemental analysis: found, C 70.97, H 6.90, N 12.42%.

Calc. for $C_{20}H_{23}N_3O_2$, C 71.19, H 6.87, N 12.45%.

IR cm^{-1} ; 3400 (NH_2), 3260 (NH), 3000-2800 (CH_2 CH_3), 1645 (quinone carbonyl), 1585 (aromatic C=C).

^{1}Hnmr: 10.8 (NH, 1H); 7.4-8.3(m) (aromatic, 7H); 3.4(q) (CH_2, 2H); 2.6(m) (CH_2, 6H); 1.1(t) (CH_3, 6H).

m/e (EI) 337 (M^+), other major fragments; 265 (M-72), 251 (M-86), 86, (M-251).

UV λ max. (ε X 10^{-3}) (HCl salt) 606 (9.59), 565 (1.01), 254 (2.69), 205 (21.00).

The hydrochloride salt was prepared as described for (1A).

3.1.3.2. Synthesis of 1-amino-5-[2'-(diethylamino)-ethylamino]-anthracene-9,10-dione (3Ci).

Compound (3Ci) was prepared and purified by the same method as for compound (2Ci) using 1-amino-5-chloroanthracene-9,10-dione (2.56g, 0.01mol) and 2-(diethylamino)ethylamine (11.6g, 0.1mol), with heating under reflux for 50 minutes, to yield the free base (1.42g, 42%), Mp 137-139°C.

TLC Rf 0.55 (EtAc/MeOH 1/1)

Elemental analysis: found, C 71.12, H 6.89, N 12.36%.

Calc. for $C_{20}H_{23}N_3O_2$, C 71.19, H 6.87, N 12.45%.

IR cm^{-1} ; 3480 (NH_2), 3325 (NH), 3000-2800 (CH_2 CH_3),

1630 (quinone carbonyl), 1565 (aromatic C=C).

^{1}Hnmr: 9.8 (NH, 1H); 6.9-7.6(m) (aromatic, 7H); 3.4(q) (CH_2, 2H); 2.6(m) (CH_2, 6H); 1.1(t) (CH_3, 6H).

m/e (EI) 337 (M^+), other major fragments; 265 (M-72), 251 (M-86), 86, (M-251).

UV λmax. (ε X 10^{-4}) (HCl salt) 507 (1.12), 280 (1.15), 230 (3.77).

The hydrochloride salt was prepared as described for (1).

3.1.3.3. Synthesis of 1-amino-5,8-bis-[2'-(diethylamino)ethylamino]-4-hydroxy-anthracene-9,10-dione (4Ci).

1,5-Diamino-4,8-dihydroxyanthracene-9,10-dione (a gift from I.C.I) and 2-(diethylamino)ethylamine (98%+) were used without further purification.

1,5-Diamino-4,8-dihydroxyanthracene-9,10-dione (2.70g, 0.01mol) and 2-(diethylamino)ethylamine (11.6g, 0.1mol) were suspended in aqueous methanol (50%, 60ml), and nitrogen (O_2 free) was bubbled through the mixture for a period of 15 minutes at 60-65°C. Sodium dithionite (4.2g, 0.02mol) was added to the mixture and heated for a further period of 22 hours under nitrogen (O_2 free).

Butan-1-ol (50ml) was then added and the mixture aerated for a period of 4-5 hours and evaporated in vacuo. The solid residue was suspended in a mixture of ethyl acetate:water (5:3, 800ml) and allowed to separate. The aqueous layer was discarded and the organic layer extracted with deionised water (3 x

300ml). The ethyl acetate layer was then dried with sodium sulphate (anhydrous), filtered and evaporated in vacuo.

The compound was purified by redissolving the residue in ethyl acetate (25ml) followed by "flash" column chromatography. The following solvent mixtures were used:

EtAc	-	250ml
EtAc/MeOH	9.5/0.5	250ml
EtAc/MeOH	9/1	250ml
EtAc/MeOH	8/2	250ml
EtAc/MeOH	6/4	500ml
EtAc/MeOH	2/8	500ml
MeOH	-	500ml

50ml fractions were collected and chromatographed on TLC sheets (solvent EtAc/MeOH, 1/1). Fractions containing a single component were pooled and evaporated in vacuo. Using the above system two components were separated but only one of these was fully characterised and found to be the desired product. This component was recrystallised from absolute ethanol to yield the free base (1.2g, 25.7%). Mp 180-182°C.

TLC Rf 0.3 (EtAc/MeOH 1/1).

Elemental analysis: found, C 66.73, H 8.02, N 14.98%.

Calc. for $C_{26}H_{37}N_5O_3$, C 66.78, H 7.91, N 14.98%.

IR cm^{-1} ; 3460 (NH, OH bonded), 3000-2800 (CH_2 CH_3), 1620 (quinone carbonyl), 1575 (aromatic C=C).

^{1}Hnmr: 9.7 (NH, 2H); 7.2-8.4(m), (aromatic, 4H);

3.6(q), (CH_2, 4H); 2.8(m), (CH_2, 12H); 1.1(t), (CH_3, 12H).

m/e (EI) 467 (M^+), other major fragments; 468 (M+1) 381 (M-86), 295, (M-172), 86, (M-381).

UV λ max. (ε X 10^{-3}) (HCl salt) 630 (9.52), 585 (8.95), 256 (24.72), 210 (16.99).

The hydrochloride salt was prepared as described for (1A).

3.1.3.4 Synthesis of 1-(4'-hydroxyphenyl)azoanthracene-9,10-dione (1C).

Fast red AL salt (anthraquinone-1-diazonium chloride, from Aldrich) and phenol were used without further purification.

Anthraquinone-1-diazonium chloride (zinc complex, 4g) was added to a solution of phenol (3.0g), in sodium hydroxide (2M, 30ml), with mechanical stirring. The resultant mixture was stirred for a further 30 minutes and allowed to stand overnight at room temperature. The resultant precipitate was filtered and sequentialy washed with sodium bicarbonate solution (2%, 3x300ml), and deionised water (6x300ml). The solid was dried in vacuo for 12 hours at 50-55°C and recrystallised from absolute ethanol to yield 1-(4'-hydroxyphenyl)azoanthracene-9,10-dione (3g), Mp 146-147°C. In this case it is not possible to calculate the percentage yield since the molecular weight of the anthraquinone-1-diazonium zinc complex is indeterminate.

TLC Rf 0.5 ($CHCl_3$).

Elemental analysis: found, C 73.56, H 3.61, N 8.52%.

Calc. for $C_{20}H_{12}N_2O_3$: C 73.16, H 3.66, N 8.53%.

