Studies on the Mechanism of Inhibition of RNA Synthesis by Template Inactivators

Nikos Panayotatos  
*Loyola University Chicago*

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STUDIES ON THE MECHANISM OF INHIBITION OF
RNA SYNTHESIS BY TEMPLATE INACTIVATORS

by
Nikos Panayotatos

A Dissertation Submitted to the Faculty
of the Graduate School
of Loyola University in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

February
1977
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I also wish to express my gratitude to my wife Kiki for her solidarity which only made this work possible.

Finally, thanks are extended to all my colleagues and especially to Laszlo Bodoni and Antonis Kindelis for their excellent companionship during the course of this work.
Nikos Panayotatos was born in Athens, Greece. In 1969 he received his Diploma in Chemistry from the University of Athens.

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TABLE OF CONTENTS

ACKNOWLEDGMENTS........................................... ii
LIFE.................................................................... iii
LIST OF TABLES............................................... vii
LIST OF ILLUSTRATIONS..................................... viii
LIST OF ABBREVIATIONS..................................... ix
CONTENTS OF THE APPENDIX............................... x
INTRODUCTION................................................ 1

1. The Pharmacological and Biological Properties of Ethidium Bromide and its Structural Analogs.......................... 3

2. The Structure and Properties of the DNA-Drug Complex.......................... 5

3. The Mechanism of Transcription.......................... 11

4. The Mechanism of Inhibition of RNA Synthesis Through Template Inactivation....... 21

MATERIALS AND METHODS.................................. 25

1. T₂ DNA Preparation...................................... 25

2. The Enzyme.............................................. 26

3. The Inhibitors.......................................... 27

4. Determination of Binding Parameters.................... 28

5. Enzymatic Activity Assays............................... 31
   a. Experiments Not Involving Rifampicin.............. 32
   b. Experiments Involving Rifampicin............... 34
RESULTS

1. The Binding of Phenanthridines to DNA ............ 37

2. The Effect of Template Concentration on Inhibition .................................. 43

3. The Effect of Drug Concentration on Enzymatic Activity ............................. 48

4. The Effect of DNA-Bound Inhibitor on RNA Synthesis ................................ 55

5. Ethidium Bromide Analogs Limit the Number of RNA Molecules that can be Initiated without Interfering Significantly with the Elongation of Initiated Chains .... 61

6. The Effect of Ethidium Bromide and DEMB on the Steps of RNA Chain Initiation .... 70
   a. Ethidium Bromide and DEMB do not Affect the Rate of the Second Initiation Step .... 71
   b. The Rate of RNA Polymerase Molecules Traversing the First Initiation Step is not Affected by the Presence of either Ethidium Bromide or DEMB .......... 76
   c. Evaluation of the Previous Results .......... 82

7. The Kinetics of Template Inactivation .......... 85
   a. Inhibition by Actinomycin D .......... 89
   b. Inhibition by DEMB .......... 92

   a. Assaying RNA Polymerase in the Presence of a Constant Ratio of Inhibitor Added per DNA Nucleotide .......... 99
   b. Assaying RNA Polymerase in the Presence of a Constant Amount of Inhibitor .......... 104
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Chemical Structure and Properties of Some Phenanthridinium Derivatives</td>
<td>39</td>
</tr>
<tr>
<td>2.</td>
<td>Comparison of the Pharmacologic and Inhibitory Effectiveness of Some Phenanthridines</td>
<td>53</td>
</tr>
<tr>
<td>3.</td>
<td>Comparison of the Inhibitory Effectiveness of the Template-Bound Forms of the Various Inhibitors</td>
<td>59</td>
</tr>
<tr>
<td>4.</td>
<td>Intercepts of Double Reciprocal Plots</td>
<td>93</td>
</tr>
</tbody>
</table>
## LIST OF ILLUSTRATIONS

### Scheme

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. The Mechanism of RNA Synthesis</td>
<td>19</td>
</tr>
</tbody>
</table>

### Figure

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Scatchard Plots for Some Phenanthridines</td>
<td>40</td>
</tr>
<tr>
<td>2. The Effect of Template Concentration on the Rate of RNA Synthesis</td>
<td>44</td>
</tr>
<tr>
<td>3. The Effect of Phenanthridine Concentration on the Rate of RNA Synthesis Relative to the Uninhibited Reaction</td>
<td>49</td>
</tr>
<tr>
<td>4. The Dependence of the Rate of RNA Synthesis on the Template-Bound Form of the Inhibitor</td>
<td>57</td>
</tr>
<tr>
<td>5. Inhibition of the Rate of Total RNA Synthesis and the Rate of RNA Chain Initiation</td>
<td>63</td>
</tr>
<tr>
<td>6. The Dependence of the Relative Rate of RNA Synthesis on Rifampicin Concentration</td>
<td>73</td>
</tr>
<tr>
<td>7. The Rate of RNA Synthesis as a Function of Pre-Incubation Time</td>
<td>77</td>
</tr>
<tr>
<td>8. Double Reciprocal Plots of the Dependence of the Rate of RNA Synthesis on the Concentration of the Template</td>
<td>90</td>
</tr>
<tr>
<td>9. A Double Reciprocal Plot of the Rate of RNA Synthesis on the Ratio of DEMB Bound Per DNA Nucleotide</td>
<td>95</td>
</tr>
<tr>
<td>10. Double Reciprocal Plots of the Rate of RNA Synthesis as a Function of Template Concentration in the Absence of Inhibitor and in the Presence of a Constant Inhibitor-to-DNA Ratio</td>
<td>102</td>
</tr>
<tr>
<td>11. Double Reciprocal Plots of the Rate of RNA Synthesis as a Function of Template Concentration in the Absence of Inhibitor and in the Presence of a Constant Amount of Inhibitor</td>
<td>105</td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>Tris-(hydroxymethyl)aminomethane chloride</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>ATP, GTP, CTP, UTP</td>
<td>The Adenosine, Guanosine, Cytidine and Uridine triphosphates, respectively</td>
</tr>
<tr>
<td>XTP</td>
<td>An equimolar mixture of the above triphosphates</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>EB, DEMB, DMEB, MAPEC, MMPB, DMNC</td>
<td>Structural analogs of ethidium bromide (see Table 1)</td>
</tr>
</tbody>
</table>

**Binding Buffer**

<table>
<thead>
<tr>
<th>Buffer components</th>
<th>Concentration</th>
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</thead>
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<tr>
<td>0.2 M KCl</td>
<td>0.04 M Tris-HCl, 0.01 M MgCl₂, 0.0001 M EDTA, 0.00032 M K2HPO4, pH 7.9 at 25°C</td>
</tr>
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**Buffer A**

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<th>Buffer components</th>
<th>Concentration</th>
</tr>
</thead>
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<tr>
<td>0.2 M KCl</td>
<td>0.04 M Tris-HCl, 0.01 M MgCl₂, 0.0001 M EDTA, 0.00032 M K2HPO4, 0.0001 M dithiothreitol, 0.4 mg/ml BSA, pH 7.9 at 25°C</td>
</tr>
</tbody>
</table>

**Buffer B**

<table>
<thead>
<tr>
<th>Buffer components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04 M Tris-HCl</td>
<td>0.02 M MgCl₂, 0.0001 M EDTA, 0.00032 M K2HPO4, 0.0001 M dithiothreitol, 0.8 mg/ml BSA, pH 7.9 at 25°C</td>
</tr>
</tbody>
</table>
CONTENTS OF THE APPENDIX

| I. Explanation of Symbols                          | 139 |
| II. The Relationship Between (S) and (S₀)          | 139 |
| III. Kinetics of Template Inactivation             | 140 |
| a. Kinetic Scheme                                  | 140 |
| b. Assumptions                                     | 140 |
| c. Equations                                       | 141 |
| d. Derivations                                     | 141 |
|   1. Binding Site Inactivation                      | 143 |
|   2. "Rate" Inhibition                             | 144 |
|   3. Mixed Binding Site Inactivation                | 145 |
|   4. Mixed Inhibition                              | 147 |
| IV. Data for Some Figures                           | 149 |
| a. Data for Figure 1                                | 149 |
| b. Data for Figure 2                                | 150 |
| c. Data for Figure 3a                               | 151 |
| d. Data for Figure 3b                               | 152 |
| e. Data for Figure 5                                | 153 |
| f. Data for Figure 8                                | 156 |
INTRODUCTION

An early step in the control of gene replication and expression is exerted through the template functions of the genome. Synthesis of RNA, in particular, appears to be controlled by cell constituents that can repress or enhance gene expression by associating with specific sites of the DNA molecule. Reduction or enhancement of transcription can also be induced artificially through the introduction of chemical agents that can interact with and alter the template capacity of the DNA molecule for nucleic acid synthesis. One such agent is ethidium bromide, a drug belonging to the family of phenanthridines, compounds characterized by the presence of a phenanthridinium ring system in the molecule. Ethidium bromide is believed to owe its pharmacological and biological activity, at least in part, to its ability to reach and bind to the DNA molecules of the cell in vivo (Waring, 1972). In vitro, ethidium bromide has been shown to bind to DNA as well and inhibit RNA synthesis as a result of this interaction (Waring, 1965). The formation of the DNA-ethidium bromide complex is accompanied by characteristic changes in the physicochemical properties of both constituents of the complex, a fact that has facilitated the study of their interaction. As a result, the DNA-ethidium bromide complex
has been the subject of numerous studies. The inhibitory effectiveness of ethidium bromide against enzymes utilizing DNA as either substrate or template has also been studied.

Despite considerable accumulation of knowledge regarding the biological properties of ethidium bromide, very little is known about the interaction of other phenanthridines with nucleic acids although the pharmacological activity of many of these structural analogs of ethidium bromide has been extensively tested. Very recently the optical and hydrodynamic properties of some phenanthridine-DNA complexes have been studied and were found to be similar to the properties of the parent compound (Waring, 1974; Aktipis and Kindelis, 1973a,b). The inhibitory effectiveness of these compounds against DNA-dependent DNA polymerase I and pancreatic deoxyribonuclease I is also comparable with that of ethidium bromide (Aktipis and Kindelis, 1974).

The subject of the present study is the mechanism of inhibition of RNA synthesis by structural analogs of ethidium bromide. Information pertinent to the factors and parameters governing RNA synthesis, as well as the mechanism of the reaction, is presented below.
1. The Pharmacological and Biological Properties of Ethidium Bromide and its Structural Analogs

The pharmacological activity of a large number of phenanthridines has been extensively investigated. The early discovery of antitrypanosomal action (Brownlee et al., 1950) was soon followed by reports on the antibacterial (Seaman and Woodbine, 1954) and antiviral (Dickinson et al., 1953) activities of these drugs and later on the antileukemic activity of ethidium bromide on experimental animals (Gosse et al., 1974). Apart from its pharmacological action ethidium bromide has been shown to exert an effect on a variety of biological functions as well (Waring, 1972). At the cellular level ethidium bromide has been found to induce petite mutation in yeast (Slonimski et al., 1968) to bind cooperatively to mitochondrial membranes (Azzi and Santato, 1971) and to eliminate plasmids from the bacterial cytoplasm (Bouanchaud et al., 1968). At the molecular level ethidium bromide has been reported to inhibit mitochondrial RNA synthesis (Zylber and Penman, 1969) and mitochondrial DNA replication in yeast (Nagley et al., 1973) while, in duck cells infected by avian sarcoma virus, ethidium bromide inhibits the appearance of closed circular viral DNA (Guntake et al., 1975). The apparent selective inhibition of exonuclear DNA's by ethidium bromide is thought to reside in the higher affinity of the drug for supercoiled DNA molecules (Waring, 1972; Hixon et al., 1975).
In vitro studies have revealed that ethidium bromide is a strong inhibitor of DNA polymerase I (Elliott, 1963; Mayer and Simpson, 1969), deoxyribonuclease I (Eron and McAuslan, 1966), RNA polymerase (Waring, 1965) and reverse transcriptase (Müller et al., 1971).

