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Inhibition of RNA Synthesis by Anthracycline Analogs

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INHIBITION OF RNA SYNTHESIS BY ANTHRACYCLINE ANALOGS

by

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VITA

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CHAPTER I

INTRODUCTION

Anthracyclines, such as daunomycin and adriamycin have been the subject of intense recent investigation. Many of these studies have been, partially at least, motivated by the demonstrated effectiveness of anthracyclines in the treatment of leukemias and other forms of cancer.

The biological and pharmacological activities of these drugs appear to be, at least in part, due to their ability to interact with DNA. Specifically, daunomycin and adriamycin can intercalate into DNA and inactivate its template function essential for nucleic acid synthesis.

Recently, several structural analogs of these antineoplastic agents have been synthesized and some of them were shown to be considerably more effective against experimental tumors than the parent compounds. The majority of recent studies of these daunomycin derivatives have indicated that the increased effectiveness of these new drugs may relate to their different modes of interaction with the target molecule, DNA.

Clearly the interaction between anthracyclines and the DNA template may be expected to influence the mechanism by which these antibiotics inhibit nucleic acid synthesis. However, very little information is available in this area. The goal of this research project is to study the mechanism by which several structurally related anthracyclines inhibit the synthesis of RNA.

I.1. Structures of Anthracycline Analogs:

Daunomycin (daunorubicin) is an antibiotic isolated from cultures of streptomyces peucetius (Di Marco et al., 1964a). This drug inhibits strongly the multiplication of bacterial and animal viruses and exhibits a high cytotoxic activity against normal and neoplastic cells (Di Marco et al., 1964b). The active form of the drug was isolated as crystalline hydrochloride with the empirical formula: $C_{27}H_{29}O_{10}N \cdot HCl$. The structure of daunomycin has been shown to be a glycosidic antibiotic constituted by a red, water insoluble, tetracyclic aglycone (daunomycinone, $C_{21}H_{18}O_8$) (Arcamone, et al., 1964a) linked to a reducing (positive Fehling, Tollens tests), water soluble amino-sugar (positive Elson-Morgan ninhydrin) named daunosamine or 2,3,6-trideoxy-3-amino-L-lyxohexose ($C_6H_{13}NO_3$) (Arcamone et al., 1964b). Mild acid hydrolysis (0.2N hydrochloric acid for 1 hr. at 90°C) can resolve the drug into its two components (Arcamone et al., 1964). The structure and absolute configuration of daunomycin and its components are now known in detail (Arcamone et al., 1968) and are presented in Figure 1a.

From the X-ray crystallographic studies of the N-bromoacetyl derivative of daunomycin, the shape of the molecule in the solid state is shown in Figure 2. The cyclohexene ring or ring D is in the "half-chair" conformation (Angiuli et al., 1971) and the C-9 is displaced from the plane of the remaining five atoms by about 7 Å and toward the C-9 hydroxyl. A more detailed picture can be found in Figure 4A. The six membered daunosamine ring was found to be in the chair conformation oriented perpendicularly to the planar chromophore.

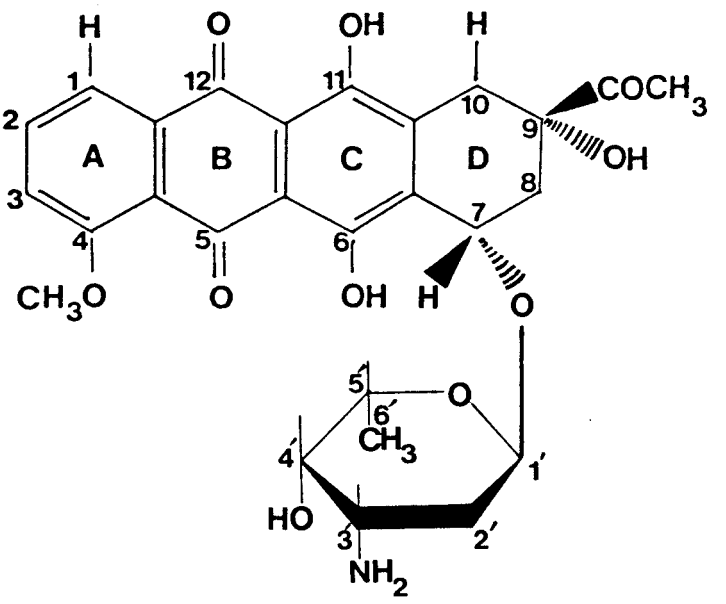
The physico-chemical characteristics of daunomycin show remarkable

Figure 1. Structures of anthracycline analogs:

(a) Daunomycin; (b) 4-demethoxydaunomycin;

(c) carminomycin; (d) aclacinomycin;

(e) marcellomycin; (f) musettamycin.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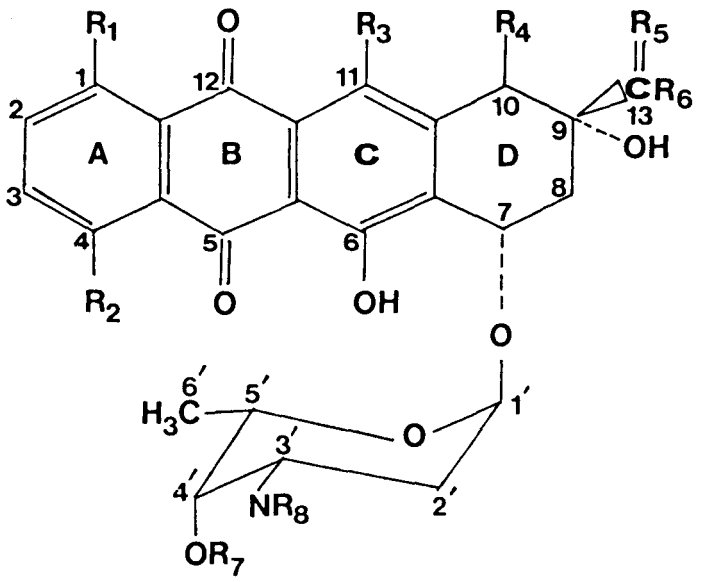
(a)

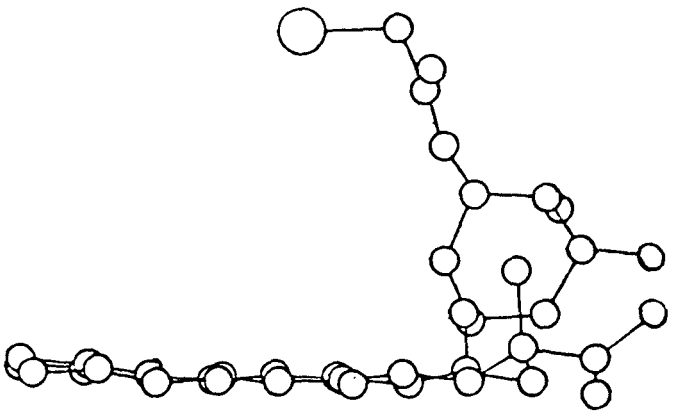


ANTHRACYCLINES	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
a) DAUNOMYCIN	H	OCH_3	OH	H	O	CH_3	H	$(H)_2$
b) 4-DEMETHOXYDAUNOMYCIN	H	H	OH	H	O	CH_3	H	$(H)_2$
c) CARMINOMYCIN	H	OH	OH	H	O	CH_3	H	$(H)_2$
d) ACLACINOMYCIN	H	OH	H	$COOCH_3$	H_2	CH_3	DF*-C**	$(CH_3)_2$
e) MARCELLOMYCIN	OH	OH	H	$COOCH_3$	H_2	CH_3	DF*-DF*	$(CH_3)_2$
f) MUSETTAMYCIN	OH	OH	H	$COOCH_3$	H_2	CH_3	DF*	$(CH_3)_2$

*DF: 2-DEOXYFUCOSE

**C: CINERULOSE

Figure 2. Three-dimensional structure of N-bromoacetyldaunomycin as indicated by X-ray analysis



similarities with those of the antibiotics classified as "Anthracyclines" (Ollis et at., 1961) and therefore it is accepted as a member of the anthracycline family (Zunino et al., 1972).

Adriamycin (14-hydroxydaunomycin) was obtained from cultures of streptomyces peucetius var. caesius (Arcamone et al., 1969a). This antibiotic gave, on acid hydrolysis, the aglycone, adriamycinone (14-hydroxydaunomycinone), and the amino sugar daunosamine. The structure and absolute configuration of adriamycin were established on the basis of chemical and spectroscopic evidence (Arcamone et al., 1969b) and are also shown in Figure 1.

The structures of the rest of the anthracycline analogs studied in this project are also shown in Figure 1 and they will be discussed separately in a latter section.

I.2. Pharmacological and Biological Properties of Daunomycin and Its Analogs

I.2.1. Antibiotic Activity

While only weakly active against some gram-positive and gram-negative bacteria and fungi (Di Marco, 1967), daunomycin shows much higher inhibitory activity against the multiplication of other bacteria such as E. coli; although different strains of E. coli have different susceptibilities (Di Marco *et al.*, 1975).

Daunomycin was found to be active against the multiplication of double-stranded DNA bacteriophage (e.g. T6) but not single-stranded DNA bacteriophage (e.g. EC9 and S13) (Calendi *et al.*, 1966). The interference is not related to a direct inactivation of the phage particles or an inhibition of their absorption to the bacterial cell (Parisi *et al.*, 1964).

The inhibitory effect of daunomycin was also demonstrated on other double-stranded DNA viruses (e.g. Herpes simplex virus) when administered before or at the early stage of infection. It is not effective after mature virions appear (Di Marco *et al.*, 1968).

Daunomycin is ineffective against the replication of animal RNA-viruses with the exception of Newcastle Disease Virus (NDV) providing that the daunomycin-NDV mixtures are exposed to light (Verini *et al.*, 1968).

By evaluation of the number of mitotic figures (mitotic index, MI) or of the proliferative activity of cells it was shown that daunomycin has a strong inhibiting effect on the *in vitro* growth of a number

of cells strains both normal and neoplastic (Di Marco et al., 1975). Cell damage induced by daunomycin is mainly nuclear, such as changes in the appearance of chromatin as well as the shape and size of the nucleoli. The changes appear soon after daunomycin addition to the medium of cell culture. During mitosis, chromosomal damage, such as fragmentations and mitotic aberrations can be observed by phase microscopy immediately after a sufficient amount of daunomycin is added (Di Marco et al., 1964b). Cytoplasmic changes, such as vacuolization, are moderate and occur only after prolonged treatment with high doses. Adriamycin also was found to induce significant alterations in membranes characterized by altered cellular agglutination ability and mitotic arrest (Murphree et at., 1976).

Daunomycin also has a strong inhibitory effect on experimental tumor cells such as ascites tumor; solid tumors show a lesser susceptibility (Di Marco et al., 1970). Adriamycin is more effective than daunomycin in increasing survival time in animals bearing Ehrlich ascites tumors (Di Marco et al., 1969) or L1210 leukemia (Sandberg et al., 1970) and exerts a high inhibitory effect on the growth of many other experimental tumors (Di Marco et al., 1971). It is under these experimental conditions that the difference between the two antibiotics is particularly evident.

I.2.2. Drug Distribution and Excretion

With the advantage of the fluorescence property of the anthracyclines, the distribution and excretion of daunomycin and adriamycin have been studied in animals (Di Fronzo and Gambetta, 1971a; Di Fonzo et al., 1971b). Both drugs are rapidly cleared from the blood stream and fixed by body tissues and organs. The two antibiotics do not appear

to cross the blood brain barrier.

As it is expected for drugs which interfere with nucleic acids synthesis (see section I.2.6.), the tissues most susceptible to the two antibiotics are rapidly proliferating such as intestinal mucosa, lymphoid organs and bone marrow (Di Marco et al., 1975).

Both drugs are excreted slowly into urine and the bile, however, adriamycin was found to be excreted much slower than daunomycin in rats and mice (Yesair et al., 1971). The calculated "concentration x time" (C x t) in the case of adriamycin equivalents has been demonstrated in all tissues to be several times greater than that found for daunomycin.

I.2.3. Drug Metabolism

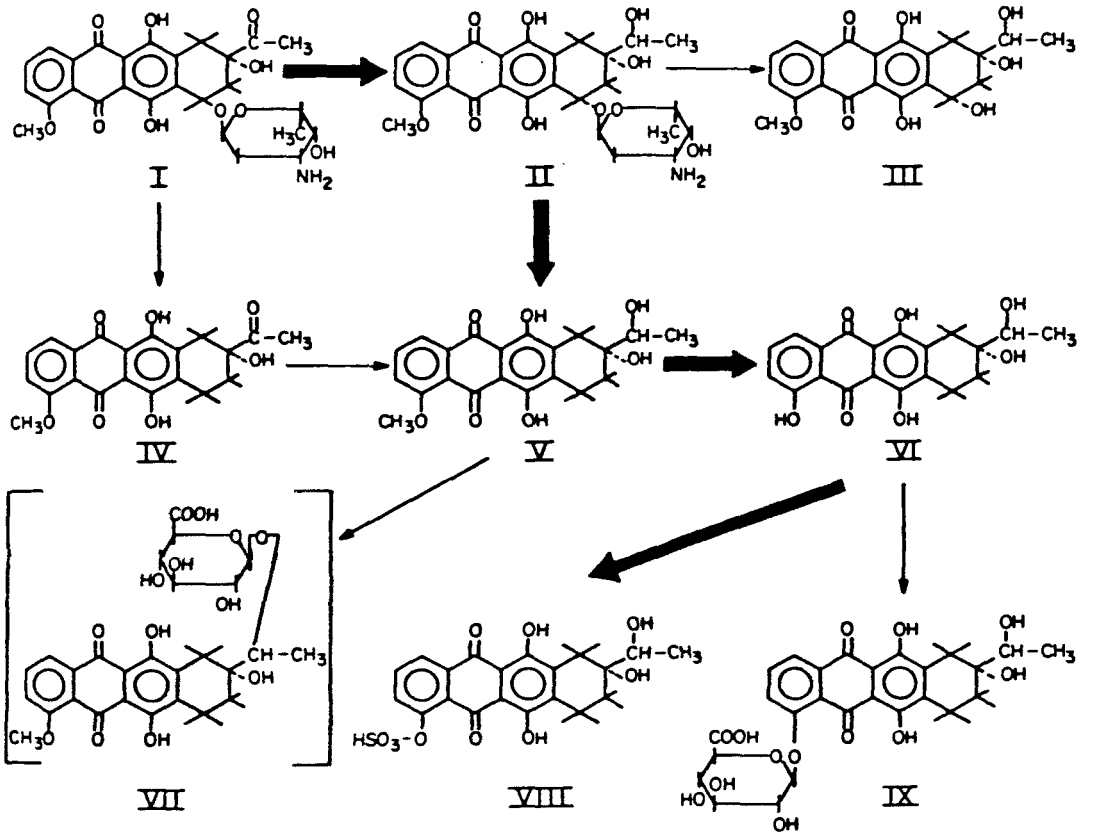
As indicated in the metabolic pathway depicted in Figure 3, daunomycin is metabolized by a aldo-keto reductase, daunomycin reductase (Bachur et al., 1971) to form daunorubicinol. This reduced product is not only the pricipal drug form excreted by human and animals (Cradock et al., 1973: Bachur et al., 1974) but also retains significant cytotoxic activity.

Daunomycin and daunorubicinol can also be further metabolized by either reductive or hydrolytic glycosidases (Bachur et al., 1972) to form aglycones which have low water but high lipid solubility. Solubilization of these aglycones can take place through O-β-glucuronidation and O-sulfation (Takanashi et al., 1975). The conjugate products can then be excreted through urine.

I.2.4. Clinical Toxicity

The most frequent signs of toxicity caused in patients by both

Figure 3. Proposed pathway for human daunmycin metabolism I. daunomycin; II, daunorubicinol; III, daunorubicinol aglycone; IV, deoxydaunorubicin aglycone; V, deoxydaunorubicinol aglycone; VI, demethyl deoxydaunorubicinol aglycone; VII, deoxydaunorubicinol aglycone-13-0-β—glucuronide; VIII, demethyl deoxydaunorubicinol aglycone-4-0-sulfate; IX, demethyl deoxydaunorubicinol aglycone-4-0 β-glucuronide.


I
II
III
IV
V
VI
VII
VIII
IX

daunomycin and adriamycin are stomatitis, alopecia, bone marrow depression, and gastrointestinal disturbances (Bernard et al., 1969). No significant kidney damage has been reported. A peculiar toxic effect of these drugs is a cardiopulmonary syndrome (Bonadonna et al., 1969) consisting of tachycardia, with or without arrhythmia, hypotension, gallop rhythm, tachypnea, and congestive heart failure, not relieved by digitalis. The mechanism of this effect has not been elucidated. Cardiac toxicity is more frequently observed when high doses (more than 25-30 mg/kg) are given, and in elderly patients (Di Marco et al., 1975). When an intermittent, rather than a daily dose schedule is used, cardiac toxicity is rarely observed.

I.2.5. Therapeutic Values

The main therapeutic indications of daunomycin and adriamycin are leukemias, especially acute lymphocytic leukemia in children. Because of the occurence of cardiac toxicity, the drugs are used, alone or in combination with other antitumor agents, mainly for remission induction and reinforcement dosing during remissions rather than for maintenance therapy (Di Marco et al., 1975). Both daunomycin and adriamycin are also active on lymphomatous neoplasms (e.g. Hodgkin's disease lymphosarcoma, reticular cell sarcoma).

Daunomycin has little or no effect on solid tumors, whereas adriamycin appears to be temporarily effective in a range of childhood tumors such as Ewing's sarcoma, neublastoma, osteogenic sarcoma and some anaplastic sarcomas (Bonadonna et al., 1970). In adults, chemotherapeutic effectiveness has been obtained in breast cancer, seminoma, ges-

tional choricocaricinoma and other soft tissue sarcomas, transitional cell carcinomas of the bladder, and lung cancer.

I.2.6. The Mode of Action of Daunomycin and Its Analogs

The fluorescence of daunomycin enables the investigation of the events which occur after the cells are incubated with the drug. It has been shown that daunomycin penetrates the cell and fixes itself in the nuclear structure (Calendi et al., 1965). The radiochemical data have confirmed that a large portion of daunomycin which enters the cell is taken up by the nucleus (Di Marco et al., 1975). In synchronized cultured cells it has been shown that the up-take of the drug occurs through the regenerative cycle, and reaches its maximum during DNA replication (Silvestrini et al., 1970).

In cultures of mammalian cells, daunomycin strongly inhibits adenine-8-C^{14} incorporation into RNA (Rusconi et al., 1966). This interference with the incorporation of labeled precursors into RNA is consistent with the findings that daunomycin inhibits in vitro DNA-dependent RNA polymerase (Di Marco et al., 1965). In cultured animal cells, RNA synthesis in the nucleolus is particularly sensitive to the drug (Angiuli et al., 1971). Autoradiographic studies carried out in vitro cultures of Hela cells (Di Marco et al., 1965; Crook et al., 1972) showed a higher inhibition of ribosomal RNA synthesis than messenger RNA synthesis. This preferential inhibition of nucleolar RNA is described in more detail in section I.2.8.1.

Inhibition of DNA synthesis by daunomycin has also been observed by following the incorporation of ^{3}H-thymidine into nucleic acids

(Rusconi et al., 1969). This inhibitory effect of daunomycin is correlated with the interference of daunomycin with the activity of DNA-dependent DNA polymerase (Hartman et al., 1964).

Studies have shown that when the daunomycin and the precursors of nucleic acids synthesis are added to the cell cultures at the same time, there are no significant differences in the relative inhibition of precursors uptake into DNA or RNA (Rusconi et al., 1969). Other autoradiographic studies have reported the influence of daunomycin on nucleic acids synthesis during different phases of cell cycle (Silvestrini et al., 1970). The effect of daunomycin inhibition is more evident on DNA synthesis which occurs in the late S phase, tentatively indentified with heterochromatin duplication and on RNA synthesis which occurs in the middle of G_1 and in the G_2 phases just before the mitotic peak (Simard, 1967). The observation (Kim et al., 1968) that the lethal effect of duanomycin on a synchronously dividing population of Hela cells is higher in S phase than in G_1 or G_2 phase suggests that the accessibility of DNA in chromosomal structures and repair processes may vary during the division cycle. Since a strong binding of daunomycin to DNA occurs only with double-stranded DNA (see section I.2.7.2.), one might assume that during the process of replication the drug could find access to DNA, bind strongly and irreversibly damage the chromosomal structure to cause cell death. In any event, the inhibition of these important metabolic events such as nucleic acid synthesis can explain the arrest of cullular reproduction. However, an immediate antimitotic activity of daunomycin (see section I.2.1.) which seems to be unrelated to the inhibition of RNA of DNA synthesis may indicate that the stiffening of DNA by the drug, which will

be discussed in the following section, may contribute to the chromosomal damage (Di Marco, 1967).

I.2.7. The Mechanism of Action of Daunomycin

Substantial amount of evidence suggest that daunomycin interferes with template activity by intercalating between the base pairs of the double helical DNA.

I.2.7.1. Spectral Evidence for Drug Binding to DNA

Initially, studies on the interaction of daunomycin with DNA (Calendi et al., 1965) revealed some physico-chemical changes upon formation of the daunomycin-DNA complex. Daunomycin absorbs in the visible range with a maximum at 475 nm. Its ultraviolet absorption spectrum shows two maxima at 233 nm and 255 nm. Daunomycin also shows a typical fluorescence spectrum with an emission at 580 nm when exicted at 485 nm.

When native or heat denatured DNA from various sources are added to an aqueous solution of daunomycin the antibiotic shows: (a) changes in visible absorption spectrum (both hypochromic, lowering of the absorbance at 475 nm, and bathochromic, shifting of its absorption maximum to 505 nm); (b) changes in ultraviolet absorption spectrum (lowering of absorbance at 233 nm and 255 nm and shifting of the two absorption maxima to a single maximum at 257 nm); (c) reduction of antibiotic fluoresence (almost complete disappearance of fluorescence) (Calendi et al., 1965). No such changes are observed when nucleotides, nucleosides, or free based are added to daunomycin solution.

Daunomycin aqueous solution is characterized by its red color.

At alkaline pH (9.4-9.8), the drug changes its color to blue due to salt formation of the compound (Calendi et al., 1965), and this color change can be prevented upon addition of DNA.

These spectral changes of daunomycin upon addition of DNA indicate that the drug binds to both native and denatured DNA. However, this binding is greatly reduced with derivatives of daunomycin where there is an alteration on amino sugar or complete removal of daunosamine, indicating that the amino group of daunosamine is actively involved in the binding process.

I.2.7.2. Evidence for Intercalation

Upon binding to daunomycin, there are also many changes in the physico-chemical properties of double-stranded DNA. These changes include a) an increase in the intrinsic viscosity of DNA (Zunino, 1971), b) a decrease in the buoyant density of DNA (Kersten et al., 1966), c) a decrease of the sedimentation coefficient of DNA (Calendi et al., 1965; Kersten et al., 1966), d) a stabilization of DNA against thermal denaturation, or in increase in the melting temperature of DNA (Zunino et al., 1972) and e) a protection of DNA from degredation by nucleolytic enzymes (nucleases) (Zeleznick et al., 1967).

According to Lerman's hypothesis on the interaction of 9-aminoacridine with DNA, and increase in the intrinsic viscosity of DNA when bound to antibiotics is accepted as a diagnostic feature of an intercalation process (Di Marco et al., 1971b). This phenomenon is attributed to a stiffening and elongation of the double-stranded DNA molecules when drugs are inserted into the hydrophobic areas between the flat base pairs

of the DNA helix (Evans et al., 1973). Besides, the planar aromatic ring system of daunomycin meets the requirement for the intercalation theory in which the drug must be "flat" to fit the space between by the base pairs (Gabby et al., 1976). The intrinsic viscosity of DNA is markedly less enhanced by all the derivatives of daunomycin with altered amino sugar than by other derivatives containing unsubstituted daunosamine (Calendi et al., 1965) indicating again that the amino group on daunosamine contributes to strengthening the intercalative binding.

In contrast to its effects on the native DNA, daunomycin causes a marked decrease in intrinsic viscosity of denatured DNA. This effect parallels the results reported for acridine dyes (Drummond et al., 1966). In the case of acridine dyes the effect of binding on the intrinsic viscosity of denatured DNA is quantitatively the same as that achieved by adding neutral electrolyte and has usually been attributed to a decrease in mutual repulsion between the charged groups of a flexible polyelectrolyte chain (e.g. phosphate groups) of DNA. The effect of daunomycin binding on denatured DNA could be explained by the effects of the positively charged amino groups of daunosamine on the mutual repulsion of the phosphate groups, suggesting that daunomycin also, like acridines, binds in the non-helical regions of DNA as in the denatured state (Zunino et al., 1972).

The decrease of the sedimentation coefficient of daunomycin-bound double-stranded DNA is explained by the uncoiling of the helix at the point of drug insertion which alters the average pitch on the helix and causes an increase in the average number of base pairs per turn (Di Marco et al., 1971b).

The increase in the melting point (Tm) of DNA upon binding to daunomycin indicates that there is an interaction between daunomycin and DNA which stabilizes the association of the two strands (Calendi et al., 1965). This increase in Tm is dependent upon the drug to DNA ratio (Zunino et al., 1972). Very little increase in Tm is observed for N-acetyl daunomycin and other derivatives with substitution in the amino sugar moiety, again indicating that the amino group is actively involved in stablizing the intercalated complex.

Daunomycin can be removed from DNA by Mg^{2+} and it can be extracted from aqueous solution of the complex by organic solvents such as phenol or n-butanol (Di Marco et al., 1972); at high ionic strengths, the binding of daunomycin to DNA is reduced (Bhuyan et al., 1965). Therefore, it seems that the complex formation involves electrostatic forces.

Formamide, a hydrogen bond breaker, was found to revert completely the binding between N-acetyl derivatives and DNA and only partially that between daunomycin and DNA (Calendi et al., 1965). This suggests that some of the bondings between the drug and DNA is of hydrogen bond type and the amino groups of the sugar moiety imparts to daunomycin a much higher binding ability by providing greater possibility for hydrogen bonding.

Other results correlate the structure of daunomycin and its derivatives with their biological activities. It has been shown that whenever the amino hydrogen of daunosamine is not substituted and the CO group of the acetyl side chain on position C-9 is not altered, the compounds are found to complex strongly to DNA and to inhibit mitotic activity, DNA synthesis and cell proliferation (Zunino et al., 1972). When

the CO group of the acetyl side chain is altered, the derivatives preserve the ability to bind to DNA but with reduced antimitiotic activity and are inactive or slightly active in inhibiting DNA synthesis and cellular proliferation. It has been shown (Arcamone et al., 1964b) that daunosamine, the amino sugar moiety of daunomycin, is an L-sugar, characterized by L-lyxo conformation. When daunosamine is substituted with α-D-glucosamine or whenever the amino group is masked, the derivatives are found to bind to DNA very weakly and to lack biological activity (Di Marco et al., 1971b). This suggests the importance of the structure and of the stereochemistry of the amino sugar moiety in the binding reaction.

All of these results have suggested two modes of binding between the antibiotic molecules and DNA: (1) the "strongly" bound antibiotic molecules are understood to be intercalated between successive base pairs of the double helix by the planar chromophore of the drug, facilitated by the positively charged amino sugar; (2) the "weakly" bound antibiotic molecules are thought to be attached to DNA by means of electrostatic interaction, involving the single-stranded DNA phosphate groups and, again, the daunosamine amino group. The shape of the isotherm (e.g. Figure 8) for the binding of daunomycin to DNA from spectrophotometric analysis (Zunino et al., 1972), the chromatographic behaviour of daunomycin on DNA-cellulose column (Zunino, 1971) and the X-ray-diffraction studies (Pigram et al., 1972) all support the intercalative model and the secondary binding of daunomycin to DNA.

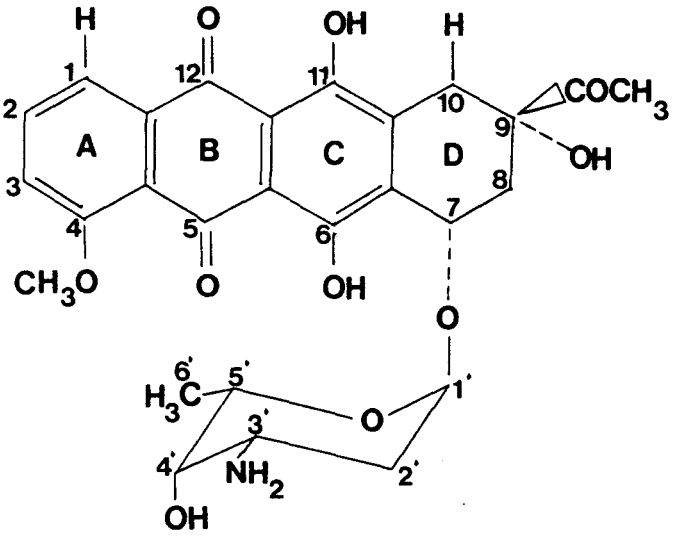
This intercalation theory can be used to explain the machanism of the inhibitory activity of daunomycin or bacterial and animal viruses

mentioned earlier. In fact, from the previously discussed data, it seems that inhibition of the function or of the replication of the viral genome requires the formation of a strong association of the antibiotics with the nucleic acids, and, from physico-chemical studies, it is known that this strong binding of drug to DNA occurs only with double-helical DNA (Zunino et al., 1972). The lack of activity on single-stranded DNA (Calendi et al., 1966) or RNA (Cohen et al., 1969) viruses appears to be a direct consequence of the inability of the drug to form this stable binding.

I.2.7.3. Models for Intercalation

An initial intercalation model was proposed in which the amino sugar of daunomycin is in the major groove of the DNA (Pigram et al., 1972). In this model the conformation of ring D of daunomycin was taken to be that found for the crystal structure of N-bromoacetyl daunomycin (Angiuli et al., 1971). In this structure C-9 is the most out of plane atom and the sugar is oriented axially with respect to ring D (Figure 4, conformation A). In this DNA-binding model, the daunomycin amino group binds to the second phosphate residue away from the intercalation site, and there is a hydrogen bond between the C-9 hydroxyl group and the first phosphate from the intercalation site (Goodman et al., 1977). A more recent analysis (Plumbridge et al., 1977) of the crystal structure of daunomycin however, suggests a slightly different preferred conformation of ring D (Figure 4, conformation B) in which C-8, rather than C-9, is the most out of plane atom and in which there is a hydrogen bond between the C-7 oxygen and C-9 hydroxyl group. The DNA-binding model, therefore,

Figure 4. The structure of daunomycin showing (a) the conformation of ring D for the crystal structure of N-bromoacetyldaunomycin, (b) that found for daunomycin, and (c) an alternative conformation for ring D.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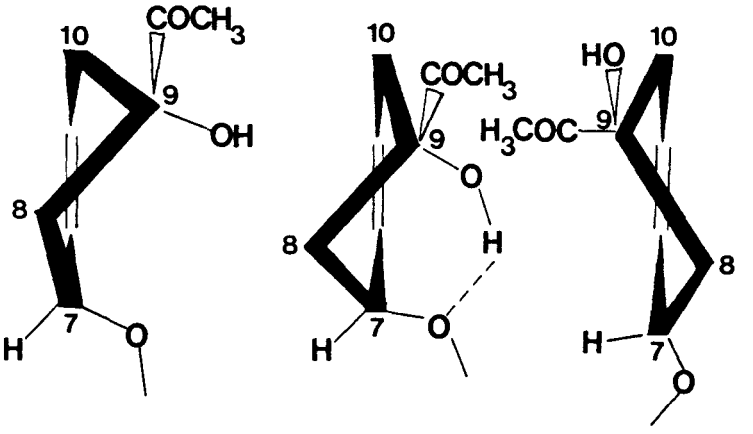
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needs to be revised to incorporated this intramolecular hydrogen bond. The conformation of ring D when the drug is bound to DNA may differ from the preferred conformation found in the crystal form. If the ring D conformation is altered so that C-8 lines behind rather than in front of the plane of the ring system as shown in Figure 4 then the amino sugar becomes oriented equatorially rather than axially (Figure 4, conformation C). Consequently there will be a spatially different interaction of the amino sugar in the major groove of the DNA (Pigram et al., 1972). The amino group will now bind to the first phosphate away from the intercalation site and there can be additional bonding in the form of hydrogen bonds between the C-4' hydroxyl group and the second phosphate from the site and between the C-9 hydroxyl and the phosphate at the intercalation site (there is no intramolecular hydrogen bond between the C-7 oxygen and the C-9 hydroxyl when the amino sugar is equatorial with respect to ring D). This latter model, therefore, provides for increased secondary stabilization of the intercalation complex. An inherent preference for daunomycin binding to A-T base pairs is also present (Plumbridge et al., 1979). In all of these models, the C-14 hydroxyl group of adriamycin can additionally hydrogen bond with the DNA. This additional bond provides the basis for the higher potency of adriamycin as compared to daunomycin in their antitumor effects (Di Marco et al., 1969).