IR Cm^{-1} ; 3400 (OH), 1620 (quinone carbonyl), 1580 (aromatic C=C).

^{1}Hnmr ($DMSOd^6$), 7.1-7.6(m), (aromatic, 11H); 5.2(s), (OH, 1H).

m/e (EI) 328 (M^+), other major fragments; 329 (M+1), 221, (M-107), 221, (M-107), 151, (M-177), 93, (M-235).

UVλ max. (ε X $10E^{-3}$) in methanol 495 (5.50), 430 (6.12), 250 (6.87).

3.1.3.5. Synthesis of 1-[2'-(diethylamino)ethylamino]-4-(2"4"-dihydroxyphenyl)azoanthracene-9,10-dione (2C).

1-Amino-4-(N',N'diethylaminoethylamino)anthraquinone (0.1g, 0.296mmol) was dissolved in hydrochloric acid (5M, 2.0ml) and cooled in a NaCl/ice bath. A solution of sodium nitrite (0.304M, 1.0ml) was also cooled and added to the amine with stirring. The resultant mixture was added to a solution of resorcinol (0.091 M) in sodium hydroxide (2M, 5ml), also at 0-5°C and stirred by shaking.

The mixture was extracted twice with dichloromethane (20ml) and the solvent discarded. The aqueous layer was then evaporated in vacuo and the last traces of water were removed by adding absolute ethanol followed by evaporation in vacuo. The residue was then suspended in chloroform (100ml), filtered to remove inorganic material, and the solution evaporated in vacuo. The resultant green solid was dissolved in 0.5ml of methanol and purified by "flash" column

chromatograpy using ethyl acetate/methanol (8/2) as eluent. Fractions (25ml) were collected and chromatographed on TLC plates; those containing a single component were pooled and evaporated in vacuo. In this case only three components were present; resorcinol, the starting amine, and the desired azo derivative. Fractions containing the azo derivative were evaporated in vacuo and the solid recrystallised from ethanol/chloroform (2/1) mixture to yield the free base (0.09g, 66%), Mp 137-138°C.

The reaction was repeated twice using 0.2g of amine to yield 0.18g, 66% and 0.20g, 73% respectively.

TLC Rf 0.3 (MeOH/EtAc 2/8)

Elemental analysis: found, C 68.15, H 5.63, N 12.22%.

Calc. for $C_{20}H_{12}N_2O_3$: C 68.12, H 5.68, N 12.23%.

IR Cm^{-1} ; 3430 (OH), 3260 (NH), 2920-2830 (CH_2 CH_3), 1640 (quinone carbonyl), 1580 (aromatic C=C).

^{1}Hnmr (DMSOd^6), 9.7 (NH, 1H); 7.2-8.3(m) (aromatic, 9H); 4.4 (OH, 2H) 3.3(q) (CH_2, 2H); 2.6(m) (CH_2, 6H); 1.1(t) (CH_3, 6H).

m/e (EI) 458 (M^+), other major fragments; 235 (M-223), 109 (M-349), 86, (M-372).

UVλ max. (ε X 10^{-3}), in chloroform 626 (3.22), 480 (4.00), 280 (5.64).

The hydrochloride salt of this compound could not be prepared as it was found that the azo compound degraded to the parent amine in the presence of HCl.

3.1.3.6. Synthesis of 1-[2'-(diethylamino)ethylamino]-5-(2"4"-dihydroxyphenyl)azoanthracene-9,10-dione (3C).

Compound (3C) was prepared by a similar method as that for compound (2C) using 1-amino-5-(N',N'diethylaminoethylamino)anthraquinone (0.1g, 0.296mmol). After coupling the diazonium chloride to resorcinol the resulting precipitate was centrifuged and the supernatant discarded. The solid was washed several times with deionised water, resuspended in cold ethanol (at 0°) and centrifuged. Ethanol was discarded and the solid dried in vacuo at 60°C for 8 hours.

Compound (3C) was purified by the same method as compound (2C) to yield the free base (0.093g, 68%), Mp 138-140°C.

The reaction was repeated once using 0.4g of amine to yield 0.37g, 68% of the product.

TLC Rf 0.3 (MeOH/EtAc 2/8)

Elemental analysis: found, C 68.09, H 5.68, N 12.25%.

Calc. for $C_{20}H_{12}N_2O_3$: C 68.12, H 5.68, N 12.23%.

IR Cm^{-1} ; 3420 (OH), 3280 (NH), 2920-2830 (CH_2 CH_3), 1620 (quinone carbonyl), 1560 (aromatic C=C).

^{1}Hnmr (DMSOd^6), 9.7 (NH, 1H); 7.4-8.3(m) (aromatic, 9H); 4.6 (OH, 2H) 3.3(q) (CH_2, 2H); 2.6(m) (CH_2, 6H); 1.1(t) (CH_3, 6H).

m/e (EI) 458 (M^+), other major fragments; 235 (M-223), 109 (M-349), 86, (M-372).

UV λ max. (ϵ X 10^{-3}), in chloroform 520 (3.20), 390

(2.90), 280 (3.55).

The hydrochloride salt of this compound could not be prepared as it was found that the azo compound degraded to the parent amine in the presence of HCl.

3.2 INTERACTION OF SUBSTITUTED ANTHRAQUINONES WITH DNA.

Calf thymus DNA (Type 1) was obtained from Sigma Chemical Company, London; pM2 DNA (cccDNA) was obtained from Boehringher Mannheim, Lewes, E.Sussex. All reagents were of Analar grade. All glass apparatus used in this work was silanised with Repelcote (Hopkins and Williams), washed twice with methanol (Fisons, H.P.L.C. grade) and dried overnight at room temperature before use. All volumetric glassware was of grade A specification. Volumes less than 0.50ml were measured using Hamilton precision glass syringes. All drugs were stored over phosphorous pentoxide under vacuume. All drug solutions were protected from light and stored at 4°C prior to use. Concentrations of all drug solutions were determined spectrophotometrically immediately before use. DNA has a variable molecular weight, and therefore the concentration of DNA in solution was expressed in terms of molarity of DNA phosphate; this was determined using an extinction value of 6600 at 260nm for DNA (phosphate). The term 'water' denotes freshly prepared water, double distilled in all glass apparatus. Unless otherwise stated Tris buffer (pH 7.40, Tris Cl 0.008M, NaCl, 0.05M) was used throughout this part of the work.