Structural analogs of ethidium bromide examined for in vitro activity on DNA polymerase I and pancreatic deoxyribonuclease were found to be strong inhibitors of both enzymes (Aktipis and Kindelis, 1974).
2. The Structure and Properties of the DNA-Drug Complex

In 1964, Fuller and Waring assigned a tentative structure to the complex of DNA with ethidium bromide based on evidence obtained through fiber x-ray diffraction studies and molecular model building. In that report, it was postulated that the planar part of the ethidium bromide molecule, i.e., the phenanthridinium ring is inserted (Table 1), in a parallel fashion to the two adjacent base pairs of double stranded DNA; i.e., present in an intercalation site. Insertion of the phenanthridinium ring system between adjacent base pairs was postulated to occur in such a fashion so as to bring the two amino groups at positions 3 and 8 of the molecule (Table 1) within bonding distance from the two phosphate groups located opposite each other on the two DNA strands. As a consequence, the intercalated molecule should be stabilized by hydrophobic forces, including \( \pi-\pi \) interactions between ethidium bromide and the neighboring bases and both hydrogen bonding and electrostatic forces between the amino groups on the drug molecule and the phosphate groups of the DNA. In order to accommodate the intercalated ethidium bromide molecule, the two adjacent base pairs had to be translocated relative to each other, resulting in a local unwinding of the double helix. The unwinding angle, i.e., the reduction of the 36° helical
rotation between two successive base pairs due to the insertion of one ethidium bromide molecule, was found to be $12^\circ$ at the point of intercalation.

The proposed model could account not only for most of the experimental evidence available at that time, but also permitted some predictions to be formulated about the properties of the complex, which have since been substantiated by experiment. Insertion of the phenthridinium ring between adjacent base pairs should stabilize the double helix as well as unwind it locally resulting in an overall length increase of the DNA molecule. Temperature transition profiles and electron microscopy in the presence and absence of ethidium bromide have verified these predictions (Waring, 1966; Freifelder, 1971). The presence of numerous ethidium bromide molecules intercalated on the DNA helix has also been found to cause an increase in the intrinsic viscosity of the macromolecule that has been attributed to the increase in the length of DNA (Douthard et al., 1973) and a decrease in the sedimentation coefficient due to the decrease in the length-specific mass of the complex relative to the uncomplexed DNA molecule (Douthard et al., 1973). The sedimentation coefficient of supercoiled double-stranded circular DNA, on the other hand, is strongly affected by increasing concentrations of bound ethidium bromide (Crawford and Waring, 1967). This behaviour has been attributed to the fact that the sedimentation coefficient of supercoiled DNA
molecules is largely determined by the number of superhelical turns in the molecule (τ). τ is in turn connected with the ratio of the number of base pairs (N) over the double helix pitch (p) by the expression: \( \tau = \alpha - \frac{N}{p} \) (Crawford and Waring, 1967) where \( \alpha \) is a topological linking number. \( \alpha \) and N are constant for any given circular DNA molecule. Therefore, when the helix pitch is altered by the binding of ethidium bromide, the number of superhelical turns and the sedimentation coefficient of the superhelical DNA will be affected. The excellent agreement between extensive theoretical and experimental considerations of this phenomenon render it the best piece of evidence in favor of the intercalation model. In fact, this method is now being used in order to test other drugs for possible intercalative binding, or in the opposite sense, in order to test new DNA molecules for circularity (Waring, 1972).

The optical properties of ethidium bromide show characteristic changes in the visible spectrum when this drug associates with DNA. Upon binding to a double-stranded nucleic acid, the absorption spectrum of ethidium bromide undergoes a bathochromic and hypochromic shift with the appearance of two well-defined isosbestic points in the visible region of the spectrum (Waring, 1965). Also the fluorescence quantum yield of ethidium bromide increases drastically (Le Pecq and Paoletti, 1967). The circular
dichroism and optical rotatory dispersion spectra of the DNA-ethidium bromide complex show characteristic bands in the visible region of the spectrum, a consequence of the presence of the absorbing drug chromophore in an asymmetric environment (Aktipis and Martz, 1970; Dalgleish et al., 1971; Aktipis and Kindelis, 1973).

The kinetics of formation of the intercalation complex between ethidium bromide and transfer-RNA have also been studied. The results are best fitted by a mechanism in which ethidium bromide intercalates following a two-step, three-component pathway. In the first step, ethidium bromide and the DNA (two components) interact to form a complex (the third component) in which ethidium bromide is bound on the outside of the DNA helix. Subsequently, ethidium bromide from this complex is inserted between the DNA base pairs in a monomolecular, rate-limiting step (Tritton and Mohr, 1971).

Studies on the kinetics of association of ethidium bromide with double-stranded DNA suggest that in addition to the above mechanism intercalated ethidium bromide molecules can also be transferred between intercalation sites in a direct exchange reaction (Bresloff and Crothers, 1975). Kinetic experiments of the association of actinomycin D and proflavine, two other well characterized intercalators, support the two-step, three-component mechanism as a general kinetic model for intercalation (Li and Crothers, 1969; Müller and Crothers, 1968).
However, some revisions to the original intercalation model may be necessary in view of the recent x-ray diffraction studies on crystalline samples of ethidium bromide double-stranded oligonucleotide complexes (Tsai et al., 1975). These studies conclude that the overall geometry of the ethidium bromide molecule in the intercalation site is in agreement with the conformation proposed by Fuller and Waring, although the drug molecule is now placed in the minor groove of the DNA and the unwinding angle of the helix is found to be 26° per intercalated drug molecule rather than the 12-14° angle predicted by the Fuller-Waring model. Recent hydrodynamic studies with double-stranded circular DNA have also resulted in a value of 26° for the unwinding angle (Wang, 1974).

The conformation and the properties of DNA complexes formed with structural analogs of ethidium bromide have received very little attention. The binding parameters of some of these compounds and ethidium bromide to double-stranded DNA have been found to be of similar magnitude to one another and the optical properties of their DNA complexes share some common features (Kindelis, 1976).

Direct evidence that ethidium bromide analogs also bind to DNA by intercalation has been obtained with circular DNA by examining the dependence of the sedimentation coefficient on the concentration of bound drug as discussed previously. From these experiments it was concluded that the ability of
the structural analogs of ethidium bromide to bind to DNA by intercalation is maintained even among compounds carrying extensive substitution on the phenanthridinium ring system (Wakelin and Waring, 1974). However, compounds lacking the amino group at position 8 of the phenanthridinium ring exhibit characteristic fluorescence and circular dichroism spectra indicating that the DNA complexes of these analogs may exist in the intercalation site in conformations different than those of ethidium bromide and the other diamino derivatives (Kindelis, 1976).
3. **The Mechanism of Transcription**

RNA synthesis catalyzed by bacterial DNA-dependent RNA polymerase and viral DNA as template is a multistep process involving initial binding of the enzyme at non-specific DNA sites followed by recognition of a specific site, formation of the first phosphodiester bond, elongation of the product chain and, finally, termination (Burgess *et al.*, 1969). With T₂ or T₄ DNA as template, RNA synthesis may only be catalyzed by holoenzyme; i.e., core enzyme consisting of the four stable subunits (α₂ββ') plus the less stable σ subunit (Burgess *et al.*, 1969). The σ subunit is necessary for the enzyme to recognize the correct initiation site on viral DNA templates (such as T₇, T₄ or T₂ DNA) and is released during chain elongation (Travers and Burgess, 1969). Although the exact mechanism of action of the σ factor is unknown at the present time it is thought that perhaps it binds to the core enzyme and may act as an allosteric effector to the holoenzyme. Chain elongation with T₇ DNA as template yields in vitro a major RNA product which is approximately 7,000 nucleotides long (Millette *et al.*, 1970), while with T₂ or T₄ DNA the average chain length is approximately 5,000 nucleotides (Richardson, 1970; Maitra and Barash, 1969). The rate of chain growth in vivo is comparable to the rate of growth in vitro, which with T₇ DNA has a value of 36 nucleotides per sec while with T₄ DNA this figure is only 22
nucleotides per sec (Bremer, 1970). Since the growth rate of RNA chains with T₂ DNA as template is slower than with T₇ DNA while the length of the transcript with either template is of comparable size, termination of chain elongation should not occur within 90 sec of assay time with T₂ DNA as template. The growth rate with T₂ DNA which is used in this work would be expected to be comparable to that of T₄ DNA, since the two molecules have been shown to possess similar chemical, biological and genetic properties (see for example Rauh, 1965). Although completion of a long transcript in vitro requires approximately 2 min, addition of one nucleotide to a growing chain requires only a small fraction of a second. This rate is very fast compared with the time required for half the enzyme to locate the initiation sites, which is 15-20 sec (Hinkle and Chamberlin, 1972).

Despite the fact that the values of the kinetic parameters governing the various steps of RNA synthesis have been determined repeatedly in many laboratories, common agreement on their exact magnitude is difficult to achieve. Due to the complexity of the reaction, these kinetic parameters are influenced by a great number of factors, such as temperature, KCl concentration, Mg²⁺ and/or Mn²⁺ concentration, the concentration of template relative to enzyme, the nature of the template, the σ-subunit content of the enzyme preparation, the nucleotide substrate concentration, the sequence of addition of the various components in the
assay mixture, the pre-incubation and incubation time, the method of collecting the product, to name only a few. Some of these factors are bound to vary from one set of conditions to another, making direct comparison of literature values very difficult.

Even a brief review of the ways in which these factors might influence the process of RNA synthesis becomes a nearly impossible task and, for most purposes, a meaningless one. Because of the enormous complexity of the system catalyzing this reaction, many factors which might facilitate the rate of one step might be deleterious to another. To give only one example, raising the salt concentration from 0.05 M to 0.2 M increases the rate of elongation, whereas, it decreases the number of RNA chains that can be initiated. At the same time, RNA polymerase molecules which complete the synthesis of one chain are released from the template and are able to reinitiate a new chain in 0.2 M KCl, but only to a smaller extent in 0.05 M KCl (Bremer, 1970). What are, then, the optimal salt conditions for RNA synthesis? The answer to this question is, naturally, that the effect of each factor should be examined separately for each step. Then, the importance of techniques which would be able to isolate the events of each particular step becomes evident.

One method for studying the events of RNA chain initiation and chain elongation independently but simultaneously has
been described by Maitra and Hurwitz (1965). This method relies on the fact that, among all the ribonucleotides to be incorporated into the RNA product, only the first one will maintain its β and γ phosphate groups intact. One could, therefore, use a nucleoside triphosphate substrate labelled on the terminal phosphate with $^{32}$P and on the purine or pyrimidine ring with $^3$H or $^{14}$C. Incorporation of $^{32}$P in the product would then be a measure of the number of chains initiated with that particular nucleotide, while incorporation of $^3$H (or $^{14}$C) would be a measure of the overall rate of RNA synthesis. The ratio of $^3$H over $^{32}$P would be a measure of the length of the product chain. Using this method, it was found that the large majority (> 90%) of all product chains are initiated with either an ATP or GTP as the first nucleotide. The ratio of chains initiating with ATP over those initiating with GTP was found to be a function of many factors and varied between 6/1 and 1/1 with intact T₄ DNA as template (Maitra et al., 1967). Denaturation of the DNA or reduced content of σ-factor for the enzyme increased the number of chains initiating with GTP. An excellent review article covering the information available on RNA synthesis prior to 1974 has recently been published (Chamberlin, 1974).