Analogs of daunomycin have been used to test the validity of the main features and the fine details of these models (Brown, 1978; Arcamone, 1977). However, these studies have not shown conclusively whether the analogs bind by the reinforced intercalation mechanism as shown in Figure 4 conformation C.

All of the models for daunomycin and DNA interactions suggests

that the binding will be more sensitive to changes in the sugar unit than in the C-9 substitutents. Consequently the effect of inversion of configuration at C-7, C-1', C-3' and C-4' and the effect of deletion of C-4' hydroxyl group, on the mechanism of binding to DNA were studied (Arcamone et al., 1976; Zunino et al., 1977; Di Marco et at., 1977; Plumbridge et al., 1978). It was found that the reinforced intercalative binding to DNA which is typical of daunomycin can also occur if epimerization is present at C-4' or if the O-methyl group is lost or if the C-9 substitute is deleted or if the 4'-hydroxyl group is absent. In the latter two cases, however, there is a reduction in affinity for DNA, supporting the suggested role of the 9-hydroxyl and 4'-hydroxyl groups in the secondary stabilization of the complex.

Epimerization at C-1' or at C-3' alters but does not abolish the intercalative mode of binding to DNA whereas epimerization at C-7 precludes intercalation of the chromophore into the helix of DNA (Plumbridge et al., 1979). All of these results tend to support the binding model in which ring D adopts conformation C in preference to the other binding models.

In a separate report (Rich et al., 1981) a crystal of two daunomycin molecules intercalating into a double helical DNA was obtained. The DNA contains two strands of a self-complementary hexanucleoside pentaphosphate, d(CpGpTpApCdG). The saturation level for this intercalated complex is one daunomycin per three base pairs.

X-ray diffraction studies revealed that the chromophore (rings A through D, Figure 5) intercalates into DNA with ring A protruding well into the major groove formed between base pairs C1-G6* and G2-C5*. Ring

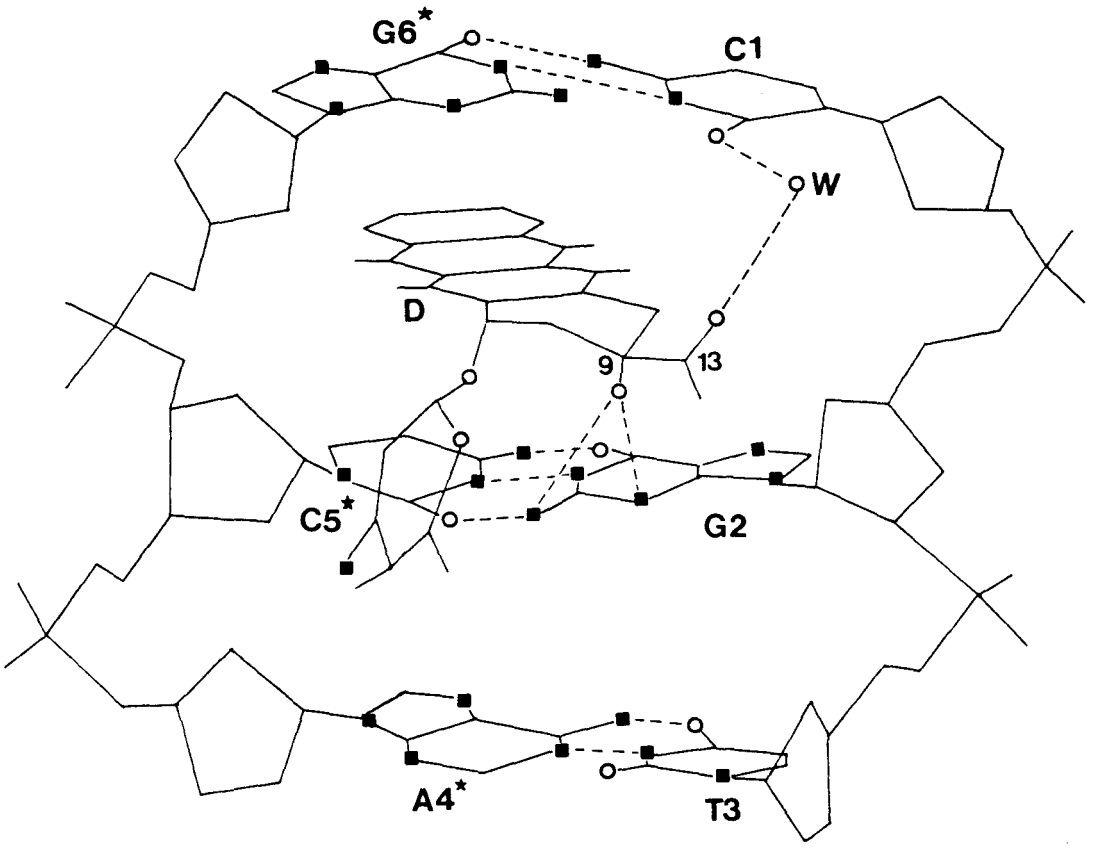
Figure 5. Intermolecular attractions of intercalated daunomycin and d(CpGpTpApCpG)

o Oxygen molecules

■ Nitrogen molecules

W Water molecule

D Daunomycin


G6*
C1
W
D
9
13
C5*
G2
A4*
T3

D of the chromophore and the attached amino sugar were found in the minor groove between base pairs G2-C5* and T3-A4*. Conformational changes for DNA molecules were also detected in this intercalated complex. Specifically, base pairs are displaced by 1.3 Å toward the major groove. An 8° unwinding of the DNA also takes place at the base pair one removed from the intercalation site.

Tight hydrogen bonding are also formed in this intercalated complex. Figure 5 shows that the hydroxyl group on 09 forms a hydrogen bond with N3 of G2 by donating its hydrogen. This hydroxyl oxygen also receives a hydrogen from another amino group of G2. In the case of adriamycin, the 14 hydroxyl group could form additional hydrogen bonding with 03' of G2. These hydrogen bonds may provide "anchoring function" holding the antibiotic to the double helix.

According to this data, ring D seems to adopt conformation A in Figure 4 which places the positively charged amino group in the minor groove where it is well separated from the negatively charged phosphate groups on either side.

I.2.8. The Mechanism of RNA and DNA Synthesis Inhibition by Daunomycin and Its Analogs

I.2.8.1. Inhibition of RNA Synthesis

The formation of a complex between template DNA and RNA polymerase is essential for the synthesis of RNA (Hinkle et al., 1972a-d). It was found that daunomycin has no effect on the activities of the RNA polymerase enzyme per se, since the pre-incubation of the enzyme with

the inhibitor in the absence of the template does not affect the activity of the polymerase (Zunino et al., 1974). Base on these information it seems that the reduction of RNA polymerase activity could then be accounted for by the loss of template activity of the drug-treated DNA. The finding that daunomycin causes a marked drop in the formation of a complex between RNA polymerase and DNA template (Mizuno et al., 1975) indicates that the binding of daunomycin to DNA may give rise to steric hindrance effects that interfere with the association of the template to RNA polymerase and thus, result in RNA synthesis inhibition. Experimental support comes from the finding that there is a competitive inhibition of daunomycin and DNA template in RNA synthesis in which inhibition can be overcome by readdition of the template (Zunino et al., 1974). On the contrary, a non-competitive relationship was established between the drug and the RNA polymerase enzyme, indicating that the inhibition is predominently due to the interaction of daunomycin with template DNA. It was also noted that Mg^{2+} could reverse the inhibitory effect of daunomycin in RNA synthesis presumably by releasing the drug from the drug-DNA complex (Calendi et al., 1965) thereby freeing the DNA template so that it can be associated with RNA polymerase for transcription. All of these results suggest that the inhibition is due to the formation of a reversible inhibitor-DNA complex which reduces the capacity of DNA to bind RNA polymerase.

The mechanism of RNA polymerase inhibition by daunomycin has also been studied in terms of the preferential binding of the drug to specific base pairs of the template, but the results from such reports have conflicted. Studies with DNA obtained from different sources

revealed that the binding of daunomycin to DNA increases as the G-C content of DNA increases (Zunino, 1971). Kersten also reported that the conformational changes of the daunomycin chromophore upon complex formation with DNA are slightly dependent on the G-C content of DNA (Kersten et al., 1971). However, inhibition studies of the DNA-dependent RNA polymerase by daunomycin (Ward et al., 1965) showed that daunomycin inhibits DNA-dependent RNA polymerase activity irregardless of the G-C content of the template used. Recent reports, from the study of the interaction between daunomycin and short segments of double helical DNA (Rich et al., 1981), also indicate that although highly specific hydrogen bonds, which serve to hold the antibiotic to the double helix, can be formed between daunomycin and G-C base pairs, such bond do not suggest great selectivity, i.e., when adenine is present instead of guanine, different but still highly stable hydrogen bonds can also be formed with daunomycin. Furthermore, results from model building (Plumbridge et al., 1979) suggest that daunomycin has a preference for A-T sequence and the stabilization of double helix by daunomycin appears to be dependent on the A-T content in DNA (Zunino et al., 1974). Other inhibition studies also showed that DNA templates which contain poly(dA·dT) are more sensitive to the action of daunomycin (Bhuyan et al., 1965). These results are supported by the observations that the inhibitory action of daunomycin is most effective at later stages of cell division (later S, G_2 and M) in mammalian system (Mizuno et al., 1975; Silvestrini et al., 1970). At this stage the drug preferentially binds to the heterochromatin fraction which is rich in adenine-thymine (Yunis et al., 1971). In addition, studies with synchronized rat fibroblasts (Silvestrini et al., 1970)

showed that the high sensitivity of RNA synthesis to daunomycin in G_2 phase is related to the fact that DNA containing poly (dA·dT) sequences are being transcribed at this time (Becker, 1972). It seems, therefore, that the extent of inhibition may be not only a function of the base composition of DNA but also of the secondary structure of DNA (Bram, 1971a) as well as the complexity of nucleotide sequence (Bram, 1971b) of the DNA segment that is being transcribed.

The regulation for gene expression (via transcription) in eukaryotic cells appears to involve multiple forms of RNA polymerase (Widnell et al., 1966). It was discovered that RNA polymerase A (or I), which is located in the nucleolus, is responsible for the synthesis of rRNA. RNA polymerase B (or II), which is located in the nucleoplasm is responsible for the general synthesis of mRNA. These enzymes can be distinguished by their sensitivity to α-amanitin; RNA polymerase A is insensitive, RNA polymerase B is completely inhibited (Chambon, 1975). Also, RNA polymerase A is activated by Mg^{2+} whereas RNA polymerase B is activited by Mn^{2+} or $(NH_4)_2SO_4$ (Barthelemy et al., 1976).

It was discovered that daunomycin preferentially inhibits the incorporation of tritiated uridine into ribosomal RNA components (Dano et al., 1972). The synthesis of heterodisperse, high molecular-weight, nucleoplasmic RNA has little sensitivity to inhibition by daunomycin, whereas the synthesis of tRNA, 5S RNA, and two small molecular-weight RNA components (Components C and D) shows medium sensitivity to daunomycin. In contrast, daunomycin inhibits the synthesis of 18S and 28S rRNA and the nuclear 45S and 32S precursors strongly and to the same extent, indicating that it is the synthesis of 45S RNA that is inhibited

and not the processing of 45S RNA to rRNA. This preferential inhibition of rRNA by daunomycin is supported by electron microscopic studies (Rusconi et al., 1964) and autoradiographic studies (Di Marco et al., 1965) in Hela cells. One explanation for this preferential inhibition is found in the findings that heat denatured DNA is more transcribed than the native DNA by RNA polymerase B (Barthelemy et al., 1976) and it takes much higher concentration of daunomycin to inhibit RNA polymerase activity using denatured DNA as template (Zunino et al., 1975). This is another piece of indirect evidence to support the intercalative theory of inhibition in which the double-stranded DNA is required for strong binding and inhibition. Native DNA, on the other hand, is a better template for RNA polymerase A and thus is inhibited by the intercalating daunomycin to a much greater extent.

Some preliminary proposals for the mechanism of RNA synthesis inhibition by daunomycin have been reported. Daunomycin has been found to reduce the formation of a complex between RNA polymerase and DNA because the drug-DNA complex provides the template a different conformation which is unable to bind RNA polymerase (Mizuno et al., 1975; Section I.2.8.1.). A later study with DNA extracted from Ehrlich Ascites tumor cells (Barthelemy et al., 1976) revealed that the presence of daunomycin also greatly increases the rate of dissociation of the enzyme-template complex. It was speculated in this study that daunomycin probably interferes with the melting of the double stranded DNA by RNA polymerase at the binding site (which transforms the enzyme-DNA complex into a stable one for RNA synthesis initiation, (Meilhac et al., 1972; also see Section I.3.4., and Figure 6)) and therefore de-stabilizes the enzyme-DNA complex

for RNA synthesis.

Since elongation of RNA strands does not seem to be affected significantly by daunomycin (Schellinx et al., 1979), it appears that once the first phosphodiester bond is formed, dissociation of daunomycin from the opened helix is fast enough not to be rate limiting.

I.2.8.2. Inhibition of DNA Synthesis

Very little effort has been made in the study of the mechanism of DNA polymerase inhibition by daunomycin or adriamycin. The currently available information is at best conflicting and inconclusive. For example, in a system using salmon spearm DNA and T4 DNA polymerase (Goodman et al., 1974; Goodman and Lee, 1977) it was determined that DNA synthesis is inhibited by adriamycin via inactivation of the template functions. Both intercalative and ionic types of binding of the drug to DNA were found to occur and the relative amounts of each type of binding may depend upon the relative drug to DNA ratio. At low drug to DNA ratio, the intercalation is favored, while at high drug to DNA ratio, the ionic interactions become additional forces for DNA synthesis inhibition.

A separate study (Tanaka et al., 1980) showed that inhibition of DNA synthesis by daunomycin and adriamycin is mainly the result of direct interaction of drug molecules with DNA polymerase rather than the result of drug impairing of the template activity of DNA due to intercalation. Apparently stronger inhibition is produced by pre-incubating the drugs with enzyme rather than with the template-primer complex.

The reason for the discrepancies between these results is not clear. It is apparent, however, that the mechanism of inhibition of

both RNA and DNA synthesis by daunomycin and its analogs are still poorly understood. Additional studies are necessary to determine the mechanism of the inhibition process and to resolve conflicting reports.

I.2.8.3. Factors Affecting the Inhibition Studies

Conflicted results have also been reported on the comparative study of the extent of inhibition by daunomycin in the incorporation of labeled precursors into RNA and DNA in vivo or in cultured cells.

In vitro studies on Ehrlich ascites tumor cells (Dano et al., 1972) and on L1210 mouse leukemia cells (Meriwether et al., 1972) showed that daunomycin inhibits DNA and RNA synthesis to a similiar degree. Other in vivo studies support this result (Rusconi et al., 1969; Meriwether et al., 1972). However, some studies showed that DNA polymerase is more sensitive to daunomycin inhibition than RNA polymerase (Mizuno et al., 1975; Momparler et al., 1976). These discrepancies may be due to the different experimental conditions employed, since studies with Hela cells demonstrated that at low daunomycin concentrations RNA synthesis is more inhibited than DNA synthesis whereas at high drug concentrations both DNA and RNA synthesis are inhibited to the same degree (Rusconi et al., 1969).

Cell population densities also affect the response of the cells to inhibition. In fact, the inhibition of RNA or DNA synthesis is lower when then cell number is relatively high even though the relative uptake or the amount of daunomycin bound to DNA remains the same as when the cell number is relatively low. Autoradiographic studies have provided evidence that, in synchronized cells, the uptake of the antibiotic also

varies according to the phase of the cell cycle (Silvestrini et al., 1963).

Discrepancies have also been reported on the relative inhibitory potency of daunomycin and adriamycin. Evidence from cell culture (Meriwether et at., 1972) and cell free systems (Zunino et al., 1975) show that even though daunomycin and adriamycin inhibit both DNA and RNA synthesis to the same extent, daunomycin appears to be a better inhibitor in cell culture than adriamycin whereas adriamycin appears to be a better inhibitor in the cell free systems. However, it was shown that the uptake of daunomycin by the cells is much greater than that of adriamycin, indicating that the actual effectiveness of adriamycin could be higher than that of daunomycin. Some in vivo studies with animals (Sandberg et al., 1970) and preliminary clinical studies (Bonadonna et al., 1970) showed a higher therapeutic index for adriamycin.

All of these studies have lead to the conclusion that the understanding of the mechanism of daunomycin action is still far from clear and that more than one mechanism of inhibition could be operating in the intact animals. For example, mechanisms involving differences in the rates of drug uptake and routes of drug excretion, differences in protein binding, differences in metabolic pathways, or different rates of metabolism in vivo for each drug probably play important roles in determining the overall drug effectiveness in vivo.

I.3. Mechanism of Transcription

DNA-dependent RNA polymerase is responsible for the synthesis of all cellular RNA and thus plays a central role in the process by which the genetic information encoded in DNA is expressed. The information presented in this section pertains to bacterial RNA polymerase, unless specified otherwise.

The prokaryotic transcriptional process catalyzed by bacterial RNA polymerase can be formally separated into several steps, including binding of the enzyme to the template, initiation steps which yield a tight complex of RNA polymerase with the promoter and opening the double-stranded structure at specific sites allowing the formation of the first phosphodiester bond. This is followed by elongation using one strand as a template to direct the synthesis of a complementary RNA molecule, and finally termination, in which the product RNA is liberated and the enzyme becomes available for the next cycle (Zillig et al., 1976).

I.3.1. Structure and Composition of Bacterial RNA Polymerase

Bacterial DNA-dependent RNA polymerase is a multisubunit enzyme. Holoenzyme monomers from various sources contain two large subunits, β' and β (molecular weight 160,000), two identical small subunits, α(molecular weight 40,000), and an initiation factor, σ(molecular weight ranging from 44,000 to 92,000 (Lill et al., 1975a; Palm et al., 1975)). Holoenzyme ($\beta'\beta\alpha_2\sigma$) can be separated into two functional parts: a core enzyme ($\beta'\beta\alpha_2$) which is able to initiate RNA synthesis but lacks the ability to initiate such synthesis at specific sites on the template (Chamberlin,

1976); and the sigma factor (σ), which enables the enzyme to select a specific initiation site on the template (Burgess, 1971) by recognizing a particular base sequence termed "promoter". The sigma factor is released during chain elongation (Travers and Burgess, 1969). The reconsitution of holoenzyme from these two components occurs rapidly when the two are mixed in solution (Berg et al., 1971) and the reconstituted holoenzyme regains the ability to initiate specific RNA chains.

I.3.2. Functions of the Subunits of RNA Polymerase

Dissociation of the enzyme with SDS or high concentration (8M) of urea leads to complete separation of all the subunits of the enzyme; these subunits can then be resolved from each other by electrophoresis or acrylamide gel or by sizing chromatography (Travers and Burgess, 1969; Heil and Zillig, 1970).

β', the most basic subunit of RNA polymerase (Rexer et al., 1975) is able to bind to DNA whereas the other subunits which are less basic, or the subassembly $\beta\alpha_2$ (Fukuda et al., 1974: Yarbrough et al., 1974) do not bind to DNA. Therefore β' has been designated as the DNA-binding subunit (Sethi et al., 1971). Heparin, a polyanionic transcription inhibitor, binds to the β' subunit (Zillig et al., 1971) and competes with DNA for a template binding site on the polymerase (Walter et al., 1967) and therefore provides further evidence for the DNA binding function of β' subunit. It has also been shown (Stetter et al., 1974) that β' contains a binding site for σ. During transcription σ is released from the active complex of RNA polymerase, template and product when the product has reached a critical length of about ten nucleotide residues (Krakow et al., 1971).

It has been suggested that σ is bound to the "product site" of the enzyme (which might involve β') and is displaced by the growing RNA strand (Zillig et al., 1976).

The understanding of the functional role of β subunit comes from the studies involving rifampicin which binds to RNA polymerase (Wehrli et al., 1968) and inhibits the initiation of RNA synthesis (Sippel et al., 1968). RNA polymerases from mutants resistant to the drug do not bind the drug and show a β subunit with an altered electrophoretic mobility (Rabussay et al., 1969). β subunit also has been found to be directly involved with streptolydigin, another antibiotic which interacts with core RNA polymerase and inhibits elongation of RNA chains (Siddhikol et al., 1969). Therefore the β subunit contains the antibiotic binding sites and is responsible for the catalytic functions (both initiation and elongation) of the enzyme.

Subunit α is present in solution as a dimer (Lill et al., 1975b) in which the two α chains are linked by a disulfite bridge (Zillig et al., 1976). The function of α is not clear at the moment, although it has been suggested that in addition to β, α and β' are required for reconstitution of the catalytically active holoenzyme (Chamberlin, 1974a) and α is required to bind β in order to activate β for drug binding (Lill et al., 1975a).

These assignments of specific functions to the various subunits are still somewhat tentative, particularly due to the lack of knowledge about the primary and three dimensional structure of the enzyme.

I.3.3. Binding of RNA Polymerase to DNA

In the presence of an excess of RNA polymerase, whether it be holo- or core-enzyme, helical DNAs such T7 can bind an amount of RNA polymerase limited only by the steric interactions between enzyme molecules (Pettijohn et al., 1967). Hence RNA polymerase possesses a substantial general affinity for DNA that is not sequence-specific (Hinkle et al., 1972a). This general affinity is called the non-specific binding and is entirely electrostatic in nature (Record et al., 1976). The binding constants for either core- or holo-enzyme are not highly temperature sensitive, indicating that the bindings do not involve opening (or melting) of DNA base pairs (Saucier and Wang, 1972). The association constant for general binding of RNA polymerase holoenzyme and core-enzyme to T7 DNA were estimated at 10^{8}-10^{9} M^{-1} and 10^{11} M^{-1} respectively under normal reaction condition in 0.01 M Mg^{2+} and 0.05 M Na^{+} (Hinkle and Chamberlin, 1972a). The substantially higher binding constant for core enzyme than that for holoenzyme suggests that binding of sigma subunit supresses the general binding affinity of core polymerase for DNA (Chamberlin, 1974b) except at promoter sequences (see next section).

Core polymerase is able to catalyze the synthesis of RNA chains but is unable to locate promoter sites to form the highly stable RNA polymerase-promoter complexes with DNA template. Therefore core-polymerase-DNA complexes are highly sensitive to attack by heparin (Hinkle et al., 1972c) and rifampicin (Bautz and Bautz, 1971), which indicates a slow rate of RNA chain initiation by these complexes. It was also found that single-stranded breaks are the primary sites for initiation by core enzyme (Hinkle et al., 1972c) probably because of the fact

that DNA-core enzyme complexes are defective in opening the DNA strands, which is necessary in transcription (Saucier and Wang, 1972).

I.3.4. Site Selection of RNA Polymerase Holoenzyme

In systems where RNA polymerase carries out selective transcription, effective binary complexes are formed primarily at promoter regions of the DNA template. The specificity of the process of "site selection" involves the binding interaction which positions RNA polymerase on the template and the subsequent interactions between the enzyme and DNA at that site that render the complex effective in the chain initiation reaction. This process of promoter "site slection" is a key point at which cellular regulation of transcription takes place.

Under appropriate reaction conditions, RNA polymerase holoenzyme and DNA template form stable binary complexes which can go on synthesizing RNA with specific 5'-termini (Pribnow, 1975). The formation of these stable complexes is dependent upon the rapid dissociation of the non-specific complexes mentioned in the last section. Studies from T7 DNA and E. coli RNA polymerase holoenzyme showed that a maximum of eight enzyme molecules are tightly bound to T7 DNA with a binding constant of 10^{12}-10^{14} M^{-1} (Hinkle et al., 1972a). A limited number of base pairs (six to seven) are opened in the complex (Saucier and Wang, 1972) with the RNA polymerase acting as a sequence-specific melting protein (Mangel and Chamberlin, 1974a). This opening process is temperature dependent and the complexes formed have been termed "open promoter complexes" (Mangel and Chamberlin, 1974b).

With T7 DNA, open promoter complexes are not formed at low temperature; as the temperature is increased, however, there is a coopera-

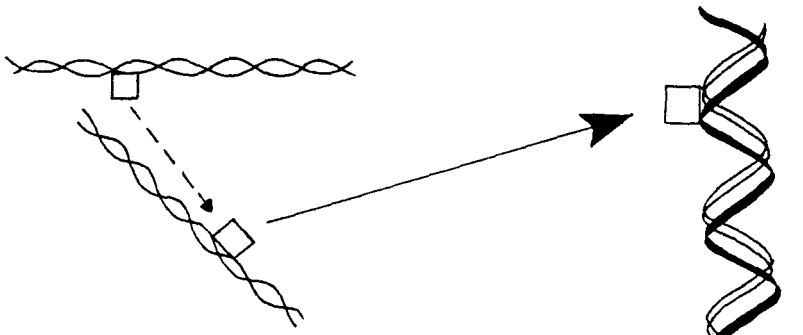
tive transition with a midpoint of about 18 °C (transition temperature), and above this temperature complex formation is eventually complete (Mangel and Chamberlin, 1974a). The enthalpy for this melting reaction is calculated at 57 Kcal per mole (Mangel and Chamberlin, 1974a). This opening is essential to the subsequent binding of the nucleoside triphosphates in the chain initiation step (Chamberlin, 1976).

In conclusion, the sequence of steps in site selection by RNA polymerase holoenzyme involves (1) reversible, weak binding to random sites on DNA to form non-specific complexes, leading to (2) attachment to a site at or near the specific site (promoter) on the T7 DNA molecule to form the closed promoter complex (I complex) and (3) a reaction to form a highly stable complex, the open promoter complex (or RS complex) with the separation of the DNA strands at or near the promoter sites (Zillig et al., 1971). The open promoter complex is able to initiate the formation of the first phosphodiester bond to form the OP complex. A scheme depicting all these pre-initiation events is presented in Figure 6.

I.3.5. Assays for the Rate of RNA Initiation

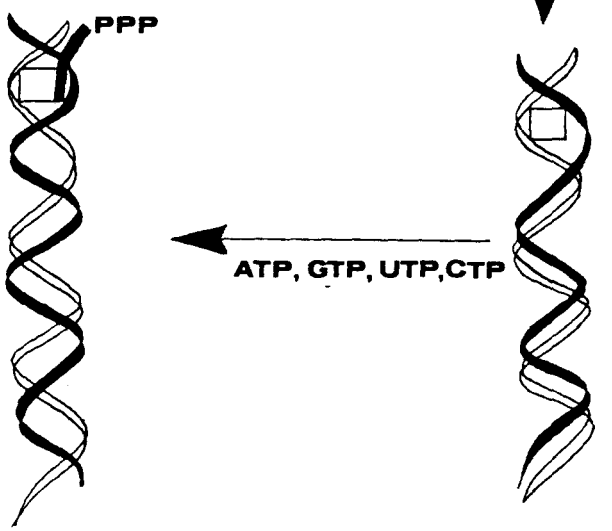
Several assays for the rate of RNA chain initiation have been developed (Mangel and Chamberlin, 1974a,b,c) by exploiting the property of the drug rifampicin to attack and inactivate RNA polymerase molecules. It was discovered that both free E. coli RNA polymerase and RNA polymerase bound to T7 DNA template are attacked by rifampicin in second-order reactions although the rate constant for the latter is 100-fold slower (Hinkle et al., 1972d). In contrast, the elongating enzyme molecules are insensitive to the attack of rifampicin (Sippel and Hartman,

Figure 6. Promoter Recognition and Initiation of RNA Synthesis



1. Intermolecular Exchange
(non-specific complexes)

2. Initial Recognition
(I complex)



4. Initiation
(OP complex)

3. Activation
(RS complex)

1968).

Another important feature in these assays is the time under which the RNA synthesis is allowed to proceed. The in vitro transcription of T7 DNA by E. coli RNA polymerase is restricted to the early region consisting of about 20% of the r-strand of the T7 duplex (Davis and Hyman, 1970), which yields an RNA product of approximately 7,000 nucleotides long (Millette et al., 1970). Since the rate of the chain growth is rapid (about 40 nucleotides per second for T7 DNA (Davis and Hyman, 1970)), termination and re-initiation could take place within a few minutes. Therefore the reaction time in these assays is limited to 90 seconds to eliminate the possibility of termination and re-initiation.

Utilizing this information two assays were developed to measure the rate of I complex to RS complex transformation and the rate of RS complex to OP complex transformation. The detailed descriptions of these assays are presented in section III.3.4.2.

I.3.6. Other Properties for Promoter Sites

The formation of open promoter complexes is affected by the structure of the RNA polymerase involved, the structure and nucleotide sequence of the DNA template, other components present in solution and, finally, the reaction temperature.

The open promoter complex has a high stability to dissociation with half-lives of many hours under standard ionic conditions (0.01 M Mg^{2+}, 0.05 M Na^{+}). At low temperature or high ionic strength, the stability of the complex is greatly reduced (Seeburg and Schaller, 1975). The RNA polymerase in open promoter complex is monomeric (Beabealashvilly

et al.,1972) and is attacked at a reduced rate by rifampicin (Hinkle and Chamberlin, 1972d) and polyanion heparin (Zillig et al., 1971). It is able to initiate an RNA chain rapidly when presented with nucleoside triphosphate substrates (Mangel and Chamberlin, 1974c).

Promoter sites display a definite specificity for a particular RNA polymerase. The T7-specific enzyme, for example, does not utilize any of the promoter sites on T3 DNA that are read by the T3-specific enzyme (Golomb and Chamberlin, 1974), nor does it read E. coli RNA polymerase promoter sites on T7 DNA. Therefore, each promoter has the sequence information essential for its utilization by a particular RNA polymerase (Chamberlin, 1976).

Promoter sites on the same DNA differ in their rates of utilization (Heyden et al., 1972). With T7 DNA, for example, a major fraction of the RNA formed with E. coli RNA polymerase at low ratios of enzyme to DNA is initiated at three closely linked promoter sites (A1, A2, A3) at the early region of the molecule (Dunn and Studier, 1973; Daussee et al., 1972). These three sites are used with approximately equal effeciency at 37°C, but at low temperature (10-15°C) or high ionic strength of the reaction mixture, site A3 is used over 50% of the time (Daussee et al., 1972). In addition to these so called "major" promoter sites, "minor" promoter sites, which are used with lower effeciency except when excess enzyme is present, are also located in the early region of the T7 genome (Minkley and Pribnow, 1973), but it is not known whether these sites are functional in vivo.

Major promoter sites can also differ from the minor sites in the strength of the melting interaction between the promoter and the RNA

polymerase. Under conditions which reduce the melting reaction (i.e., low temperature) a major promoter will be read at a reduced rate and becomes a minor promoter (Richardson, 1975). At higher temperatures or in the presence of DNA de-stabilizing agents these promoters are activated and begin to be read at a substantial rate (Richardson, 1975; Surzycki, 1976). However, for some minor promoters, conditions that facilitate the melting interaction do not alter the relative rate of reading (Golomb, 1974b).

For certain promoter sites, such as that governing the lac operon, transcription by E. coli RNA polymerase alone is very slow unless a cyclic AMP receptor protein is added along with cyclic AMP (Chen et al., 1971; Anderson et al., 1974). The cyclic AMP receptor protein was found to reduce the melting temperature of DNA (Makanishi et al., 1974). This positive control protein is also known to be essential for transcription in vivo (Chamberlin, 1976).

Repressor proteins suppress transcription at specific promoter sites and block the formation of open promoter complexes by making the promoter inaccessable to RNA polymerase (Squires et al., 1976) or acting as anti-melting agents (Wang et al., 1974; Von Hippel et al., 1975).

I.4. Purpose and Scope for This Research Project

Recently the correlation between biological properties and pharmacological effectiveness of a series of anthracyclines has been examined. Many attempts have been made to establish a relationship between the effects of anthracycline bind to DNA and the resulting stabilization of the double helix versus the antitumor activities of the drugs.