3.2.1. Effect of DNA on the spectral properties of drug.

3.2.1.1. Effect of DNA on absorbance of light by drug in the visible region of the spectrum.

A $5x10^{-5}$ M solution of the drug was prepared in Tris buffer and 5.0ml of this solution was added to each of six 10ml volumetric flasks. DNA solution (about $2.5x10^{-3}$ M) in the same buffer was added to give a range of DNA:drug ratios from 0 to 15:1. The solutions were then made to volume with Tris buffer.

The spectra of the solutions were recorded sequentially against a buffer blank, between 150nm below and 150nm above the λ max of the drug in the visible region of the spectrum, and were superimposed on each other.

3.2.1.2. Effect of DNA on absorbance of light by drug in the UV region of the spectrum.

A solution of drug ($1x10^{-4}$ M), and a range of DNA solutions were prepared in tris buffer (ranging from $5x10^{-5}$M to $5x10^{-4}$M). Appropriate DNA solution (precisely 2.0ml) was placed in both sample and reference cuvette, thus ensuring that the concentration of DNA in sample and reference beam were identical. Drug solution (precisely 1.0ml) and buffer (precisely 1.0ml) were added to the sample and reference cuvette respectively, and the solutions mixed carefully. The

spectra were recorded sequentially from 150nm above the λ max in the visible region to 190nm and were superimposed as before. The spectrum of free drug was recorded by replacing DNA solution with buffer.

3.2.1.3. Spectrophotometric titration studies.

Spectrophotometric titrations were performed at 25°C using a Perkin Elmer 552S spectrophotometer fitted with a thermostatted 5-cell holder and cell programmer. Fixed volumes (3.00ml) of the drug solution (approximately 5 x 10^{-5}M) were transfered to each of three matched quartz cuvettes and buffer was (3.00ml) placed in the reference cell as a blank. The cuvettes were protected from light to minimise photodecomposition of the drug.

Aliquots (12 x 20, 4 x 40, and 7 x 100µl) of DNA solution (2.5 x 10^{-3}M) were added sequentially to each of the cuvettes (drug and blank) using a Hamilton 100µl syringe. After each addition the mixture was carefully stirred and left to stand for 5 minutes to allow DNA/drug complex to equilibrate. After equilibration, the absorbance of the mixture was measured at the λ max of the free drug. Aliquots of DNA solution were added until there was no significant change in the observed extinction coefficient (ε_{obs}). The data for each cell were treated separately to allow for minor variations in the pathlengths. The values for the extinction of the free drug (ε_{f}) were calculated by dividing the initial absorbance of each cell by the free drug concentration. The binding parameters K and n were

determined from a Scatchard plot of [(bound drug concn./DNA concn.) /free drug concn.] vs (bound drug concn./DNA concn.); the free and bound drug concentrations for each point being determined from the fractional decrease in extinction at that point.

3.2.1.4 Dissociation kinetics study of DNA-drug complexes by stopped-flow spectrophotometry.

Solutions of daunorubicin (a gift from Farmitalia Carlo Erba, Milan, Italy), doxorubicin (Sigma Chemical Company, Poole, Dorset, UK) and each of the anthraquinones (compounds 1A-4A, and compounds 1B-4B) were prepared in tris (0.015M) NaCl (0.2M) buffer pH 7.4, containing calf-thymus DNA (Type I) at a drug to DNAp ratio of exactly 0.10. DNAp is the concentration of DNAp calculated as DNA phosphate from the absorbance of the solution at 260nm (ε260=6,600).

The dissociation rate constant for a particular DNA/drug complex was determined at 20°, 25° and 37°C as follows. The appropriate solution was placed in one reservoir of a SF3 series Stopped Flow spectrometer (Hi-Tech Scientific Limited, Salisbury, UK). Sodium dodecyl sulphate (SDS), 0.2%, in the same buffer was placed in the other reservoir. The reservoirs were connected to two separate syringes and to the mixing chamber via a three way tap (figure 51). Both syringes were driven at the same rate, using compressed air, to deliver each solution to the mixing chamber; on stopping the flow, the absorbance, at the λ max of the free drug, was monitored and the output recorded on a Tectronix storage oscilloscope.