In 1974, new methods were developed allowing further resolution of the events that take place during chain initiation (Mangel and Chamberlin, 1974a; b; c). This
achievement was accomplished by exploiting the property of the drug rifampicin to attack and inactivate RNA polymerase molecules at greatly different rates, depending upon whether these enzyme molecules are going through initiation, elongation or are free in solution. Template-bound RNA polymerase molecules are inactivated approximately 100 times slower than free enzyme molecules while elongating polymerase molecules are truly resistant to rifampicin. The second order rate constant of rifampicin attack on holoenzyme-T_7 DNA complex is $3.5 \times 10^3 M^{-1} s^{-1}$ (Hinkle et al., 1972). Mangel and Chamberlin formed complexes of E. coli RNA polymerase with bacteriophage T_7 DNA at 37°C in the absence of ribonucleotide substrates. Then, the reaction was initiated by the addition of a mixture of the four ribonucleotides with rifampicin and was allowed to proceed for 90 sec. In this period of time, the enzyme-DNA binary complex (RS) can have one of two fates: a) it may be inactivated by rifampicin; or b) it may react with a nucleotide and form a ternary initiation complex (OP) which is resistant to rifampicin and forms product uninhibited. If this scheme is correct, Mangel and Chamberlin argue, the ratio of the rate of the reaction in the absence of rifampicin over the rate of the reaction in its presence plotted as a function of increasing rifampicin concentrations should be a straight line of characteristic slope and
intercept: The intercept on the rate axis should be a measure of the number of RNA polymerase molecules which are able to escape rifampicin and form product, while the slope should be equal to the ratio of the second-order rate constant of rifampicin attack on the binary enzyme-DNA complex (RS), over the rate constant of the transformation of the RS complex to the rifampicin-resistant ternary complex (OP). Experiments conducted under a variety of nucleotide concentrations verified these predictions and allowed the calculation of a value of 5 s⁻¹ for the pseudo-first order rate constant governing the transformation of the RS to the OP complex with T₇ DNA at 0.4 mM XTP (Mangel and Chamberlin, 1974).

However, the results of similar experiments, conducted under various temperature and ionic strength conditions, indicated that another state (I) might exist for the enzyme-DNA binary complex, from which the enzyme could rapidly equilibrate with the RS complex. Formation of the I complex is favored over the RS complex at low (< 10°C) temperature and high (> 0.1 M KCl) salt concentrations (Mangel and Chamberlin, 1974b; c). In order to measure the rate of transformation of the I to the RS complex, I complexes were formed by incubating E. coli RNA polymerase and T₇ DNA at 0°C under low salt (< 0.05 M KCl) conditions. Subsequently, the mixtures were transferred into a 37°C bath for increasing periods of time (pre-incubation). RNA synthesis was then
initiated by the addition of a mixture of rifampicin and
the four ribonucleotide substrates and terminated 90 sec
later. With increasing pre-incubation time, an increasing
fraction of RNA polymerase molecules would find the necessary
time and be transformed from the I to the RS complex. Then,
the fraction of enzyme molecules still at I complexes would
be inactivated by rifampicin upon addition of the rifampicin-
nucleotide mixture, while the majority of the RS complexes
would be able to initiate an RNA chain. It was indeed
shown that the rate of rifampicin attack on I complexes is
much faster than the rate of transformation of I to RS
(Chamberlin and Ring, unpublished observations, cited by
Chamberlin, 1974). Consequently, Mangel and Chamberlin
argued and demonstrated experimentally a plot of the rate
of the reaction as a function of pre-incubation time must
yield an exponential curve having a slope equal to the
first order rate constant of the transformation of the I
to the RS complex. The half-life time of this transforma-
tion was found to be approximately 22 sec under the
conditions of their assay.

Based on the evidence obtained with these methods,
Mangel and Chamberlin postulated that at least two steps
may be distinguished in the process of RNA chain initiation
between the time the initial binding of the enzyme at the
specific recognition site on the DNA template takes place
and the subsequent formation of the first phosphodiester bond (Scheme I). In the first initiation step, an enzyme molecule already bound on a specific DNA recognition site in a highly stable rifampicin-sensitive binary complex (I) transforms into a rapidly-starting (RS) complex. This transformation occurs via a temperature dependent, rate-limiting transition that may involve partial unwinding of the DNA helix as well as translocation of the enzyme along the template. In the second initiation step the binary (RS) complex forms a rifampicin-resistant ternary initiation complex (OP) by forming the first phosphodiester bond. This latter step does not seem to involve translocation of the enzyme along the template (Heyden et al., 1975).

The methodology of Mangel and Chamberlin was described in some detail here not only because of its significance but also because it is being used in this work in order to study the effect of various inhibitors of RNA synthesis on each initiation step.

Application of one of these methods had already allowed direct measurement of the effect of the number of superhelical turns of a closed circular DNA on the second initiation step. It turns out that superhelicity does not have any significant effect on the second initiation step (Richardson, 1975). On the other hand, the presence of superhelical turns on the molecule appears to greatly
Scheme I. The mechanism of RNA synthesis. The line represents a hypothetical template molecule and the dark discs an RNA polymerase molecule. For schematic purposes, different DNA sites have been assigned to each complex and are depicted either in the "closed," \[\text{\#} \]; or in the "open," \[\text{\#} \], configuration.

<table>
<thead>
<tr>
<th>NS</th>
<th>I</th>
<th>RS</th>
<th>OP</th>
<th>EL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-specific binding - formation of non-specific complex NS</td>
<td>Recognition - formation of the I complex</td>
<td>First initiation step - formation of the RS complex - DNA strand separation</td>
<td>Second initiation step - formation of the first phosphodiester bond, ternary OP complex</td>
<td>Elongation steps - formation of the elongation complexes EL</td>
</tr>
</tbody>
</table>

Description

NS  Non-specific binding - formation of non-specific complex NS
I   Recognition - formation of the I complex
RS  First initiation step - formation of the RS complex - DNA strand separation
OP  Second initiation step - formation of the first phosphodiester bond, ternary OP complex
EL  Elongation steps - formation of the elongation complexes EL
facilitate the temperature dependent transition through step 1 (Hayashi and Hayashi, 1971). However, it is not known whether superhelicity affects the number or the rate of enzyme molecules going through the first initiation step.

These recent advances in the understanding of the mechanism of RNA synthesis in vitro have provided clues as to the possible mode of action of the various transcriptional control elements in vivo. For instance, one type of a DNA-binding protein (the CAP protein) has been identified which facilitates the initiation of transcription in vivo. Binding of the CAP protein to the DNA induces a structural transition of the template which may facilitate the formation of the (RS) complex (Dickson et al., 1975).
4. The Mechanism of Inhibition of RNA Synthesis Through Template Inactivation

Chemical agents that attack and permanently modify the primary and/or the secondary structure of the DNA are known to interfere with the template properties of the macromolecule (Waring, 1972; Bücher and Sie, 1969). Inhibition of RNA synthesis, however, may also result from the interaction of the DNA molecule with compounds that bind reversibly and alter the conformation of the double helix only for that period of time during which they remain associated with the template. For example, compounds that bind to DNA by intercalation between base pairs like ethidium bromide, proflavine or actinomycin D, are strong but reversible inhibitors of RNA synthesis. These compounds however exert their inhibitory effect on RNA polymerase through various modes of action. Specifically, although ethidium bromide, proflavine and actinomycin D all inhibit the overall rate of RNA synthesis, each one of these compounds does so by affecting different steps of the overall reaction. Actinomycin D up to a given concentration interferes only with RNA chain elongation (Sentenac et al., 1968; Hyman and Davidson, 1970) while ethidium bromide affects almost exclusively chain initiation (Richardson, 1973a). Proflavine has been found to inhibit both steps
and to approximately the same extent (Hurwitz et al., 1962; 1967; Richardson, 1966a and b).

Ethidium bromide has been the best studied initiation inhibitor to date. The activity of this compound as an inhibitor of RNA synthesis has been found to vary with the type of template employed and this variation does not appear to result from the small differences noted in the affinity of this compound for each different DNA template (Richardson, 1973b).

Binding of ethidium bromide on the DNA-template at concentrations causing total inhibition of RNA synthesis does not appear to interfere with the binding of RNA polymerase at non-specific sites on the template (Richardson, 1973a). On the other hand, ethidium bromide has been found to inhibit the formation of the strong complexes of RNA polymerase with specific sites of the template, as evidenced by the decrease in the amount of complexes which are retained on a cellulose nitrate filter in the presence of inhibitor (Richardson, 1973a). This inhibition of the strong binding of RNA polymerase by ethidium bromide appears to result from the inability of the enzyme to form complexes with the specific sites of the template in the presence of ethidium bromide molecules intercalated in the vicinity of those sites. RNA polymerase, however, has the ability to displace ethidium bromide from its strong
binding sites while ethidium bromide, which has a much weaker affinity for the template than the enzyme, is unable to induce dissociation of the enzyme from its strong (cellulose nitrate non-filterable) complexes (Richardson, 1973a).

However, no quantitative correlation has been attempted between the degree to which specific binding of the polymerase to the template is inhibited and the inhibition of RNA chain initiation (Richardson, 1973a). Moreover, it is not known which one of the specific enzyme-DNA complexes described in the previous section (Scheme I) is inhibited by the template-bound form of the drug. Neither is it known whether ethidium bromide inhibits by causing a reduction in the rate of formation of specific enzyme-DNA complexes or by causing a decrease in the number of such complexes that can be formed in the presence of this inhibitor (or perhaps, by a combination of the above two modes of inhibition).

A study of the effects of ethidium bromide and some of its structural analogs on the number and the rate of RNA polymerase molecules involved in product formation has been the main goal of this investigation. Since at least five individual steps are presently recognized in RNA synthesis (Scheme I) the effect of each inhibitor on the number and the rate of RNA polymerase molecules traversing
each one of these steps has separately been studied. The results indicate that ethidium bromide and its structural analogs inhibit RNA synthesis as a result of their presence in the vicinity of a specific DNA site which must be recognized by RNA polymerase in order for the first productive enzyme-DNA complex to be formed.
MATERIALS AND METHODS

Nucleoside triphosphates (sodium salts) were purchased either from Sigma or from P-L Biochemicals specified as at least 98% pure. Tritium or $^{32}$P-labelled nucleotides were purchased either as freeze-dried ammonium salts or as 50% ethanolic solutions (Amersham-Searle). In the latter case, the ethanol was blown off under a stream of nitrogen before use. Both tritium and $^{32}$P-labelled nucleotides were purchased at a specific activity of more than 15 Ci/mmol and were diluted to the desired specific activity with unlabelled material. Calf thymus DNA was purchased from Worthington Biochemical Corporation. Bacteriophage $T_2$ was obtained from Miles Laboratories at a concentration of $5-10 \times 10^{12}$ virus particles per ml and a titer of $2.1 \times 10^{12}$ plaque forming units per ml.

1. $T_2$ DNA Preparation

The method described by Bautz and Dunn (1971) was used for the isolation of $T_2$ DNA.

Six milliliters of the commercial virus suspension was dialyzed against 2.0 l of 0.1 M sodium phosphate buffer pH 7.2 for three hours. The dialyzate was transferred to a 15 ml conical glass tube and 0.11 volume (0.66 ml) of a freshly prepared 10% sodium dodecyl sulfate solution was added. The mixture was brought to 65°C for 3 min, then
rapidly chilled in ice and 1.0 M KCl was added to the solution to make it 0.3 M in KCl. After 15 min centrifugation in the cold (IEC SB206 rotor, 27,000 g), an equal volume of freshly distilled phenol (saturated with 0.1 M sodium phosphate buffer pH 7.2 and containing 0.08% 8-hydroxyquinoline) was added to the supernatant and the mixture was rocked for 14 min at a frequency of 40 rpm/min. Centrifugation for 15 min in an IEC clinical, swinging bucket centrifuge at top speed separated the phenol layer (yellow) which was discarded with the help of a Pasteur pipette, from the buffer layer which was dialyzed against 4.0 L 0.01 M Tris-HCl, 0.01 M KCl, 0.00005 M EDTA pH 7.9 with three changes for four days. The purified T₂ DNA solution was stored at -20°C in five 2 ml aliquots. The concentration of the product (0.0010 M in nucleotide) was determined spectrophotometrically assuming a molar extinction coefficient of 6500 at 260 nm and a nucleotide mean molecular weight of 357 (Rubenstein et al., 1961). The A₂₆₀/₂₈₀ ratio of the product was 1.80 and the A₂₆₀/₂₃₀ ratio was 2.1, indicating very low protein contamination (Thomas and Abelson, 1966).

2. The Enzyme

**Escherichia coli** K-12 DNA-dependent RNA polymerase (EC 2.7.7.6) was purchased from Sigma Chemical Co. as a 50% glycerol solution having a specific activity of 396
units per mg protein (2.1 mg protein/ml of solution) using the unit definition of Burgess (1969) with calf-thymus DNA as template. Enzymatic activity assays carried out in the laboratory under identical conditions yielded a value of 800 units per ml of solution with calf-thymus DNA as template, but a value of 1600 units per ml of solution with T₂ DNA as template. Higher enzymatic activity with T₂ DNA, as compared to calf-thymus DNA, is indicative of an enzyme having a high content of σ-subunit (Burgess, 1969). Indeed, the rifampicin experiments described below (section 6a of Results) indicate that the RNA polymerase had approximately 65% of the activity expected if all enzymatic protein were active holoenzyme (Mangel and Chamberlin, 1974a).