Studies involving large number of structural analogs of daunomycin have shown that the strength of DNA binding correlates in general with antitumor activity (Nakanishi et al., 1974). Since the proposed mode of binding of anthracycline to DNA is intercalation, certain predictions regarding the DNA affinities of various daunomycin derivatives have been made on the basis of the structure of the resulting anthracycline-DNA complex. For example, the C-4' hydroxyl of the anthracycline sugar moiety was found not to take part in any interactions and this suggests that epimerization at C-4' would produce derivatives with no alteration in their DNA binding ability (Neidle, 1977). This indeed was found to be the case with 4'-epiadriamycin which intercalates to DNA with the same affinity as its parent compound (Plumbridge and Brown, 1978) and has equivalent activity against experimental tumors as that of adriamycin (Arcamone et al., 1975). It was also predicted that since the C-4 methoxy carbon atom of daunomycin is not co-planar to the heterocyclic ring, removal of this group should allow deeper insertion of the ring into the intercalation site and therefore should increase the affinity to DNA (Zunino et al., 1976). Indeed 4-demethoxy derivatives

of daunomycin have been found to enhance the thermal stability of DNA to a larger degree than the parent compounds (Zunino et al., 1976). Furthermore 4-demethoxydaunomycin is as effective against various experimental tumors as the parent compound but at doses four to eight times lower than those effective for daunomycin (Arcamone et al., 1976). Similarly, 4-demethoxyadriamycin and 4-demethoxy-4'-epiadriamycin are effective against the same experimental tumors but at doses ten times lower than those effective for adriamycin (Di Marco et al., 1978). Therefore, the present argument is that the structure of anthracyclines greatly influence their binding properties to DNA and these binding properties in turn correlate with the antitumor activities of these drugs (i.e., stronger binding increases antitumor effectiveness of the drug).

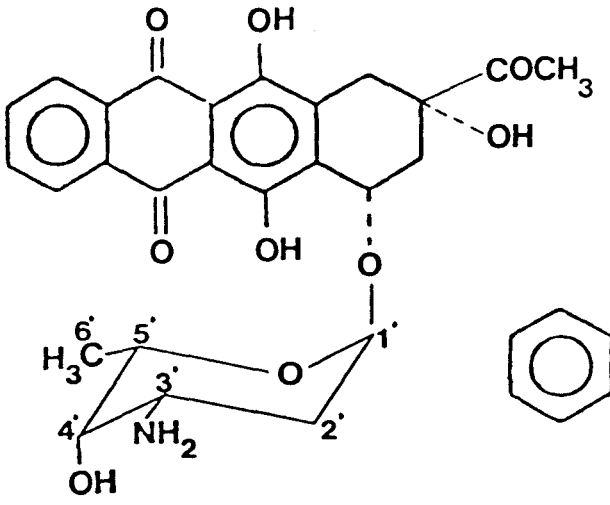
Although there is very little doubt that DNA binding affects the antitumor activities of anthracyclines, there are now some indications that the strengths of association between anthracyclines and DNA (i.e., the association constant) may not be the most appropriate parameters to explain the correlation. For instance, the higher potency of 4-demethoxydaunomycin compared to daunomycin is not consistent with the similarity of the apparent association constants of these drugs with DNA, which are $2.4 \pm 0.3 \times 10^6\ M^{-1}$ and $3.3 \pm 0.8 \times 10^6\ M^{-1}$ respectively (Zunino et al., 1976). Other findings have shown that upon binding to DNA, anthracyclines stabilize the double helix (Zunino et al., 1972). This suggests that the extent of double helix stabilization may provide a better approach to correlate the biochemical and pharmacological activities of anthracyclines. In fact 4-demethoxydaunomycin has been found to be more effective in stabilizing the double helix than the parent

compound (Zunino et al., 1976). It should be noted that although this property is partially dependent on the strength of association between DNA and the drug, it is not strictly proportional to it.

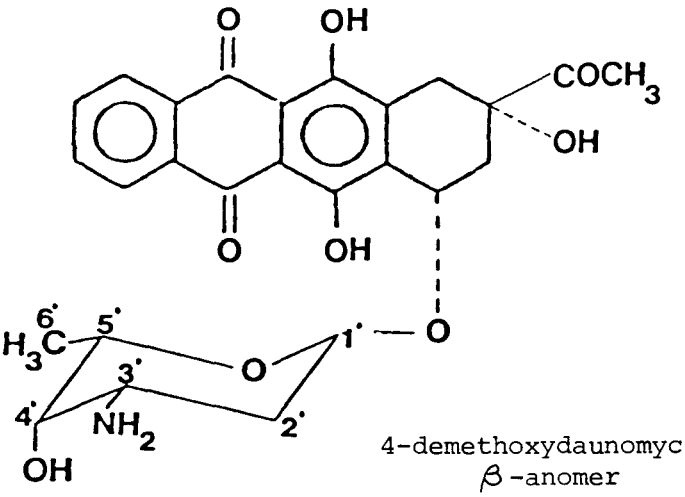
Furthermore, studies on the effectiveness of the four stereoisomeric 4-demethoxydaunomycin derivatives (Figure 7) as potential antitumor agents have shown that inversions of configuration at position 1 of the amino sugar and at positions C-7 and C-9 of the heterocyclic ring markedly decrease demethoxydaunomycin binding to DNA, with the β-anomers binding less strongly. In terms of double helix stabilization, the α-anomers are most effective with the 7,9-bis-epi derivatives producing the least amount of stabilization (Zunino et al., 1976). However, the inhibitory effects of these series of isomers on the in vitro synthesis of RNA correspond more closely to their stabilizing effects on the double helix rather than their binding affinities to DNA.

Recently anthracyclines such as carminomycin, aclacinomycin, marcellomycin, and musettamycin (Figure 1c, d, e, and f) have been isolated or synthesized (Nettleton et al., 1977; Oki et al., 1975). Carminomycin has a similar structure with daunomycin while the other three anthracyclines are characterized by the presence of a carbomethoxy group at C-10 position and a glycosidic side chain containing from two to three sugar residues. The amino group of the primary sugar residue for these new anthracyclines is also substituted. It has been shown that daunomycin, adriamycin and carminomycin inhibit RNA synthesis at comparable concentrations (DuVernay et al., 1980) whereas aclacinomycin, marcellomycin and musettamycin are more effective against RNA synthesis than the previous group (Crooke et al., 1977; DuVernay et al., 1979). However,

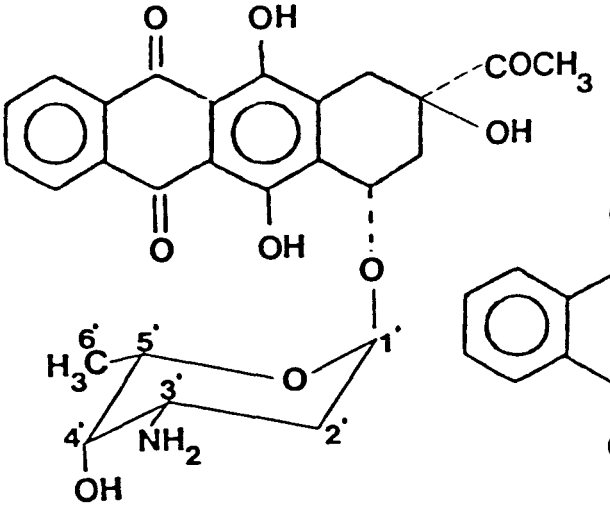
Figure 7. Stereoisomers of 4-demethoxydaunomycin



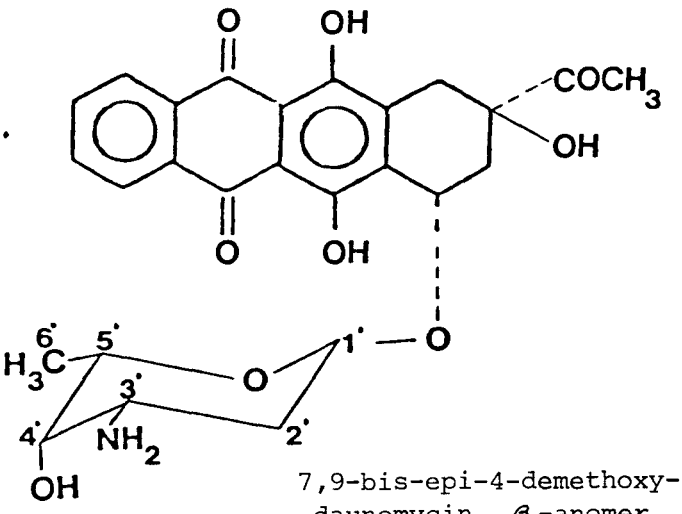
4-demethoxydaunomycin
α-anomer



4-demethoxydaunomycin
β-anomer



7,9-bis-epi-4-demethoxy-
daunomycin α-anomer



7,9-bis-epi-4-demethoxy-
daunomycin β-anomer

the mechanisms of RNA synthesis inhibition by these drugs remain unknown.

The mechanisms of RNA synthesis inhibition by ethidium bromide and its analogs have been thoroughly studied (Aktipis and Panayotatos, 1977). These drugs were found to inhibit primarily the initiation process of RNA synthesis without affecting elongation to a significant extent. Subsequently the effects of these drugs on the rate of RNA polymerase-DNA binary complex transformation processes prior to RNA synthesis initiation were carefully examined. It was concluded that even though no significant differences were observed in the mechanism by which these drugs inhibit RNA synthesis, the relative inhibitory effectiveness of ethidium and its analogs depended not only on their relative binding affinities to DNA but on the individual structural characteristics of these drugs as well. Thus, it is reasonable to expect that structural differences between related anthracyclines may also influence the mechanism of inhibition of RNA synthesis to different extents, and that such differences may not all be due simply to differences in the affinity with which each anthracycline associates with the DNA template.

Previous attempts to correlate the biochemical properties of anthracyclines, in terms of their DNA binding affinities and stabilization of double helical structures, with the pharmacological effectiveness of these drugs have failed to provide consistent results. In addition, the mechanism by which anthracyclines inhibit RNA synthesis has generally been overlooked in these studies. It is the goal of this dissertation to examine the mechanism by which anthracyclines inhibit RNA synthesis. Once the mechanism of RNA synthesis inhibition by each anthracycline analog is determined, it would also be of interest to examine whether a correla-

tion between the mechanism of inhibition and the template affinities of these various anthracyclines can be established.

CHAPTER II

MATERIALS AND METHODS

II.1. Materials

Bacteriophage T7 (host: E. coli B) was a generous gift from Dr. D. Hinkle of the University of Rochester (Rochester, New York 14627). The phage had a titer of 3.5×10^{11} plaque forming units per ml.

E. coli B thymine requiring mutant strain was a generous gift from Dr. Robert V. Miller of Loyola University of Chicago (Maywood, Illinois 60153).

DNA-dependent RNA polymerase (Nucleosidetriphosphate: RNA nucleotidyltransferase; EC No. 2.7.7.6) from E. coli B., strain K-12 was purchased from Sigma Chemical Company (St. Louis, Missouri 63178) as a solution in 50% glycerol and 50% 0.01 M Tris buffer, pH 7.9, containing 0.1 M NaCl, 0.1 mM Dithiothreitol and 0.1 mM EDTA. The enzyme (Lots 108C-04491 and 100F-00781) had specific activities of 1195 and 750 units/mg, and consisted of 2.9 mg proten/ml and 3.5 mg protein/ml, respectively. One unit of enzyme will incorporate 1 nMole of labeled ATP into an acid insoluble product in 10 minutes at pH 7.9 at 37°C, using Calf thymus DNA as template (Burgess, 1969).

Daunomycin ((8S-cis)-8-acetyl-10-(β-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxyl-1-methoxy-5,12-naphthacenedione), a red crystalline solid, was purchased from Boehringer Mannehim Biochemicals (Indianapolis, Indiana 46250) as hydrochloride, with a molecular weight of 564.0 daltons (Lot No. 1457501).

4-demethoxydaunomycin (Lot No. GDA 2652/61) was a generous gift from Dr. Federico Arcamone of Farmitalia Carloerba, Milano, Italy. Crystalline solids of aclacinomycin (yellow, Lot No. 80F108), carminomycin (orange, Lot No. 80F401), marcellomycin (dark red, Lot No. C36145-S369-G55) and musettamycin (dark red, Lot No. C36145-S420-G25) were generous gifts from Dr. J.L. MacBeth of Bristol Laboratories (Syracuse, New York 13201). Marcellomycin and musettamycin were provided as free bases. Rifampicin (m.w. 823 daltons) was purchased from Sigma.

White crystalline sodium salts of nucleoside triphosphates; ATP, CTP, GTP, and UTP were purchased from P-L Biochemicals, Inc. (Milwaukee, Wisconsin 53205). The commercial preparations were at least 97% pure according to the manufacturer's specifications.

[5,6-^{3}H] uridine-5'-triphosphate, tetrasodium salt (specific activity: 25 Ci/mmol) in ethanol:water (1:1) solution was purchased from ICN Pharmaceuticals, Inc. (Irvine, California 92715). The solvent was removed by directing a gentle stream of nitrogen gas onto the surface of the warmed solution (not exceeding 30°C) before use. [Methyl-^{3}H] thymine agueous solution (specific activity 30 Ci/mmol) was purchased from Amersham Corporation (Arlington Heights, Illinois 60005). All radioactive materials were over 99% pure according to the manufacture's specifications.

Nitrocellulose membrane filters (BA85, 24 mm, 0.45 micron) were purchased from American Scientific Products (McGaw Park, Illinois 60085). Whatman glass microfibre filters (GF/C, 2.4 cm) were purchased from Sargent-welch (Skokie, Illinois 60077).

II.2. Instruments:

The following instruments were used in this study:

Cary model 15 Recording Spectrophotometer (Varian, Cary Instruments Division, Monrovia, California 91016).

Beckman model L5-65 Ultracentrifuge (Beckman Instruments, Inc. Fullerton, California 92634).

Beckman model LS 7500 Liquid Scintillation Counter with Microprocessor (Beckman Instruments, Inc. Fullerton, California 92634).

Sorvall RC-5B Refreigerated Superspeed Centrifuge (Du Pont Instruments, Wilmington, Delaware 19898).

Barnstead Laboratory Sterilizer (Sybron Coporation, Downers Grove, Illinois 60515).

Corning model 12-B Research pH Meter (Corning Scientific Instruments, Medfield, Massachusetts 02052).

Mettler P163 Balance (Mettler Instrument Corporation, Highstown, New Jersey 08520).

Perkin-Elmer model 320 Spectrophotometer (Perkin-Elmer Corporation, Norwalk, Connecticut 03016).

Pipetman Adjustable Pipet models P-1000, P-200, P-20 (Rainin Instrument Co. Inc. Woburn, Massachusetts 01801).

Forma model 2563 Shaker Bath (Forma Scientific, Marietta, Ohio 40133).

II.3. Preparation of Working Solutions

II.3.1. Preparation of T7 DNA

Bacteriophage T7 was grown essentially as described by C.C. Richardson (Richardson, 1966). E. coli B was grown in a TCG medium (Thomas and Abelson, 1966) (0.8 ml, 0.10 M Na_2SO_4; 450 ml H_2O; 0.5 ml, 1.0 M $MgSO_4$; 1.0 ml, 25% NaCl; 0.1 ml, 0.5 M $CaCl_2$; 1.5 ml, 1 mM $FeCl_3$; 3.2 ml, 0.1 M KH_2PO_4; 50.0 ml, 1.0 M Tris-HCl, pH 7.4; 5.0 ml, 10% glucose; 5.0 ml, 5% Casamino acids) in a culture flask with rapid swirling at 37°C. T7 phages were added to the medium at a multiplicity of 0.1 when E. coli B had reached log phase. Shaking was continued for 1 hour at 37° C to permit complete lysis of the cells. The lysate was centrifuged for 15 minutes at 10,000 g and the precipitate was discarded.

A rapid bacteriophage sedmientation method (Yamamoto and Alberts, 1970) was employed to purify and collect the phage particles. The crude lysate was cooled to 4° C and enough NaCl was added to bring its concentration up to 0.5 M. Polyethylene glycol (PEG 6000) 6% (w/v) was added into the crude lysate and the solution was let stand for at least 1 hour at 4° C. The phage pellet was collected after centrifugation at 10,000 g for 15 minutes and was resuspended in a minimum amount (1 ml) of sterile 1 M NaCl, 0.01 M Tris buffer (pH 8.0). The phage suspension was then purified by CsCl density gradient equilibrium centrifugation. Sterile CsCl solutions of densities 1.1, 1.3, 1.5 and 1.7 g/ml (5 ml each) were added in succession by introduction at the bottom of the tube. The phage suspension was then layered on top of the CsCl solutions. After centrifugation for 3 hours at 22,000 rpm and then 12 hours at

35,000 rpm the purified phage was collected and dialyzed overnight against 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 M NaCl. Over 95% of the pure phages were reported to be recovered using the PEG precipitation method (Yamamoto and Alberts, 1970).

T7 DNA samples were prepared by phenol extraction (Thomas and Abelson, 1966) of purified T7 phages. The purified phage solution was diluted to an optical density between 5 and 15 in a sterilized test tube and was shaken (60 rev/min) with equal volume of freshly distilled purified phenol (saturated with a 0.1 M potassium phosphate buffer, pH 7.5, containing 0.1 M NaCl) for 45 minutes at room temperature. The tube was then cooled to 4° C and centrifuged for 5 minutes at 3,000 rpm in an IEC clinical centrifuge to separate the two phases. The phenol layer (the bottom one) was removed with a Pasteur pipet and the DNA solution was dialyzed exhausively against the original buffer and finally against 0.01 M Tris-HCl buffer (pH 7.8) containing 0.05 M KCl. This T7 DNA preparation was used without further purification.

^{3}H-labeled T7 DNA was isolated from phages grown in the presence of [methyl-^{3}H] thymine using thymine-requiring mutant of E. coli B as host in a M-9 minimal medium (1 g NH_4Cl, 3 g KH_2PO_4, 6 g $NaHPO_4$, 4 g glucose and 1 ml of 1 M $MgSO_4$ in one liter of water) supplemented with 2μg unlabeled thymine/ml. ^{3}H-thymine (30 Ci/mmol) was added to the culture 1 hour before infection. The steps for purification of the ^{3}H-labeled phage particles and the extractions of ^{3}H-labeled T7 DNA were identical as before.

The concentrations of T7 DNA, when designated as DNA-P, are expressed as equivalents of nucleotide phosphorus, and were determined

spectrophotometrically using a molar extinction coefficient of 6750 with respect to phosphorus at 260 nm (Richardson, 1966). The A_{260}/A_{280} ratio of the purified T7 DNA was 1.91 and the A_{260}/A_{230} ratio was 2.35, indicating low protein contamination (Thomas and Abelson, 1966).

II.3.2. Preparation of Drug Solution

Working solutions of all anthracyclines were prepared by dissolving these compounds in water. Aqueous solutions of marcellomycin and musettamycin were prepared by wetting the drug crystals with DMSO, followed by addition of water. Drug concentrations were determined spectrophotometrically using the molar extinction coefficients (Liter$\cdot$Mole$^{-1}\cdot$cm^{-1}) listed in Table 1. Fresh drug solutions were prepared for each experiment.

Rifampicin stock solutions were prepared by dissolving 1 mg of rifampicin into 10 ml of 0.04 M Tris-HCl buffer, pH 7.9. The stock solutions were further diluted with the buffer to the desirable concentrantions in each experiment.

II.3.3. Preparation of Nucleotide Substrates

Unlabeled nucleoside triphosphate solutions were prepared by dissolving ATP, CTP, GTP and UTP sodium salts (5 mM each) in 0.04 M Tris-HCl buffer, pH 7.9. Radioactively labeled substrates were made by dissolving nitrogen-dried (section II.1.) ^{3}H-UTP (specific activity 0.02 µCi/nmole) in unlabeled nucleoside triphosphate solutions. These substrate preparations were used immediately and no substrate solutions were used after two weeks of preparation.

II.3.4. Preparation of RNA Polymerase Solution

It has been shown that under 37° C temperature, transcription of T7 DNA by E. coli RNA polymerase takes place primarily at three major promoter sites at the left end of the genome (Stahl and Chamberlin, 1977). Since under the conditions mentioned above, for every four molecules of E. coli RNA polymerase only one will bind to the major promoter site on T7 DNA, 12 molecules of E. coli RNA polymerase are required to saturate the three type A promoter sites on one molecule of T7 DNA. Therefore, unless specified otherwise, a ratio of 12 polymerase molecules per T7 DNA molecule was used in all the experiments in this study.

RNA polymerase obtained commercially was diluted with buffer solutions specified in each experiment so that during the assay approximately 1 μg of enzyme protein was mixed with 170 uM DNA-P to meet the molar ratio of 12 to 1.

II.4. Methods

II.4.1. Determinations of Binding Parameters for Anthracyclines

In order to study the effect of anthracyclines on RNA synthesis at the molecular level, a quantitative measurement of the drug bound to T7 DNA under the assay conditions and temperatures must be obtained for each drug.

The interaction of a large polymeric molecule DNA with small molecules of drug involves multiple binding sites so that DNA interacts simultaneously with more than one drug molecule. Each binding site on DNA is characterized by an apparent association constant, K_{app}= [occupied sites]/[unoccupied sites] [free drug]. If n is the maximum number of binding sites per DNA nucleotide and r_b is the number of drug molecules bound per DNA nucleotide at a free drug concentration of $[I_f]$ (moles/l), then for one type of site and in the absence of mutual interaction, the following equation could be obtained.

$$\frac{r_b}{I_f} = K_{app} \cdot n - K_{app} \cdot r_b \qquad (1)$$

Scatchard plots (Scatchard, 1949) are constructed by plotting r_b/I_f vs r_b which yield a straight line with a slope of $-K_{app}$. The values of the apparent association constant (K_{app}) and the maximum number of binding sites per DNA nucleotide (n) can then be obtained from the slopes and the intercepts.

The spectrophotometric method of Peacocke and Skerett (Peacocke and Skerett, 1956) was used to determine the apparent binding constant

and the maximum number of binding sites per DNA nucleotide for each drug on T7 DNA. This method is based on the observation that when ligands bind to nucleic acids, there is a change in the absorption spectrum of the ligand and this change is dependent upon the concentration of the nucleic acids.

If the total concentrations of DNA and drug are denoted as [DNA] and $[I_o]$ respectively, then the equation

$$[I_f] + r_b[DNA] = [I_o] \qquad (2)$$

can be derived in which $r_b[DNA]$ is the amount of drug bound to DNA, (or $[I_b]$). If A_o denotes the absorbance of free drug (when $r_b = 0$, or [DNA] = 0, all drug molecules are in free form and A_o becomes the absorbance obtained when only drug molecules are present in solution), A_b denotes the absorbance of the bound drug (when all drug molecules are bound, then $[I_f] = 0$ and $r_b[DNA] = [I_o]$), and A_x is the absorbance of a mixture of free and bound drug, then

$$A_x = \frac{[I_f]}{[I_o]} A_o + \frac{r_b \cdot [DNA]}{[I_o]} A_b. \qquad (3)$$

If b is the fraction of total drug bound to DNA or

$$b = \frac{r_b \cdot [DNA]}{[I_o]} \qquad (4)$$

then from equations (2), (3) and (4), equation (5) can be derived.

$$b = \frac{A_o - A_x}{A_o - A_b} \qquad (5)$$

The amount of drug molecules in free form ($[I_f]$) can also be obtained from equations (2) and (4). Therefore

$$[I_f] = [I_o] - [I_b] = [I_o] - b \cdot [I_o] = [I_o] \cdot (1-b) \quad (6)$$

In the binding experiments, T7 DNA was mixed with a binding buffer (0.04 M Tris-HCl, pH 7.9, 0.02 M $MgCl_2$, 0.01 M 2-mercaptoethanol). The concentration of DNA-phosphorus was determined spectrophotometrically with a Perkin-Elmer 320 spectrophotometer at 260 nm, using a molecular extinction coefficient of 6750. Solutions of increasing DNA to drug ratios were prepared by adding increasing amounts of drug. The reaction mixtures were brought to the same final volume (2 ml) with binding buffer. Identical preparations containing equal DNA concentration but no drug were also prepared and used as reference.

Since only limited amounts of T7 DNA (approximately 1.5 mM) was obtained from each preparation, the amount of drug (approximately 20 μM) was chosen so that at the highest DNA to drug ratio, a maximal change in the visible spectrum due to drug binding to DNA could be obtained. One of the helpful features of the Perkin-Elmer 320 spectrophotometer was that its absorbance scale could be adjusted as desired. Therefore, the absorbance at λ_{max} for the solution containing total drug concentration $[I_o]$, but in the absence of DNA, could be expanded to full scale, thus providing more accurate readings of the absorbance.

The reaction solutions were incubated for ten minutes at 37° C for thermal equilibration and the difference absorption spectra were obtained versus the corresponding reference solutions.

The absorbance of each sample (A_x) and total drug concentration in the absence of DNA (A_o) were obtained from the recorded spectra at a wavelength chosen so that $A_o - A_x$ was maximum. The absorbance of drug totally bound to DNA (A_b) could be obtained from the absorbance of a

solution prepared with very high DNA concentration, assuming that all drug molecules are bound under this condition. However, since high DNA concentration required for this purpose caused scattering and introduced error into the subsequent calculations, the graphic method of Li and Crothers (Li and Crothers, 1969) was used to obtain A_b. This method showed that a plot of $1/A_o-A_x$ vs $1/[DNA]-[I_o]$ yielded a straight line with an intercept on the ordinate equal to $1/A_o-A_b$. From the value of the intercept, A_b may then be calculated.

II.4.2. Nitrocellulose Filter Assay for the Binding of RNA Polymerase to DNA

Binding of RNA polymerase to DNA was measured using a modification of the procedure of Jones and Berg (Jones and Berg, 1966). RNA polymerase solution was prepared by diluting commercial enzyme preparation with binding buffer (10 mM Tris, pH 8.0; 10 mM $MgCl_2$; 50 mM NaCl; 10 mM 2-mercaptoethanol; 1 mM EDTA; and 1 mg BSA/ml). The reaction mixture contained 10 µl of ^{3}H-T7 DNA (final concentration of 170 µM DNA-P), 80 µl of binding buffer with or without desired amount of inhibitors, and 1 µg of enzyme in 10 ul of enzyme solution so that a molar ratio of 12 is reached for enzyme and DNA (section II.3.4.). Reaction was initiated by addition of enzyme. After incubation at 37° C for 10 minutes, reactions were diluted to 1.0 ml with binding buffer and filtered immediately through 24 mm Schleicher and Schuell BA85 nitrocellulose filter disks with gentle suction (5 cm of Hg, which produced a flow rate of approximately 4 ml/min). The filter disks were then dried and the radioactivity determined in toluene containing 0.4% 2,5-diaphenyloxazole in a liquid scientillation counter. The amount of radioactivity

collected on the filter disks in the absence of any inhibitors was used as control. A minimum amount (~3%) of radioactivity was attributed to non-specific binding.

II.4.3. RNA Polymerase Assays

DNA-dependent RNA polymerase activity was determined by measuring the incorporation of radioactive ribouncleotide substrates into ribonucleic acid insoluble in trichloroacetic acid (TCA).

Assay reaction mixtures consisting of buffer solution with or without inhibitors, T7 DNA, RNA polymerase solution, and radioactive nucleotide substrates were introduced into 12 x 75 mm disposable glass test tubes. The exact amount of each component was measured with Pipetman adjustable pipets. The test tubes and all the glasswares used in the experiments were pre-heated at 180° C for at least 48 hours prior to use to inactivate any possible contaminating ribounclease activity. Disposable plastic gloves were also worn to avoid contamination by human skin ribonuclease (Rovozzo, 1973).

II.4.3.1. Assay for RNA Polymerase Activity Under the Influence of Anthracyclines

In experiments designed to measure the relative inhibitory potency of all anthracyclines on the overall RNA synthesis in the presence of a constant amount of DNA (170 μM DNA-P), the enzyme concentration (1 μg/sample) was prepared so as to meet the desired enzyme to DNA ratio of 12 to 1 (Section II.3.4.).

In these experiments, 35 μl of 2.5 times concentrated buffer A solution (final concentration: 0.04 M Tris-HCl, pH 7.9; 0.02 M $MgCl_2$;

0.01 M 2-mercaptoethanol; 1 mg/ml BSA) was mixed with 20 µl of 170 uM (final concentration) of T7 DNA and kept at 0 °C. 15 ul of anthracycline solution (or water when used as control) of desired concentration was added to the mixture and the solution was transferred to a 37° C bath for 5 minutes. 10 µl of enzyme solution containing 5 µl of buffer (2.5 times its final concentration), 1 µg of commercial enzyme protein and deionized water was then added. After 7 minutes of preincubation (i.e., after the formation of RNA polymerase-DNA binary complex was completed) the RNA synthesis reaction was initiated by the rapid addition of 10 µl solution containing 5 mM (10 times the final concentration) each of ATP, GTP, CTP and ^{3}H-UTP (specific activity: 0.02 µCi/nmol) plus 10 µl of 0.04 M Tris buffer, pH 8.0. After 90 seconds of incubation at 37° C the reaction was terminated with 1 ml, freshly prepared ice-cold, 5% trichloroacetic acid solution containing 1 M KCl and 0.05 M of sodium pyrophosphate.

After mixing and standing in ice for 10 minutes the insoluble material was collected by filtration through a Watman GF/C (2.4 cm) filter disk with gentle suction. The filter disk was washed with 20 ml of ice cold 5% TCA solution containing 1.0 M KCl and 0.05 M $Na_2P_2O_4$ followed by 30 ml of 95% ethanol, dried overnight (or at 95° C for 5 minutes) and counted for radioactivity in 10 ml of toluene containing 0.4% 2,5-diphenyloxazole in a Beckman Liquid Scientillation Counter.

The radioactivity absorbed and retained by the filter disks and the radioactivity trapped within the precipitated components of the assay mixture was determined from the residual radioactivity of blanks which did not contain any enzyme. The blank values were usually less

than 250 cpm.

Experiments designed to measure the inhibition of ribonucleic acid chain initiation were conducted as described as above except that a nucleoside triphosphate solution containing γ-[^{32}P]-ATP, CTP, GTP, and [5,6-^{3}H]-UTP (Specific activities: 1 μCi/nmol and 0.02 μCi/nmol, respectivity) at a concentration of 5 mM for each nucleoside Triphosphate (ten times the final concentration) was used.

Special procedures for terminating the reaction and for washing were used for experiments involving ^{32}P incorporation in order to reduce the high background due to unincorporated radioactivity. Ractions were terminated by the addition of 0.5 ml ice-cold 0.1 M sodium pyrophosphate solution containing 20 mM ATP and 2 mM UTP and 0.5 ml of ice-cold 10% trichloracetic acid solution containing 1 M KCl. The insoluble material collected on the filter disks was soaked in 50 ml of ice-cold 5% trichloracetic acid solution containing 1 M KCl and 0.01 M sodium pyrophosphate for 1 hour, and further soaked in 40 ml of ice-cold 95% ethanol for 20 minutes. The filter disks were dried as before.

Simultaneous counting of ^{3}H and ^{32}P activities were performed in Beckman Model LS 7500 Liquid Scintillation Counter using a pre-set condition under which less than 2% activity spill-over between ^{3}H and ^{32}P could be detacted.

II.4.3.2. Assay for RNA Polymerase Activity Involving Rifampicin

Rifampicin was used in the experiments designed to measure the rate constants governing the initiation steps of RNA synthesis. All of the experiments involving rifampicin were conducted in buffer B (0.2 M

Tris-HCl, pH 7.9; 0.01 M $MgCl_2$; 0.05 M 2-mercaptoethanol, 5 mg BSA/ml).

II.4.3.2.1. Inactivation of RNA Polymerase-DNA Complex by Rifampicin

In experiments designed to study the kinetics of rifampicin inactivation of DNA-RNA polymerase complex, the DNA-RNA polymerase complexes were formed as described previously (Section II.3.4.1.) in the presence or absence of anthracyclines. After 7 minutes of incubation at 37° C, 10 µl of rifampicin solution with a final concentration of 0.312 µg/ml was added. Incubation was continued for a designated period of time and the RNA synthesis reaction was initiated by adding 10 µl of radioactively labeled substrates. The reaction was terminated 90 seconds later and the product was processed as described previously (Section II.4.3.1.). Under these conditions, the amount of RNA formed should be a direct measure of the amount of active RNA polymerase bound to DNA complexes. The number of complexes at t = 0 is experimentally defined as the amount of RNA synthesis obtained in 90 seconds when both rifampicin and the four ribonucleoside triphosphates were added to the complexes at the same time.