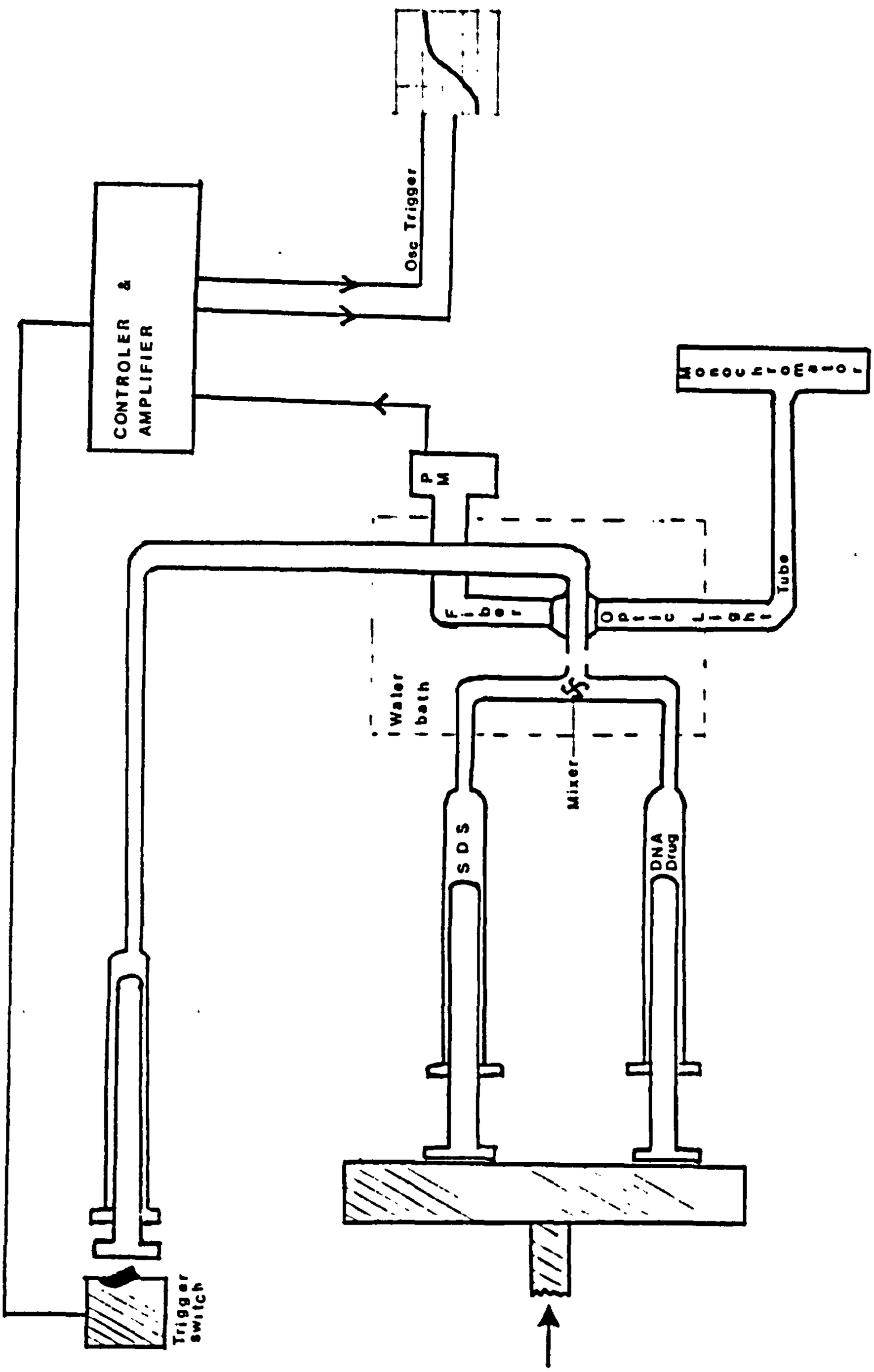

Figure 51: Schematic diagram of stopped flow apparatus.

The increase in extinction was monitored over a period of about 6 half lives. In each case at least eight data points were collected and fitted to a single exponential and to a biexponential equation using a microcomputer programme. In all cases the data could be fit to a single exponential with a correlation coefficient of 0.99 or better: the data did not fit a biexponential model (correlation coefficient less than 0.89).

3.2.2. Effect of drug on the binding of ethidium to DNA.

3.2.2.1. The effect of drug on the fluorescence enhancement of ethidium bromide due to binding to DNA.

A solution of drug ($2.0x10^{-6}$M) in Tris buffer was prepared, two further solutions containing DNA ($2.0x10^{-5}$M) and DNA and drug ($2.0x10^{-5}$M and $2.0x10^{-6}$M respectively) were prepared.

Buffer (precisely 2.0ml) was placed in one cuvette and the same volume of each of the above solutions were placed in three separate cuvettes; buffer and drug solutions acting as controls. The cuvettes were placed in a jacketted cell holder of a Perkin Elmer LS5 spectrofluorimeter and fluorescence intensities, (λ excitation, 476nm, λ emission, 596nm), of the solutions were determined at 25°C.

Aliquots, (6 x 10µl, 5 x 20µl, 6 x 40µl, and 6 x 100µl), of ethidium solution ($2.0x10^{-5}$M) were

sequentially added to each of the four cuvettes and readings taken after mixing and allowing to stand for 1 minute. Fluorescence intensities were corrected for changes in volume.

3.2.2.2. Determination of fluorescence polarisation of ethidium bromide when bound to DNA and irradiated with polarised light.

A solution of ethidium bromide (2.0×10^{-6}M) was prepared in tris buffer. Tris buffer (2.00ml) was placed in one cuvette and ethidium bromide solution (2.00ml) in each of 3 cuvettes. The cuvettes were placed in a jacketted cell holder of a Perkin Elmer LS5 spectrofluorimeter fitted with a manual polarisation accessory and fluorescence intensities, (λ excitation, 476nm, λ emission, 596nm), of the solutions were determined at 25°C.

The fluorescence readings were converted into polarisation values using the equation:

$$P_{corrected} = \frac{FII_{(v)} - FL_{(v)} \times (FII_{(H)}/FL_{(H)})}{FII_{(v)} + FL_{(v)} \times (FII_{(H)}/FL_{(H)})}$$

Where $P_{corrected}$ is the corrected polarisation, F represents fluorescence intensity, II denotes that the emission polariser is parallel to to the vertical component of the polarised light, L denotes that the emission polariser is perpendicular to the vertical component of the polarised light $_{(v)}$ indicates the direction of polarisation (vertical) of the light <u>incident</u> on the sample, and $_{(H)}$ indicates the direction

of polarisation (horizontal) of the light <u>incident</u> on the sample.

The above equation may be rewritten as:

$$P_{corrected} = \frac{F_{(0/0)} - F_{(0/90)} \times (F_{(90/0)}/F_{(90/90)})}{F_{(0/0)} + F_{(0/90)} \times (F_{(90/0)}/F_{(90/90)})}$$

Where F represents the fluorescence intensity, and the subscripted values in brackets represent the position in ° of excitation and emission filters respectively.

Aliquots, (6 x 10µl, 5 x 20µl, 6 x 40µl, and 6 x 100µl), of DNA solution (2.0×10^{-4}M) were sequentially added to the cuvettes and the polarisation of fluorescence determined. The readings were corrected for changes in volume.

3.2.2.3. The effect of drug on the fluorescence polarisation of ethidium bound to DNA.

A solution of ethidium bromide (2×10^{-5}M) was prepared in tris buffer and 1.00ml of this solution placed in each of twenty two, 10ml volumetric flasks. A solution of DNA (2×10^{-3}M) was prepared and sufficient volume added to each of the eleven flasks, to give a DNA:ethidium ratio of 4:1 precisely.

A solution of drug (2×10^{-4}M) was prepared and added to the eleven flasks containing DNA and ethidium, to give a range of concentrations of the drug from 0 to 8.0×10^{-6}M. Similar volumes were added to the rest of the flasks; the latter acting as controls. The polarisation of fluoresence, for each of the solutions was determined three times and the above experiment

repeated three times.

The % of ethidium bound to DNA was determined using the following equation:

$$\% \text{ binding} = \frac{P_{sample} - P_{control}}{P_{max}} \times 100$$

where P_{sample} is the polarisation of DNA+ethidium+drug, $P_{control}$ is the polarisation of drug+ethidium and P_{max} is the polarisation of DNA+ethidium (4:1).

3.2.3. Effect of drug on the physical properties of DNA.

3.2.3.1. Effect of the drug on thermal denaturation properties of DNA.

A solution (precisely 3.0ml) of an appropriate concentration of drug in buffer, and water (6.0ml) were added to a 10ml volumetric flask and the solution sonicated for 15 minutes to remove dissolved air.