3. The Inhibitors

Actinomycin D was obtained from Calbiochem. Rifampicin was obtained from Sigma. Ethidium bromide was purchased from Calbiochem. Ethidium bromide analogs were the generous gift of Dr. T. I. Watkins of Boots Company Ltd., Nottingham, England. The chemical formulae, molecular weights, molar extinction coefficients and DNA binding parameters of these compounds are given in Table 1 (see p. 39). The molar extinction coefficients shown in this Table are based on the formula weight of the compounds and were determined from the absorption spectra of carefully prepared solutions at a concentration of 1-2 mg/25 ml in 0.04 M Tris-HCl pH 7.9 at
room temperature. More detailed studies of the optical properties of the DNA complexes of these compounds are described by Kindelis (1976).

4. Determination of Binding Parameters

The spectrophotometric method of Peacocke and Skerett (1956) was used to determine the binding constant and the maximum number of binding sites per DNA nucleotide for each drug on calf-thymus DNA.

In a typical experiment, calf-thymus DNA (2-3 mg/ml) was dissolved under slow stirring at 0-5°C in binding buffer consisting of 0.2 M KCl, 0.04 M Tris-HCl, 0.01 M MgCl₂, 0.1 mM EDTA, 0.32 mM K₂HPO₄, pH 7.9. Then, the solution was dialyzed overnight against binding buffer at 0-5°C, and filtered by suction through a GF/C filter (Whatman). The concentration of DNA in the filtrate was determined spectrophotometrically in the Cary 15 at 260 nm, using a molar extinction coefficient of 6600 (Mahler et al., 1964).

Solutions of increasing DNA/drug ratio were prepared by adding increasing amounts of the DNA solution into constant amounts of the drug solution in binding buffer and bringing the mixture to the same final volume (10 ml) with buffer. Identical solutions containing DNA, but no inhibitor, were also prepared and used as reference in the double-beam Cary 15 spectrophotometer. The total drug concentration (Iₒ) was determined from the absorption
spectrum of a solution prepared as above, but in the absence of DNA. The amount of drug (around 20 μM) was chosen so that the absorbance at $\lambda_{\text{max}}$ of the resulting solution would be approximately 1.0. After mixing, each solution was transferred into a 10.00 cm path, water jacketed optical cell (Hellma) that was kept at 37°C with the help of a Lauda K-2R constant temperature circulator, and after thermal equilibration the difference absorption spectrum was recorded vs. the corresponding reference solution. The absorption spectra of the DNA complexes of all the drugs examined displayed the qualitative features described by Kindelis (1976) for the complexes of the same compounds formed under different ionic environment. In every case sharp isobestic points were observed in the area 380-400 nm and 460-500 nm.

The absorbance of each sample ($A_x$) and that of the "total" drug ($A_0$) were read off the chart paper at a given wavelength, chosen for best accuracy so that $A_0 - A_x$ is maximum.

The absorbance of the completely bound drug ($A_b$) was calculated with the graphic method of Li and Crothers (1969) who have demonstrated that a plot of $1/A_0 - A_x$ vs. $1/[\text{DNA}]-[I_0]$ yields a straight line with an intercept on the ordinate equal to $1/A_0 - A_b$. From the value of this intercept, $A_b$ may be calculated. Determination of $A_b$ with this graphic method is much more accurate than the alternative
method of reading off the chart paper the absorbance of a solution prepared with very high DNA concentration (at very high DNA concentration all of the drug is assumed to be bound; i.e., \( A_x = A_b \)). Concentrated DNA solutions required to saturate the drug at 37°C in 0.2 M KCl absorb light strongly, probably due to scattering and introduce error in the determination of absorbance.

From these values, the fraction of DNA-bound inhibitor \( b \) was calculated using equation (1).

\[
b = \frac{A_o - A_x}{A_o - A_b} \tag{1}
\]

The molar ratio of inhibitor bound to DNA per nucleotide \( r_b \) as well as the concentration of "free," unbound inhibitor \( I_f \) and bound inhibitor \( I_b \) were calculated using equations (2) and (3), respectively.

\[
r_b = b \cdot \frac{[I_o]}{[\text{DNA}]} \tag{2}
\]

\[
[I_f] = [I_o] - [I_b] = [I_o] - b \cdot [I_o] = [I_o] \cdot (1 - b) \tag{3}
\]

Then, the values of the apparent dissociation constant \( k_{app} \) and the maximum number of binding sites per DNA nucleotide \( n \) were obtained from the slopes and intercepts of plots constructed according to Scatchard (1949) and described by equation (4).
5. **Enzymatic Activity Assays**

In this work, enzymatic activity was always determined by measuring the incorporation of radioactivity from a radioactively labelled nucleoside triphosphate substrate into material insoluble in 5% trichloroacetic acid.

A typical reaction mixture consisted of a buffer solution, DNA, RNA polymerase, the inhibitors (if any) and an equimolar mixture of the four nucleoside triphosphates with one, or more, of them radioactively labelled. These components were transferred with fixed needle Hamilton syringes into 10 x 75 mm disposable glass test tubes fitted loosely with plastic caps. (A Hamilton syringe fitted with an Oxford pipette disposable plastic tip was used for transferring the DNA in order to avoid any nicking or breaking due to shear.) The test tubes had been kept at 140°C for at least 48 hrs prior to use, in order to inactivate any contaminating ribonuclease activity. The same treatment was given to all glassware used to prepare assay buffers. Alternatively, containers were washed with a dilute solution of NaOH which destroys ribonuclease. Care was exercised to handle all equipment with plastic disposable gloves. The two main types of experiments conducted under these conditions are described below.
a. **Experiments Not Involving Rifampicin**

In experiments designed to study the effect of inhibitor on the rate of RNA synthesis in the presence of a constant amount of DNA, the concentration of T₂ DNA was 30 μM. This concentration was chosen so as to correspond to the minimum amount of DNA required in order to saturate the activity of 4 μg of RNA polymerase under the conditions described below. This amount of DNA corresponds to a molar ratio of enzyme to T₂ DNA initiation sites of the order of 25 since the number of such sites per T₄ DNA molecule is approximately 22 (Bautz and Bautz, 1970).

In these experiments, 37 μl of 2.5X concentrated assay buffer A was mixed with the appropriate amounts of T₂ DNA to yield final concentrations of 0.2 M KCl, 0.04 M Tris-HCl, 0.01 M MgCl₂, 0.1 mM EDTA, 0.32 mM K₂HPO₄, 0.1 mM dithiothreitol, 0.4 mg/ml BSA, at pH 7.9 and 25°C. Inhibitor (if any) was added and the solution was brought up to 84 μl with deionized distilled water at 0-5°C. The mixture was transferred to a 37°C bath and, after 6 min, the enzyme (4 μg) was added in 8 μl of a solution consisting of 3 μl of assay buffer A (concentrated 2.5 times), 3 μl of deionized water and 2 μl of the commercial enzyme solution. Seven minutes later the reaction was initiated by the rapid addition of a solution consisting of 0.8 mM each of ATP, GTP and CTP and 0.4 mM 8-[³H]-UTP (specific activity 0.010 μCi/nmol or 0.1 μCi/nmol depending upon the experiment)
in 8 μl of water adjusted to pH 7.9 with 1.0 M Tris-base. The reaction was allowed to proceed for exactly 7.0 min and then it was terminated through the rapid addition of 0.5 ml of an ice cold solution containing 0.1 M sodium pyrophosphate and 2 mM UTP (or 0.02 M ATP or GTP for the initiation experiments) followed by 0.5 ml of ice cold 11% TCA - 1.0 M KCl - 0.01 M sodium pyrophosphate solution.

After mixing and standing in ice for at least 10 min the insoluble material was collected by filtration through a Whatman (2.4 cm diameter) GF/C filter. The filter was rinsed with 15 ml of ice cold 5% TCA - 1.0 M KCl - 0.01 M sodium pyrophosphate solution followed by 30 ml of 95% ethanol, was dried under suction and at 105°C for 3 min and, finally, counted under 0.4% 2,5-diphenyloxazole in toluene solution in the Beckman LS counter.

Experiments designed to measure the inhibition of γ-32P-incorporation, which is a measure of the inhibition of RNA chain initiation, were conducted exactly as described above, with γ-32P-labelled ATP or GTP (specific activity 1.0 μCi/nmol) and final nucleoside triphosphate concentration 0.2 mM in ATP, GTP, CTP, and 8-[3H]-UTP (specific activity 0.01 μCi/nmol).

A specific washing procedure for experiments involving incorporation of 32P was employed in order to decrease the background of unincorporated radioactivity. The filters were washed with 100 ml of the TCA-KCl-pyrophosphate solution
mentioned above followed by 60 ml of 80% ethanol and were subsequently stirred before drying and counting. Identical \(^3\text{H}\)-cpm were recovered after this treatment indicating that product is not lost.

Simultaneous counting of samples containing both \(^{32}\text{P}\) and \(^3\text{H}\) was effected with the Beckman Isoset system with a gain setting of 3.0. Under these conditions no Tritium spill-over could be detected in the \(^{32}\text{P}\)-above-\(^3\text{H}\) window, while less than 2% spill-over from \(^{32}\text{P}\) was detectable in the \(^3\text{H}\)-below-\(^14\text{C}\) window.

b. Experiments Involving Rifampicin

As described in the Introduction, these experiments were designed in order to measure the effects of various inhibitors on the rate constants governing the initiation steps of RNA synthesis. All experiments were conducted in buffer B consisting of 0.04 M Tris-HCl, 0.02 M MgCl\(_2\), 0.1 mM EDTA, 0.32 mM K\(_2\)HPO\(_4\), 0.1 mM dithiothreitol, 0.8 mg/ml BSA pH 7.9 at 25°C (Mangel and Chamberlin, 1974a).

Experiments measuring the rate of RNA synthesis as a function of preincubation time were conducted as follows. The buffer solution (37 µl) at 2.5 times the final desired concentration was mixed with the desired amount of inhibitor, template (50 µM T\(_2\) DNA) and deionized water to a final volume of 72 µl. After standing in ice for at least 10 min, the enzyme (4 µg) was added in 8 µl of a solution
consisting of 3 μl buffer B (2.5 times concentrated), 3 μl deionized water and 2 μl of the commercial enzyme preparation. After 10 more minutes at 0°C the test tube containing the above mixture was transferred into a 30°C bath for the desired time. The reaction was then initiated by the rapid addition of an XTP-rifampicin mixture in 20 μl 0.04 M Tris-HCl pH 7.9. This mixture had been equilibrated at room temperature and was prepared so as to result in final assay concentrations of 30 μg/ml rifampicin and 0.4 mM XTP (specific activity 0.10 μCi/nmol 8-[^3]H]-ATP). For the control reaction rifampicin was omitted and an equal volume of deionized water was used instead. After exactly 1.5 min of incubation at 30°C the reaction was terminated and each mixture was processed as described under section 5a.

In experiments designed to measure the rate of RNA synthesis as a function of rifampicin concentration the buffer solution, the inhibitor, the DNA (90 μM) and deionized water were mixed in the same manner as described above. Then the enzyme (2 μg in 8 μl) was added and the mixture was preincubated for 7 min at 30°C. The reaction was initiated by the addition of an XTP-rifampicin mixture prepared so as to result in 0.4 mM XTP (specific activity 0.10 μCi/nmol 8-[^3]H]-ATP) while providing the desired final rifampicin concentration. After 1.5 min at 30°C the reaction was terminated and each mixture was processed as described above. In these experiments the enzyme to T2 DNA
initiation sites ratio was either 15 or 40, assuming that 65% of the protein is active holoenzyme. This ratio is higher than the ratio of 1 used by Mangel and Chamberlin (1974) with T7 DNA. The use of excess enzyme ensures that the total number of initiation sites participates in product formation.