II.4.3.2.2. Assay for the Rate of RNA Synthesis as a Function of Rifampicin Concentration

In experiments designed to measure the rate of RNA synthesis as a function of rifampicin concentration, the enzyme-DNA complexes in the presence or absence of anthracyclines were prepared as described previously (Section II.4.3.1.). RNA synthesis was initiated by the addition of 20 µl of a substrate-rifampicin mixture (10 µl substrate, 10 µl rifampicin in 0.04 Tris-HCl, pH 7.9) prepared so as to give the desired

final rifampicin concentrations. The reaction was then terminated and the product processed as described previously (Section II.4.3.1.).

II.4.3.2.3. Assay for the Rate of RNA Synthesis as a Function of Pre-incubation Time

In experiments designed to measure the rate of RNA synthesis as a function of pre-incubation time, the drug-DNA complexes were prepared as described previously (Section II.4.3.1.) but at 0° C. After addition of the desired amount of enzyme, the reaction mixture was transferred to a water bath at 37° C for the appropriate period of time (i.e., for various pre-incubation times) and RNA synthesis was initiated by the addition of substrate-rifampicin mixtures. The termination and product processing steps were identical as described previously (Section II.4.3.1.)

II.4.3.2.4. Assay for the Rate of RNA Synthesis as a Function of Pre-incubation Temperature

In experiments designed to measure the rate of RNA synthesis as a function of pre-incubation temperature, the drug-enzyme-DNA complexes were prepared as described previously (Section II.4.3.1.) but at 0° C. Then the complexes were transferred to a water bath at different temperatures for pre-incubation for 7 minutes. The RNA synthesis reaction was initiated at 37° C and the subsequent reaction and product were treated as described previously (Section II.4.3.1.).

The background values for experiments involving rifampicin were determined from reaction mixtures prepared identically with each experiment but in the absence of the enzyme. These values were usually less than 750 cpm. Higher background vales (from 750 to 1000 cpm) were

obtained when daunomycin was present together with rifampicin. The reason for this higher background is not known but it may be attributable to interaction among rifampicin, daunomycin and the radioactive substrates.

CHAPTER III

EXPERIMENTAL RESULTS

III.1. The Binding of Anthracyclines to T7 DNA

In order to study the effects of anthracyclines on RNA synthesis, the binding parameters for each drug on DNA must be obtained under ionic and temperature conditions identical to those employed in the assays for RNA polymerase activities.

Binding experiments for each anthracycline analog were conducted as described under Methods (Section II.4.1.). Preliminary results indicated that BSA had no effect on the extent of binding and in subsequent measurements BSA was omitted from the buffer. In these measurements, the concentration of the drug was kept constant at approximately 20 μM, while the concentration of T7 DNA was varied.

Typical spectrophotometric titrations of anthracyclines with DNA are shown in Figure 8 which demonstrates the effect of increasing amount of DNA on the absorption spectra of anthracyclines. Under the specified conditions, free daunomycin exhibits three absorption maxima at 502, 535 and 575 nm as shown in Figure 8a. This significant change in absorption spectrum from that mentioned in Section I.2.7.1. is due to the presence of $MgCl_2$. Although high concentration of Mg^{2+} was reported to release anthracyclines form the drug-DNA complex (Calendi et al., 1965) the concentrrion of $MgCl_2$ present in this study is significantly lower.

Upon addition of T7 DNA, the intensity at all absorption maxima

Figure 8a. Visible Absorption Spectra for daunomycin-T7 DNA Complex

Experiments were conducted with a Perkin-Elmer 320 spectrophotometer. 18.8 μM of daunomycin in binding buffer (0.04 M Tris-HCl, pH 7.9, 0.02 M $MgCl_2$, 0.01 M 2-mercaptoethanol) was mixed with increasing amount of T7 DNA as described in Methods (Section II.4.1.). The concentrations of T7 DNA are a) 0 μM, b) 88 μM, c) 114 μM, d) 168 μM, e) 204 μM, f) 243 μM, g) 288 μM, h) 390 μM.

Measurements were carried out at 37° C. A minimum of three determinations of binding parameters were made for each anthracycline-DNA combination. Each binding determination consisted a minimum of 9 different DNA concentrations in duplicate samples. Only a portion of the data is shown.

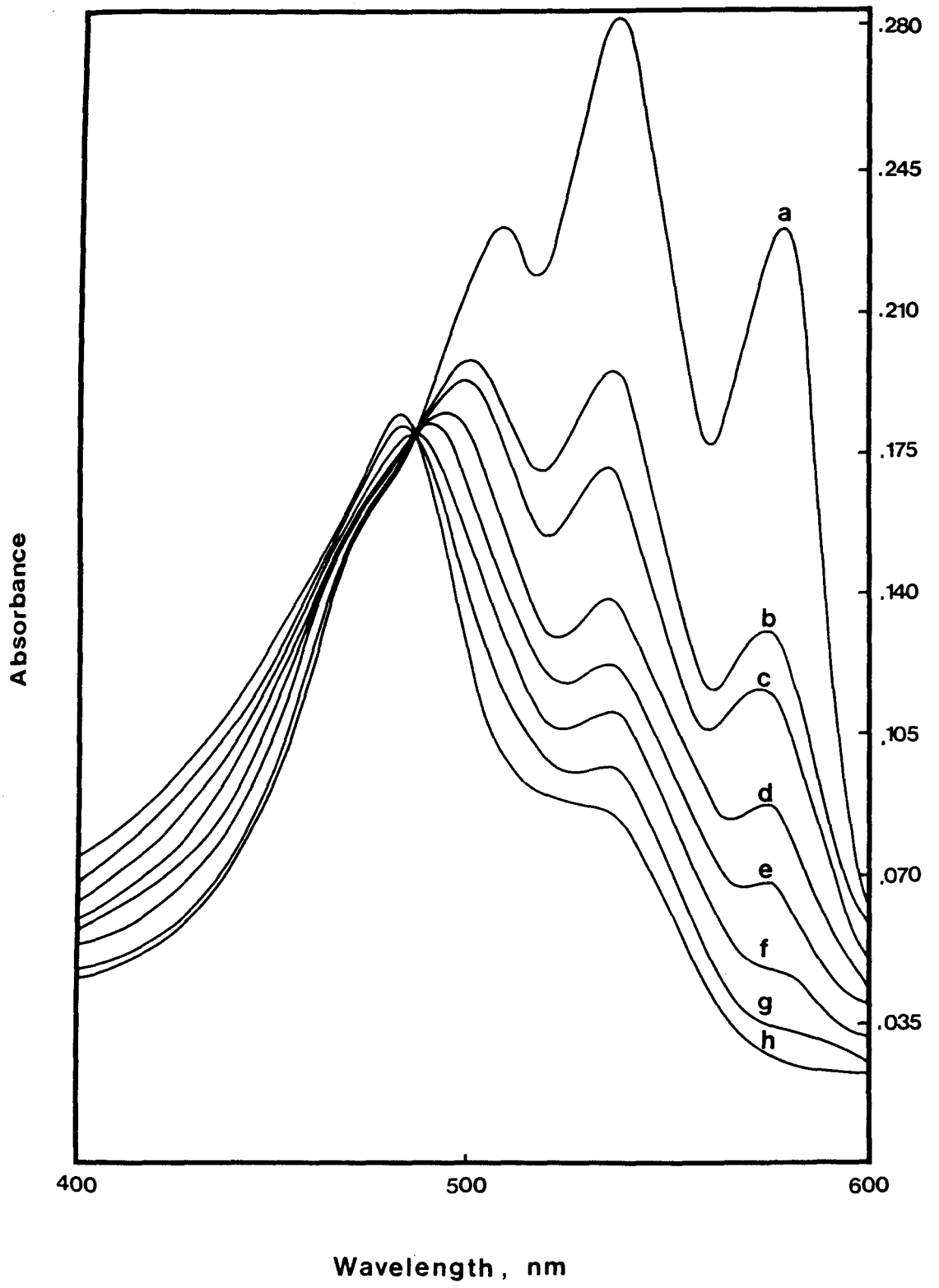
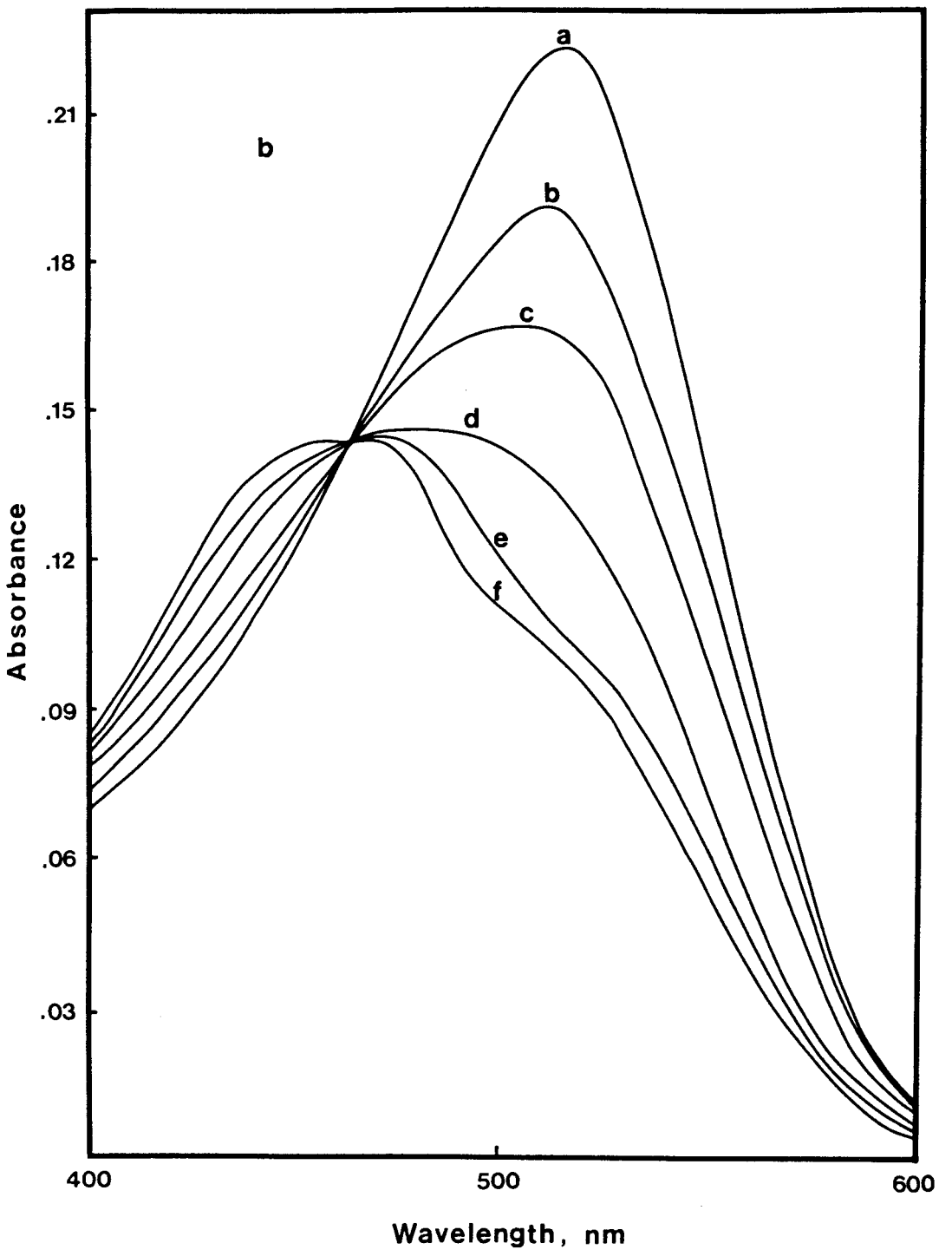

.280
.245
.210
.175
.140
.105
.070
.035
a
b
c
d
e
f
g
h
400
500
600
Absorbance
Wavelength, nm

Figure 8b. Visible Absorption Spectra for Aclacinomycin-T7 DNA Complex

20 μM of aclacinomycin solution in binding buffer was mixed with increasing amount of T7 DNA as described in Figure 8a. The concentrations of DNA are a) 0 μM, b) 48 μM, c) 96 μM, d) 175 μM, e) 242 μM, f) 390 μM.


a
b
b
c
d
e
f
.21
.18
.15
.12
.09
.06
.03
Absorbance
400
500
600
Wavelength, nm

decreases and the two maxima at longer wavelength tend to disappear more quickly than the one at 502 nm, which became predominant at the highest DNA to drug ratio employed. The largest changes in absorbance were observed at 535 and 575 nm and the absorbances at these wavelengths were used in the calculation of binding parameters.

Only one clear isosbestic point is observed for daunomycin. If there had been a DNA concentration range in which the spectra of the drug-DNA complex had exhibited an isosbestic point while in another DNA concentration range no isosbestic point had been observed, this would have been a clear indication of the presence of more than two spectral species. This is not the case for daunomycin, therefore the spectra of daunomycin were evaluated under the assumption that only two spectral species are present in the solutions; i.e.,the free drug form and the DNA-bound drug form. This assumption is further supported by the findings that the intrinsic association constants determined from the spectral changes at 535 and 575 nm are in good agreement with one another. The absorption spectra for the rest of the anthracycline analogs, with the exception of aclacinomycin, exhibit a similar pattern.

Figure 8b shows the absorption spectra of aclacinomycin under the same experimental condition. Like that of daunomycin, the intensity of the absorption maximum decreases upon addition of DNA. The absorption maximum of aclacinomycin is also shifted from 515 nm at zero DNA to drug ratio to 450 nm at maximal DNA to drug ratio. An isosbestic point is presnet at 460 nm. As is the case with daunomycin, the presence of an isosbestic point indicates that only two spectral species of aclacinomycin are present; the DNA-bound drug and the free drug.

The largest changes of absorbance were obtained at 517 nm and therefore, absorbances at this wavelength were used in calculating the binding parameters for this drug.

The values for r_b (the number of drug molecules bound per DNA nucleotide) and I_f (the concentration of free drug) for each anthracycline analogs studied were calculated according to equations (4) and (6) respectively. The association constant (k_{app}) and the maximum number of binding sites per DNA nucleotide (n) for each drug were obtained from the slopes and intercepts of Scatchard plots constructed according to equation (1).

The Scatchard plots for each anthracycline studied are shown in Figures 9a and 9b and the calculated values of k_{app} and n for each drug are listed in Table 1.

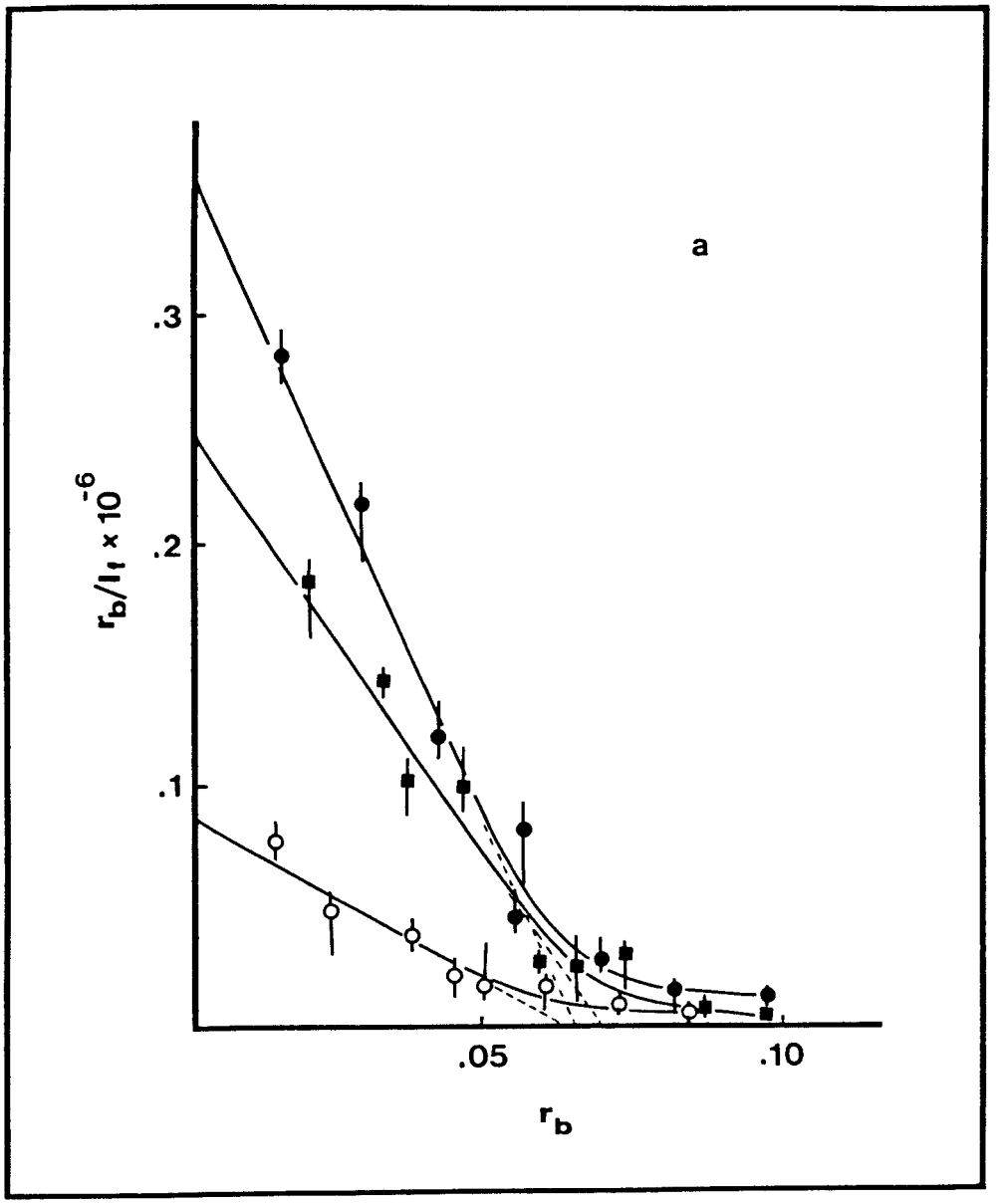
In Figure 9a, at lower DNA to drug ratio, the Scatchard plots shown deviations from the straight line and therefore, the intercept on the abscissa were made by extrapolations of the linear portions of the plots (Chambon, 1975). The deviations indicate the existence of two distinct processes of unequal strength for the binding of anthracyclines to DNA. At low DNA to drug ratio, the drugs are able to bind to DNA through electrostatic interactions at secondary binding sites in addition to the intercalative mode of binding which occurs at primary binding sites when the DNA to drug ratio is high. This interpretation is consistent with that of previous studies based on Calf thymus DNA (Zunino et al., 1972) and model building (Section I.2.7.2.). The absence of deviation for the aclacinomycin group of drugs in Figure 9b indicates that no significant amount of secondary binding is present. This observation is consistent

Figure 9. Scatchard Plots for Some Anthracyclines

The Scatchard variables of r_b (the number of ligands bound per nucleotide) and I_f (the molar concentration of free drug) were calculated from the data obtained from spectrophotometric titrations (Figure 8) according to the method of Peacocke and Skerett, 1956.

a) 4-demethoxydaunomycin -●-, daunomycin -■-, carminomycin -o-.
b) aclacinomycin -●-, marcellomycin -■-, musettamycin -o-.

The lines were drawn using the method of linear regression with the aid of Apple II computer. Each value represents the mean ± the standard deviation of three experiments in duplicate samples.


a
.3
.2
.1
$r_b/I_f \times 10^{-6}$
.05
.10
r_b

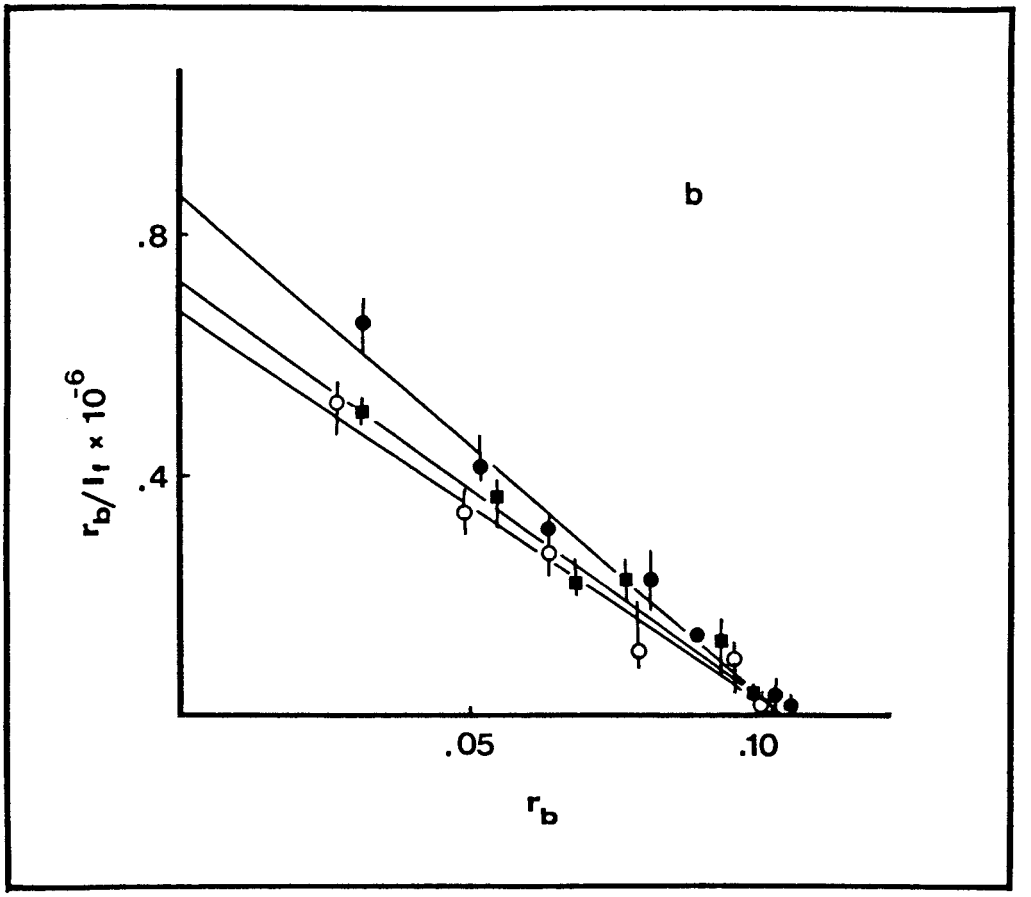

b
.8
.4
$r_b/I_f \times 10^{-6}$
.05
.10
r_b

Table I

Binding Parameters for DNA Interaction with Anthracyclines

Anthracyclines	K_{app} (10^6 M^{-1})	n
Group I:		
daunomycin	3.63 ± 0.21	0.070 ± 0.050
4-demethoxydaunomycin	5.28 ± 0.36	0.070 ± 0.002
carminomycin	1.31 ± 0.16	0.065 ± 0.001
Group II:		
aclacinomycin	8.92 ± 0.54	0.100 ± 0.007
marcellomycin	6.84 ± 0.61	0.100 ± 0.005
musettamycin	5.59 ± 0.26	0.110 ± 0.005

Binding parameters were determined from plots of r_b/I_f versus r_b, where K_{app} (the apparent association constant) is the negative slope and n (the apparent number of binding sites per nucleotide) is the intercept on the r_b-axis of the corresponding Scatchard plot. Each value represents the mean ± the standard deviation.

with the data obtained from fluoresence quenching studies which also showed only one type of binding between marcellomycin and various type of DNA (Du Vernay et al., 1979a). As mentioned previously (Section I.2.7.2.) any modification of the amino group would reduce the secondary binding. In the case of group II anthracycline, this is probably due to the inability of the masked amino group to either develop a positive charge to form secondary binding, or, even if the charge can develop, its effect is weakened by the presence of the di-methyl substitution. In any event, the secondary binding is insignificant in the binding of group II anthracyclines to DNA.

In the subsequent study of the inhibition of RNA synthesis by anthracycline analogs it is essential to realize that the true inhibitor of the RNA synthesis system is the DNA-bound form of the drug (Section III.3.2.). Since from the results of the binding studies anthracyclines may bind to DNA in two different forms; i.e., intercalation and secondary binding, their effect on RNA synthesis may become very complicated to study. Therefore in order to understand the mechanism of inhibition of RNA synthesis by each DNA-bound anthracycline molecule in terms of intercalation alone, one has to make sure that intercalation is the only mode of interaction between the drug and DNA. This can be achieved by limiting the number of DNA-bound drug molecules (r_b) under 0.05 per DNA nucleotide, which, according to the Scatchard plot, should eliminate the possibility of secondary binding. Under this condition, the resulting inhibitory effect of anthracyclines in terms of DNA binding can only be attributable to intercalation alone.

There is approximately a 3-fold decrease in the binding constants and the number of binding sites when the data from the present study are compared with that reported in the literature (Zunino et al., 1980; Rich et al., 1981). This apparent discrepancy may be explained by the presence of Mg^{2+}, which has been reported to reduce the normal binding of daunomycin to DNA (Di Marco et al., 1972; Bhuyan et al., 1965).

Based on the shapes of the Scatchard plots, the differences in binding constants and the number of drug molecules bound per DNA nucleotide, the anthracyclines studied in this project could be categorized into two groups; i.e., (I) the daunomycin group, which includes daunomycin, 4-demethoxydaunomycin and carminomycin and (II) the aclacinomycin group, which includes aclacinomycin, marcellomycin and musettamycin. This distinction is also correlated to the basic structure of the anthracyclines; i.e., those without extra sugar groups on the daunosamine (the daunomycin group), and those with one or more sugar residues attached to rhodosamine (the aclacinomycin group).

Generally speaking, group II anthracycline molecules bind to T7 DNA with greater affinity and more of them can bind per DNA nucleotide. The increase of affinities of anthracyclines to DNA has been attributed to the presence of C-10 subsitution and extra residues on the glycosidic side chain (DuVernay et al., 1979a,b) despite the fact that the amino group on the primary sugar residue has been masked by two bulky methyl groups. The effects of other substitutions (i.e., at C-1, C-11, and C-13) are not clear at this moment.

Differences in binding parameters are also noted among group I anthracyclines. The calculated binding constants for daunomycin and

4-demethoxydaunomycin are found to be 3.63×10^6 M^{-1} and 5.28×10^6 M^{-1} respectively. This result shows a higher DNA binding ability for 4-demethoxydaunomycin and is in disagreement with the binding constant obtained previously on Calf thymus DNA (Zunino et al., 1976) which suggested a stronger binding for daunomycin. However, the present result is consistent with the expectation based on the structural analysis that 4-demethoxydaunomycin should have a higher affinity for DNA due to the absence of the bulky methoxy group on C-4 of daunomycin (see page 48).

III.2. Assays for the Most Appropriate Conditions to be Used in Subsequent Experiments

In order to establish the assay conditions for the inhibition studies, the RNA synthesis by DNA-dependent RNA polymerase as a function of reaction time was studied using T7 DNA as template. The amount of RNA synthesized was plotted against the reaction time and the results are shown in Figure 10.

From Figure 10 it is evident that for the first 10 minutes, RNA synthesis is linear with respect to incubation time, then it slows down considerably and finally reaches a plateau at around 60 minutes. The rate of the reaction follows roughly first order kinetics as can be seen from the linear plot in Figure 11. A reaction time of 90 seconds was used in all subsequent assays to eliminate reinitiation (see Methods), and this reaction time falls well within the limit of 10 minutes beyond which substrate depletion may occur.

The effect of template concentration on RNA synthesis was also studied and the results are presented in Figure 12. The reaction seems to follow Michaelis-Menton kinetics and the Lineweaver-Burk plot is pre-

Figure 10. The Kinetics of RNA Synthesis

170 μM of T7 DNA was pre-incubated at 37° C with 1 μg of RNA polymerase for 8 minutes. The initation was started by the addition of 0.5 mM of ^{3}H-labeled nucleotide substrate. The reaction was terminated according to the time specified and the radioactive product was collected and processed as described in the Methods (Section II.4.3.1.).