DNA solution (Calf thymus), approximately 2.5 x 10^{-3}M in buffer, (precisely 0.60ml), was added to the contents of the flask, the solution made to volume with water, and mixed gently (final buffer concentration; 0.018M NaCl, 0.00288 M Tris Cl; DNA:drug ratio of exactly 10:1). The solution (2.0ml) was placed in a quartz cuvette with a ground glass stopper. The cuvette was placed in an electrically thermostatted cell holder of a Perkin Elmer 552S Spectrophotometer fitted with a temperature programmer. The absorbance of the solution at 260nm was recorded as the temperature was raised

from 58 to 110°C (0.5°C/min) except for compound (2A) and compound (4A) where the absorbance was monitored at the isosbestic point (268nm and 245nm respectively). This was repeated for three further samples and the mean Tm calculated.

The mean Tm of DNA in the absence of drug from 4 similar determinations in the same buffer was subtracted to give the ΔTm value.

3.2.3.2. Effect of drug on covalently closed circular DNA (PM-2).

PM-2 DNA was dissolved in tris buffer, pH 7.4, (0.05M tris-Cl buffer containing 0.002M EDTA, 0.018M NaCl and 0.02M sodium acetate) to give a solution of approximately 100µg ml^{-1}. Appropriate volumes of drug solution (100µg ml^{-1}) were added to each of eight 5ml volumes of 1% Agarose Type I (Sigma) in the same buffer, at 45°C, such as to give final concentration range of drug from 0.00 to 1.00µg ml^{-1}. Each solution was poured into a 125mm x 5.8mm glass tube with one end sealed with nescofilm, and the ends of the resultant gels trimmed when cool, the nescofilm being replaced with cotton gauze. An aliquot (20µl) of PM-2 DNA solution containing drug at 5:1 molar ratio of DNA(P)/drug was layered onto the top of the gel followed by 20µl of 20% sucrose and bromophenol blue. Electrophoresis was performed at 45V (5 milliamps per tube) at 20-25°C for 3-4 hours until the bromophenol blue had migrated to about half the length of the gel. The gels were removed, stained with ethidium bromide

(4.0µg ml^{-1}) in the same buffer, destained overnight with the same buffer and photographed. The critical concentration was determined, this being the concentration at which the ccc-DNA virtually co-migrates with nicked PM-2 DNA in the sample.

3.3. METABOLISM OF AZOANTHRAQUINONES USING RAT LIVER FRACTIONS IN VITRO.

3.3.1. Subcellular fractionation of rat liver.

A male rat was killed by decapitation, the abdomen was cut open and the liver quickly excised and rinsed free of excess blood by immersion in a beaker of ice cold 0.15M NaCl 0.05M phosphate pH7.4 buffer. All subsequent stages were carried out at a temperature of 0-4°C.

After gently blotting, to remove fluid, the liver was weighed and cut into small pieces and placed into a glass Potter Elveljehm homogeniser tube (30ml) containing approximately 15-20ml of the above buffer. The pestle was attached to an electric motor and the liver homogenised by repeated passings of the pestle through the homogenate, while the pestle was driven at a suitable speed (800-1000 rpm). The final concentration of the homogenate was adjusted so that the liver was suspended at a final concentration of 20% w/v. The homogenate was centrifuged at 10,000 x g for 30 minutes, the supernatant was decanted and centrifuged at 100,000 x g for 60 minutes. The resultant supernatant was cleared of the fatty surface layer and termed cytosol. The pellet was resuspended in

the same buffer (0.15M NaCl 0.05M phosphate pH 7.4) and termed microsomal fraction (final protein content 2.0mg/ml determined by the method of Lowry et al., 1951).

3.3.2. Metabolism of azoanthraquinones by rat liver fractions In vitro.

The metabolism of the azoanthraquinones (1C-3C) was examined in vitro by the following method.

A solution of the appropriate azoanthraquinone (1mg/ml, 0.1ml), in ethanol was added to each of five tubes containing 1.0ml of microsomal (or cytosolic) fraction, NADPH* regenerating system (0.5ml, see appendix 1), and 0.4ml of buffer at 0°C. Appropriate controls (five of each) were also set up containing drug (0.1ml, 1mg/ml) and either:

heat denatured microsomes (or cytosol), 1.0ml + NADPH regenerating system (0.5ml) + Buffer 0.4ml or,

microsomes (or cytosol), 1.0ml + Buffer (0.9ml), or,

NADPH regenerating system (0.5ml) + Buffer (1.4ml).

Nitrogen (O_2free) was bubbled through the mixtures for 15 minutes and the tubes sealed.

The tubes were incubated in a water bath (37°C) for 0, 0.5, 1.0, 2.0, and 4.0 hours. Sodium hydroxide solution (2.0ml, 5M) and dichloromethane (1.0ml) were added at the end of incubation period and centrifuged.

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*In the case of cytosolic fraction NADH (100mg/ml, 0.5ml) was used instead of NADPH regenerating system.

Dichloromethane was then carefully removed and an aliquot (10µl) spotted onto a TLC sheet together with a solution of appropriate azoanthraquinone and aminoathraquinone. In all cases the solvent system was same as that used for synthetic work (see section 3.1.3).

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4. SUMMARY AND CONCLUSIONS

Cancer is a leading cause of death in Western countries. To date, complete cure for the majority of tumours has not been attained, despite the introduction of a number of treatments, including surgery, radiotherapy and chemotherapy. A major problem in the treatment of cancer has been a failure to selectively eradicate <u>all</u> tumour cells. Surgery, in combination with radiotherapy and (or) chemotherapy, has proved to be useful in the treatment of solid tumours; however the treatment of metastases and leukaemia is only possible by the use of radiotherapy and chemotherapy. A major problem with the currently available chemotherapeutic agents is their severe toxicity and rapid development of tumour resistance to these agents.

Since the late 1940's, many antitumour agents have been discovered; mainly by large scale empirical screening programmes. An alternative approach to develop more effective antitumour agents is to study the mechanisms by which the existing drugs act and to attempt to improve the cytotoxic activity by modification of the structure. This work has been concerned with the design of potential antitumour drugs based on the mode of action of the anthracycline antibiotics doxorubicin and daunorubicin. Both doxorubicin and daunorubicin are potent antileukaemic agents. Doxorubicin also shows activity against a broad spectrum of solid tumours (Weirnik, 1980). These anthracyclines are known to intercalate between the base-pairs of double-stranded DNA (Neidle, 1980), and this is currently believed to be the principal

mechanism by which they exert their cytotoxic action.

A considerable amount of information has been accumulated on the nature of intercalation of drug molecules into DNA since Lerman first described the intercalation model in 1963. The intercalation site is now well defined and the structural features necessary for a molecule to intercalate into DNA are also known. These requirements include a planar tri- or tetra-cyclic ring system with a positively charged group (such as $^{+}NH_3$), either in the ring system or in a side chain, to attract the drug to the anionic helix (Waring, 1981). The side chain may be orientated in such a way as to stabilise the interaction once the drug has intercalated, by binding electrostatically to the phosphate residues on the helix, or by other bonding interactions.