In all experiments measuring incorporation of radioactivity into 5% TCA insoluble product, background values were determined by treating identically a complete reaction mixture not containing enzyme. Background values were less than 200 cpm in experiments involving 3H and less than 700 cpm in experiments using 32p.
RESULTS

1. The Binding of Phenanthridines to DNA

The binding of ethidium bromide and a number of structural analogs of this phenanthridine to DNA has been extensively studied (Kindelis, 1976; Waring, 1974).

However, in order to obtain a quantitative measure of the distribution of the drug between the template-bound and the free forms under the ionic environment and temperature conditions of the assays used for determining rates of RNA synthesis, the binding parameters for each drug must be determined under those conditions.

However, three of the compounds shown to inhibit RNA synthesis exhibited complex spectroscopic characteristics (Kindelis, personal communication). The reasons for such a behaviour could be attributed to the fact that complexes of DMNC with DNA were only partially soluble, MMPB was not completely soluble at the desired concentrations and DDEB appeared to aggregate at high concentrations.

Binding experiments leading to the calculations of $r_b$ (the molar ratio of drug bound per DNA nucleotide) and $I_f$ (the concentration of free drug) were conducted as described under the Methods. The values of the apparent dissociation constant ($k_{app}$) and the maximum number of binding sites per DNA nucleotide ($n$) were obtained from
the slopes and intercepts of plots constructed according to Scatchard (1949) and described by the equation

\[ \frac{r_b}{I_f} = n \cdot k_{app}^{-1} - k_{app}^{-1} \cdot r_b \]  

(4)

The Scatchard plots for EM, MAPEC, DEMB and DMEB are shown in Figure 1a and b. The values of \( k_{app} \) and \( n \), calculated for each drug are listed in Table 1. Comparison of the values in Table 1 and inspection of the plots in Figure 1 allow the following conclusions to be drawn:

a) The intrinsic affinities for DNA of all four drugs as well as the corresponding number of binding sites are similar in magnitude.

b) Only one type of binding, i.e., binding to primary sites in which the bound drug molecules are intercalated between neighbouring base pairs is evident. Drug bound weakly to secondary sites on the outside of the DNA helix is not present to an appreciable extent as indicated by the linearity of the Scatchard plots and the magnitude of \( n \). This binding behaviour is expected at relatively high (0.2 M KCl) ionic strengths (Wariny, 1965b).
<table>
<thead>
<tr>
<th>Systematic Name</th>
<th>Abbreviation</th>
<th>MW</th>
<th>$E_{\text{max}}$</th>
<th>$k_{\text{diss}}$ (μM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,8-diamino-5-ethyl-6-phenyl-phenanthridinium Bromide</td>
<td>EM</td>
<td>394</td>
<td>5600379</td>
<td>18.9</td>
<td>0.19</td>
</tr>
<tr>
<td>3,8-diamino-5-ethyl-6-methyl-phenanthridinium Bromide</td>
<td>DMEB</td>
<td>332</td>
<td>3900463</td>
<td>32.2</td>
<td>0.19</td>
</tr>
<tr>
<td>3,8-diamino-6-ethyl-5-methyl-phenanthridinium Bromide</td>
<td>DEMB</td>
<td>332</td>
<td>4700463</td>
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<td>0.18</td>
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<tr>
<td>3,8-diamino-5,6-diethyl-phenanthridinium Bromide</td>
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<td>346</td>
<td>4100463</td>
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<tr>
<td>3,8-diamino-5-methyl-6-nonyl-phenanthridinium Chloride</td>
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<td>4900467</td>
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<td>---</td>
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<tr>
<td>8-amino-6-p-aminophenyl-5-ethyl-phenanthridinium Chloride</td>
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<td>4800431</td>
<td>32.4</td>
<td>0.17</td>
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<tr>
<td>8-amino-5-methyl-6-phenyl-phenanthridinium Bromide</td>
<td>MMPB</td>
<td>365</td>
<td>3650430</td>
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</table>

![Chemical Structure](attachment:chemical_structure.png)
Figure 1. Scatchard plots for some phenanthridines. a) EB, -○--; MAPEC, -▲-. b) DMEB, -■--; DEMB, -△-.
Experiments were conducted at 37°C in binding buffer (0.2 M KCl, 0.04 M Tris-HCl, 0.01 M MgCl₂, 0.1 mM EDTA, 0.32 mM K₂HPO₄, pH 7.9 at 25°C) with calf-thymus DNA as described under Methods. The amount of the DNA was varied between zero and 6 mM while the concentration of each phenanthridine was chosen so as to yield an absorbance reading of 1.0 at the maximum wavelength.
\( \frac{r_b}{I_r} \times 10^{-4} \)

\( r_b \)

(a)
2. The Effect of Template Concentration on Inhibition

Waring (1965a) has demonstrated that ethidium bromide inhibits RNA synthesis competitively with the template, by determining the level of enzymatic activity as a function of template concentration in the absence and in the presence of a constant amount of inhibitor. In the present study, similar experiments were performed with MAPEC, DEMB, and DMEB, the structural analogs of ethidium bromide, and the data were plotted as activity$^{-1}$ vs. [DNA]$^{-1}$. In each of the double reciprocal plots shown in Figure 2a, b and c, the lines for the inhibited and uninhibited reactions are crossing over on the activity axis indicating that the inhibitory effect of the drug would be completely alleviated at infinite DNA concentrations. It may also be noted that in the presence of inhibitor the double reciprocal plots shown in Figure 2a, b and c are curvilinear, a characteristic which becomes more pronounced at the higher drug concentrations. This apparently abnormal behaviour, which has previously been noted by Waring (1965a) will be discussed in a later section.
Figure 2. The effect of template concentration on the rate of RNA synthesis either in the absence of inhibitor, -●-, or in the presence of the following amounts of inhibitor: a) 14.0 μM MAPEC; b) 11.0 μM DMEB; and c) 15.0 μM DEMB. Experiments were conducted in 0.1 ml of assay buffer A containing 0.8 mM each of ATP, GTP and CTP, 0.4 mM 8-[³H]-UTP (0.1 μCi/nmol), 4 μg RNA polymerase and increasing amounts of T₂ DNA. Reaction time was 7 min at 37°C.
3. The Effect of Drug Concentration on Enzymatic Activity

In order to study in more detail the inhibitory effect of the phenanthridines on RNA synthesis, experiments were performed in which increasing amounts of drug were mixed with constant amounts of DNA, enzyme and substrate [XTP] and assayed for RNA polymerase activity.

The phenanthridinium derivatives studied may be characterized as strong inhibitors of RNA polymerase, since inhibitor concentrations as low as 50 \( \mu M \) were found to cause complete inhibition of RNA synthesis. Plots of the data as activity (per cent) vs. inhibitor concentration (Figure 3a and b) reveal that the activity curves of all compounds studied displayed the same overall shape. Enzymatic activity gradually decreases with increasing concentration of inhibitor up to a certain point where a sharp decline in activity is observed with a small increase in inhibitor concentration, followed by a much slower further decrease in activity which eventually reaches the zero (per cent) level at very high inhibitor concentrations (above 50 \( \mu M \)). These qualitative similarities seem to suggest a mechanism of inhibitory action common to all drugs.

However, important quantitative differences are also evident. Ethidium bromide, which causes 40% inhibition at
Figure 3. The effect of phenanthridine concentration on the rate of RNA synthesis relative to the uninhibited reaction. The maximum activity (100%) corresponds to the incorporation of $2.5 \times 10^4$ cpm into product insoluble into 5% TCA. Reactions were conducted in 0.1 ml of assay buffer A containing 0.8 mM each of ATP, GTP, and CTP, 0.4 mM $\beta$-$[3^H]$-UTP (0.01 μCi/nmol), 4 μg RNA polymerase and 30 μM T2 DNA for 7 min at 37°C.

a) Each bar represents the standard deviation of the mean of twelve determinations for EB, -○--; MAPEC, -▲--; DMEB, -o-; DEMB, -■-. Standard deviation of the mean (σ) was calculated using the formula

$$\sigma = \sqrt{\frac{\sum x^2}{N} - \left(\frac{\sum x}{N}\right)^2}$$

where x is the experimental value and N is the number of values.

b) The experimental points are less accurate than those of Figure 7a due to the fact that complexes of DMNC, -o-, with DNA were partly soluble in the assay mixture; MMPB, -●-, was not completely soluble at the desired concentration; DDEB, -▼-, appears to aggregate at high concentrations.
a concentration of only 4.5 \( \mu M \) as compared with DEMB, for which 14.5 \( \mu M \) are required to reach the same level of inhibition, appears as the strongest inhibitor. Table 2 lists the drug concentration for which 40% inhibition is reached. The values are determined from the plots in Figure 3a and b. It may be observed that maximum inhibitory capacity appears to be associated with the presence of large hydrophobic groups at position 6 of the phenanthridinium ring. The effect of the relatively large, hydrophobic phenyl or nonyl groups, in the case of EB and DMNC respectively, is particularly apparent if the inhibitory effectiveness of these two drugs is compared with that of DDEB, DMEB or DEMB, i.e., compounds that carry the smaller methyl or ethyl groups at the same position. Only 4.5 \( \mu M \) and 7.0 \( \mu M \) of EB and DMNC respectively are required for 40% inhibition, which is two to three times less than the concentrations of DDEB (12.5 \( \mu M \)), DMEB (13.5 \( \mu M \)) or DEMB (14.5 \( \mu M \)) required for the same inhibitory effect.

The inhibitory effectiveness of MMPB relative to EB can only be tentatively assessed due to the apparent solubility problem associated with the former compound (see legend to Figure 3b). Specifically, it appears that the absence of an amino group at position 3 of the molecule renders the compound a much weaker inhibitor. Again much less EB (4.5 \( \mu M \)) is required than MMPB (11.0 \( \mu M \)) in order
Table 2. Comparison of the Pharmacologic and Inhibitory Effectiveness of some Phenanthridines

<table>
<thead>
<tr>
<th>Compound</th>
<th>Trypanocidal Activity* (Dimidium = 1)</th>
<th>Concentration Resulting in 40% Inhibition (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB</td>
<td>10.00</td>
<td>4.5</td>
</tr>
<tr>
<td>DMNC</td>
<td>Active**</td>
<td>7.0</td>
</tr>
<tr>
<td>MAPEC</td>
<td>0.50</td>
<td>9.3</td>
</tr>
<tr>
<td>MMPB</td>
<td></td>
<td>11.0</td>
</tr>
<tr>
<td>DDEB</td>
<td>0.22</td>
<td>12.5</td>
</tr>
<tr>
<td>DMEB</td>
<td>0.12</td>
<td>13.5</td>
</tr>
<tr>
<td>DEMB</td>
<td>0.10</td>
<td>14.5</td>
</tr>
</tbody>
</table>

*Data compiled from the Median Curative Dose determinations of Woolfe, G., (1956a, b).

**Active indicates trypanocidal activity below 0.10 relative to dimidium.
to inhibit RNA synthesis to the same extent (Table 2).
The relative inhibitory strength of EB and MAPEC, another
derivative lacking the amino group at position 3 but
carrying a second amino group at the para position of the
phenyl substituent, seems to support the same conclusion,
i.e., the absence of the amino group at position 3 renders
a compound a weaker inhibitor than ethidium bromide.
4. The Effect of DNA-bound Inhibitor on RNA Synthesis

Ethidium bromide has been shown to inhibit RNA synthesis by inactivating template molecules, not enzyme molecules, as a result of the association of the drug with the DNA template (Waring, 1965). Consequently, the active form of ethidium bromide as an inhibitor of RNA synthesis is the DNA-bound form of the drug. An appropriate means of expressing quantitatively the concentration of the active form of ethidium is in terms of the ratio of drug molecules bound per DNA nucleotide ($r_b$). This ratio, however, does not depend linearly on the total drug concentration (see equation 5, section 7 of Results), a factor which may become particularly important in comparative studies of the inhibitory effectiveness of the structural analogs of ethidium bromide which have similar, but not identical affinities for the DNA template.

In order to establish that the differential inhibitory capacity of the phenanthridines is not simply the result of their different affinities for the DNA template, the dependence of RNA polymerase activity was determined as a function of $r_b$ of each drug.