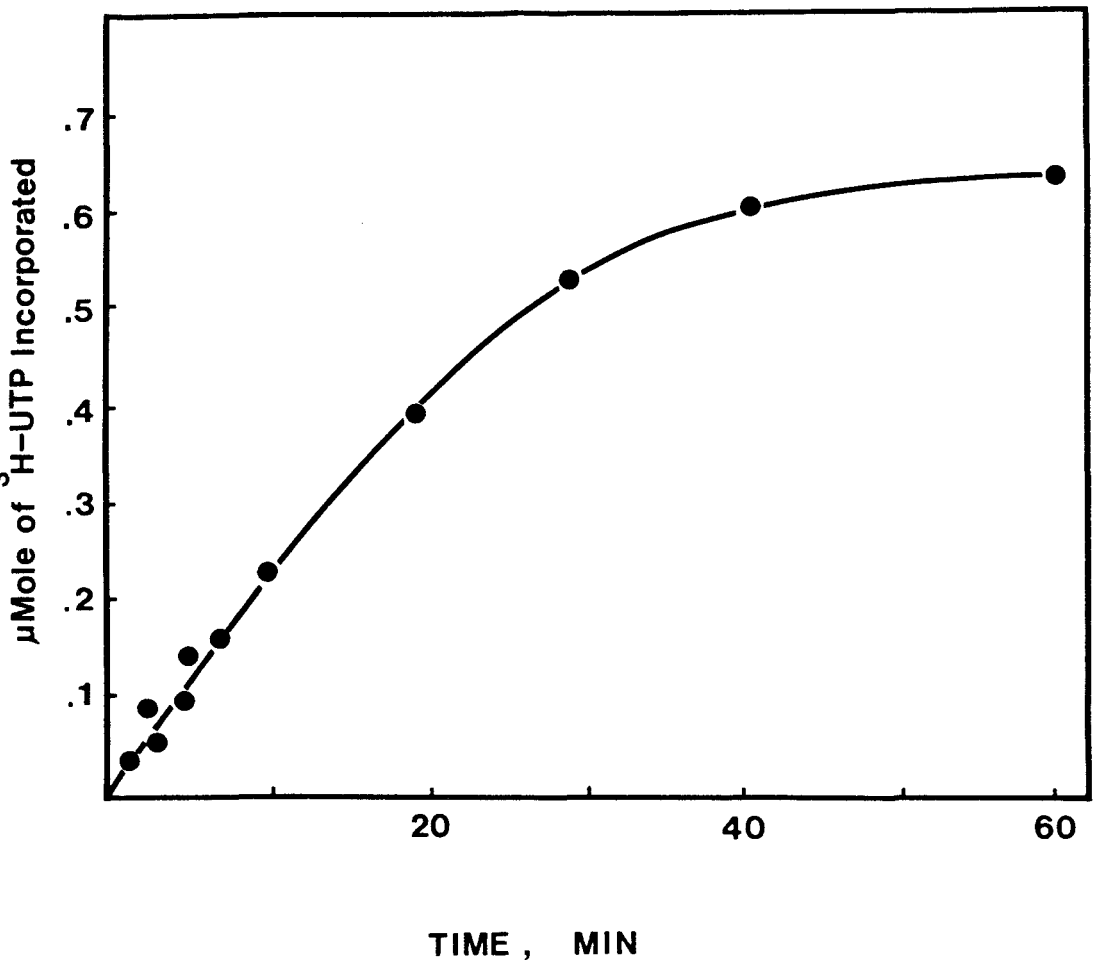

μMole of ^{3}H-UTP Incorporated
.7
.6
.5
.4
.3
.2
.1
20
40
60
TIME , MIN

Figure 11. First Order Plot of the Effect of Reaction Time on RNA Synthesis

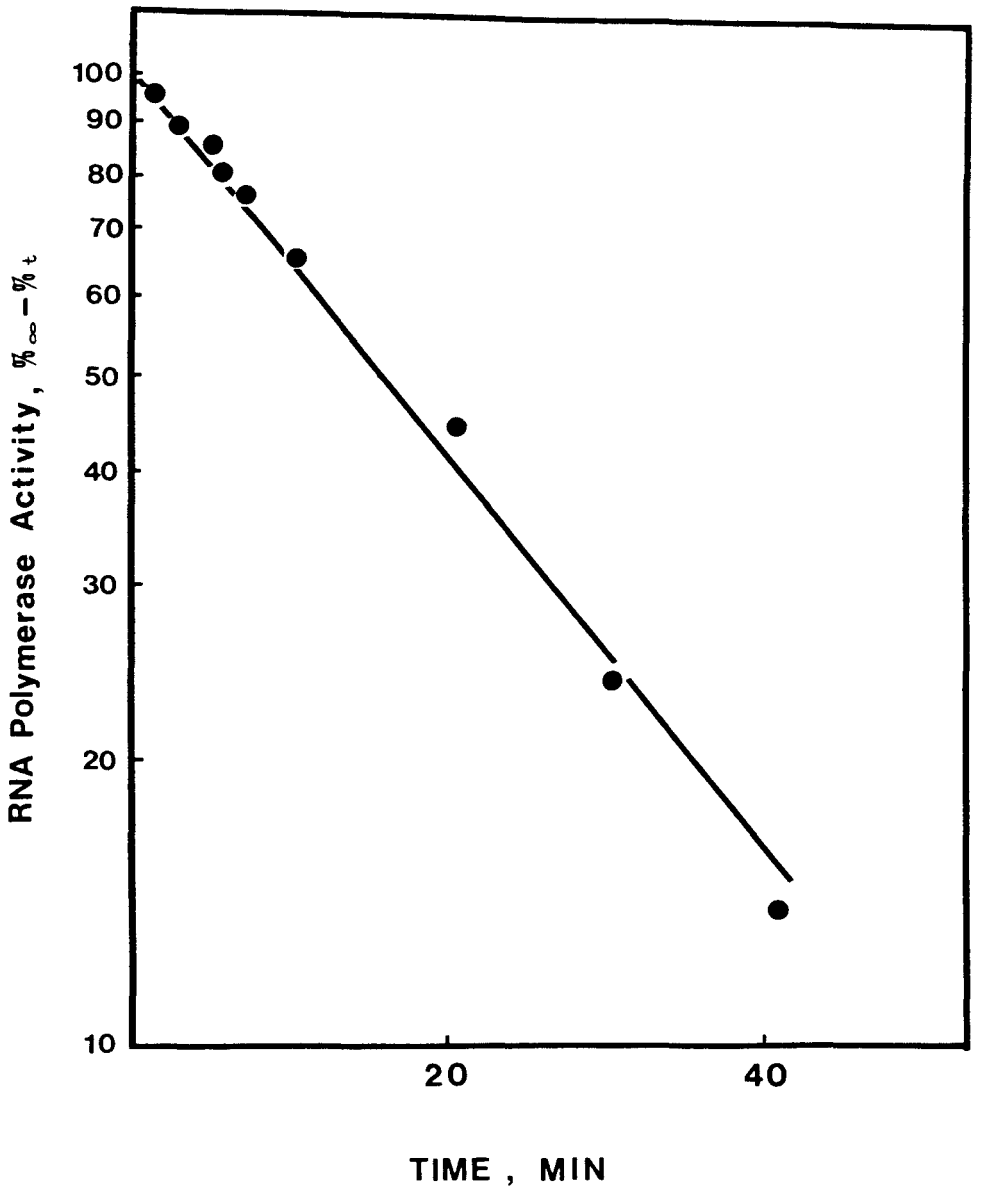
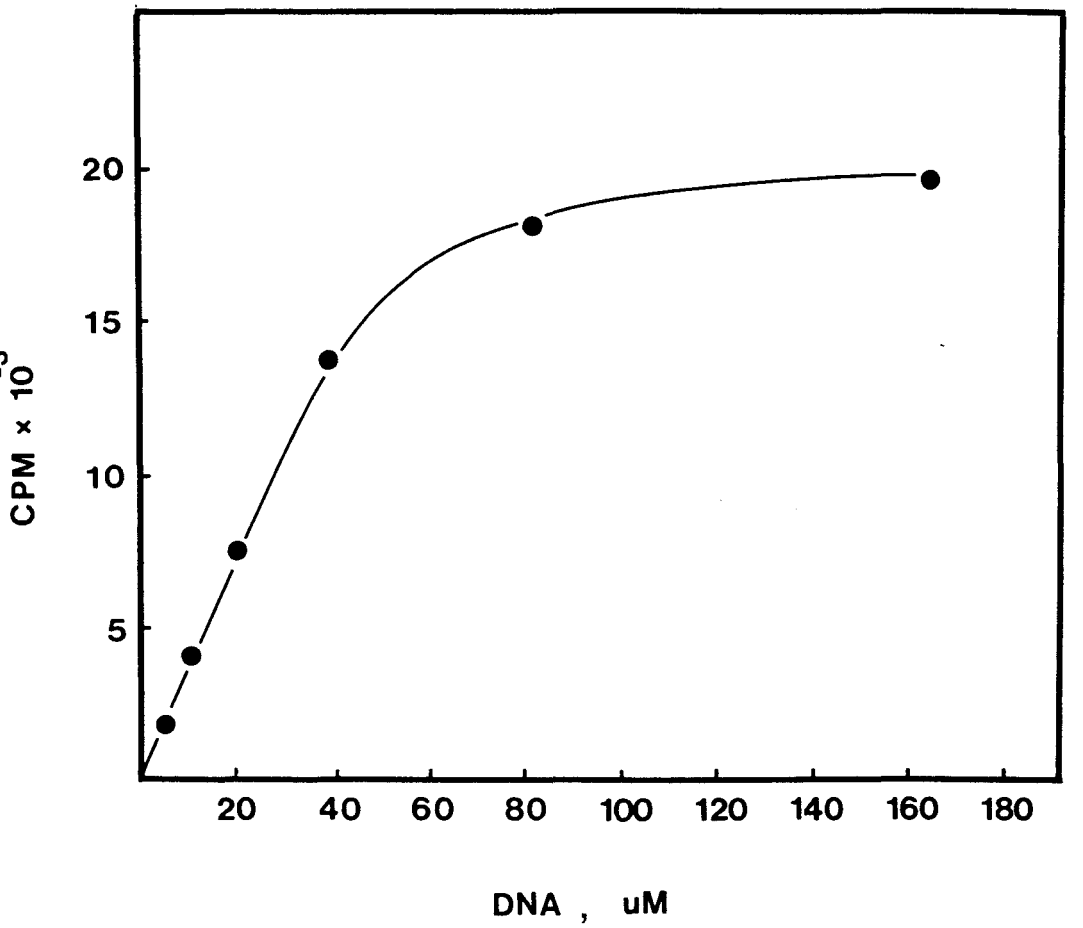

RNA Polymerase Activity, $\%_\infty - \%_t$
100
90
80
70
60
50
40
30
20
10
20
40
TIME , MIN

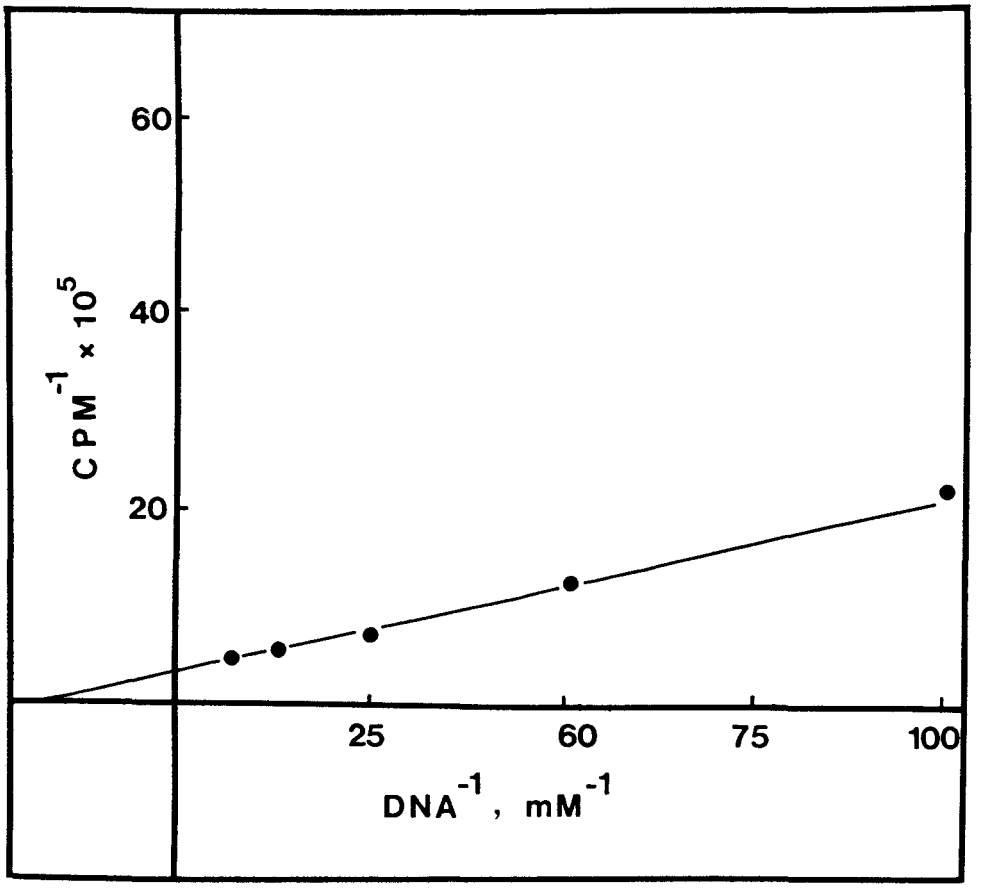
Figure 12. The Effect of Template Concentration on RNA Synthesis.

Various amount of T7 DNA were mixed with RNA polymerase for 8 minutes. The reaction was then initiated by rapid addition of 5 mM of ^{3}H-labeled substrates and terminated 90 seconds later. RNA polymerase activity is expressed as radioactivity retained on filter papers.


20
15
10
5
CPM x 10^-3
20
40
60
80
100
120
140
160
180
DNA , uM

sented in Figure 13. It is also apparent from Figure 12 that the maximum amount or incorporation of radioactivity occurs at 170 μM of DNA-P when 5 mM (initial concentration) of labeled nucleotide solution is used. Therefore, for subsequent experiments DNA concentration of 170 μM and an enzyme to DNA molar ratio of 12 were used to ensure the highest amount of incorporation of radioactive materials under the specific ^{3}H-labeled nucleotide concentration.

Figure 13. Linewaver-Burk Plot of RNA Polymerase Activity as A Function of Template Concentration


60
40
20
CPM^{-1} x 10^{5}
25
60
75
100
DNA^{-1}, mM^{-1}

III.3. The Inhibition of RNA Polymerase Activity By Anthracyclines

III.3.1. The Relative Potency of Anthracyclines on the Overall Inhibition of RNA Synthesis

The relative inhibitory activity of anthracyclines were studied by determining the level of RNA polymerase activities in the presence and absence of the drugs. Increasing amounts of drug were mixed with constant amounts of DNA, enzyme and nucleotide substrates and assayed for RNA polymerase activity, which was expressed as the amount of radioactive materials incorporated after a specific reaction time. The inhibitory activity of each drug was expressed as the percent residual enzyme activity determined by the radioactive materials synthesized, under the influence of increasing amount of anthracyclines, over that in the absence of drug.

In Figure 14, percent radioactivities are plotted vs the total added drug to DNA-P ratio. Clearly this ratio of drug over DNA-P does not provide any information as to the amount of drug that is actually bound to DNA.

From Figure 14 the effectiveness of each anthracycline in the inhibition of the overall RNA synthesis can be obtained. The added drug to DNA-P ratios which cause 50% inhibition of RNA synthesis for the different anthracyclines are listed in Table II.

In general, the ability of each anthracycline to inhibit RNA synthesis rises sharply with increasing amounts of added drug until roughly a 0.1 added drug to DNA ratio is reached. Beyond this point the increase in inhibition slows down and finally reaches a maximum. However, even though the inhibitory curves for all the drugs studied have a similar

Figure 14. The Overall Inhibitory Effectiveness of Anthracycline Analogs on T7 RNA Synthesis

Enzyme-template binary complexes were formed by mixing 1 μg of RNA polymerase with 170 uM of DNA in the presence of desired amount of daunomycin (●), 4-demethoxydaunomycin (○), carminomycin (▲), aclacinomycin (△), marcellomycin (□), and musettamycin (■) at 37° C, so that the added drug to DNA ratios were maintained within the range of 0 to 0.4. RNA synthesis was then initiated with the addition of ribonucleotide substrate (0.5 mM each of ATP, GTP, CTP and ^{3}H-UTP, 0.02 μCi/nmol) and terminated 90 seconds later with 5% TCA. The acid insoluble RNA product was collected on a filter paper and counted for radioactivity in a liquid scintillation counter. The amount of radioactivity obtained at zero drug concentration was used as 100% RNA polymerase activity. Each value represents the mean ± the standard deviation of six experiments.

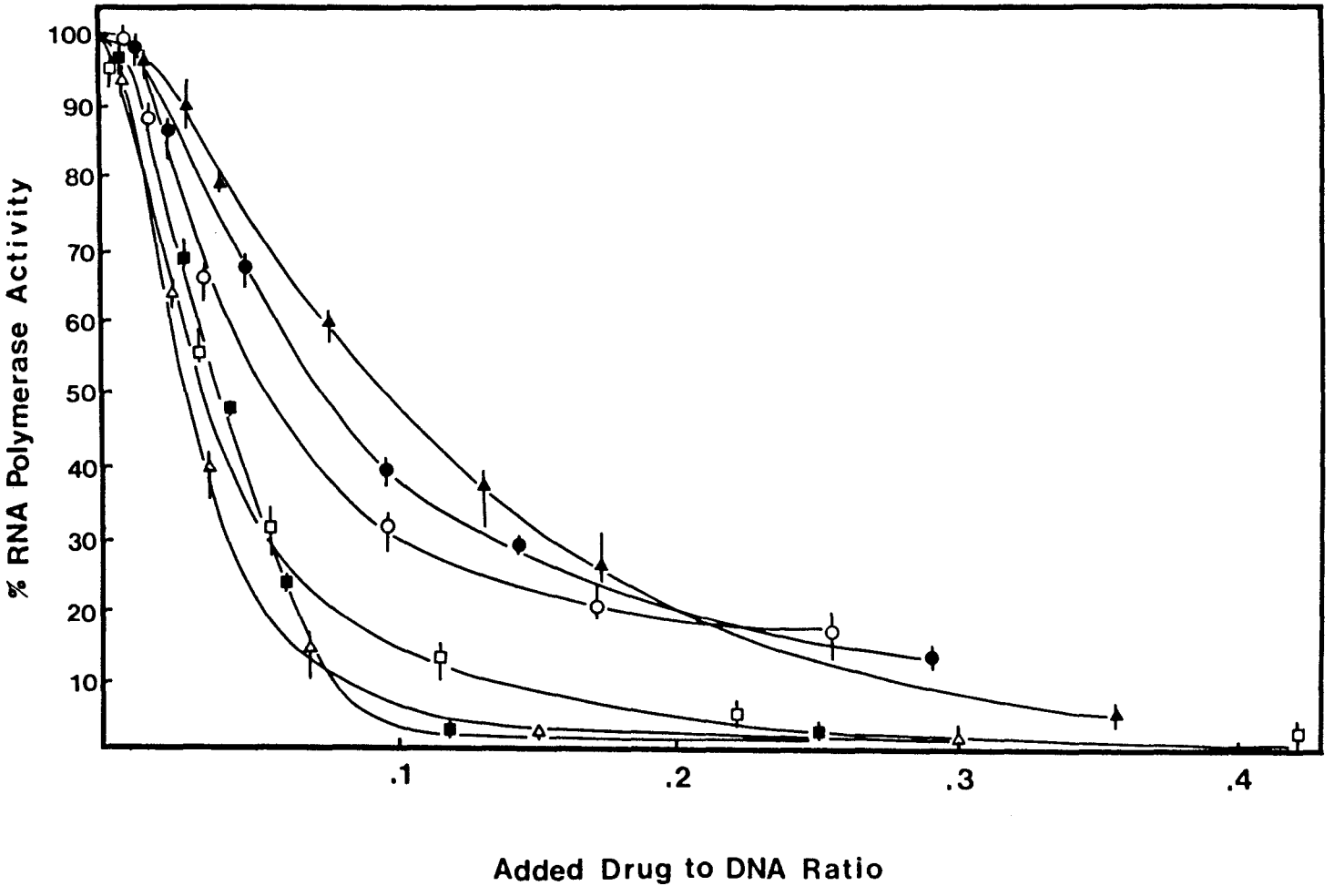

% RNA Polymerase Activity
100
90
80
70
60
50
40
30
20
10
.1
.2
.3
.4
Added Drug to DNA Ratio

Table II

Comparison of the Inhibitory Effectiveness of Some Anthracyclines towards RNA Synthesis

Anthracycline analogs	Added drug/DNA ratio resulting in 50% inhibition	IC_{50} (uM)
Group I:		
daunomycin	0.078	13.3
4-demethoxydaunomycin	0.058	9.9
carminomycin	0.110	18.7
Group II:		
aclacinomycin	0.030	5.1
marcellomycin	0.033	5.6
musettamycin	0.043	7.3

shape, they may be separated into two groups according to the structure of the compounds mentioned before.

Important quantitative differences in the inhibitory behavior of anthracyclines are evident from Table II. For the same ratio of added drug to DNA (i.e., IC_{50}), the aclacinomycin group is generally more effective (twice as effective on the average) than the daunomycin group. This higher effectiveness against RNA synthesis for group II anthracyclines is consistent with the result from in vitro studies with tumor cells (Crooke et al., 1977).

Results from the binding study (Table I) show that more drug molecules from group II anthracyclines can bind per unit DNA than group I drugs. This is indicated by the smaller size of binding sites (1/n, with n being the number of binding sites per unit DNA) required for group II anthracyclines. In addition, group II drugs can bind to DNA with greater affinity. Since previous reports indicated that RNA synthesis was directly related to the amount of DNA-RNA polymerase stable complexes formed (Hinkle and Chamberlin, 1972 a-d), it is logical that when identical amount of drugs from group I and group II anthracyclines are added to a constant amount of DNA, more drug molecules from group II can bind to DNA with greater affinities and thus inhibit the formation of the DNA-RNA polymerase complex to a greater extent.

The relative effectiveness of anthracyclines toward RNA synthesis inhibition within each group can also be evaluated in terms of their binding parameters. For example, the differences in the extent of inhibition between members of group I anthracyclines follow closely with their binding affinities. Specifically, in the case of 4-demethoxydaunomycin, the

removal of the methoxy group on C-4 increases both the binding and inhibitory effectiveness of the drug. From the x-ray diffraction study of the crystals of the intercalated complex of daunomycin and DNA (Rich et al., 1981) it is evident that ring A which contains the methoxy group penetrates completely through the base pairs and hangs out on the other side into the major groove. Removal of this bulky methoxy group may aid the penetrating process, thereby stabilize the intercalated complex. On this basis one might expect that carminomycin also should be a better binding drug than daunomycin. However, carminomycin was found to bind less strongly to DNA and to inhibit RNA synthesis less than daunomycin. Similar results have also been obtained in the comparative study of DNA binding between carminomycin and adriamycin (DuVernay et al., 1980). It is possible that inter- or intra-molecular hydrogen bonding involving the C-4 hydroxyl group of carminomycin might be present which could interfere with the intercalation of the aglycone of this compound.

No significant differences in either the binding effectiveness or the RNA synthesis inhibitory effect were found among members of group II anthracycline probably because no significant differences are present in the substitution on the aglycone of these drugs.

In any event, there seems to be a structural-activity relationship among the two groups of anthracyclines studied. Substitutions on C-10 and C-4' provide group II anthracyclines with better binding affinities to DNA and therefore stronger inhibitory effect on overall RNA synthesis over that of group I drugs. Furthermore, C-4 substitutions for group I anthracyclines play an important role in changing anthracycline binding to DNA and the ability to inhibit RNA synthesis.

III.3.2. The Effect of DNA-Bound Anthracyclines on RNA Synthesis

Daunomycin has been shown to inhibit RNA synthesis by reducing the availability of DNA template for binding RNA polymerase. This reduced availability is a consequence of daunomycin intercalating to DNA (Mizuno et al., 1975). Daunomycin has no effect on the activity of RNA polymerase per se (Zunino, et al., 1974). Therefore the form of the drug in solution which is active in the inhibition of RNA synthesis is the DNA-bound form, and Figure 15, which is a plot of the RNA polymerase activity vs DNA-bound drug concentration (i.e., r_b, Section II.4.1.), is essential for providing information regarding differences on the inhibition of RNA synthesis by anthracycline on a molecular level.

The corresponding r_b value for each added drug to DNA ratio in Figure 14 was obtained using equations (1) and (2) and subsequently Figure 14 was converted into Figure 15. In this figure, the relative effectiveness of each anthracycline analog expressed in terms of DNA-bound concentration (r_b) toward inhibition of RNA synthesis, are compared. The r_b values for each anthracycline analog which cause 50% RNA inhibition are listed in Table III.

The results in Figure 15 appear strikingly different from those shown in Figure 14. Specifically, on the basis of equal amount of drug molecules bound to T7 DNA, the relative inhibitory effectiveness of group I and group II anthracyclines are reversed, i.e., the members of the daunomycin group are more effective in inhibiting RNA synthesis than the members of the aclacinomycin group. This finding indicates that identical numbers of bound drug molecules per unit DNA have different inhibitory effects on the ability of RNA polymerase to utilize DNA as template for

Figure 15. The Inhibiroty Effectiveness of DNA-Bound Anthracyclines Toward RNA synthesis

RNA polymerase activity is plotted verses the number of drug molecules bound to each DNA nucleotide (r_b).

daunomycin (●); 4-demethoxydaunomycin (○); carminomycin (▲); aclacinomycin (△); marcellomycin (□); musettamycin (■).

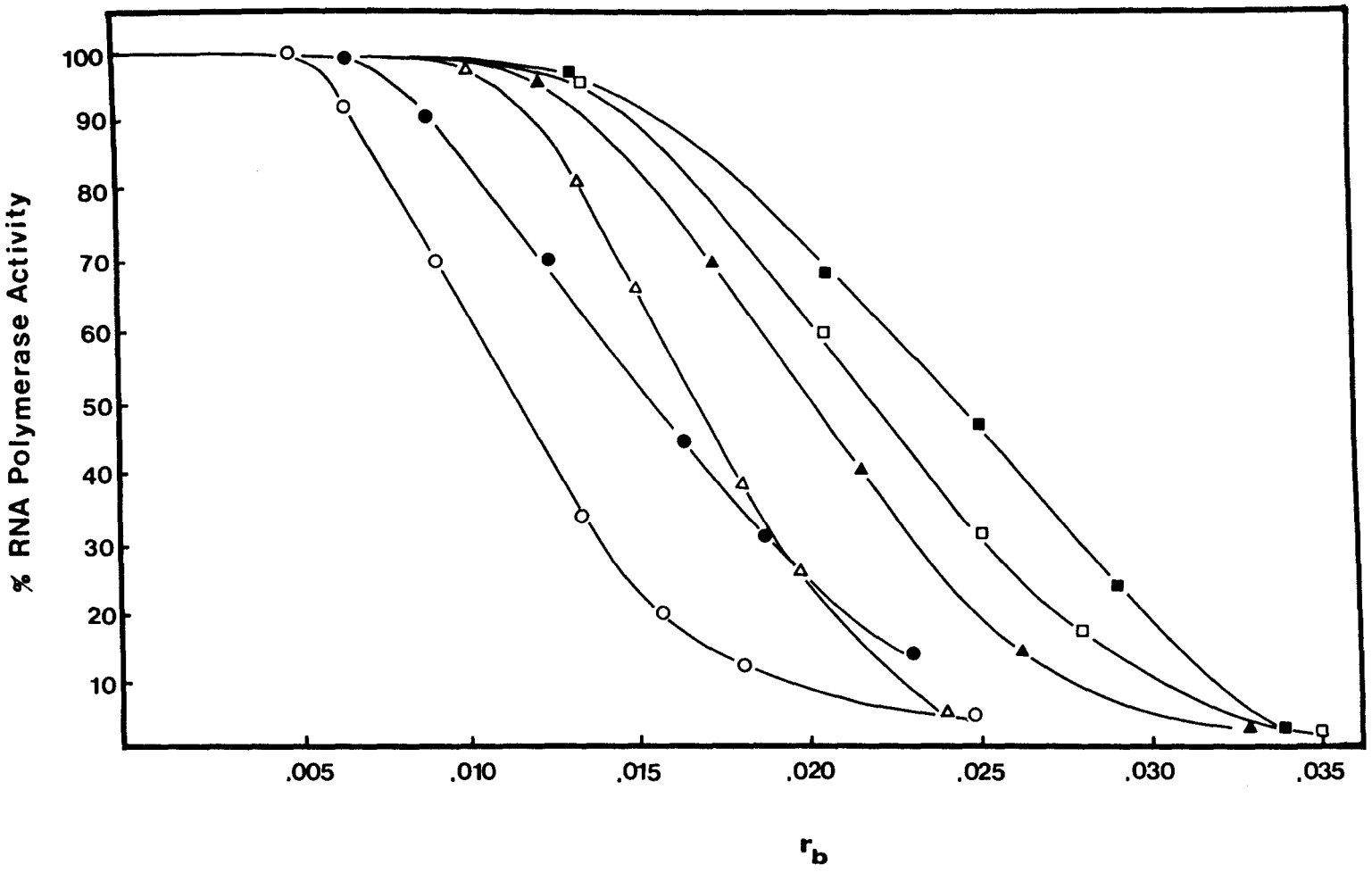

% RNA Polymerase Activity
100
90
80
70
60
50
40
30
20
10
.005
.010
.015
.020
.025
.030
.035
r_b

Table III

Comparison of the Inhibotory Abilities of Anthracyclines based on the Concentration of DNA-bound Drug

Anthracyclines	r_b at which 50% inhibition is observed
Group I:	
daunomycin	0.015
4-demethoxydaunomycin	0.011
carminomycin	0.016
Group II:	
aclacinomycin	0.020
marcellomycin	0.022
musettamycin	0.024

RNA synthesis. Consequently, the inhibitory effectiveness of these anthracyclines is determined not only by the affinity of each drug to DNA but may also by the specific structure of the drug molecules from each group of anthracyclines.

Besides the binding of RNA polymerase to DNA, another area which directly affects RNA synthesis is the mechanism by which RNA polymerase carried out its catalytic functions. The mechanism of DNA synthesis has been shown to be sensitive to the structures of the intercalated anthracyclines (Goodman and Lee, 1977; Section I.2.8.2.). It is likely, therefore, that different structures of the intercalated anthracycline analogs might also affect the mechanism of RNA synthesis to a different extent.

At this point it should be noted that the concentration of anthracyclines at which the inhibition of RNA synthesis is studied (i.e., $r_b <$ 0.035) is far less than the level for secondary binding to occur ($r_b >$ 0.05). Therefore the inhibitory effect of the drug studied is not due to the result of secondary binding.

III.3.3. The Effect of Template Concentration on the Overall RNA Synthesis Inhibition By Anthracycline Analogs

Previous studies have demonstrated that daunomycin inhibits RNA synthesis competitively with the template and the inhibition can be overcome by re-addition of the template (Zunino et al., 1974). In the present study, RNA synthesis under the influence of anthracyclines was studied by determining the level of RNA polymerase activity as a function of template concentration in the presence and absence of a constant amount of various inhibitors. Inhibitor concentrations were chosen so as to cause 50% inhibition of overall RNA synthesis as determined in Section III.3.1.

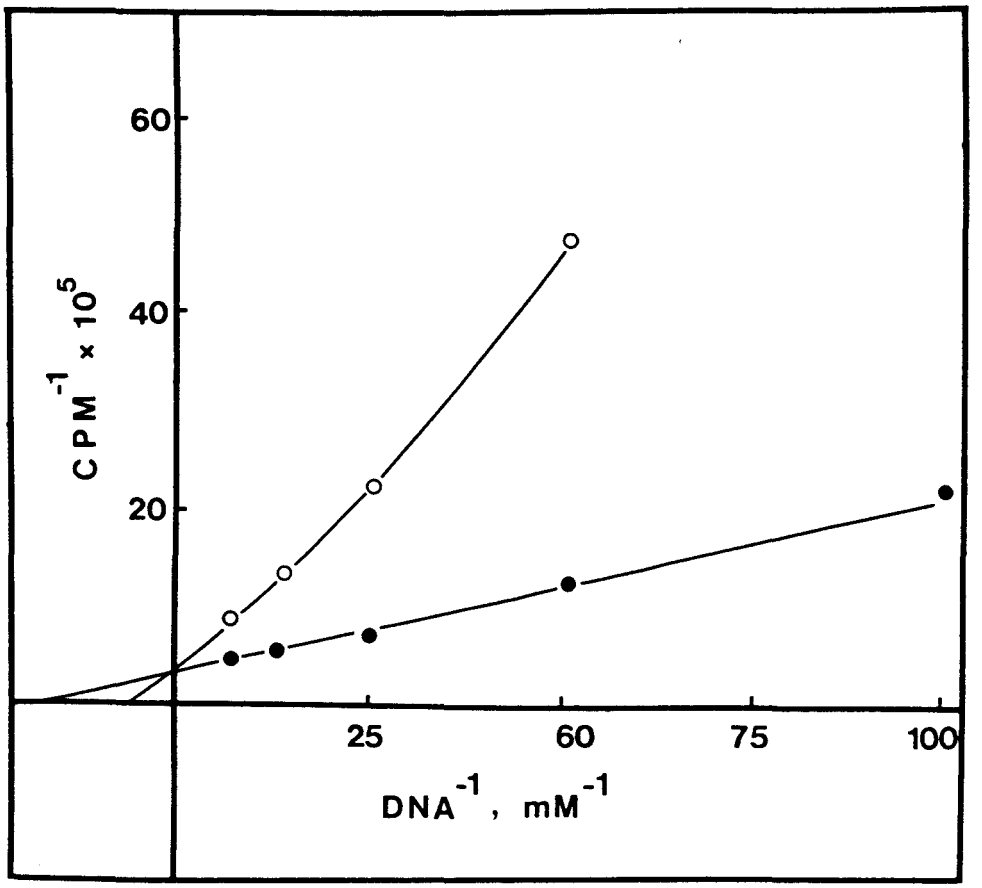
The results from two representative members of group I anthracyclines (daunomycin and carminomycin) and one from group II anthracyclines (aclacinomycin) are presented as Lineweaver-Burk plots in Figure 16a, b and c. The lines for the inhibited and un-inhibited reactions cross over on the coordinate indicating competitive inhibition for all the anthracycline analogs studied. Clearly the inhibitory effects of each drug can be completely abolished when the concentration of DNA is in large excess relative to that of the inhibitor. In addition, pre-incubation of the drugs with RNA polymerase was found to have no effect on the inhibition.