In an attempt to mimic the planar unit of doxorubicin, the anthraquinone chromophore present in this antibiotic was selected as the chromophore for the compounds prepared in this work. Several aminoalkyl-substituted anthraquinones have been previously synthesised (Müller, et al., 1971; Double and Brown, 1975; Zee-Cheng and Cheng, 1978; Murdock, et al., 1979). However only a limited variation of substitution pattern has been incorporated in these studies. Furthermore there is a lack of a systematic study of the nature of interaction of anthraquinones with DNA. This work has investigated these issues.

In this work three series of substituted

anthraquinones were prepared. The first series consisted of four compounds (1A-4A) substituted with a 2'-(diethylamino)ethylamino side chain in the 1-, 1,4-, 1,5-, and 1,8- positions of the anthraquinone chromophore. The second series consisted of four compounds (1B-4B) substituted with a 2'-(hydroxy-ethylamino)ethylamino side chain in the 1-, 1,4-, 1,5-, and 1,8- positions of the anthraquinone chromophore. The third series of anthraquinones consisted of azoanthraquinone prodrugs (1C-3C) and their potential aminoanthraquinone metabolites (2Ci-4Ci).

The effect of substitution pattern on the nature and affinity of binding of the two series of 1-, 1,4-, 1,5-, and 1,8- substituted anthraquinones to DNA was examined in detail (summarised in table 10) and correlated with computer graphics modelling studies (performed by S. Islam and S. Neidle) which examined interaction of these synthesised anthraquinones with DNA. The methods used to evaluate the DNA binding of the anthraquinones were selected to allow both quantitation and characterisation of the DNA/drug interaction. Doxorubicin was also used as a reference intercalating agent.

All compounds prepared in this work (except the pro-drugs 2C & 3C), were shown to intercalate into DNA since they all showed the following properties:

1. significant hypochromic and bathochromic shifts of the absorbance maxima of the drug, and the presence of an isosbestic point, on binding to

DNA;

2. competitive displacement of a known intercalating agent (ethidium bromide) from DNA, by the compound under test, analysed by competitive fluorescence polarisation assay;

3. a significant stabilisation of the DNA helix to thermal denaturation in the presence of drug,

4. unwinding of covalently closed circular DNA (pM2) caused by drug, analysed by gel electrophoresis.

Taken individually, none of these results alone is sufficient to unequivocally prove intercalation of drug occurs, but all these effects are typical of those seen with other proven intercalating agents, including anthracyclines (Plumbridge and Brown, 1979). When considered together, the data (table 10) provide strong evidence of an intercalative interaction of the anthraquinones (excluding the pro-drugs) with DNA.

All compouds, with the exception of the pro-drugs (2C & 3C), were found to intercalate into DNA, and in addition they showed qualitatively similar effects to those observed with doxorubicin. Having shown that intercalation occurs, the affinity of the drug for DNA was then quantified by spectrophotometric titration with Scatchard plots (table 10). The compound with greatest affinity for DNA was shown to be, 1,5-bis-[2'(Diethylamino)ethylamino]anthraquinone (2A), as evidenced by spectrophotometric titration (K=3.97 X

Compound	On binding to DNA: % dec. in ε	ΔTm[a] (°C)	Kx 10^{-6} M^{-1} (n)[b]	ϕ[c]	Conc. to give 50% ethidium binding (μM)	(k_{-1})[d] $(20°C)$	IC(50)[e] HeLa	CCRF
Doxoru-bicin	35.0	16.3	4.42 (0.19)	14.3	0.70	1.84	1.0	-
1A (1-)[f]	37.0	8.8	1.48 (0.20)	10.6	7.50	>150	51.0	5.02
2A (1,5-)	27.0	25.1	3.97 (0.21)	14.2	0.85	0.91	9.0	1.77
3A (1,8-)	32.0	9.5	1.71 (0.20)	14.3	3.50	6.99	27.0	2.55
4A (1,4-)	42.0	20.0	3.17 (0.22)	14.2	0.35	3.73	25.0	3.34
1B (1-)	32.0	8.0	1.03 (0.19)	12.46	5.00	>127	17.0	5.48
2B (1,5-)	32.0	23.6	3.20 (0.22)	13.51	0.90	1.39	2.6	1.44
3B (1,8-)	33.0	18.2	2.35 (0.22)	11.65	0.90	3.69	5.7	1.23
4B (1,4-)	38.0	19.5	2.83 (0.19)	18.02	0.50	3.82	1.0	0.21
2Ci	25.0	8.9	ND	10.7	2.60	ND	ND	4.82
2C	0.0	0.1	ND	0	>40.0	ND	ND	10.48
3Ci	33.0	7.2	ND	10.7	2.50	ND	ND	4.82
3C	0.0	0.2	ND	0	>40.0	ND	ND	23.58

a The difference between the melting temperature of DNA in presence and absence of drug at a 10:1 DNA/drug ratio.

b Affinity constant (K), and the number of binding sites per DNA phosphate group (n).

c Unwinding of ccc DNA per bound drug molecule in °.

d First order dissociation rate constant (k_{-1} S^{-1}).

e Concentration of drug (μM) required to inhibit cell proliferation by 50%.

f Substitution pattern.

ND Not determined.

Table 10 : Summary of DNA binding and *in vitro* antiproliferative properties of substituted anthraquinones and their potential pro-drugs.

10^6 M^{-1}), and Δ Tm value (25.1°); indeed the Δ Tm value is considerably greater than that obtained for doxorubicin (16.3°). However the affinity of compound 2A for DNA is lower than that obtained for doxorubicin (K=4.42 X 10^6 M^{-1}). Compounds 1A (1-substited, K=1.48 X 10^6 M^{-1}) and 3A (1,8-bis- substituted, K=1.71 X 10^6 M^{-1}) show a significantly lower affinity for DNA than compound 2A; however the corresponding 1,4-bis-substituted analogue (4A, K=3.17 X 10^6 M^{-1}) shows similar affinity for DNA as compound 2A. Similar differences in the DNA binding of 2'-(hydroxy-ethylamino)ethylamino-substituted anthraquinones (B series) were shown.