The values of $r_b$ were calculated for each drug using equation 4, section 1 of Results, and were subsequently employed in plots of RNA polymerase activity (%) as a function of $r_b$. In Figure 4, the enzymatic activity appears
to depend linearly on $r_b$ in the range between 90% and 15% with either ethidium bromide or its derivatives as the inhibitor. A similar observation regarding the linear dependence of RNA polymerase activity on the $r_b$ of ethidium bromide has also been made by Waring (1965a).

The compounds included in Figure 4 exhibit clear differences in their inhibitory capacity against RNA polymerase. Comparison of the values of enzymatic activity remaining at a value of $r_b = 0.04$ reveals that EB is the strongest inhibitor among these compounds, effecting a 54% decrease in enzymatic activity, while at the same value of $r_b$ MAPEC, DEMB and DMEB decrease the activity of RNA polymerase by 49%, 26% and 17% respectively (Table 3). These results indicate that identical numbers of bound inhibitor molecules per molecule of DNA have different inhibitory effects on the ability of RNA polymerase to utilize this DNA as a template for RNA synthesis. Consequently, the inhibitory effectiveness of these phenanthridines is determined not only by the affinity of each compound for the template, but also by the specific structure of each intercalated inhibitor molecule.

Table 3 also lists the values of $r_b$ of each drug which result in total inhibition of RNA synthesis ($r_{10}$) as obtained by extrapolating the linear portion of the curves in Figure 4 to zero activity. From these values it would appear that on the average, intercalation of one inhibitor
Figure 4. Dependence of the rate of RNA synthesis on the concentration of the template-bound form of the inhibitor. EB, -•--; MAPEC, -▲--; DMEB, -○--; DEMB, -■-. Plots constructed on the basis of the inhibition data shown in Figure 3a.
Table 3. Comparison of the Inhibitory Effectiveness of the Template-Bound Forms of the Various Inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$r_b$, 40%</th>
<th>$r_I$, 100%</th>
<th>Decrease in Activity (%) at $r_b = 0.04$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB</td>
<td>0.032</td>
<td>0.068</td>
<td>54</td>
</tr>
<tr>
<td>MAPEC</td>
<td>0.036</td>
<td>0.058</td>
<td>49</td>
</tr>
<tr>
<td>DEMB</td>
<td>0.048</td>
<td>0.077</td>
<td>26</td>
</tr>
<tr>
<td>DMEB</td>
<td>0.051</td>
<td>0.080</td>
<td>17</td>
</tr>
</tbody>
</table>

$r_b$, 40% is the ratio of bound inhibitor per DNA nucleotide required for 40% inhibition.

$r_I$, 100% is the ratio of bound inhibitor per DNA nucleotide that effects total inhibition. $r_I$, 100% is obtained by extrapolating the linear portion of the curves shown in Figure 4 to zero activity (%).
molecule per seven base pairs can completely abolish the template properties of the DNA when measured by this method. The significance of this observation with respect to the mechanism of template inactivation will become apparent in a later section.
5. Ethidium Bromide Analogs Limit the Number of RNA Molecules that can be Initiated without Interfering Significantly with the Elongation of Initiated Chains

One method for determining whether a given compound inhibits RNA synthesis by interfering with RNA chain initiation and/or chain elongation is to study the incorporation of radioactivity from two different substrates simultaneously, i.e., the incorporation of a $\gamma$-[\textsuperscript{32}P]-labelled purine triphosphate nucleoside and a [\textsuperscript{3}H] or [\textsuperscript{14}C] labelled nucleotide triphosphate as described by Maitra et al. (1967).

In order to determine whether the structural analogs of ethidium bromide inhibit one or both steps involved in RNA chain synthesis, the simultaneous incorporation of radioactivity from a $\gamma$-[\textsuperscript{32}P]-ATP - [\textsuperscript{3}H]-UTP and a $\gamma$-[\textsuperscript{32}P]-GTP - [\textsuperscript{3}H]-UTP system was measured as a function of inhibitor concentration. The results are shown in Figure 5a, b, c and d. The decline in product formation in the presence of increasing amounts of inhibitor (as evidenced by [\textsuperscript{3}H] incorporation) loosely parallels the decline in the number of chains being initiated with either ATP (as evidenced by [\textsuperscript{32}P] incorporation from $\gamma$-[\textsuperscript{32}P]-ATP) or GTP (as evidenced by [\textsuperscript{32}P] incorporation from $\gamma$-[\textsuperscript{32}P]-GTP) as the first nucleotide. This behaviour indicates that ethidium bromide analogs inhibit RNA synthesis mainly by limiting the number
of chains that can be initiated rather than by reducing the growth rate of initiated chains.

Using this method with circular DNA from *Pseudomonas phage* 2 as template, Richardson (1973a) found that ethidium bromide inhibits RNA chain initiation without interfering with chain elongation. However, with a linear template, ethidium bromide appeared to inhibit to a significant extent chain elongation as well, especially at high inhibitor concentrations.

The extent to which each drug inhibits (in addition to its primary effect on chain initiation) chain elongation is quantitatively depicted in the insets to Figure 5a, b, c and d. Theoretically the ratio $[{}^{3}H]/[{}^{32}P]$ is a measure of the degree of polymerization of the average product chain. In the extreme case characterized by inhibition of initiation and the absence of any effect of the inhibitor on the rate of chain elongation, the degree of polymerization of the product should remain constant with increasing inhibitor concentration, i.e., the $[{}^{3}H]/[{}^{32}P]$ ratio should exhibit a slope of zero. In the case where inhibition is exerted only at the level of chain elongation, a line with a negative slope should be obtained. The slope of this line should be equal to that of the activity vs. inhibitor concentration curve. Accordingly, compounds inhibiting both steps of RNA synthesis would be expected to yield lines of negative slopes, the steepness of which would be a
Figure 5. Inhibition of the rate of total RNA synthesis and the rate of RNA chain initiation. $[^{3}\text{H}]-\text{UMP}$ incorporation, -$\circ$-; $\gamma-[^{32}\text{P}]-\text{ATP}$ incorporation, -$\bullet$-; $\gamma-[^{32}\text{P}]-\text{GTP}$ incorporation, -$\triangle$-; for a) EB; b) MAPEC; c) DMEB and d) DEMB. In each case, the ratio of $[^{3}\text{H}]$ vs. $[^{32}\text{P}]$ incorporation, -$\circ$-, was calculated using the formula

$$\frac{\%[^{3}\text{H}]}{\%[^{32}\text{P}]} = \frac{5 \times \%[^{3}\text{H}]-\text{UMP}}{4 \times \%\gamma-[^{32}\text{P}]-\text{ATP} + \%\gamma-[^{32}\text{P}]-\text{GTP}}$$

This formula is used because, in the absence of inhibitor, four RNA molecules are initiated with ATP for every molecule initiated with GTP as determined by the molar ratio of A-starters to G-starters. This ratio was calculated from these data under conditions of identical UMP incorporation. $2.5 \times 10^4$ cpm of $[^{3}\text{H}]-\text{UMP}$, $5 \times 10^3$ cpm of $\gamma-[^{32}\text{P}]-\text{ATP}$ and $2 \times 10^3$ cpm of $\gamma-[^{32}\text{P}]-\text{GTP}$ were incorporated in the absence of inhibitor. Each bracket represents standard deviation of the mean of six determinations. The DNA concentration was 30 $\mu\text{M}$. The specific activity of $\gamma-[^{32}\text{P}]-\text{labelled nucleotides}$ was 1.0 $\mu\text{Ci/nmol}$ and that of $8-[^{3}\text{H}]-\text{UTP}$ was 0.01 $\mu\text{Ci/nmol}$. 
(b) MAPEC.
(d)
measure of the relative degree of inhibition of the initiation and elongation steps.

The two strongest inhibitors, EB and MAPEC, appear to have, in addition to their major effect against RNA chain initiation, a minor effect on the rate of chain elongation as well. The negative slope observed in the inset to Figure 5a indicates that with a linear DNA such as T₂ DNA as template high concentrations of ethidium bromide decrease to some extent the rate of elongation although the main effect is still exerted on the number of chains that can be initiated. This result is in agreement with previous reports (Richardson, 1973). MAPEC appears to inhibit chain elongation to a lesser degree than ethidium bromide as indicated by the reduced slope of the curve in the inset to Figure 5b. On the other hand, both DMEB and DEMB appear to behave essentially as initiation inhibitors and have a very small, if any, effect on RNA chain elongation (Figure 5c and d). DMEB and DEMB may therefore be used most profitably in studies of the mechanism of inhibition of RNA synthesis by chain initiation inhibitors.

Some information, albeit tentative, about the relative number of RNA molecules starting with ATP vs. GTP may be obtained from the activity curves in Figure 5a, b, c and d. Under the conditions of these experiments a ratio of A to G starters of A was observed. This ratio which depends largely on experimental conditions is within the range of
1-6 observed with holoenzyme and T₄ DNA as template (Maitra et al., 1967). Under experimental conditions different from those used here, a ratio of 1.3 was obtained with T₂ DNA as template (Maitra et al., 1967). It may be observed that in every case the incorporation of radioactivity from γ-[³²P]-GTP declines faster with increasing inhibitor concentration than the incorporation from γ-[³²P]-ATP. It thus appears as if the initiation of RNA molecules which carry GTP as their terminal 5' nucleotide (G-starters) is inhibited more effectively than RNA molecules with an ATP as their 5' terminal nucleotide (A-starters). This finding, which pertains to a linear DNA, is in contrast to a previous report (Richardson, 1973) that A-starters are inhibited by EB more effectively than G-starters when a circular DNA template from Pseudomonas phage Z is used. It is possible that the physical state of the template is responsible for the preferential inhibition of A-starters by ethidium bromide when the superhelical PM2 DNA is used as template. However, the relatively high experimental error associated with measurements of [³²P] incorporation in experiments of this type requires a cautious interpretation of small differences in the exact values of the activity curves as those observed in Figure 5a, b, c and d.
6. The Effect of Ethidium Bromide and DEMB on the Steps of RNA Chain Initiation

Inhibitors of DNA-dependent RNA polymerase may be expected to exert their effect at one or more of the various steps involved in RNA synthesis. Inhibition may be the result of an alteration in either the rate or the number of enzyme molecules traversing any given step or a combination of the two effects. In the previous section it was shown that EB and DEMB inhibit RNA synthesis by limiting the number of enzyme molecules that can form the first phosphodiester bond of the product without significantly affecting the rate of polymerization of the initiated chains. This limitation in the number of chains growing in the presence of EB or DEMB results from the inhibition of a step prior to the formation of the first phosphodiester bond and may, in turn, reflect an effect of the inhibitor on the rate and/or the number of enzyme molecules traversing that prior step or steps.