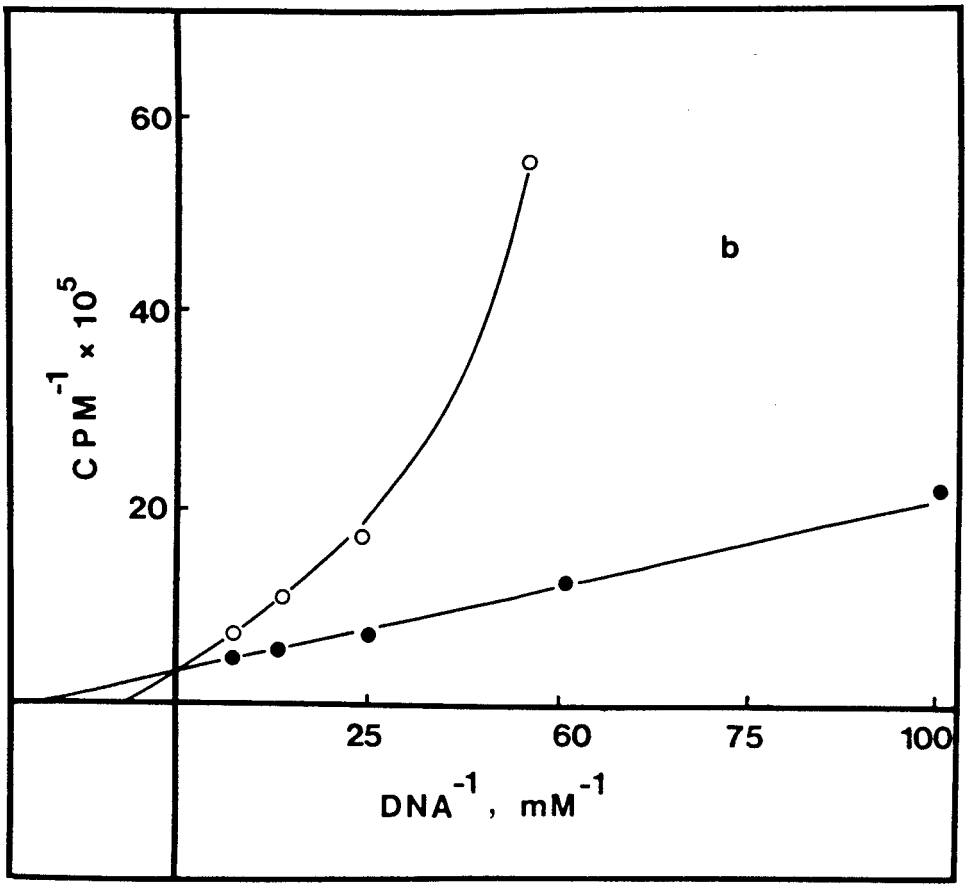
It is also apparent that the Lineweaver-Burk plots for the inhibited reactions deviate from linearity. This deviation is more pronounced at higher drug to DNA ratios. This surprising behavior is due to the fact that in the presence of a constant amount of intercalators, the increase in DNA concentration causes a gradual decrease in r_b (the number of DNA bound drug molecules per DNA nucleotide) as indicated by equation (7) (Section III.3.2.). Since the actual concentration of the inhibitors should be expressed in terms of r_b rather than $[I_o]$, a gradual decrease in r_b also gradually reduces the slope of the inhibited plot in Figure 16, and results in curvilinearity. A similar observation has been reported in the study of the interactions between phenanthridines and DNA (Aktipis and Panayotatos, 1981).

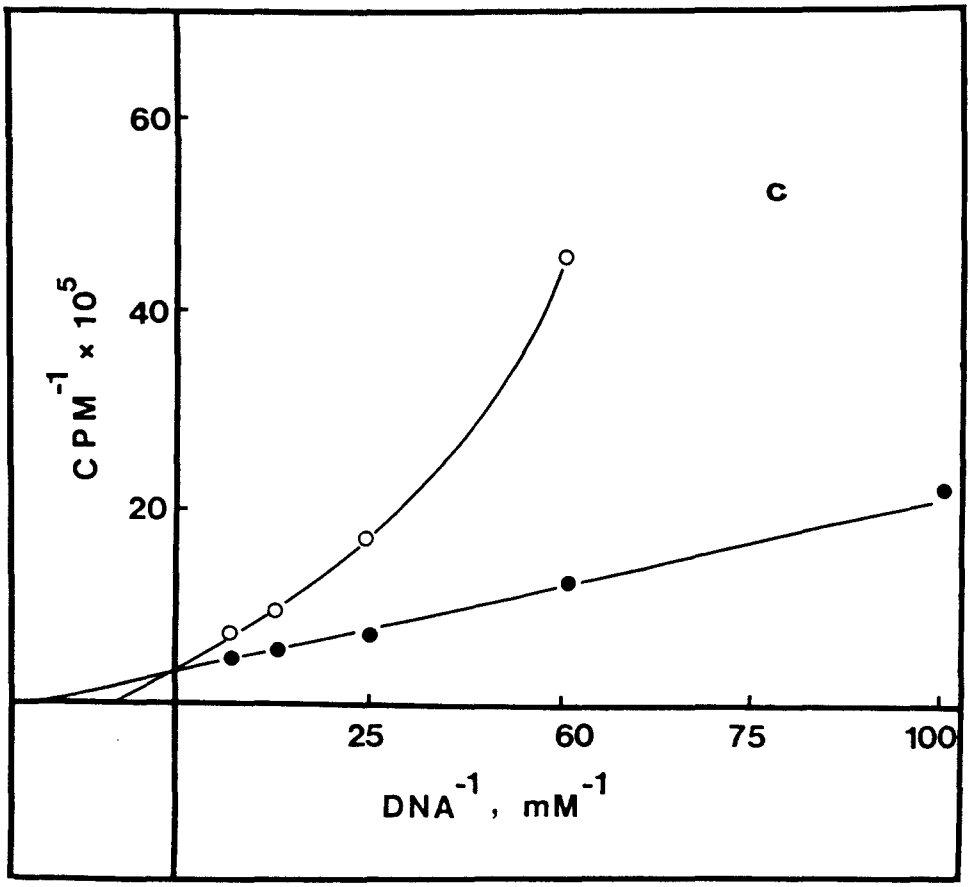
In any event, this result demonstrates that anthracyclines studied in this project competitively inhibit RNA synthesis and is consistent with the proposal that the inhibition of RNA synthesis by anthracycline is primarily due to the inactivation of the template (Zunino et al., 1974).

Figure 16. The Effect of Template Concentration on RNA Synthesis

The amount of RNA synthesized was measured in the absence of intercalators (●), or in the presence (○) of (a) 13.3 μM of daunomycin, (b) 18.7 μM of carminomycin, and (c) 5.1 μM of aclacinomycin.


60
40
20
CPM^{-1} x 10^5
25
60
75
100
DNA^{-1}, mM^{-1}


60
40
20
CPM^{-1} x 10^5
b
25
60
75
100
DNA^{-1}, mM^{-1}


60
40
20
c
CPM^{-1} x 10^5
25
60
75
100
DNA^{-1}, mM^{-1}

III.3.4. Mechanisms of Inhibition of RNA Synthesis By Anthracycline Analogs

The results presented in the preceding pages suggest that the mechanisms by which the two groups of anthracycline analogs inhibit RNA synthesis may be different despite the fact that they all inhibit RNA synthesis competitively. Previous reports on the mechanism of RNA synthesis inhibition by daunomycin have suggested that daunomycin inhibits RNA synthesis initiation without significantly affecting elongation due to the relatively rapid dissociation of the drug from DNA (Schellinx et al., 1979). However, this conclusion is clearly in contradiction with a previous report in which daunomycin was found to affect the elognation of RNA chains (Zunino et al., 1974). Since the initiation of RNA synthesis from T7 DNA by E. coli RNA polymerase has been studied thoroughly and the different steps in the events prior to the formation of the first phosphodiester bond have been elucidated (Section I.3.5.), it is of interest to examine the effect (if any) of each anthracycline analog on the events that precede the formation of the first phosphodiester bond as well as its effect on chain elongation.

III.3.4.1. The Effect of Anthracycline Analogs on the Formation of DNA-RNA Polymerase Complex

Conflicting results have been reported concerning the ability of daunomycin to inhibit the formation of DNA-RNA polymerase stable binary complexes which are necessary prior to the formation of the first phosphodiester bond (Section I.2.8.1.). In this study the effect of anthracyclines on the formation of DNA-RNA polymerase binary complexes were examined by measuring the radioactivity retained on filter paper using a method devised by Jones and Berg in 1966. This method is based on the

observation that when a buffered solution containing DNA and RNA polymerase is subjected to filtering, only the DNA-RNA polymerase binary complexes are retained on the filter if a controlled rate of filteration is used. Any unassociated molecules of either free DNA or RNA polymerase are filtered through. In the present study, the amounts of ^{3}H-T7 DNA and RNA polymerase were kept constant while increasing amounts of various inhibitors were added. The experimental details are presented in the Methods (Section II.4.2.)

Figure 17 shows the results of the effect of anthracyclines on the formation of DNA-RNA polymerase binary complexes. It is evident that as the amounts of inhibitors increase fewer DNA-RNA polymerase complexes are formed as indicated by the decreasing amount of radioactivity retained on the filters. The shape of the inhibitory curves is similar for all the anthracycline analogs studied. However, with the aclacinomycin group, an almost linear relationship is noted between the effectiveness of the members of the group in dissociating the enzyme-DNA complex and drug concentration up to low concentrations of the drug. The inhibition of the binary complex formation by members of the daunomycin group, on the other hand is relatively low until an added drug to DNA ratio of about 0.04 is reached. Beyond this point inhibition increases fairly rapidly with increasing amount of added drug. However, the increase is not as rapid as that noted for the aclacinomycin group.

The added drug to DNA ratio for each drug at which only 50% of the binary complex is formed are listed in Table IV. Comparison of the data in Table II and Table IV indicates that within group II anthracyclines the relative inhibitory effect of each drug against complex formation

Figure 17. The Effectiveness of Anthracyclines on the Dissociation of the DNA-RNA Polymerase Binary Complex: Amount of Complex Determined by the Nitrocellulose Filter Assay

10 µl, 170 µM of ^{3}H-T7 DNA in 80 ul binding buffer with or without desired amount of rifampicin or anthracyclines, was mixed with 10 µl (containing 1 µg) of RNA polymerase. After incubating at 37° C for 10 minutes, the mixture was diluted to 1.0 ml with binding buffer and filtered through a nitrocellulose filter paper. The radioactivity collected on the filter is proportional to the amount of DNA-RNA polymerase binary complexes formed.

Results obtained: without anthracycline but in the presence of rifampicin (✕); in the presence of daunomycin (●); 4-demethoxydaunomycin (○); carminomycin (▲); aclacinomycin (△); marcellomycin (□); musettamycin (■). Each value represents the mean ± the standard deviation of four experiments.

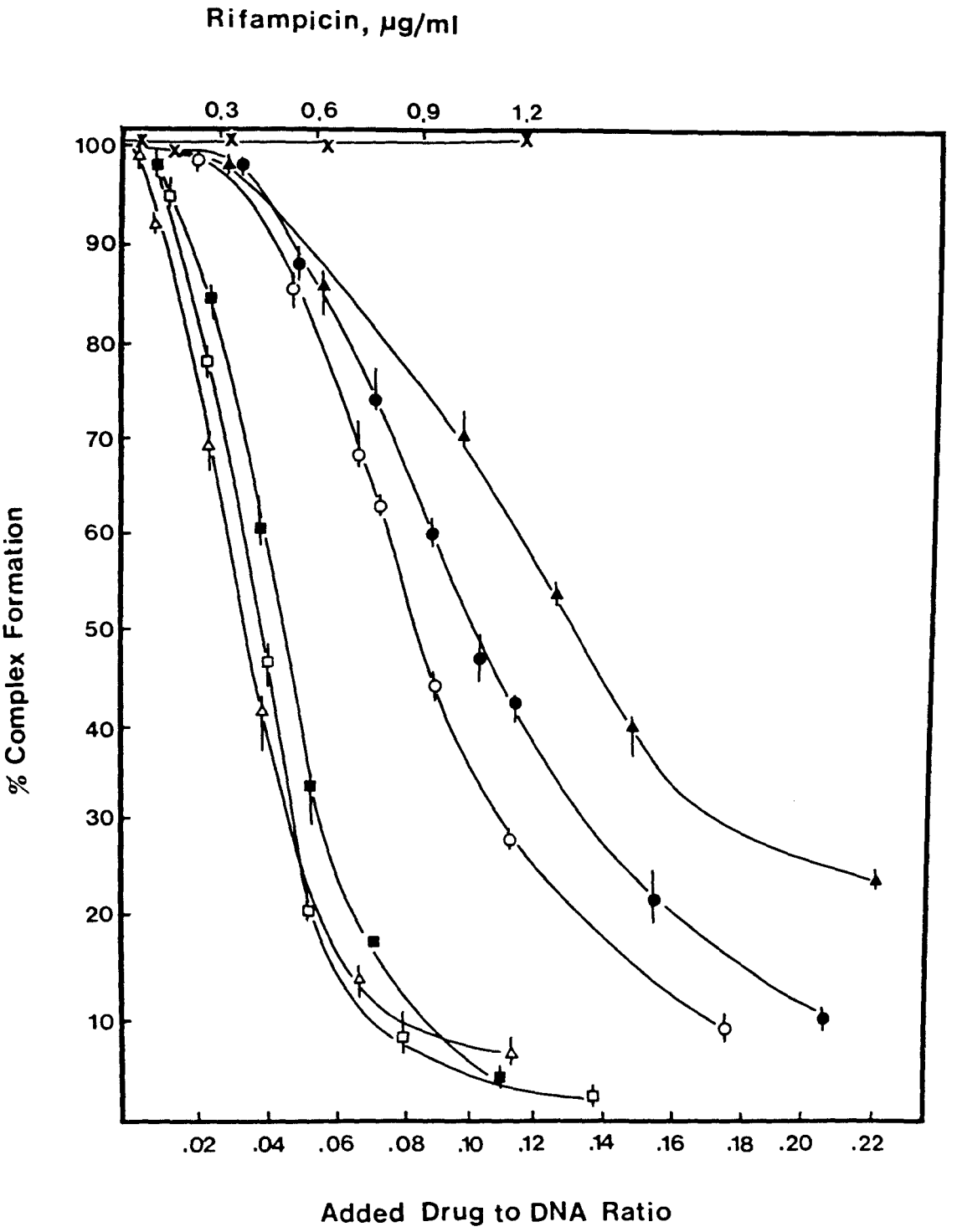

Rifampicin, µg/ml
0.3
0.6
0.9
1.2
100
90
80
70
60
50
40
30
20
10
% Complex Formation
.02
.04
.06
.08
.10
.12
.14
.16
.18
.20
.22
Added Drug to DNA Ratio

Table IV

Effect of Anthracyclines on the Formation of the DNA-RNA Polymerase Complex

Anthracyclines	added drug to DNA ratio which causes 50% inhibition
Group I:	
daunomycin	0.096
4-demethoxydaunomycin	0.085
carminomycin	0.140
Group II:	
aclacinomycin	0.035
marcellomycin	0.035
musettamycin	0.045

closely parallel that of RNA synthesis inhibition. This observation seems to suggest that the overall RNA synthesis inhibition by group II anthracyclines is a direct result of their abilities to inhibit template-enzyme complex formation. In contrast, the RNA synthesis inhibition by group I anthracyclines can only be partially explained by their complex inhibition abilities. For example, the drug to DNA ratios for daunomycin which cause 50% of inhibition in RNA synthesis and enzyme-template complex formation are 0.078 and 0.096 respectively. Other factor must also exist to contribute to the total RNA synthesis inhibition abilities of group I anthracyclines in addition to their inhibition of the DNA-enzyme complex formation.

This study also shows that rifampicin, which attacks RNA polymerase, does not prevent RNA polymerase from binding to T7 DNA. This is indicated by the fact that 100% radioactivity was recovered even in the presence of the highest rifampicin concentration tested (1.25 ug/ml) in the experiment. Other experiments (data not shown) carried out in the presence of rifampicin with and without RNA polymerase show that only DNA-enzyme binary complexes are retained on the filters, DNA with or without rifampicin is filtered through.

III.3.4.2. The Effect of Anthracycline Analogs on Events Prior to the Formation of the First Phosphodiester Bond

As described in Figure 5, the interaction of T7 DNA and E. coli RNA polymerase leading to the formation of the first phosphodiester bond consists of three sequential events: (1) reversible, weak binding of RNA polymerase to T7 DNA at random sites, (2) enzyme attachment to a site at or near a specific promoter site to form the I complex, (3)

transformation of the I complex to a highly stable RS complex, from which a new complex (the OP complex) can be formed with the formation of the first phosphodiester bond.

In the following experiments, the rates of each of these two complex transformations were studied under the influence of anthracycline analogs.

III.3.4.2.1. The Rate of RS to OP Complex Transformation

The antibiotic rifampicin, which has been found to inhibit RNA synthesis initiation without affecting elongation (Mangel and Chamberlin, 1974c), is used in the assay for studying the rate of RS to OP complex transformation. In addition, rifampicin does not block the formation of RS complex, and does not increase the rate of dissociation of this complex (Section III.3.4.1.). In fact, the ribonucleoside triphosphate substrates are in competition with rifampicin so that once the first phosphodiester bond is formed, the RS complex transforms into OP complex and becomes insensitive to rifampicin (Mangel and Chamberlin, 1974c).

The rate of rifampicin attack on the highly stable T7 DNA-RNA polymerase holoenzyme complex (RS complex) has been shown to obey second order kinetics (Hinkle et al., 1972d). Therefore, if RNA polymerase-T7 DNA binary complexes are formed in the presence of anthracyclines and then challenged simultaneously with a mixture of rifampicin and the four ribonucleoside triphosphates, two competing reactions (equations 7 and 8 below) would take place:

(1) Inactivation by rifampicin:

$$C + R \xrightarrow{k_2} C^r \qquad (7)$$

(2) RNA chain initiation:

$$C + nXTP \xrightarrow{k^*} C^* \qquad (8)$$

In these equations C represents the concentration of RNA polymerase holoenzyme-T7 DNA binary complex: C^r represents the concentration of the binary complexes inactivated by rifampicin and consequently unable to initiate a RNA chain; C* represents the binary complexes which have initiated an RNA chain and are therefore insensitive to rifampicin; k_2 is the second order rate constant for rifampicin attack on RNA polymerase-T7 complexes, and k* is an apparent first order rate constant for RNA chain initiation at a particular concentration of ribouncleoside triphosphates.

With the assumptions that the binding of rifampicin to holoenzyme in the binary complex is irreversible; that RNA chain initiation by the binary complexes is irreversible and that during the assay, concentration of rifampicin and the ribonucleoside triphosphates remain constant, and with the use of the conservation equation:

$$C^r + C^* = C_o \qquad (9)$$

(where C_o is the concentration of binary complexes at t = 0,) the following relationship can be derived from equations (7) to (9):

$$\frac{C^r}{C^*} = \frac{k_2 \cdot [R]}{k^*} \qquad (10)$$

The second order rate constant for rifampicin attack on holoenzyme-T7 DNA complexes (k_2) is 3.5×10^3 $M^{-1}s^{-1}$ (Hinkle et al., 1972d); thus, only an assay for measuring C_o and C* is required to determine the apparent

first order rate constant for RNA chain initiation (k*). With this assay, C_o is experimentally defined as the amount of RNA synthesized in 90 seconds (Second II.4.3.2.1.) in the absence of rifampicin, and C* is the amount of RNA synthesized under identical conditions but in the presence of rifampicin.

To calculate the rate of RNA chain initiation, equation (10) can be transposed utilized equation (9) to yield equation (11),

$$\frac{C_o}{C^*} = \frac{k_2\,[R]}{k^*} + 1 \qquad (11)$$

where C_o/C^* represents the reciprocal of % RNA polymerase activity. At various concentrations of rifampicin, a graph of the reciprocal of % RNA polymerase activity vs the rifampicin concentration should result in a straight line with a characteristic slope. The slope should be equal to the rate of the second-order rate constant of rifampicin attack on the binary complex (RS) over the rate constant of the transformation of the RS complex to the rifampicin-insensitive ternary complex (OP), or k_2/k^*.

In order to study the effect of anthracycline analogs on the rate of RS to OP complex transformation, identical assays can be performed in the presence and absence of anthracyclines. If anthracyclines change the rate of RNA synthesis initiation (i.e., the transformation of RS to OP complex), the slope of the C_o/C^* vs [R] plot should be different assuming that the rifampicin attack on RNA polymerase is not affected by the presence of the intercalating anthracyclines.

Since the rate of rifampicin attack on the RNA polymerase in RS complex, k_2, is crucial in the determination of the rate of RNA chain initiation, it must be assumed that k_2 is not affected by the presence

of anthracyclines in order to use the rifampicin technique in the study of the effect of anthracycline analogs on the rate of RS to OP complex transformation.

In ascertaining that anthracyclines have no effect on k_2, 4-demethoxydaunomycin and aclacinomycin were chosen to represent group I and group II anthracyclines respectively. Daunomycin was not chosen for these experiments because it appears to interact with rifampicin, forming a non-specific complex which is responsible for retaining radioactive material on the filters thus giving erroneously high counts.

The assays used for determining the rate constants of rifampicin inactivation of the RS complex in the presence and absence of the two anthracycline analogs are described in the Methods (Section II.4.3.2.1.). The results are presented in Figure 18a, b and c.

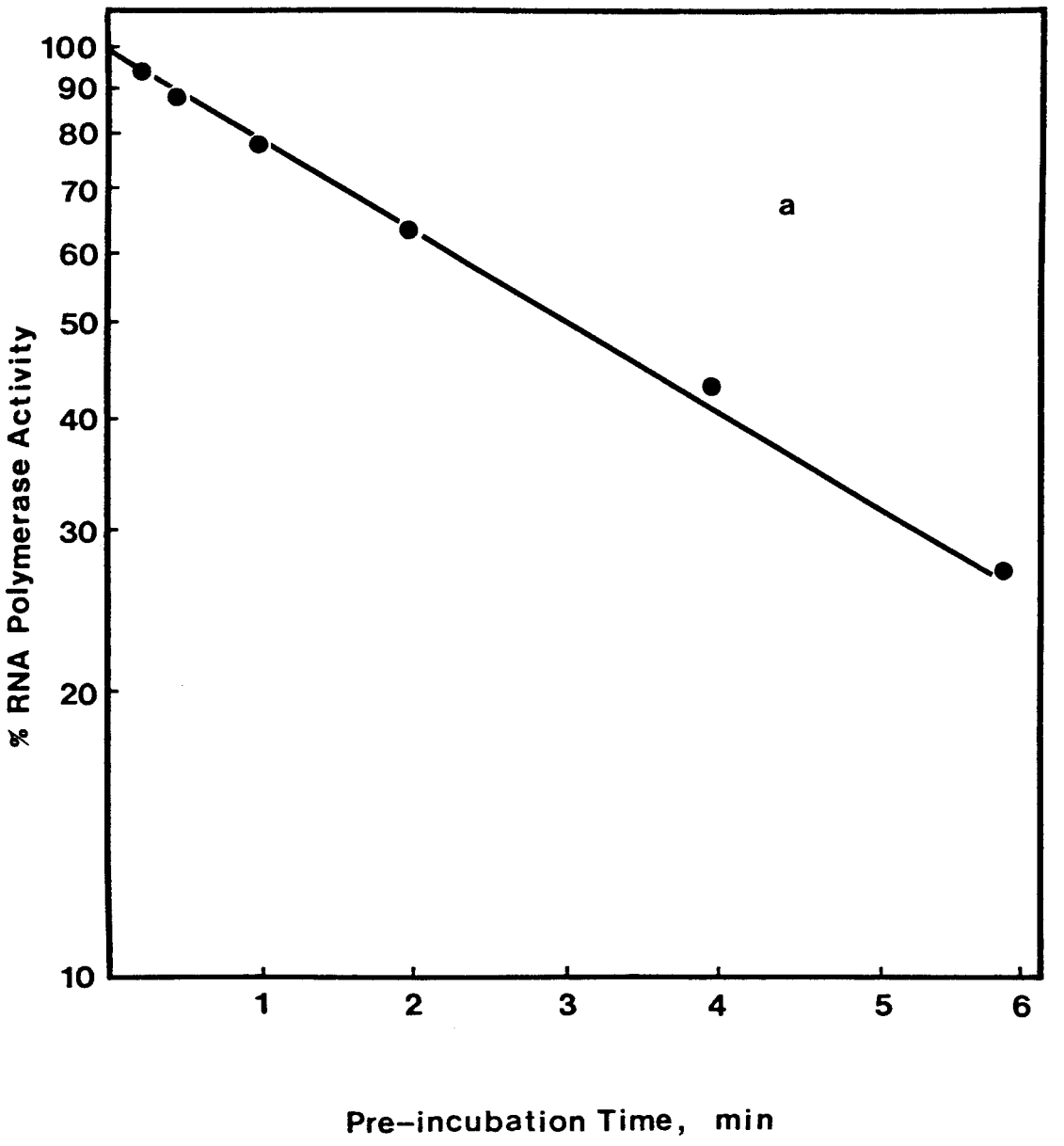
Under the assays conditions, where rifampicin is present in great excess over RNA polymerase (over 10 fold), the kinetics for inactivation of the enzyme in the binary complex by rifampicin are pseudo first-order, as indicated by the first order rate plot in Figure 18. In other words, although the binding of rifampicin to RNA polymerase is a second-order reaction, under conditions where the concentration of unbound antibiotic can be considered constant during the reaction $k_{obs} = k_2 \times$ [rifampicin], where k_{obs} is the apparent rate constant for the binding of rifampicin to RNA polymerase and k_2 is the true second-order rate constant for the reaction.

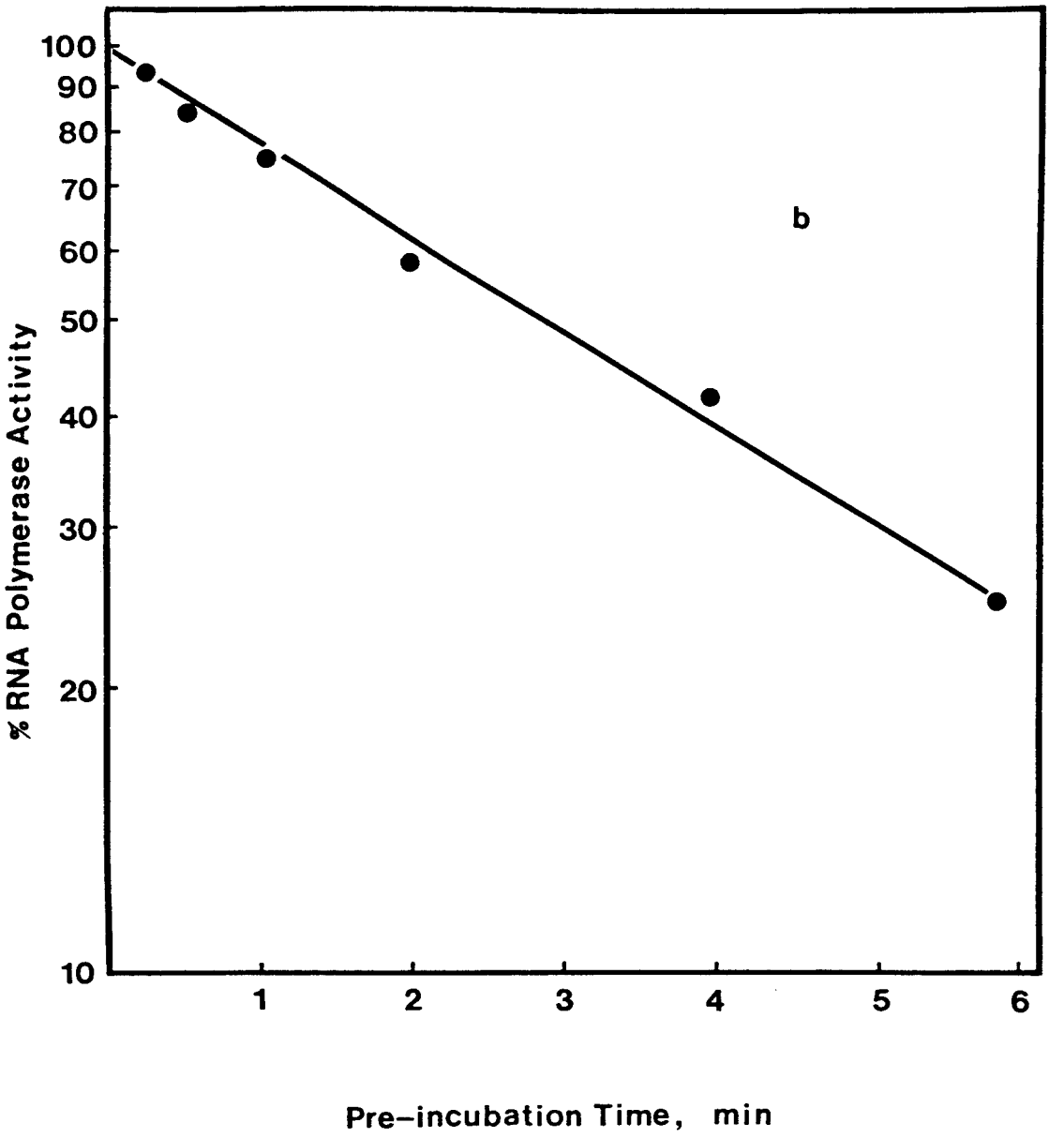
In the presence of 4-demethoxydaunomycin and aclacinomycin, RNA polymerase in the enzyme-DNA binary complex has a half-life of approximately 3 minutes. This half-life time is nearly identical with that

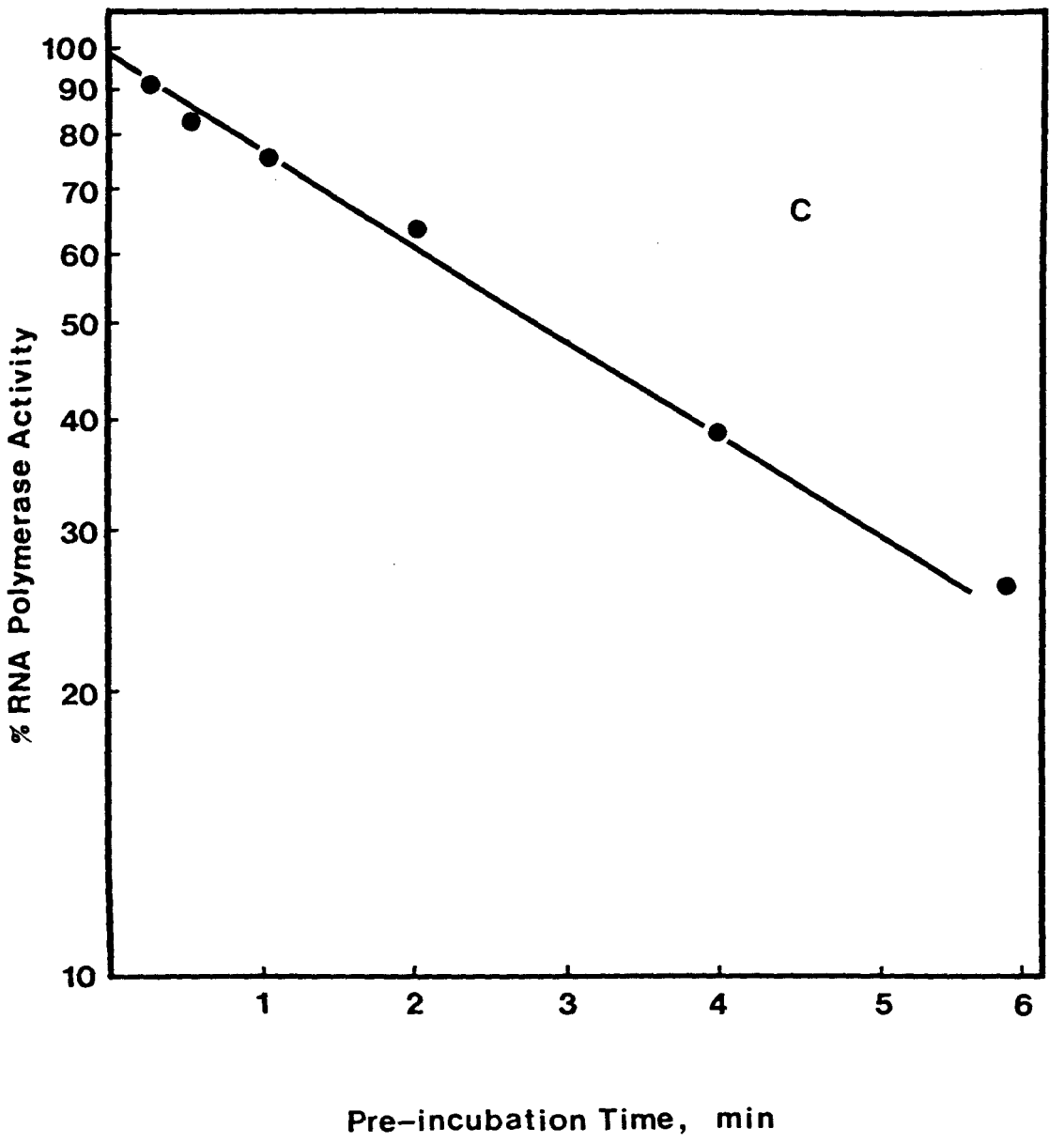
Figure 18. Kinetics of Inactivation of RNA Polymerase-T7 DNA Complex by Rifampicin at 37° C

Enzyme-template binary complexes were formed by adding 1 µg of RNA polymerase in 10 µl of enzyme solution (Section II.4.3.) to a mixture of 35 µl of buffer, 20 µl of DNA (170 µM) and 15 µl of H_2O or anthracycline solution. After 7 minutes of incubation, 10 µl of H_2O with or without 0.38 µM of rifampicin was added. Incubation was continued for the indicated time intervals, then RNA synthesis was initiated by the addition of 10 µl of 0.5 mM each of GTP, ATP, CTP and ^3H-labeled UTP. The reaction was terminated 90 seconds later and the acid-insoluble radioactivity was determined.

a) Incubation in rifampicin only; b) incubation in 9.9 µM of 4-demethoxydaunomycin; c) incubation in 5.1 µM of aclacinomycin.