From computer graphics modelling studies of the interaction of anthraquinones (A series) with DNA (performed by S.A. Islam and S. Neidle), it was shown that compound 1A (1-substituted) can be intercalated into DNA from either the major or minor groove direction. The low energy state of the anthraquinone intercalated into the d(CpG) model for DNA was calculated to be between -305 and -312.1 Kcal $mole^{-1}$. The 1,8-bis-substituted anthraquinone (3A) can only intercalate from the major groove side, with the chromophore arranged parallel to the base pairs. The low energy state of the anthraquinone intercalated into DNA was -338.2 Kcal $mole^{-1}$. Compound 2A has an enhanced energy of interaction, due to favourable dispersion factors. One side-chain resides in the major groove, and the other is in the minor groove. Compound 2A could only bind to DNA by this "straddling". In order to

accomodate the 1,5-substituted compound (2A), DNA-breathing (transient base pair unstacking) has to occur to allow the docking of the drug molecule into the receptor site. In this case the low energy state of the anthraquinone intercalated into DNA was calculated to be -353.6 Kcal $mole^{-1}$; of which at least 20-25 Kcal $mole^{-1}$ is needed, in addition to the total intermolecular energy of the DNA-drug complex. The dissociation of compound 2A from its intercalation site would involve prior disruption of the base base pairs not only immediately at, but also adjacent to, the site. Therefore it would be expected that compound 2A would dissociate less readily than compounds 1A and 3A. Furthermore compound 2A would be expected to have some preference for A-T regions, since these have a higher population of transient base-pair disruption. The latter has recently been confirmed experimentally by other workers using NMR techniques (Feigon et al., 1984). The 1,4-bis-substituted derivative (4A) was found to only bind satisfactorily in a perpendicular orientation with the side chains in the major groove. The low energy state of the anthraquinone intercalated into DNA was calculated to be -345.5 Kcal $mole^{-1}$. However compound 4A can also intercalate into DNA by "straddling"; this type of binding is only possible after full geometric optimisation with the low energy state of the anthraquinone on interaction being -373.4 Kcal $mole^{-1}$.

Results from stopped-flow spectrophotometry were consistent with these findings. The dissociation rate

constants of both series of anthraquinones from DNA, (table 10) were in the order 1- (1A & 1B);>> 1,8- (3A & 3B);> 1,4- (4A & 4B);> daunorubicin and doxorubicin > 1,5- (2A & 2B) substituted anthraquinones. The 1,5-disubstituted anthraquinones (2A & 2B) thus showed the slowest rate of dissociation from DNA; the DNA-anthraquinone complexes dissociating more slowly than the DNA complexes of the anthracyclines daunorubicin and doxorubicin. This would be expected considering the computer graphics modelling studies which show DNA breathing is necessary to allow the subsequent release of drug (2A) from DNA.

The 2'-(diethylamino)ethylamino-substituted anthraquinones (A series) showed a greater affinity for DNA than the corresponding 2'-(hydroxyethylamino)-ethylamino substituted anthraquinones (B series), with the exception of the 1,8-disubstituted compounds (3A and 3B). However the 2'-(hydroxyethylamino)ethylamino substituted anthraquinones were more cytotoxic against HeLa and CCRF human leukaemia cells <u>in vitro</u> (tested by M. Partridge, Leicester Polytechnic and by Lilly Research Centre, Windlesham) than the 2'-(diethyl-amino)ethylamino-substituted anthraquinones. For example, IC_{50} values of 1.0 and 25.0μM respectively were obtained for compounds 4B and 4A against HeLa cells; however the affinity constants (K) were 2.83 and 3.17 X $10^{6}M^{-1}$ respectively. This apparent lack of correlation between DNA binding and <u>in vitro</u> biological activity, of the two series of anthraquinones, may be due to differences in cellular uptake of the drug.

However, if the DNA binding data for compounds within each of the two series of anthraquinones are compared, then there is a correlation between IC_{50} and DNA binding ability. Both the affinity for DNA, and antiproliferative activity, of the first series of compounds (1A-4A) is in the order 1,5-> 1,4-> 1,8-> 1-substituted anthraquinone. In the case of the second series (1B-4B) the affinity for DNA is in the same order as for the first series but the antiproliferative activity of the compounds is 1,4-> 1,5-> 1,8-> 1-substituted anthraquinone, this may probably be due to differences in the protein binding of these drugs.

From the compounds prepared in this work, the 1,5-bis-[2'(diethylamino)ethylamino]anthraquinone (2A) has the highest affinity for DNA but is not the most potent cytotoxic compound in the in vitro screens employed here. This may be due to insufficient uptake of drug by the cells, as previously described. The in vitro antiproliferative activity of substituted anthraquinones against HeLa cells are in the order 4B> 2B> 3B> 2A> 1B> 4A> 3A> 1A. It is apparent from these preliminary results, that the 2'-(hydroxyethyl-amino)ethylamino anthraquinones (1B-4B) are significantly more cytotoxic than the corresponding 2'-(diethylamino)ethylamino anthraquinones (1A-4A). To date only the first series compounds prepared have been evaluated for antitumour properties against P-388 leukaemia in mice in vivo; they were shown to lack cytotoxic activity. These results show that the substituted anthraquinones (A series) would be

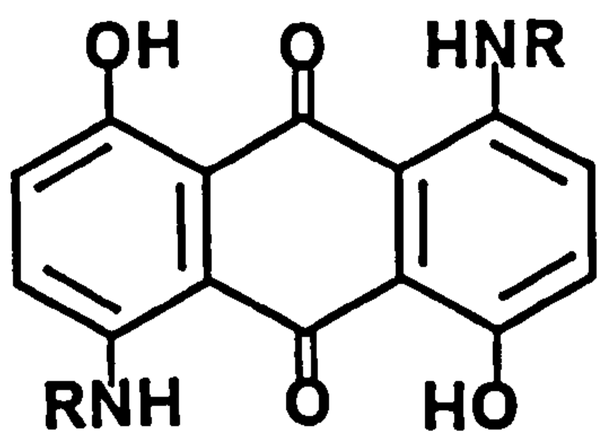
cytotoxic if sufficient drug concentrations were attained at the tumour site.

It has so far been shown that it is possible to design compounds which have significantly high affinity for DNA, and that these compounds can also be cytotoxic providing the drug can be delivered to the site of action; the next logical step was to target the drug to specific tumour cells. This approach also should allow reduction of the toxicity of the drugs. In this work, model anthraquinone pro-drugs were prepared which were anticipated to be selectively activated by a target organ. The target organ chosen, in this case, was the liver since at present only a limited number of chemotherapeutic agents have shown to be effective against liver tumours; furthermore the single most effective agent against hepatocellular carcinoma is doxorubicin, a known intercalating agent. The pro-drugs (2C-3C) were so designed that they would not intercalate into DNA but, when metabolised by intracellular enzymes, they would yield the intercalating anthraquinones (2Ci-3Ci). It was found that azotization of 1-amino-4(or5)-[2'(diethylamino)-ethylamino]anthraquinone with subsequent coupling to resorcinol yielded a compound which did not intercalate into DNA, in accord with its design. The pro-drugs (2C & 3C) were unable to stabilise DNA to thermal denaturation, to displace ethidium from DNA, or to unwind cccDNA. Since the anthraquinone pro-drugs did not show significant DNA binding, the affinity of the drugs for DNA, could not be satisfactorlly determined.