As described in the Introduction, two major steps are presently recognized in the process of RNA chain initiation by DNA-dependent RNA polymerase. Methods which allow the independent determination of the number and the rate of enzyme molecules which are traversing each of these steps have also been developed (Mangel and Chamberlin, 1974a and c). These methods when used in the absence and in the
presence of an inhibitor, such as ethidium bromide or DEMB, which have been shown not to inhibit chain elongation appreciably, can provide information concerning the effect of the inhibitor on either one or both of these parameters.

a. **Ethidium Bromide and DEMB do not Affect the Rate of the Second Initiation Step**

The effect of the two intercalating drugs ethidium bromide and DEMB on the rate and the number of RNA polymerase molecules traversing the second initiation step (see Introduction) was studied by determining the level of enzymatic activity as a function of rifampicin concentration in the presence and in the absence of these inhibitors.*

The results of these experiments were analyzed according to the equation described by Chamberlin (1974):

\[
\frac{C_T - C^*}{C^*} = k_2 \cdot \frac{k^*}{[\text{rifampicin}]}.
\]

where \( C_T \) and \( C^* \) are the total binary RNA polymerase-DNA complex concentration and the concentration of binary complexes able to initiate an RNA chain respectively; and \( k_2 \)

*Heretoforth the terms inhibitor, inhibited reaction, etc., will be reserved only for intercalating compounds such as EM, DEMB, actinomycin D, etc. Rifampicin, although a strong inhibitor of RNA polymerase in itself, is merely being used as a tool in these methods and will always be designated by its full name.
and $k^*$ are the rate constants for rifampicin attack on binary complexes and RNA chain initiation respectively. Since, under our experimental conditions, the measured rates in the presence and the absence of inhibitor ($v$ and $V_{\text{max}}$ respectively) are proportional to the concentration of the complexes, one can therefore write:

$$\frac{C_T - C^*}{C^*} = \frac{V_{\text{max}} - v}{v} = \frac{k_2}{k^*}. \text{[rifampicin]}$$

The results plotted in each case as the ratio of the rate of RNA synthesis without rifampicin ($V_{\text{max}}$), divided by the rate of RNA synthesis with rifampicin ($v$) vs. rifampicin concentration are shown in Figure 6. The slope of each line is a measure of the ratio of the rate constant of rifampicin attack on the enzyme over the rate constant of chain initiation through the second initiation step (Mangel and Chamberlin, 1974a). If we assume that the rate of rifampicin attack on the enzyme is the same in the presence as well as in the absence of inhibitor, then the slope of each line in Figure 6 is a measure of the rate constant of initiation through the second initiation step. The near identity of the slopes for the inhibited and uninhibited reactions indicates that the presence of either EB or DEMB has no effect on the rate constant of RNA polymerase molecules which proceed from their RS complexes to form the first phosphodiester bond. These
Figure 6. The dependence of the relative rate of RNA synthesis on rifampicin concentration. The rate of incorporation of radioactivity into product in the absence of both inhibitors and rifampicin ($V_{max}$) was $8.4 \times 10^3$ cpm. $v$ is the rate of the reaction in the presence of rifampicin only, -•--; in the presence of rifampicin and an $r_b = 0.035$ for EB, -□--; or in the presence of an $r_b = 0.50$ for DEMB, -○-. Enzymatic activity was assayed in 0.1 ml of assay buffer B containing 0.4 mM XTP (0.1 μCi/nmol $8^{-[3}H{-]}$-ATP), 2 μg RNA polymerase and 90 μM T2 DNA. Reaction conditions were 1.5 min at 30°C. The dotted line corresponds to the theoretical curve that would be obtained using the equation on page 72 in the presence of an inhibitor causing a 25% decrease in the rate of the control reaction ($v$) without affecting the number of active enzyme molecules. The slope of this curve relative to the control reaction is a measure of the sensitivity of this method and demonstrates that all experimental curves in this Figure are parallel to one another within experimental error.
results also indicate that it is unlikely that either EB or DEMB affect the rate constant of rifampicin attack on RNA polymerase. Otherwise, these inhibitors would have to exert two equal and opposite effects on the rate constants governing initiation and rifampicin attack; i.e., two distinct reactions among different reactants and sites on the enzyme. It is established that rifampicin attacks the site responsible for nucleotide binding (Wu and Goldwaith, 1969), while the DNA bound inhibitors would be thought to interfere with the template binding site of the enzyme.

The intercept of each line on the $V_{\text{max}}/v$ axis of Figure 6 is, on the other hand, a measure of the maximum number of RNA polymerase molecules which are able to transform the RS complex to the rifampicin-resistant ternary complex (Mangel and Chamberlin, 1974a). In the presence of EB at an $r_b$ of 0.035 and DEMB at an $r_b$ of 0.050, the value for this intercept is 36γ and 47% respectively of the value of the uninhibited reaction which indicates that both inhibitors strongly limit the number of enzyme molecules that can traverse the second initiation step. It should be emphasized, however, that the decrease in the $V_{\text{max}}/v$ intercept may be the result of either one of two possible causes. One possibility is that EB and DEMB may limit the number of enzyme molecules that can initiate directly by preventing the formation of
the RS complex. Alternatively, EB and DEMB may act by inhibiting a step prior to the formation of the RS complex. In either case, identical experimental results would be expected since the method employed merely measures the effect of the inhibitor on the maximum number of enzyme molecules that can form RS complexes irrespective of the origin of the effect. Further experimentation is therefore necessary in order to distinguish between these two possibilities.

b. The Rate of RNA Polymerase Molecules Traversing the First Initiation Step is not Affected by the Presence of Either Ethidium Bromide or DEMB

The effect of EB and DEMB on the rate and the number of RNA polymerase molecules traversing the first initiation step was studied by determining enzymatic activity as a function of pre-incubation time of the I complex in the presence and in the absence of rifampicin and one of the intercalating inhibitors (Methods). The results plotted as activity (cpm) vs. pre-incubation time are shown in Figure 7a and b. It is apparent that the curves in Figure 7a and b display the same overall shape; i.e., a rapid increase in the initial rate of incorporation of radioactivity is observed during the first two minutes of pre-incubation time, which is followed by a much more gradual increase of this rate at longer times. The control
Figure 7. The rate of RNA synthesis (cpm) as a function of pre-incubation time (sec) in the absence, -o-, and in the presence, -●-, of 30 μg/ml of rifampicin. a) in the presence of an $r_b = 0.035$ of EB with, -▲-, or without, -▲-, 30 μg/ml rifampicin; b) in the presence of an $r_b = 0.050$ of DEMB with, -▲-, or without, -▲-, 30 μg/ml rifampicin; c) a composite plot of (a) and (b) showing the dependence of the logarithm of enzymatic activity (%) at infinite pre-incubation time, minus enzymatic activity (%) at time (t) vs. the pre-incubation time (t). Activity (%) is measured as the cpm incorporated in the presence of rifampicin divided by the cpm incorporated in its absence when no inhibitor was present, -o-, or when EB, -▲-, or DEMB, -●-, was present. The activity levels corresponding to 160 sec of pre-incubation time are taken as the activity level at infinite time. Enzymatic activity was assayed in 0.1 ml buffer B containing 50 μM $T_2$ DNA and 40 μg/ml RNA polymerase as described under Methods. The nucleoside triphosphate concentration was 0.4 mM (0.1 μCi/nmol 8-[3H]-ATP).
pre-incubation time, sec
(c)

**Activity (\% of control)**

**Pre-incubation time (sec)**

- EB
- DEMB
reaction, obtained in the absence of both rifampicin and inhibitor (see footnote on page 71), reaches maximum activity within the first minute of pre-incubation time, as expected (Mangel and Chamberlin, 1974c).

The gradual rise in activity observed between two and thirty minutes of pre-incubation time appears to be faster in the presence of both DEMB and rifampicin (or, to a lesser extent, in the presence of EB and rifampicin) than the rise in activity in the presence of rifampicin alone. As it will be discussed later, this apparent paradox may be due to the ability of the enzyme to overcome the inhibitory effect of DEMB (or EB) presumably by displacing at a very slow rate the intercalated drug from sites suitable for specific enzyme binding on the template.

Figure 7c was constructed for the purpose of determining the half-life time of the I complex in the presence and in the absence of inhibitor. The incorporation of radioactivity (%) at infinite pre-incubation time ($t_\infty$) minus the incorporation of radioactivity (%) at time ($t$) is shown in a logarithmic scale as a function of pre-incubation time ($t$). (Incorporation of radioactivity per cent is the cpm incorporated in the presence of rifampicin divided by the cpm incorporated in its absence.) (Mangel and Chamberlin, 1974c). The results obtained in the absence of inhibitor as well as those obtained in the presence of either DEMB or EB yield lines of similar slopes.
and intercepts indicating that the presence of the intercalated inhibitor has little effect on the rate of transformation of the I complex into the RS complex.

On the other hand, the data shown in Figure 7a and b reveal that addition of EB or DEMB together with rifampicin in the enzymatic assay mixture drastically decreases the level of incorporation of radioactivity by RNA polymerase far below the level obtained when rifampicin is added alone.

These data indicate that both DEMB and EB have a strong effect on the enzymatic activity of RNA polymerase above and beyond that exerted by rifampicin. The fact that the half-life time of the I complex is approximately the same in the presence of rifampicin, rifampicin-plus-DEMB and rifampicin-plus-EB indicates that neither DEMB nor EB significantly affect the rate at which RNA polymerase molecules can traverse the first initiation step. Therefore, the decreased level of activity in the presence of the inhibitor would have to be attributed to a decrease in the number of enzyme molecules that can form product.

c. Evaluation of the Previous Results

Ethidium bromide and DEMB have been found not to affect the rate constants of the elongation or the initiation steps although they strongly limit the number of enzyme molecules that can initiate a chain. Yet, EB and DEMB do not inhibit
each and every one of these steps, but they rather interfere with only one step and the effect is being carried over to the others. Two facts allow this conclusion to be drawn. First, concentrations of EB (0.035 r_b) and DEMB (0.050 r_b) that were shown to cause approximately 50% inhibition of the overall enzymatic activity are found to produce roughly the same degree of inhibition when each step is studied independently of the other. Second, the methods employed for the study of the effect of inhibitor on each initiation step merely measure the number of enzyme molecules that are traversing each step in the presence and in the absence of inhibitor, without providing any information as to which particular step or steps are inhibited. Inhibition of either the recognition or the elongation step, for example, could limit the number of enzyme molecules able to form product equally well. This fact, however, does not complicate the determination of the rate constants of the initiation and elongation steps which were specifically shown not to be affected by the presence of either EB or DEMB. It may therefore be concluded that EB and DEMB do not affect the rate constants of RNA polymerase traversing the initiation or the elongation steps, while at the same time, these compounds inhibit one of the steps involved in RNA synthesis. The one step inhibited could be either the recognition step or one of the initiation steps with the restriction that the primary mechanism of inhibition must exclude an effect of
the inhibitor on the rate constants of the two initiation steps. Formation of the non-specific complex has already been shown not to be inhibited by EB (Richardson, 1973), and the rate of chain elongation was shown in section 5 not to be significantly affected by either EB or DEMB.
7. **The Kinetics of Template Inactivation**

In the previous sections evidence was presented that DEMB exerts its inhibitory effect at a single step of RNA synthesis by interfering with the formation of an enzyme-DNA productive complex. However, DEMB may inhibit the formation of this complex through any one (or a combination) or two distinct, general mechanisms. On one hand, intercalation of the drug at a specific site of the template might distort the conformation of that site so that the enzyme would not be able to recognize the site and bind on it productively. In this case, the number of enzyme molecules able to form product in the presence of DEMB would be decreased although those enzyme molecules able to recognize the binding site would be expected to synthesize product at a rate unaffected by the presence of the inhibitor. On the other hand, the presence of drug at a specific site of the template might allow recognition by the enzyme to take place, albeit at a slower rate. In this case, the same number of enzyme molecules could be active in the presence of drug as in its absence, although in the presence of inhibitor these active enzyme molecules would be expected to form product at a reduced rate.

In order to distinguish between these two alternative mechanisms, the effect of the inhibitor on the rate of formation of productive complexes must be determined.
independently of the effect of the inhibitor on the number of enzyme molecules which are able to form complexes of this type.

In 1970, Shih and Bonner demonstrated that computer fitting of experimental data of RNA polymerase activity as a function of the concentration of the DNA template yielded satisfactory fitting to an equation formally analogous to the Michaelis and Menten equation, provided that the enzyme concentration is kept constant. It was thus shown that the DNA template can kinetically be described as a substrate. Accordingly, and for the purpose of deriving kinetic equations, compounds believed to inhibit RNA synthesis by interacting only with the template (substrate) may be treated as substrate inactivators, i.e., compounds that exert their inhibitory effect by lowering the effective concentration of the substrate.