100
90
80
70
60
50
40
30
20
10
% RNA Polymerase Activity
a
1
2
3
4
5
6
Pre-incubation Time, min


100
90
80
70
60
50
40
30
20
10
% RNA Polymerase Activity
b
1
2
3
4
5
6
Pre-incubation Time, min


100
90
80
70
60
50
40
30
20
10
% RNA Polymerase Activity
C
1
2
3
4
5
6
Pre-incubation Time, min

obtained in the absence of anthracyclines and is in good agreement with that reported by Hinkle under comparable conditions (Hinkle and Chamberlin, 1972d). This identical half-life for RNA polymerase under the influence of various anthracyclines indicates that the rate constant of rifampicin attack on the enzyme in the RS complex is not affected by either group I or group II anthracyclines.

The effect of different anthracycline analogs on the rate of RS to OP complex transformation are shown in Figure 19a, and b. For these experiments the concentrations of anthracyclines were chosen so as to cause 50% inhibition of RNA synthesis as determined in Section III.3.1.

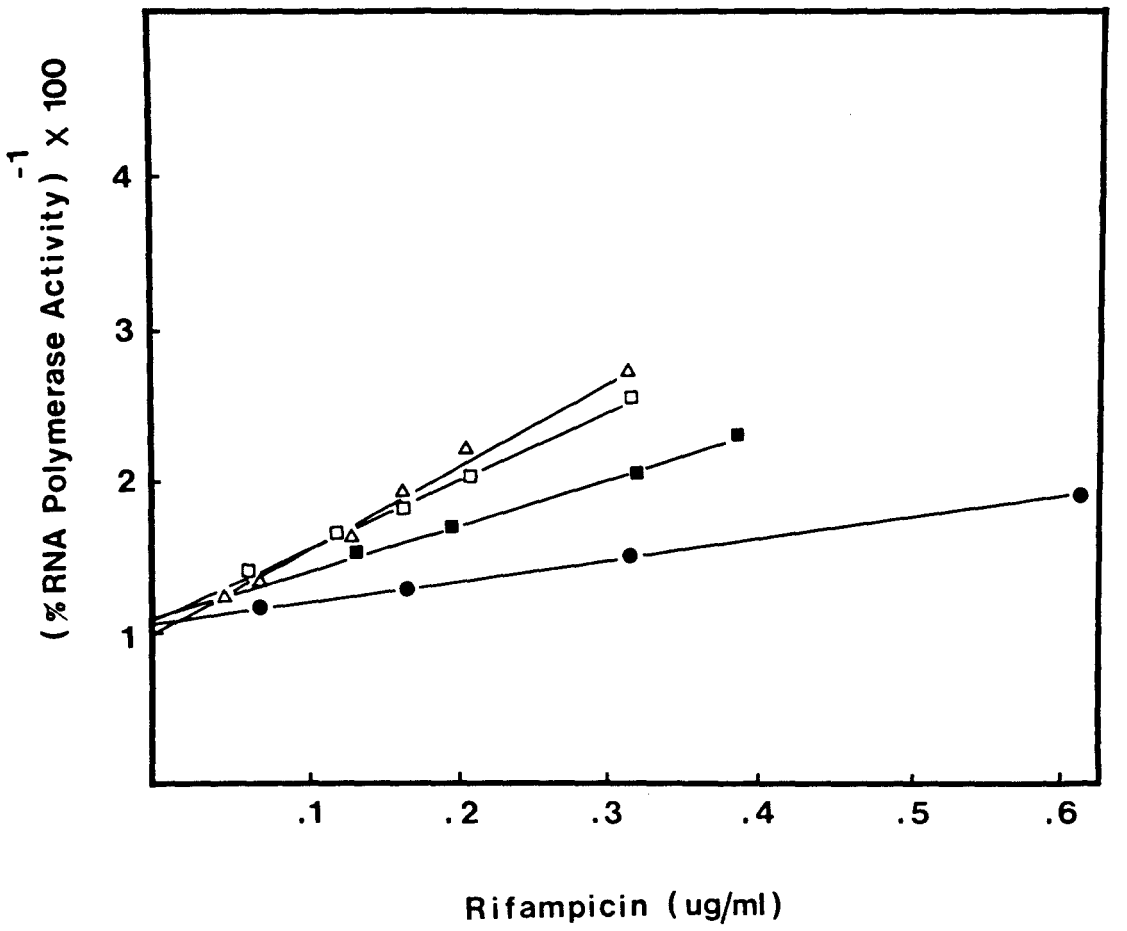
From the comparison of the slopes of the plots of enzyme activity vs rifampicin concentration, it is evident that group II anthracyclines show a striking difference in the way they affect the RS to OP transformation as compared to group II anthracyclines. Specifically, group II anthracyclines exhibit basically identical slopes with the slopes obtained in their absence. Since k_2 is not affected by the presence of these drugs (Figure 18), this result indicates that the presence of type II anthracyclines has no effect on the rate constant of RNA polymerase molecules which proceed from the RS complex to form the first phosphodiester bond. A similar conclusion has been drawn in a previous study about the effect of Ethidium Bromide on the rate of RS to OP transformation (Aktipis and Panayotatos, 1977).

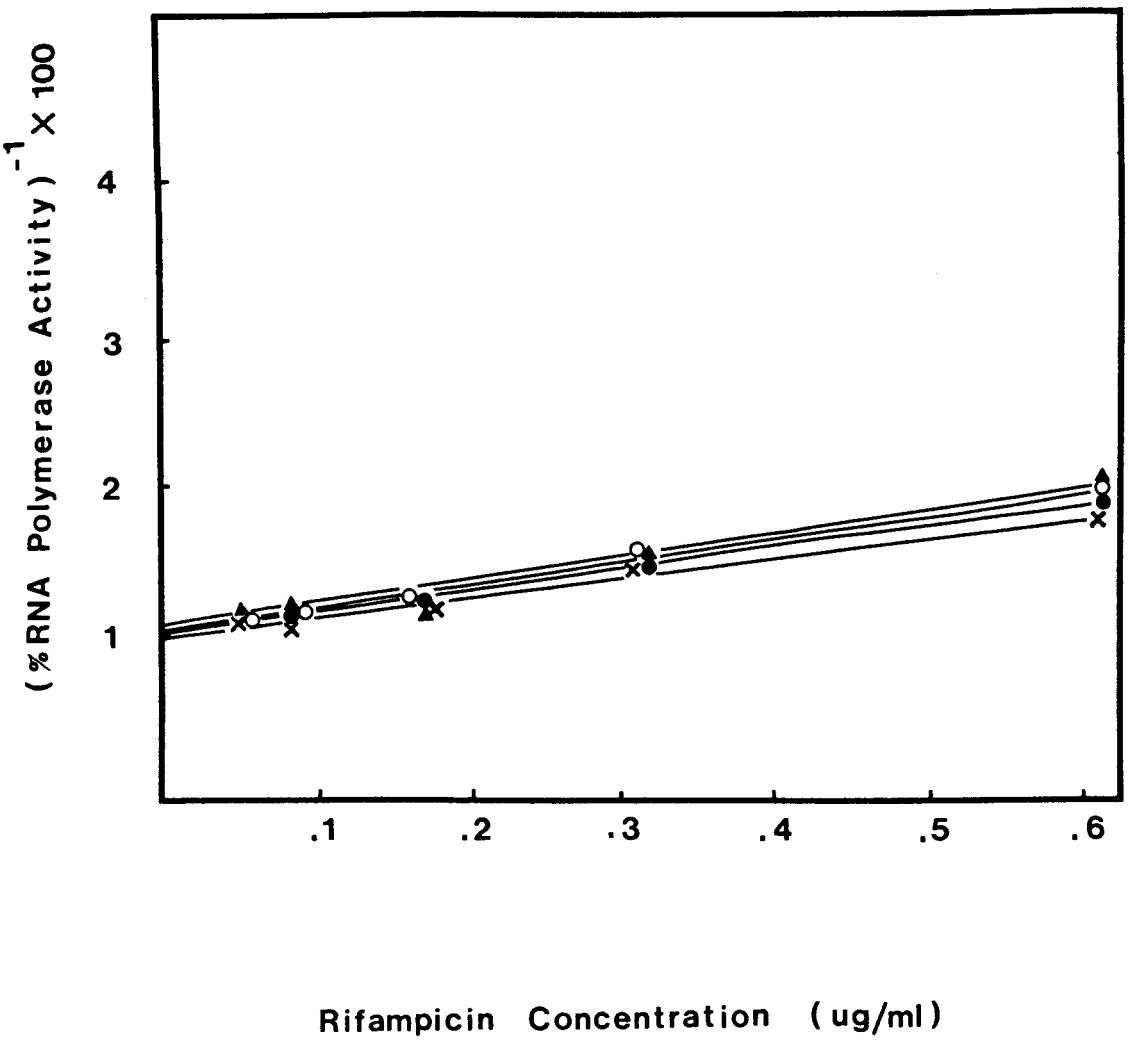
In contrast, group I anthracyclines produce an increase in the k_2/k^* (the slope) as compared to reactions in the absence of these drugs. Since these drugs do not affect k_2 (Figure 18), it is clear that they inhibit the rate of RS to OP complex transformation. Other inhibitors

Figure 19. The Effect of Anthracycline Analogs on the Rate of RNA Synthesis Initiation

The rate of RNA chain initiation was measured as described in Section II.4.3.2.2. The DNA and RNA polymerase binary complexes were incubated in the absence (●) and presence of anthracycline analogs at 37° C (■ , daunomycin; ▵ , 4-demethoxydaunomycin; □ , carminomycin; ○ , aclacinomycin; ▲ , marcellomycin; ✕, musettamycin). RNA synthesis was initiated with the subsequent simultaneous addition of rifampicin and ribonucleotide substrates. The reaction products were treated as described in Section II.4.3.

The slope in this figure represents the ratio of the rate of rifampicin attack on the RS complexes to the rate of RNA chain initiation, k_2/k^*.


(% RNA Polymerase Activity)$^{-1}$ X 100
4
3
2
1
.1
.2
.3
.4
.5
.6
Rifampicin (ug/ml)


(%RNA Polymerase Activity)$^{-1}$ X 100
4
3
2
1
.1
.2
.3
.4
.5
.6
Rifampicin Concentration (ug/ml)

which interfere with the RS to OP transformation do not appear to have been reported in the literature.

III.3.4.2.2. The Rate of I to RS Complex Transformation

Evidence has been presented (Mangel and Chamberlin, 1974a) that RNA polymerase molecules bind to T7 DNA at or near the T7 early promoter region in a conformation from which rapid RNA chain initiation is not possible. Enzyme molecules in this state (I complex) are in equilibrium with enzyme molecules in the RS conformation from which rapid RNA chain initiation can take place. This equilibrium is temperature dependent; formation of I complex is favored at low temperatures (lower than 10° C). The isolation of I complex has lead to the conclusion that all E. coli RNA polymerase molecules bind initially to T7 early promoter sites in the I conformation in which the DNA helix remains in a double helical state. The enzyme molecules then carry out melting functions to separate the strands and form the RS complex allowing enzyme the access to the base pairing residues of the DNA template. Therefore, this I to RS complex transformation is an another essential step prior to the actual initiation of an RNA chain. This tranformation in the presence of anthracyclines was studied in the following manner.

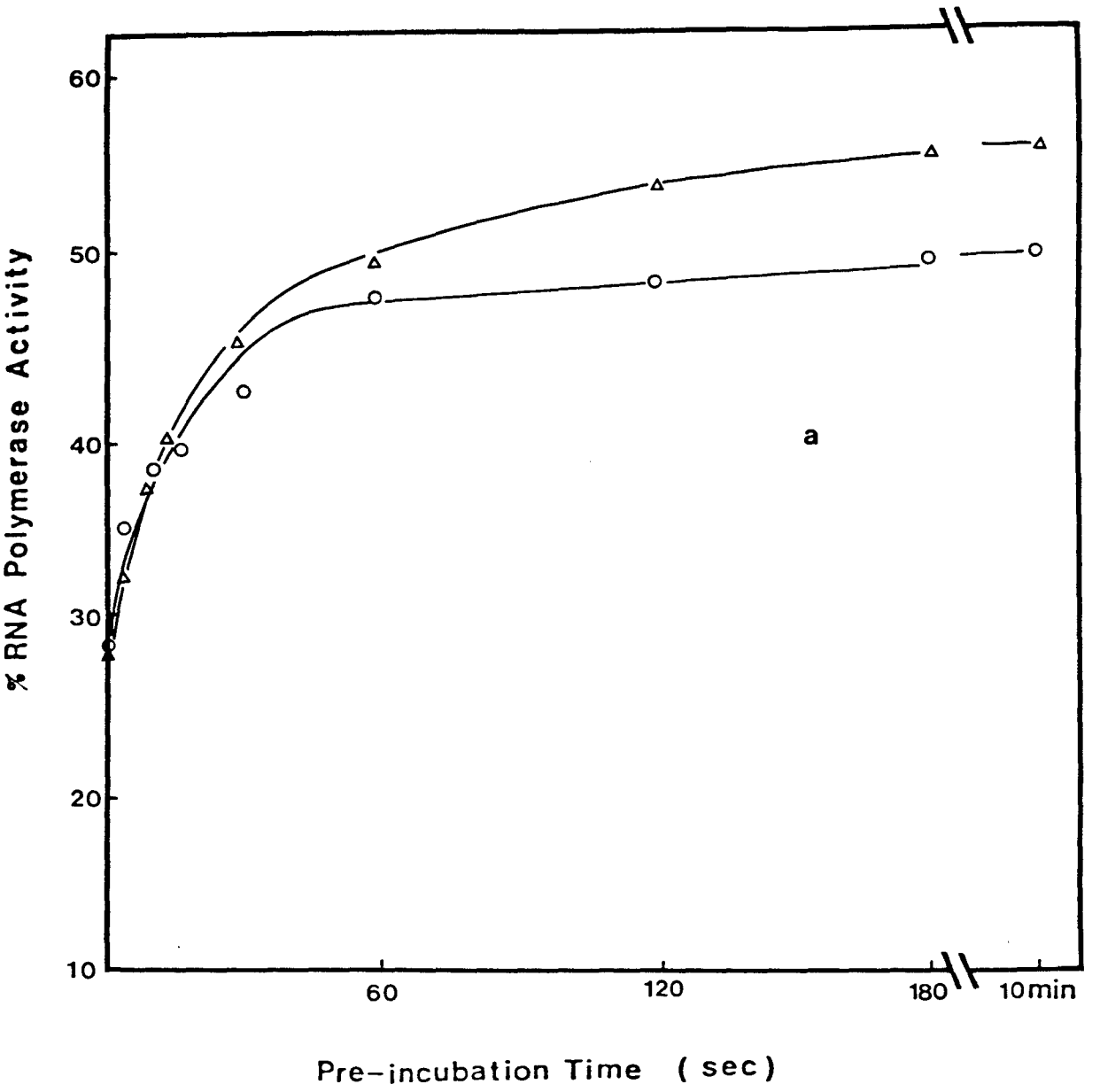
The effect of anthracycline analogs on the rate of I to RS complex transformation is shown in Figure 20 in which % RNA polymerase activity is plotted as a function of pre-incubation time of the I complex in the presence and absence of rifampicin. RNA polymerase % activity was determined by dividing the radioactivity recovered either in the presence of rifampicin or in the presence of both rifampicin and anthracyclines

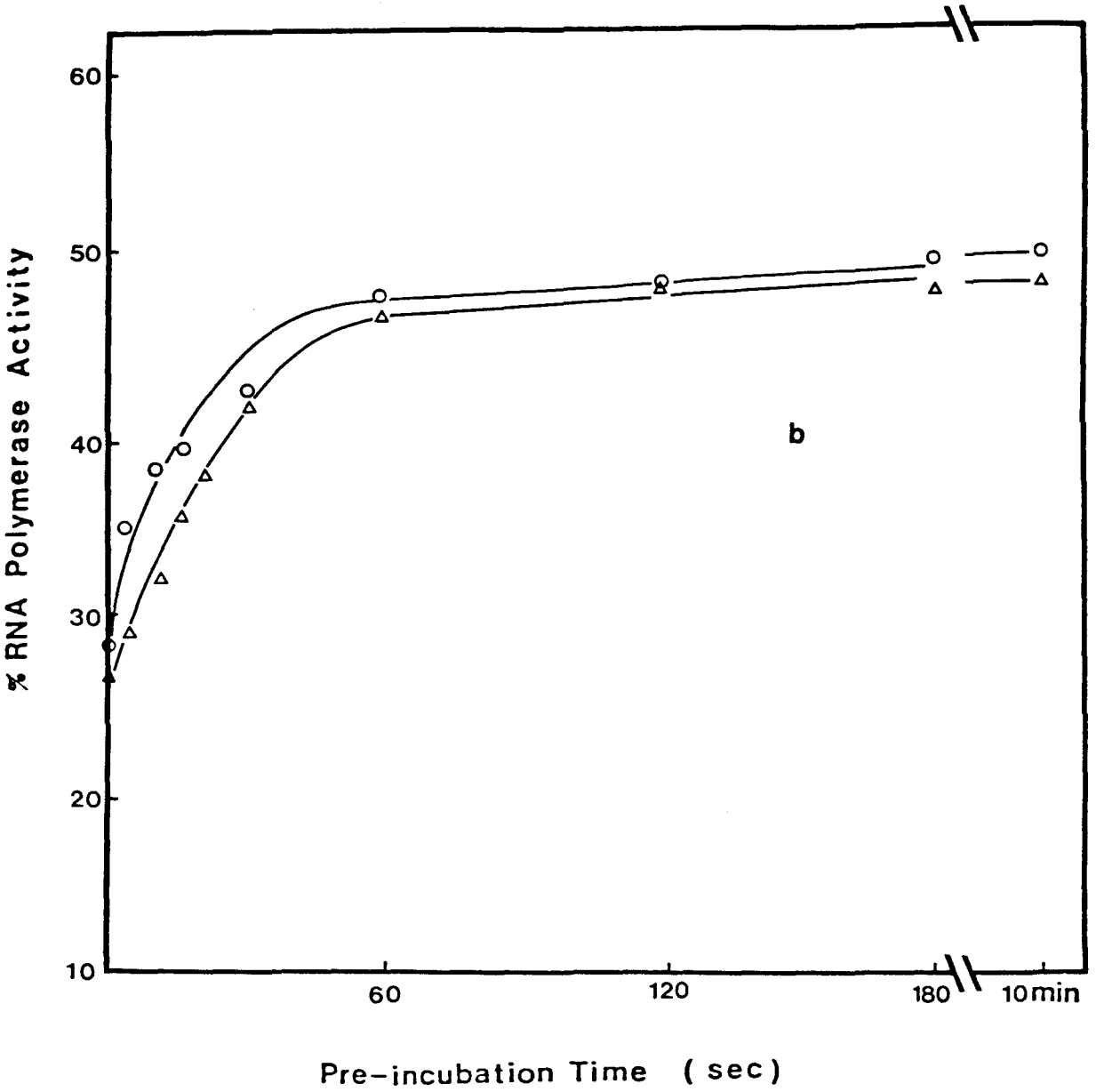
Figure 20. The Effect of Anthracycline Analogs on the Rate of Transformation of I to RS Complexes

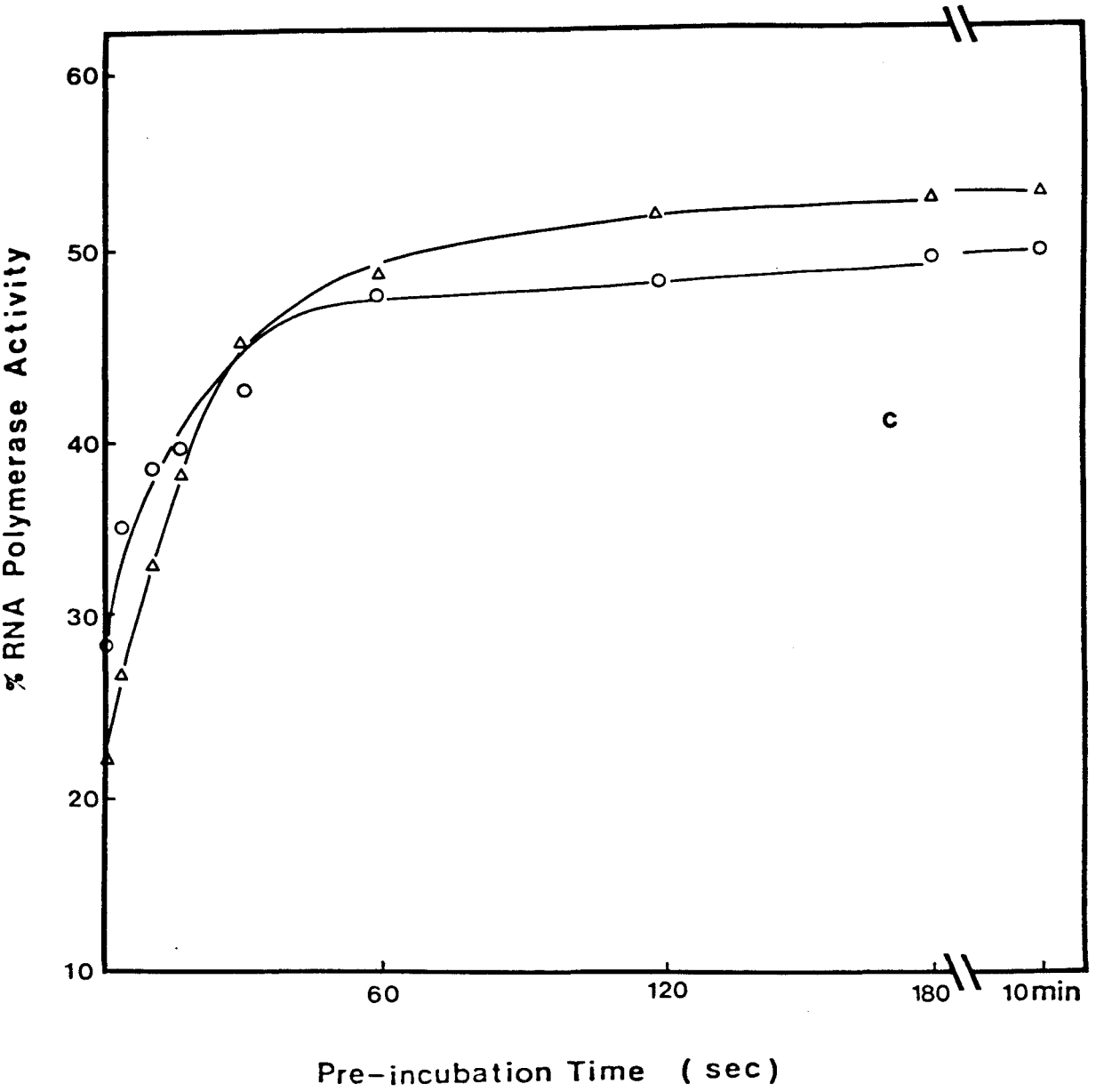
Binary Complexes between T7 DNA and RNA polymerase were formed at 0° C in the absence (O) and presence (Δ) of anthracyclines. The reaction mixture was brought up to 37° C for the indicated pre-incubation times before the addition of radioactive nucleotide substrate with or without rifampicin. The reaction was terminated 90 seconds later and the product was processed as described in Section II.4.3.

The results are expressed as % RNA polymerase activity. This is the ratio of RNA synthesized in the presence of rifampicin (0.156 μg/ml) only (O), or in the presence of both rifampicin and anthracycline (Δ), over that when no rifampicin is present.

a) In the presence of 13.3 μM of daunomycin; b) in the presence of 9.9 μM of 4-demethoxydaunomycin; c) in the presence of 5.1 μM of aclacinomycin.


% RNA Polymerase Activity
60
50
40
30
20
10
60
120
180
10min
a
Pre-incubation Time (sec)


60
50
40
30
20
10
% RNA Polymerase Activity
b
60
120
180
10min
Pre-incubation Time (sec)


60
50
40
30
20
10
% RNA Polymerase Activity
c
60
120
180
10min
Pre-incubation Time (sec)

with corresponding controls obtained in the absence of rifampicin.

I complex was formed by incubating RNA polymerase with T7 DNA at 0° C under low salt condition either in the presence or the absence of anthracyclines. Daunomycin and 4-demethoxydaunomycin were chosen to represent group I anthracyclines while aclacinomycin was chosen to represent group II anthracyclines.

As can be seen from Figure 20, for the first 60 seconds the amount of I complex which can be transformed into RS complex and escape the attack of rifampicin increases rapidly as the pre-incubation time increases. Beyond this initial surge, the increase of radioactivity incorporated slows down rapidly and reaches a plateau at around 3 minutes.

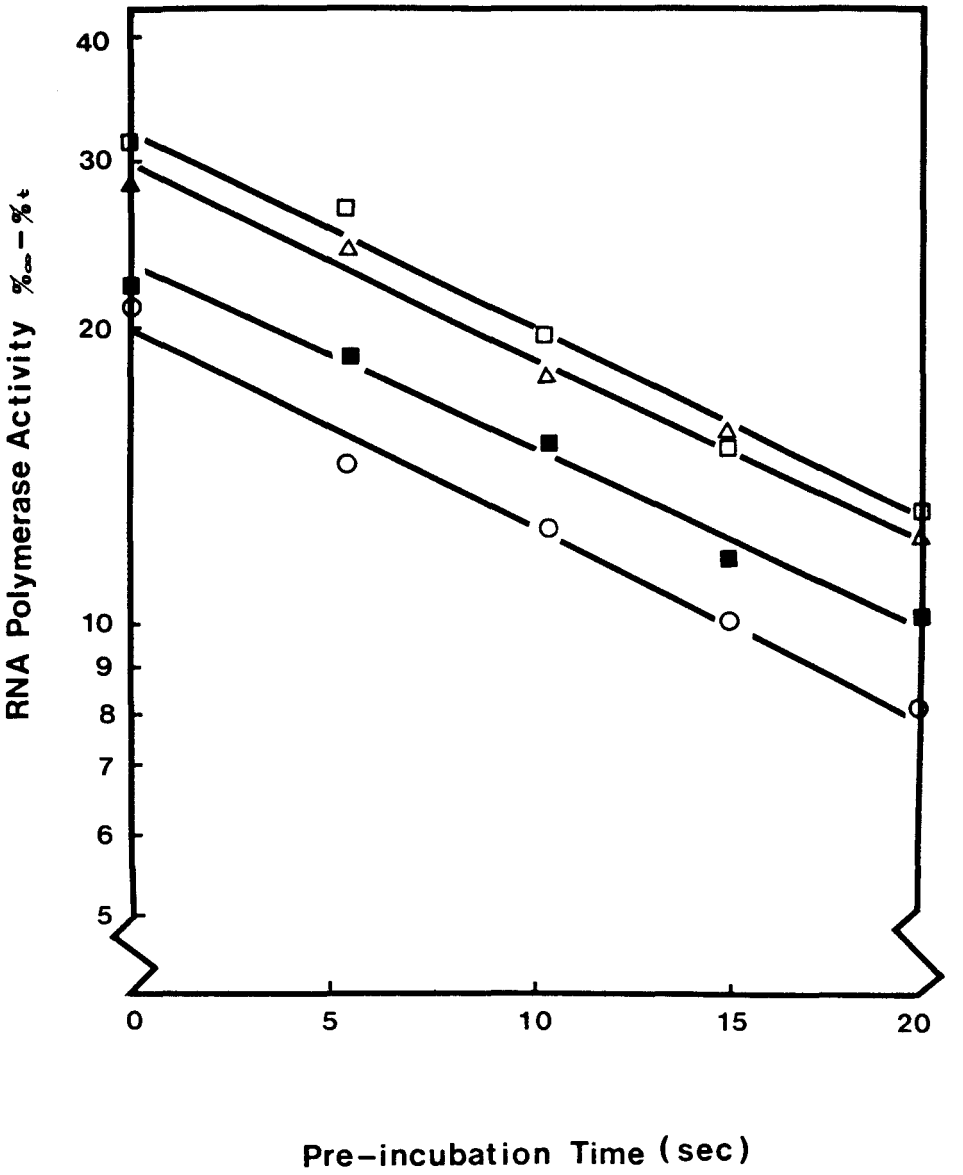
The half-life time for the I complex in the presence and absence of anthracyclines can be obtained by plotting the percent of RNA polymerase activity at infinite pre-incubation time (t_∞) minus the RNA polymerase activity at time (t) on a logarithmic scale against pre-incubation time. The results are presented in Figure 21 which shows similar half-lives for all the anthracyclines tested, indicating that anthracyclines exert no significant effect on the rate of I to RS complex transformation.

III.3.4.2.3. The Effect of Anthracyclines on the Transition Temperature of I to RS Complex Transformation

It has been reported that the equilibrium between the I and RS complexes is altered by temperature in a manner characteristic of a highly cooperative process (Mangel and Chamberlin, 1974a). An enthalpy of 57 Kcal per mole has been calculated for the formation of RS complex with T7 DNA and a transition temperature of 18° C has been determined.

Figure 21. Half-life of the Transformation of the I to RS Complex

Binary complexes between T7 DNA and RNA polymerase were formed at 0° C in the absence (O) and presence of 13.3 μM of daunomycin (Δ); 9.9 μM of 4-demethoxydaunomycin (■), and 5.1 μM of aclacinomycin(□) respectively. The reaction mixture was then brought to 37° C for the indicated pre-incubation times before initiation. The reaction was stopped 90 seconds later and the product was processed as described in Section II.4.3.

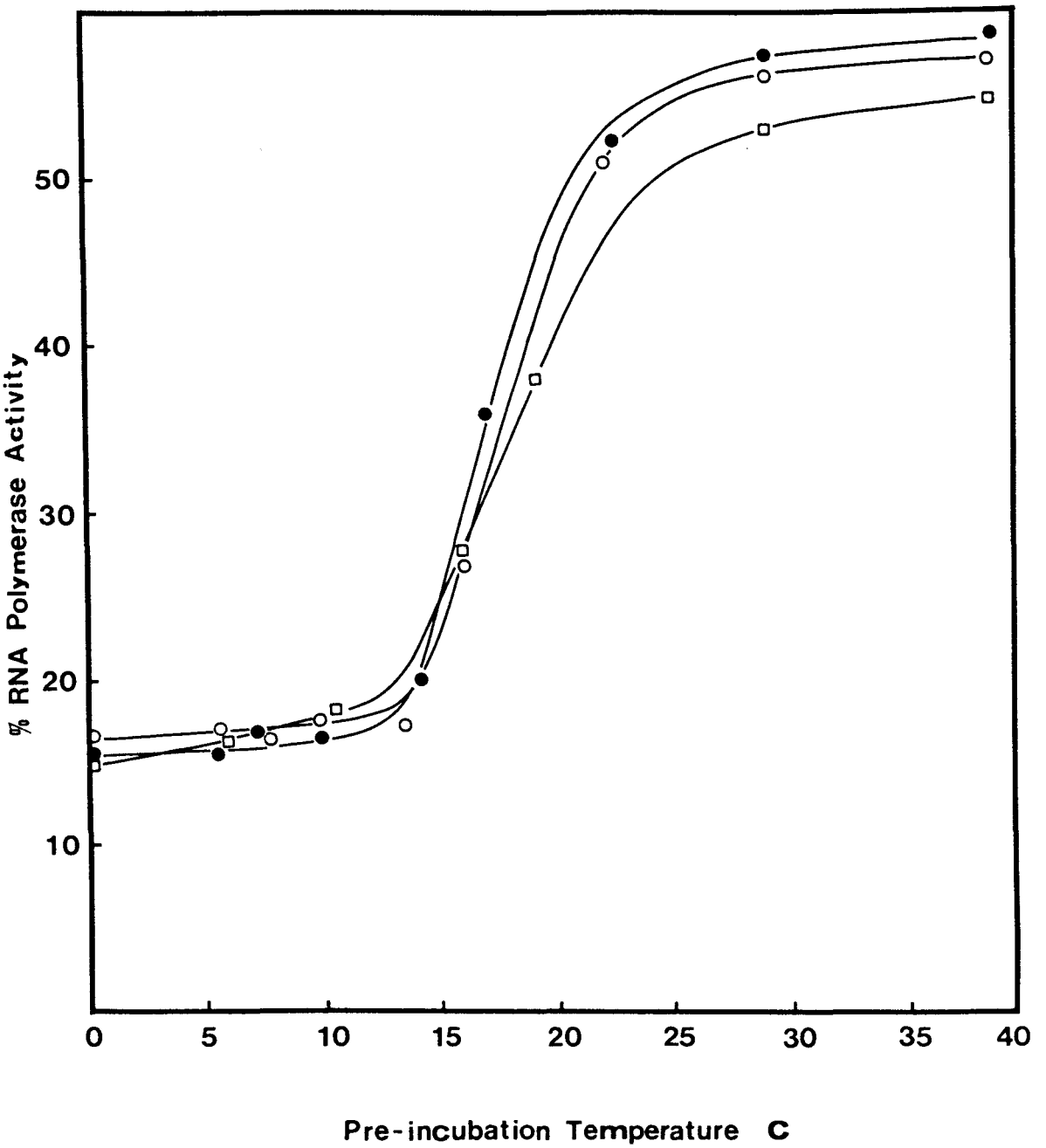

RNA Polymerase Activity $\%_\infty - \%_t$
40
30
20
10
9
8
7
6
5
0
5
10
15
20
Pre-incubation Time (sec)

If the rate of I to RS complex transformation is indeed not altered by the presence of anthracyclines as concluded from previous experiments, then the temperature dependency of the rate constant should not be affected by the drugs either. This temperature dependency could be studied by determining the transition temperatures of I to RS complex transformation in the presence of anthracycline analogs.