However, the potential "metabolites" (2Ci & 3Ci) of compounds 2C and 3C were shown to intercalate into DNA. To determine if the design was valid, the effect of cytosolic and microsomal preparations from rat liver, on the pro-drugs was investigated. The pro-drugs were indeed shown to be metabolised by rat liver microsomes and cytosol to the aminoanthraquinones confirming the validity of the approach. Preliminary in vitro antiproliferative studies against CCRF human leukaemia show that the pro-drugs are significantly less cytotoxic than the corresponding aminoanthraquinones. The aminoanthraquinones (2Ci & 3Ci) are as active as the 1-substituted anthraquinones (1A & 1B). This was as predicted from DNA binding studies since the anthraquinone pro-drugs did not bind to DNA. The low activity shown by the pro-drugs may be due to the reduction of the azoanthraquinone moieties to the corresponding amines.

From the above results, the following suggestions for the future development of compounds of this type may be made. It would be of interest to see if there are any differences in uptake and intracellular distribution of the substituted anthraquinones, and to relate these properties to lipid solubility, and cytotoxic action of the compounds in vitro and in vivo. It is clear from this work that 1,5-disubstituted anthraquinones have a greater affinity for DNA (and also dissociate less readily), than the corresponding 1,4- (and 1,8)-disubstituted anthraquinones. By analogy with the 1,4-disubstituted

anthraquinones where it was shown that 5,8-dihydroxy derivatives have a greater antitumour activity than the parent compounds (Murdock et al., 1979); the 4,8-dihydroxy analogues of compounds 2A & 2B would be expected to have a greater activity than the parent compounds.



From computer graphics studies of the interaction of the A series of anthraquinones it was observed that the terminal methyl group could lie in the vicinity of the edges of the base pairs. Appropriate substitution at these methyl positions could result in participation in specific interactions.

In this work, azo derivatives of 1-amino-4(or 5)-[2'(diethylamino)ethylamino]anthraquinones have been prepared which do not intercalate. It was shown that these anthraquinones may be metabolised to the parent amino anthraquinones, which are capable of binding to DNA. In order to show if these the compounds have selective activity, further studies of the distribution and metabolism of these anthraquinones in vivo are needed. The monosubstituted anthraquinones have insufficiently high affinity for DNA when compared to the disubstituted anthraquinones. It would therefore be advantageous to prepare azo derivatives of 1-amino-disubstituted anthraquinones in order to

exploit this enhanced affinity for DNA. This work has thus shown significant differences in DNA binding properties of substituted anthraquinones, and has identified structural requirements for optimum DNA binding in the series. The feasibility of azoanthraquinones as pro-drugs has been demonstrated. The work can therefore form a basis for a more rational design of such compounds.

All the compounds prepared in this work were designed as potential antitumour agents. However it may also be of interest to screen these anthraquinones for activity against other organisms; for example the anthracycline antibiotic daunorubicin, has been shown to possess trypanocidal action (Williamson et al., 1981). Furthermore, anthraquinones similar to those prepared here have been shown to possess antiviral, (Stringfellow et al., 1979), antileishmanial, (Schnur et al., 1983), and antiamoebic properties (Fabio et al., 1978; Winkelmann and Raether, 1979). In addition they have been shown to have activity against some Trichomonas species (Winkelmann & Raeter, 1979).

Ametantrone (Compound 4B) has been shown to inhibit lipid peroxidation and, since a free radical mechanism has been postulated for the initiation of lipid peroxidation and inflammatory processes, it would be of interest to examine whether simple anthraquinones may be useful as antiinflammatory agents.

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ADVANCED STUDIES UNDERTAKEN IN CONNECTION WITH THE PROGRAMME OF RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENT OF THE THE DEGREE OF DOCTOR OF PHILOSOPHY.

The candidate has attended courses on design of experiments, and information retrieval during the first year, and ancillary lectures in computer programming, statistics and electronics during the second and third years of the research programme. The candidate has attended lectures and seminars on drug metabolism, spectroscopy, and the use of modern chromatographic techniques.

The candidate has also attended and contributed to postgraduate seminars and colloquia held within the department, covering most aspects of the pharmaceutical sciences. The candidate has also delivered a lecture at the graduate symposium, Durham University (July, 1984) and has presented a poster at the Biochemical Society Meeting, (July, 1982).

The candidate's directed reading has included advanced aspects of organic chemistry, biochemistry, and spectrophotometry.

APPENDIX 1

Preparation of NADPH regenerating system

NADP	16.2mg
Glucose-6-Phosphate	26mg
Glucose-6-Phosphate dehydrogenase	60units
Magnesium Chloride Soln. ($MgCl_2 \cdot 6H_2O$ 1G/ml)	0.100ml
Phosphate buffer to	10ml

The solution was prepared immediately prior to use and stored in an ice bath.

PUBLICATIONS FROM THIS WORK.

1. "Computer graphics in rational anticancer drug design", S.A.Islam, R.A.Kuroda, S.Neidle, J.R.Brown, B.M.Gandecha and L.H.Patterson, Biochem. Soc. Trans. 10, 501, (1982).

2. "Effect of daunorubicin and amitoxantrone in vivo on hepatic drug-metabolising enzymes and involvement in lipid peroxidation". L.H.Patterson, B.M.Gandecha, and J.R.Brown, Brit. J. Pharmacol., 77, 490P (1982).

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6. "Comparision of DNA-binding of substituted anthraquinones by computer graphics and by solution studies.", J.R.Brown, S.Islam, S.Neidle, B.M.Gandecha, and L.H.Patterson., Brit. Pharmaceutical Conference 1983.

7. "Dissociation kinetics of DNA-anthracycline and DNA-anthraquinone complexes by Stopped-Flow Spectrophotometry.", B.M.Gandecha, J.R.Brown, and M.R.Crampton., Biochem. Pharmacol. (In press).

8. "Comparative computer graphics and solution studies of the DNA interaction of substituted anthraquinones based on doxorubicin and mitoxantrone.", S.Islam, S.Neidle, B.M.Gandecha, M.Partridge, L.H.Patterson, and J.R.Brown., J.Med.Chem. (In Press).