In this experiment, a constant amount of T7 DNA was mixed at 0° C with RNA polymerase in the presence and absence of daunomycin and aclacinomycin, representing group I and II anthracyclines respectively. The I complexes formed were then pre-incubated at various temperatures for 7 minutes before being challenged with rifampicin and substrates at 37° C. The results are presented in Figure 22 in which the % RNA polymerase activity in the presence and absence of each anthracycline is plotted vs pre-incubation temperatures. As expected, the transition temperature of approximately 18° C remains unchanged regardless of whether or not intercalators are present. This transition temperature is in close agreement with that reported previously by Mangel (Mangel and Chamberlin, 1974a). The nearly identical transition temperatures irregardless of the presence or absence of anthracyclines provides an independent confirmation of the previous conclusion that anthracyclines have little effect on I to RS complex transformation.

Figure 22. The Effect of Anthracycline Analogs on the Transition Temperature of I to RS Complex Transformation

Binary complexes were formed between T7 DNA and RNA polymerase at various temperatures in the absence or presence of anthracyclines representing each group. The reaction mixture was then incubated at the indicated temperatures for 7 minutes before initiation at 37° C. The results are expressed as % RNA polymerase activity which is the ratio of the amount of RNA synthesized in the absence of anthracycline (●) but in presence of rifampicin over that in the absence of both anthracycline and rifampicin. The same experiment was repeated in the presence of daunomycin (13.3 μM, ○) and aclacinomycin (5.1 μM, ■).


% RNA Polymerase Activity
10
20
30
40
50
0
5
10
15
20
25
30
35
40
Pre-incubation Temperature C

III.3.4.3. Comparison of the overall RNA synthesis inhibition and the inhibition of RNA chain initiation by anthracyclines

The inhibitory effectiveness of daunomycin and aclacinomycin towards RNA chain initiation was also examined by assaying the simultaneous incorporation of ^{32}P-ATP and ^{3}H-UTP (Section II.4.3.1.). The amount of γ-^{32}P-ATP incorporated indicates the number of RNA chains that can be initated in the presence of the drugs while the amount of 3H incorporated measures the rate of overall RNA synthesis. In Figure 23 the amount of RNA synthesized in terms of the RNA polymerase activity is plotted versus the added drug to DNA ratio for daunomycin and aclacinomycin.

It may be noted that the overall inhibition of RNA synthesis by daunomycin is much stronger than the inhibition of the number of RNA chains that can be initiated. Since γ-32P incorporation measures the events of RNA initiation up to the formation of the first phosphodiester bond, the difference seen between ^{32}P and ^{3}H incorporation can only be attributed to the subsequent inhibition of RNA chain elongation. Therefore at drug to DNA ratio of 0.175 at least 17% of the overall inhibition of RNA synthesis by daunomycin is due to the inhibitory effect of the drug on chain elongation. Aclacinomycin, on the other hand, does not affect chain elongation as indicated by the fact that the inhibition of the overall RNA synthesis coincides with the inhibition of the chain initiation.

The data obtained from the filter binding assay (which measures the amount of RNA polymerase-DNA complex formed in the presence of anthracyclines) and the data of ^{32}P and ^{3}H incorporation are expressed in terms of r_b (the number of drug molecules bound per DNA phosphate) a shown in

Figure 23. Inhibition of RNA synthesis by daunomycin and aclacinomycin assayed by the simultaneous incorporation of ^{32}P-ATP and ^{3}H-UTP

RNA polymerase and T7 DNA complexes were formed at 37° C in the presence of various amounts of daunomycin and aclacinomycin. RNA synthesis was then initiated by the rapid addition of nucleoside triphosphate mixture containing γ-^{32}P-ATP, CTP, GTP and 5,6-^{3}H-UTP (specific activities: 1 μCi/nmol and 0.02 μCi/nmol, respectively) at a concentration of 5 mM for each nucleoside triphosphate.

The amount of RNA synthesized in the presence of daunomycin in terms of ^{3}H (●) or ^{32}P (○) incorporation, or in the presence of aclacinomycin in terms of ^{3}H (▲) or ^{32}P (△) incorporation, is expressed as % RNA polymerase activity versus the added drug to DNA ratio.

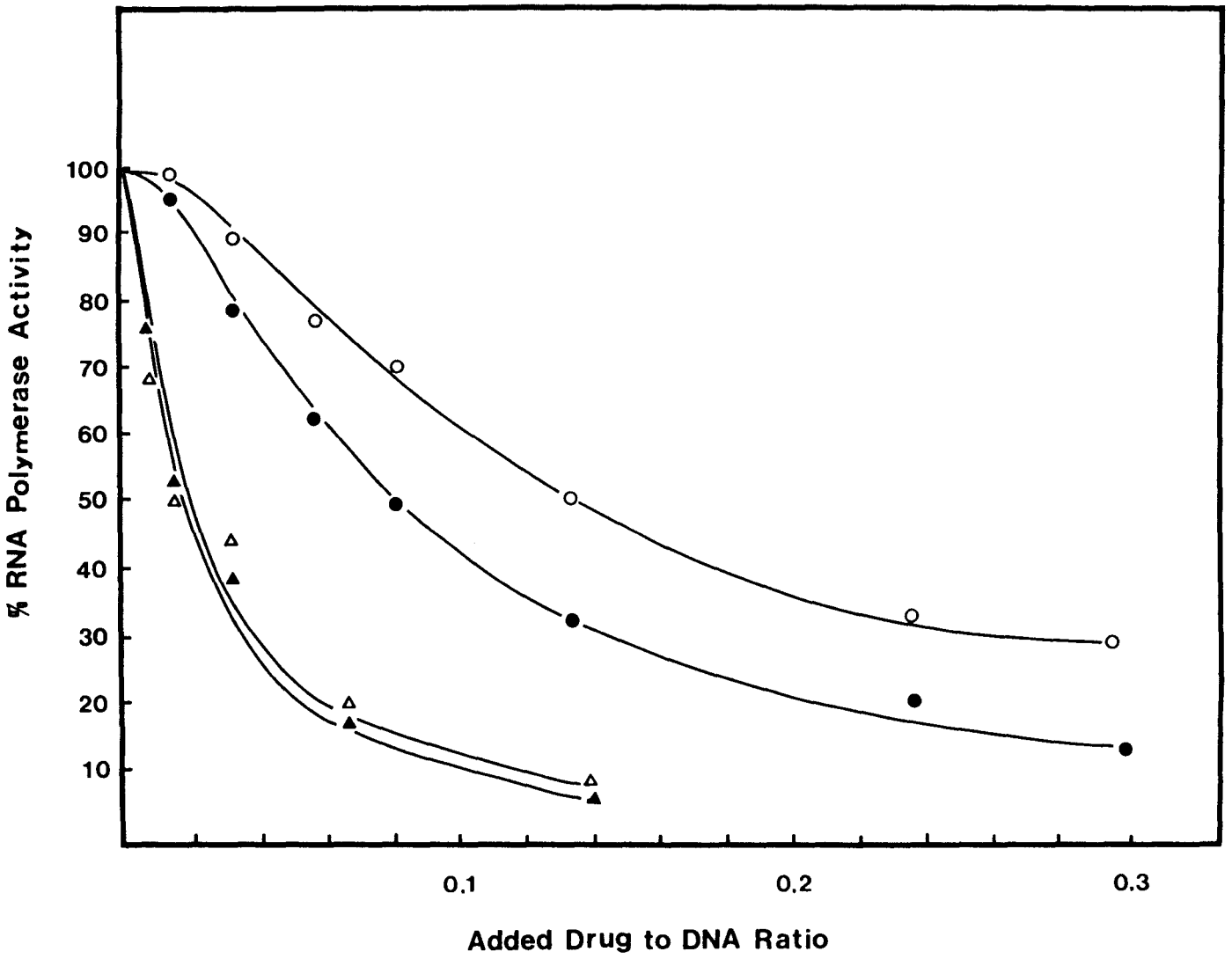
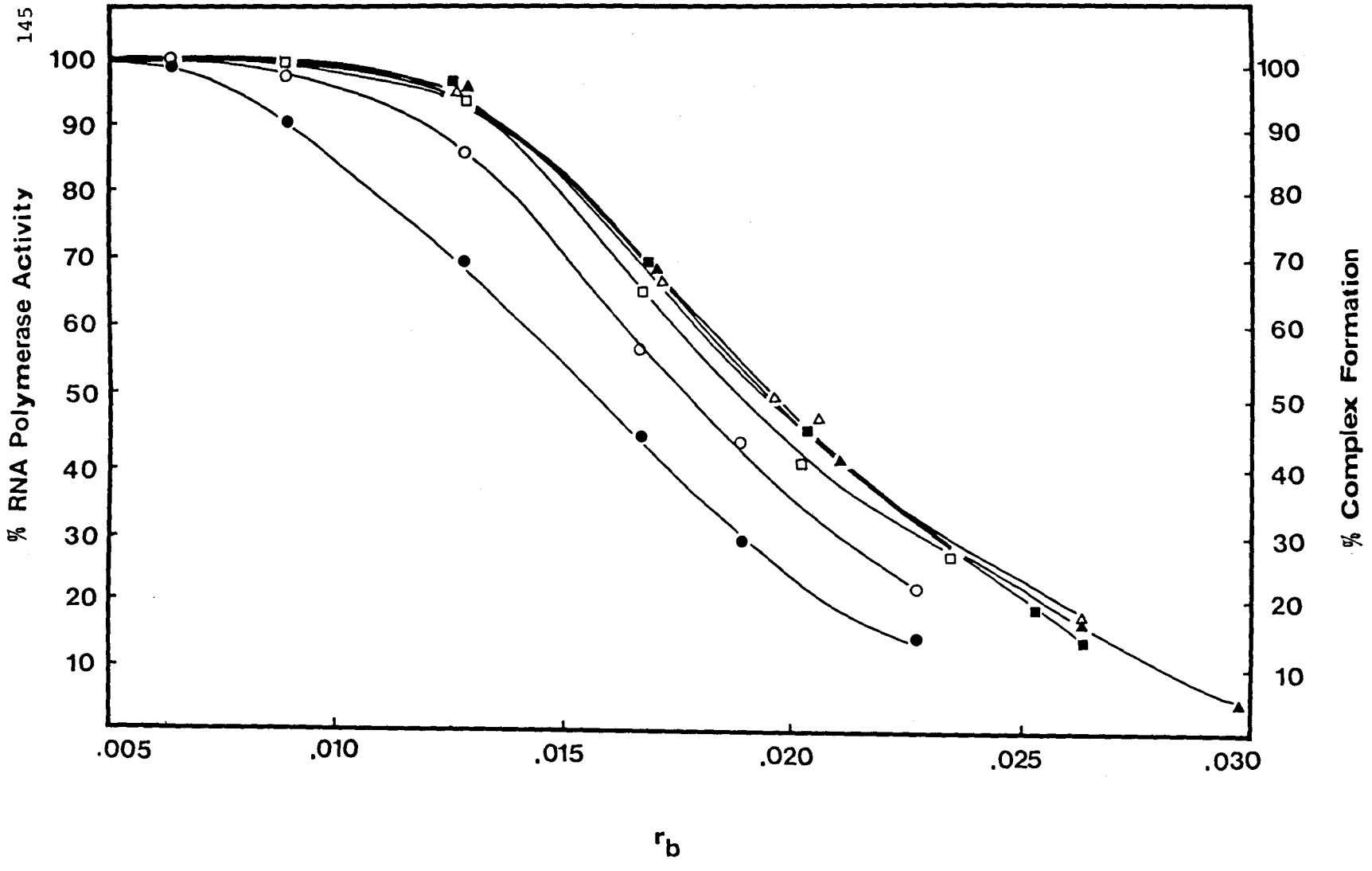

% RNA Polymerase Activity
100
90
80
70
60
50
40
30
20
10
0.1
0.2
0.3
Added Drug to DNA Ratio

Figure 24. These data indicate that under the influence of aclacinomycin the level of RNA polymerase-DNA complex formation, the level of overall RNA synthesis and the level of RNA chain initiation all closely follow each other. This however is not the case for daunomycin. It is clear that the inhibitory effect of aclacinomycin on RNA synthesis can be totally explained by its effect on the binding of the enzyme to DNA. No other step in the RNA synthesis process is affected by this drug. For daunomycin, the situation is more complicated. At an r_b of 0.015 a 13% difference between the inhibition of the formation of the complex and the inhibition of the RNA chains is noted. This effect is attributed to daunomycin inhibition of the RS to OP complex transformation, i.e., inhibition of the rate of the formation of the first phosphodiester bond. However, there is also an additional 17% difference between the inhibition of the overall RNA synthesis and the inhibition of the initiation of the RNA chains. This difference can only be attributed to the inhibition of RNA chain elongation.

The overall inhibition of RNA synthesis by daunomycin may be divided into two main categories; 1) the inhibition of enzyme-DNA complex formation and 2) the inhibition of the rate of RNA synthesis which includes the inhibition of RS to OP complex transformation and elongation. The relative amount of inhibition of the rate of RNA synthesis attributable to either the inhibition of RS to OP complex transformation or the inhibition of chain elongation is dependent on the concentration of daunomycin. At r_b = 0.009, 17% of the rate inhibition is due to the inhibition of RS to OP complex transformation while 83% is due to inhibition of elongation. At r_b = 0.013, 27% and 73% of rate inhibition is due to in-

Figure 24. Composite plot of % RNA polymerase activity and % of I complex formation in the presence of daunomycin and aclacinomycin

The % RNA polymerase activity in the presence of daunomycin in terms of ^{3}H (●) or ^{32}P (○) incorporation and the % of enzyme-DNA complex formation (□) are plotted versus r_b (the number of drug molecules bound per unit DNA). The same plots are also constructed for aclacinomycin in terms of ^{3}H (▲), ^{32}P (△) incorporation and the % of enzyme-DNA complex formation (■).


% RNA Polymerase Activity
% Complex Formation
100
90
80
70
60
50
40
30
20
10
.005
.010
.015
.020
.025
.030
r_b

hibition of RS to OP complex transformation and elongation, respectively. The inhibition of RS to OP complex transformation relative to the inhibition of elongation increases further at even higher r_b values (i.e., at r_b = 0.015 which causes 43% and 57% for inhibition of complex transformation and elongation, respectively). This observation indicates that at high daunomycin concentration, the inhibition of RS to OP complex transformation becomes more significant. A similar effect may have also been noted previously from the studies of Miracil D (Bodoni, 1978). In this report Miracil D was found to inhibit the initiation at high r_b values in addition to its inhibitory effect on elongation but at lower r_b values Miracil D was found to inhibit only elongation.

CHAPTER IV

DISCUSSION

The anthracycline analogs examined in this study can be divided into two groups according to their structural characteristics. The structural differences between anthracyclines in group I (daunomycin, 4-demethoxydaunomycin and carminomycin) and group II (aclacinomycin, marcellomycin and musettamycin) anthracyclines are shown in Figure 1 and are discussed in Section I.4. The major differences relate to the nature of the substituents at C-10 and C-13 of the aglycone and the nature of the glycosidic side chain. These structural differences have been shown in this study to influence not only the binding affinities of these drugs to DNA but also the mechanism by which they inhibit RNA synthesis in vitro.

RNA synthesis was studied at an RNA polymerase to DNA molar ratio of 12 which ensured that only the major promoter sites on DNA template were utilized (Figure 10, Section III.2.). Under this condition, both daunomycin and aclacinomycin were found to inhibit the in vitro DNA-dependent RNA polymerase activity competitively (Fig. 16, Section III.3.2.) at DNA concentration which corresponded to maximum incorporation of radioactive substrate (Fig. 12, Section III.2.). This competitive mode of inhibition of RNA polymerase by anthracyclines is consistent with the proposal (Section I.2.8.1.) that the inhibition of RNA polymerase activity by anthracyclines is primarily due to the inactivation of the DNA template. This conclusion is also supported by the observation that preincubation of RNA polymerase with the inhibitors has no effect on the

level of RNA synthesis (Section III.3.2.).

It has been proposed that the inhibition of RNA polymerase by anthracyclines results from intercalation (Section I.2.7.2.). However, the precise mechanism by which the intercalators inhibit the template activity of DNA remains unclear. In this investigation, it has been shown that all the anthracycline analogs examined reduce substantially the binding of RNA polymerase at the major promoter sites of T7 DNA (Fig. 17, Section III.3.4.1.). It is likely that RNA polymerase is prevented from binding to the template because the enzyme binding sites are already occupied by the intercalators, or due to steric hindrance by the molecules of the bound drug in the vicinity of the enzyme binding site.

There are substantial differences in the effectiveness of group I and II anthracyclines as inhibitors for the formation of the enzyme-template binary complex. Specifically, the drug to DNA ratios which causes 50% reduction in the formation of the enzyme-template binary complex are approximately 0.09 and 0.04 for group I and II anthracyclines respectively (Table IV, Section 3.4.1.), indicating that group II drugs are at least twice as effective in inhibiting the formation of the enzyme-template binary complex than group I drugs.

The differences in the binding abilities between group I and II anthracyclines to DNA have been attributed to several major structural differences between these drugs. Specifically, the additional sugar residues on the glycosidic side chain and the presence of a C-10 carbomethoxy substituent in group II anthracyclines increases the binding affinities of anthracyclines to DNA (Du Vernay et al., 1979a), while the presence of a substituted amino group on the primary sugar significantly

reduces binding (Du Vernay et al., 1979b). The enhanced binding of group II anthracyclines to DNA, in terms of both the association constant and the number of binding sites per unit DNA, is evident under the conditions employed in the present study (Table I, Section III.1.). It appears that both the carbomethoxy residue and the additional sugar residue of group II anthracyclines are involved in increasing the stability of the intercalated complex to an extent which compensates for the loss of binding due to the presence of substituted amino groups. It is likely that the carbomethoxy component and the additional sugar residues offer greater opportunity for additional hydrogen bonding and better Vander Waals interactions between the intercalators and the DNA template. However, group II anthracyclines also lack a carbonyl group at position C-13 which has been claimed to be involved in a hydrogen bonding with DNA to further stabilize the intercalated complex (Section I.2.7.3.). Lack of this hydrogen bond may seriously compromise the stability of the complex formed by group II anthracyclines. This may explain the observation that when the ability to bind DNA and prevent the formation of enzyme-DNA complex is compared between daunomycin (group I) and aclacinomycin (group II) in terms of r_b (the number of drug molecules bound per unit DNA), group I drugs become just as effective as group II drugs (Fig. 24, Section, III. 3.4.3.). This observation indicates that each DNA-bound molecule of daunomycin is just as effective in preventing the formation of complex as is each molecule of aclacinomycin. The difference between the two drugs seen in terms of their added drug to DNA ratio may be attributed to the fact that the number of binding sites for group II drugs are greater than that for group I drugs. Therefore, when the same concentra-

tion of daunomycin or aclacinomycin is added to the DNA more molecules of group II anthracyclines can bind to DNA and thus prevent the formation of enzyme-DNA complex to a higher extent. The differences in the binding constants of these anthracyclines to DNA seem to play a minor role in determining their effectiveness as inhibitors toward the formation of the enzyme-DNA complex.

Since the binding of RNA polymerase to DNA at major promoter sites leads to the formation of an enzyme-template binary complex which is a necessary for the subsequent events in specific RNA synthesis initiation (Section I.3.4.), it may be proposed that the reduction in the formation of this enzyme-template binary complex by anthracyclines accounts for the resulting inhibition of RNA synthesis. However, when the effectiveness of anthracyclines in reducing enzyme-template complex formation (Fig. 17 and Table IV, Section III.3.4.1.) is compared with the effectiveness of these drugs as inhibitors of RNA synthesis (Fig. 14 and Table II, Section III.1.), some interesting discrepancies become apparent. While it is clear that the extent of enzyme-template complex inhibition by group II anthracyclines parallels the extent of RNA synthesis inhibition, i.e., the drug to DNA ratio which causes 50% of inhibition in both the process of complex formation as well as RNA synthesis is similar for group II antibiotics (e.g., 0.033 for marcellomycin), this is not the case for group I anthracyclines. From the same comparison for group I drugs it becomes evident that a higher drug concentration is required (for example, 4-demethoxydaunomycin at a ratio of 0.058 prevents the synthesis of RNA by 50% whereas in order to prevent the formation of the binary enzyme-template complex to the same extent 4-demethoxydaunomycin

must be present at a much higher ratio, i.e., 0.085) so that the formation of binary complex can be inhibited to the same extent as the synthesis of RNA. In other words, RNA synthesis inhibition by group I anthracyclines can be explained only partially by the effect of these drugs on binary complex formation. Therefore the differential inhibitory capacity of the anthracyclines is not simply the result of their different affinities for DNA for group I anthracyclines. Other factors must also be involved in the mechanism of the inhibition of RNA synthesis.

The study of interactions between DNA and drugs is not a simple task because DNA contains multiple binding sites for drug molecules (Table I, Section III.1.). The structures of the drugs also influence the strength of the binding (Table I). Since anthracyclines inhibit the synthesis of RNA by reducing the availability of DNA template sites for the binding of RNA polymerase, the form of the drug in solutions which behaves as an inhibitor is the DNA-bound form. The appropriate means of expressing quantitatively the concentration of DNA-bound drug is in terms of the ratio of drug molecules bound per DNA nucleotide, (r_b) (Section III.3.3.). Since r_b does not depend linearly on the total drug concentration (Section III.3.3.), the availability of r_b values is essential for the comparative study of the inhibitory effectiveness of anthracycline analogs which have different affinities toward the DNA template.

When the dependence of RNA polymerase activity was determined as a function of r_b for each drug (Fig. 15, Section III.3.3.), a different picture for the relative effectiveness of the various anthracyclines studied became apparent. Specifically, group I anthracyclines are more effective inhibitors of RNA synthesis than group II drugs. These results

also indicate that identical numbers of bound inhibitor molecules per molecule of DNA have different inhibitory effects on the activity of RNA polymerase. Consequently, the inhibitory effectiveness of group I anthracyclines is determined not only by the affinity of each compound towards DNA, as in the case with group II anthracyclines, but also by the specific structures of the intercalated molecules.

What is the basis for the differential inhibitory effect on RNA synthesis between group I and II anthracyclines? An answer to this question can be found by comparing the rates of transformation of the binary enzyme-DNA complex during the events leading to the initiation of RNA synthesis in the presence of group I and II inhibitors.

E. coli RNA polymerase molecules bind to T7 DNA at or near promoter sites to form I complex (Section I.3.4.). I complexes are then transformed through an energy requiring step involving the separation of the double helical strands of DNA to form RS complexes. In the presence of nucleoside triphosphate substrates, the RS complexes can be transformed to OP complexes with the formation of the first phosphodiester bond. The rates of I to RS and RS to OP transformation can be measured (Section III.3.4.2.).

In the present study the effect of anthracyclines on the rates of these transformations was examined. The results clearly indicate that in the presence of group II anthracyclines, the number of RS complexes is reduced (Fig. 17, Section III.3.4.1.) but the rate of RS to OP complex transformation is not affected (Fig. 19, Section III.3.4.2.1.). The reduced number of RS complex could be the consequence of either a reduced number of I complexes or a reduced rate of I to RS transformation, or it

may be the result of both. Since I complexes in the process of their transformation to RS complexes are not affected by the presence of group II anthracyclines (Fig. 20-22, Section III.3.4.2.2.), the only explanation for the reduced number of RS complexes that can be offered is that the number of I complexes is substantially reduced by group II anthracyclines. This observation leads to the conclusion that inhibition of RNA synthesis by group II anthracyclines is entirely due to the effect of these drugs in preventing the formation of enzyme-template binary complex. This conclusion is also consistent with the findings (Fig. 24, Section III.3.4.3.) that the extent of enzyme-DNA complex inhibition, the extent of inhibition of RNA initiation (RS to OP complex transformation) and the extent of inhibition of overall RNA synthesis are all identical in the presence of aclacinomycin (a group II anthracycline).

On the contrary, group I anthracyclines reduce the number of RS complexes (Fig. 17, Section III.3.4.1.) as well as the rate of RS to OP transformation (Fig. 19, Section III.3.4.2.1.) but they do not affect the rate of I to RS transformation (Fig. 20-22, Section III.3.4.2.2-3.). By the same analogy described above for group II drugs, it seems that group I anthracyclines also reduce the number of I complexes. In addition, however, once I complexes have proceeded to become RS complexes, the subsequent RS to OP transformation is also affected by group I anthracyclines, probably due to the interference of these drugs with the formation of the first phosphodiester bond. (At this point it should be noted that the effect of daunomycin on RS to OP complex transformation is not an artifact due to its inhibition on chain elongation. Miracil D, which has been shown to be a chain elongation inhibitor at low drug concentra-

tion, has no effect on the rate of RS to OP transformation when measured in an identical assay utilizing rifampicin (data not shown).

Finally, a comparison of the rates of initiation of RNA chains and overall RNA synthesis (Fig. 23, Section III.3.4.3.) shows that daunomycin (group I anthracycline) also inhibits the elongation of RNA chains. Furthermore, the relative amount of RNA synthesis inhibition by daunomycin due to either the inhibition of the RS to OP complex transformation or of the elongation of RNA chains is dependent on the concentration of the drug (Section III.3.4.3.). At high drug concentration the inhibition of RS to OP transformation becomes more significant. These additional steps of inhibition explain the observation cited previously that inhibition of the enzyme-template complex by group I anthracyclines can only partially account for the inhibition of RNA synthesis.

Numerous attempts have been made (Section I.4.) to establish a relationship between the structures of anthracycline analogs and their properties. It is quite apparent from the results of this study that the structural differences between group I and II anthracyclines contribute to the differences in the tendencies of the members of the two groups to bind DNA. The latter property directly affects the effectiveness of anthracyclines as inhibitors of RNA synthesis. More specifically, the structural differences among these drugs appears to affect the mechanism of initiation of RNA synthesis, i.e., the various members of group I anthracyclines affect RS to OP transformation and elongation, while group II anthracyclines do not.

It has been established that the amino group on the sugar residue of daunomycin is positively charged at neutral pH. This charged amino

group plays an important role in the formation of a stable intercalated complex between daunomycin and DNA (Di Marco. et al., 1971) by having electrostatic interaction with the negatively charged phosphate backbone of DNA. Alteration of this amino group (either by elimination or substitution) may result in a significant reduction of intercalation. It has also been proposed that at very high drug concentration, this positively charged amino group is responsible for the secondary binding of the drug with the phosphate backbone of the DNA helix. In this case, drug molecules, rather than intercalating into DNA, are located on the outside of the double helix (Section I.2.7.2.). This secondary binding takes place presumably after the intercalation sites have been saturated at high drug concentration.

In the present study, the potential for secondary binding for group I anthracyclines is evident from the Scatchard plots (Fig. 9a, Section III.1.) at very high drug concentrations. This finding is consistent with the presence of an unsubstituted amino group on the primary sugar residue. Group II anthracyclines carry a substituted amino group and do not form secondary binding as is evident from the linearity of the corresponding Scatchard plots (Fig. 9b, Section III.1.). However, since the assays for the inhibition of RNA synthesis by anthracyclines were carried out at drug concentrations far below (below r_b = 0.035, Fig. 15) the level at which secondary binding takes place (above r_b = 0.05, Fig. 9a), any differences seen between the mechanisms of RNA synthesis (e.g. RS to OP transformation) by group I and group II drugs cannot be attributed to secondary binding.

A possible cause for the additional step of inhibition of RS to OP

transformation by group I drugs then, is the presence of electrostatic interaction between their amino groups and the phosphate backbone of DNA in the intercalated complex. This electrostatic interaction may impair the catalytic function of the RNA polymerase by interfering at the catalytic site in a manner which inhibits the formation of the first phosphodiester bond. The same electrostatic interaction within the intercalated complex may also retard the rate of elongation.

In contrast, group II anthracyclines, which do not carry a positively charged amino group and are therefore unable to interact electrostatically with DNA phosphate, apparently interfere only with the formation of I complexes and have no effect on the formation of the first phosphodiester bond.

The results presented in this dissertation suggests that the unsubstituted amino group on the primary sugar residue of anthracyclines may play an imporant role in determining the mechanism by which anthracyclines inhibit RNA synthesis. Both group I and group II anthracyclines inhibit RNA synthesis by interfering with the formation of a specific template-RNA polymerase binary complex (I complex). However, in addition to inhibiting the formation of the I complex, group I anthracyclines interfere with the transformation of the rapidly starting complex (RS complex) to the OP complex, (i.e., a complex containing the first phosphodiester bond of the nacent RNA chain) and with the elongation of RNA chains.

CHAPTER V

SUMMARY

The in vitro synthesis of RNA catalyzed by E. coli DNA-dependent RNA polymerase using T7 DNA as template is inhibited by anthracycline analogs.

The initiation step of RNA synthesis is a multi-step process involving 1) the binding of the enzyme molecules to the non-specific binding sites on the template, 2) the binding of the enzyme molecules to major promoters to form I complex, 3) the melting of the double-stranded DNA at enzyme binding site to form RS complex and 4) the formation of the first phosphodiester bond.

Anthracyclines apparently inhibit RNA synthesis by intercalating into DNA double helix and interfering with template function. In the present study two groups of anthracyclines which differ in the substituents at C-10 of the aglycone and the amino group of the primary sugar moiety, were examined to determine their relative effectiveness in binding to DNA and in inhibiting the synthesis of RNA.

When identical amounts of anthracycline from each group are added to DNA, group II anthracyclines (which carry a carbomethoxy group at C-10 and a substituted amino group on the sugar moiety) inhibit overall RNA synthesis to a greater extent than group I drugs. This higher inhibitory effectiveness of group II anthracyclines towards overall RNA synthesis is the result of their better binding for DNA. However, when comparisons are made with the same number of molecules from each group of anthracyclines bound to DNA, group I anthracyclines show a higher effectiveness as inhi-

bitors of RNA synthesis. This enhanced inhibitor activity is attributed to a distinctive feature in the mechanism by which group I anthracyclines inhibit RNA synthesis. Group I anthracyclines reduce not only the number of I complexes that can be formed, but also the rate of transformation of these complexes from RS to OP form as well as the rate of RNA chain elongation. In contrast, group II anthracyclines act only by limiting the number of I complexes.

In conclusion, it appears that group I anthracyclines inhibit the formation of I complex through intercalation and the rate of RS to OP transformation and RNA chain elongation through electrostatic interactions within the intercalated complex, thus acting as multiple inhibitors. In contrast, group II anthracyclines, which carry substituted amino groups, and are thus unable to form similar ionic interactions, inhibit, as a result of intercalation, only the formation of I complex.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the ewquirements for the degree of Doctor of Philosophy.

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