For simple enzymatic systems, inhibition via substrate inactivation has been described (Westley, 1969). However, quantitative treatment of the inactivation of a large template carrying many potential inhibitor binding sites would require a more carefully defined and, therefore, more complex description. Nevertheless, the quantitative description of such a system is greatly facilitated in view of the fact that compounds such as EB and actinomycin D have been shown to interfere with the template function of the DNA but not to act directly on the enzyme. Accordingly,
a general hypothesis can be introduced specifying that in a DNA-drug inhibition system, a DNA-inhibitor (SI) complex may form while an enzyme-inhibitor (EI) complex is not formed. Then, the only additional assumptions to be made regarding this system should concern the properties of any possible ternary enzyme-substrate-inhibitor (ESI) complex. Thus, it may be assumed in turn, that (1) an ESI complex does not form; (2) an ESI complex does form but it cannot lead to product; (3) an ESI complex does form and does lead to product, but it does so as fast as the ES complex; or (4) that an ESI complex does form and does lead to product, but it does so at a rate different than that of the ES complex. For each of these cases, equations can be derived describing the dependence of enzymatic activity on the concentration of DNA as shown in the Appendix. In the derivation of these equations it is assumed that all complex formations preceding the chain elongation are rapid with respect to the elongation step, in agreement with all previous experimental observations. These equations predict that double reciprocal plots of RNA polymerase activity vs. substrate concentration (DNA), constructed on the basis of experimental points obtained in the presence of a constant $r_b$ of inhibitor, should produce straight lines with intercepts on both axes characteristic of the mechanism of inhibition.
In order to test the suitability of the equations describing quantitatively the effect of a rate inhibitor, actinomycin D was used, which has previously been shown to inhibit the rate of RNA chain elongation without affecting the number of chains that are being initiated (Hyman and Davidson, 1970). At the same time, in a similar but separate experiment, the kinetic behaviour of DEMB was tested using the method described below.

Reaction mixtures were prepared in which the concentration of DNA and that of the inhibitor (actinomycin D or DEMB) were adjusted so as to produce a constant ratio of bound drug to DNA nucleotide \( r_b \) as the absolute values of concentration for both reagents were being increased. The amount of inhibitor mixed with a given amount of DNA in order to obtain a constant \( r_b \) value was calculated using equation 5, which is directly derived from the Scatchard equation (equation 4, section 1 of the Results).

\[
[I_0] = [DNA] \cdot r_b + \frac{k_{diss} \cdot r_b}{n - r_b}
\]  

(5)

where \([I_0]\) is the concentration of the inhibitor to be mixed with the concentration of DNA, \([DNA]\).

The binding parameters \( k_{diss} \) and \( n \) shown in Table 1 were used for DEMB. For actinomycin D, the values reported by Müller and Crothers (1968) were used, adjusted according to Hyman and Davidson (1970), i.e., \( k_{diss} = 0.7 \ \mu M, \ n = \)
The binding parameters for actinomycin D were adjusted by taking into account the difference in the temperature between the experimental conditions reported by Müller and Crothers (1968) and those employed for the RNA polymerase assays.

The reaction mixtures used to assay RNA polymerase in the presence of DEMB or actinomycin D were processed as described previously (Methods) and the results were fitted into double reciprocal plots of activity vs. substrate concentration [DNA] as shown in Figure 8.

a. Inhibition by Actinomycin D

In the presence of constant \( r_b \) of actinomycin D, the intercept in Figure 8 on the reciprocal activity axis (\( V_{\text{int}} \)) appears to be lower than the \( V_{\text{int}} \) of the uninhibited reaction while the intercept on the reciprocal substrate axis (\( K_{\text{int}} \)) has the same value with the uninhibited reaction. This effect of the inhibitor on the values of \( V_{\text{int}} \) and \( K_{\text{int}} \) is predicted only for "rate inhibitors" as shown in Table 5. Therefore, among the various mechanisms examined, actinomycin D appears to fit only the mechanism predicted by equation 13 of the Appendix. This equation describes the kinetic behaviour of compounds that inhibit RNA synthesis by forming, with a productive enzyme-DNA complex, a ternary complex which cannot break down to form product. In other words, actinomycin D does not interfere with the number of
Figure 8. Double reciprocal plots of the dependence of the rate of RNA synthesis on the concentration of the template in the absence of inhibitor, -○-, and in the presence of DEMB at an $r_b = 0.055$, -■-, or actinomycin D at an $r_b = 0.00085$, -○-. Each experimental point represents the mean of three determinations; standard deviation of the mean was less than 9%. RNA polymerase (4 μg) was assayed in 0.1 ml of buffer A containing 0.8 mM each of ATP, GTP and CTP and 0.4 mM 8-[$^3$H]-UTP (0.1 μCi/nmol). Reactions were carried out for 7 min at 37°C as described under Methods.
enzyme molecules forming productive ES complexes, although it eventually combines with such a binary complex to form an inactive ternary ESI complex. Since actinomycin D inhibits the rate of chain elongation, the large majority of the ESI complexes must be formed between the inhibitor and the propagating binary complexes. Formation of the ESI complex forces the enzyme to terminate synthesis since the ESI complex cannot break down to form product.

This model is in agreement with the well accepted view that actinomycin D inhibits RNA synthesis by decreasing the growth rate of the RNA chains without interfering with the number of RNA chains which are being initiated (Maitra et al., 1967; Hyman and Davidson, 1970).

b. Inhibition by DEMB

In the presence of a constant $r_b$ of DEMB the intercept of the plot shown in Figure 8 on the reciprocal substrate axis ($K_{int}$) appears to be larger than the $K_{int}$ of the uninhibited reaction, while the intercept on the reciprocal activity axis ($V_{int}$) has the same value with the uninhibited reaction. This effect of DEMB on the values of $K_{int}$ and $V_{int}$ is distinct from the pattern predicted for actinomycin D by equation 13, but appears to be in general agreement with the pattern predicted by either equation 11 or equation 15 of the Appendix (see also Table 4).
Table 4. Intercepts of Double Reciprocal Plots

<table>
<thead>
<tr>
<th>Type</th>
<th>Intercept on Velocity Axis ((V_{int}))</th>
<th>Intercept on DNA Substrate Axis ((K_{int}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Inhibition</td>
<td>1</td>
<td>(\frac{1}{K'1})</td>
</tr>
<tr>
<td>Binding Site Inactivation</td>
<td>(\frac{1}{V_{max}})</td>
<td>(\frac{1-r_b}{K'1})</td>
</tr>
<tr>
<td>&quot;Rate&quot; Inhibition</td>
<td>(\frac{1}{V_{max}(1-r_b)})</td>
<td>(\frac{1}{K'1})</td>
</tr>
<tr>
<td>Mixed Binding Site Inactivation</td>
<td>(\frac{1}{V_{max}})</td>
<td>(\frac{1-r_b}{K'1} + \frac{K_3\cdot r_b}{K'1})</td>
</tr>
<tr>
<td>Mixed Inhibition</td>
<td>(\frac{(1-r_b) + K_3\cdot K'1\cdot r_b}{V_{max}(1-r_b) + V_{max}\cdot K_3\cdot r_b})</td>
<td>(\frac{1-r_b + K_3\cdot r_b}{K'1})</td>
</tr>
</tbody>
</table>
In order to distinguish graphically the pattern predicted by equation 11 from the pattern predicted by equation 15, the set of experimental data obtained in the presence of constant DNA concentration described in section 4 was used. For experiments carried out in the presence of constant DNA concentrations equation 11, but not equation 15, predicts that a plot of

\[
\frac{V_{\text{max}}}{v} \text{ vs. } \frac{1}{1 - r_b}
\]

should yield a straight line having an intercept of 1.0 on the activity axis. Accordingly, if DEMB follows the mechanism corresponding to equation 11, then a plot constructed with the experimental data obtained in section 4 should yield the pattern predicted by this equation. Nevertheless, when this plot is constructed, a curve of increasing slope instead of the expected straight line is obtained (Figure 9). However, when the same data are plotted as

\[
\frac{V_{\text{max}}}{v} \text{ vs. } \frac{1}{1 - \frac{r_b}{r_I}}
\]

(where \(r_I\) has the value shown in Table 3) a straight line is obtained with an intercept of approximately 1.0 on the activity axis (Figure 9). This latter plot is not the
Figure 9. A double reciprocal plot of the dependence of the rate of RNA synthesis on the ratio of DEMB bound per DNA nucleotide \((1 - r_b)\), -o-; or the same ratio divided by the ratio of DEMB bound per DNA nucleotide that results in complete inhibition \((1 - r_b/r_I)\), -•-. The graphs are replots of the data shown in Figure 4.
\[ \frac{V_{\text{max}}}{V} \] vs \[(1 - r_b)^{-1}\]

DEMB

\[ \frac{1}{1 - \frac{r_b}{R_i}} \] vs \[(1 - r_b)^{-1}\]
result of an arbitrary modification of equation 11. Rather, it results from a normalization which must be introduced in order to account for: a) the uncertainty regarding the exact size of the DNA length unit which is being inactivated by a single inhibitor molecule; b) the distribution of the inhibitor on the specific promoter sites relative to the rest of the DNA molecule; and c) the fact that the usual expressions of $r_b$ in terms of inhibitor molecules bound per DNA nucleotide, DNA base pair, DNA molecule, and so forth are purely arbitrary.

As evidenced by the results shown in Figure 9, introduction of the value of $r_I$ into equation 11 can, at least mathematically, compensate for the lack of specific information on these important aspects of the mechanism of template inactivation. Therefore, the value of $r_I$ must be introduced not only into equation 11, but also into equation 16 as shown by equations 11' and 16', respectively.

\[
\frac{V_{\text{max}}}{v} = 1 + \frac{K_I'}{1 - \frac{r_b}{r_I}} \cdot \frac{1}{S_0} \quad (11')
\]

\[
\frac{V_{\text{max}}}{v} = 1 + \frac{K_I'}{1 - \frac{r_b}{r_I}} \cdot \frac{1}{S_0} \quad (16')
\]
One additional test for the necessity of introducing \( r_I \) into equations 11 and 16 is the fact that for the limiting case \( r_b = r_I \), equations 11' and 16' yield \( v = 0 \) and for the case \( r_b = 0 \), the term containing the inhibitor concentration disappears and equations 11' and 16' become identical with the equation for the uninhibited reaction. Furthermore, plots of \( \frac{v_{\text{max}}}{v} \) vs. \( \frac{1}{1 - \frac{r_b}{r_I}} \) for the phenanthridines shown in Table 3, also yield straight lines if, and only if, their corresponding \( r_I \) values shown in Table 3 are introduced into equation 11' (Figures not shown).

Equations 11' and 16' differ only by the factor \( \frac{K_1-K_3}{K_1} \) which appears in the denominator of equation 16'. Accordingly, the two equations remain distinct only if the value of \( K_3 \) is not negligible relative to the value of \( K_1 \). For values of \( K_3 \) which are less than 10% of \( K_1 \), equations 11' and 16' may thus appear indistinguishable. Consequently, among the various mechanisms of inhibition, DEMB (and most probably by analogy EB, MAPEC and DEMB as well) appear to act by interfering with the formation of the first productive enzyme-DNA complex I.
8. Alternative Kinetic Methods

In the previous section it was demonstrated that the reciprocal of RNA polymerase activity depends linearly on the reciprocal of DNA concentration in the absence of inhibitor or in the presence of a constant ratio of inhibitor bound per DNA nucleotide ($r_b$). However alternative kinetic methods have been extensively used and reported in the literature. In these methods the experimental data are also plotted in the form of the reciprocal of activity vs. the reciprocal of DNA concentration, but the experiments are conducted in the presence of either a constant amount of inhibitor or a constant ratio of inhibitor added per DNA nucleotide rather than a constant ratio of inhibitor bound per DNA nucleotide.

In order to determine the suitability of these alternative kinetic methods for describing quantitatively the inhibition of RNA polymerase by intercalating drugs, experiments were conducted under conditions of constant drug concentration and constant added drug-to-DNA ratio and the results were presented in double reciprocal plots of activity vs. DNA concentration as described below.

a. Assaying RNA Polymerase in the Presence of a Constant Ratio of Inhibitor Added per DNA Nucleotide

In the specific case of an inhibitor like ethidium
bromide, DEMB or actinomycin D which were shown to act by altering the template properties of the DNA, the enzymatic activity must be assayed with an inhibitor-complexed template whose composition remains constant, as the parameter under study is varied. Constant template composition may readily be achieved experimentally when the template properties of chromatin for instance, which is a mixture of DNA template with inhibiting histone proteins, are being studied (Shih and Bonner, 1970; Cedar and Felsenfeld, 1973; Keshgegian and Furth, 1972). The high affinity of the histones for DNA apparently results to an approximately constant ratio of bound protein per DNA nucleotide as the concentrations of the DNA and protein components are increased in a fixed ratio. However when this method is adopted for the study of complexes of DNA with compounds having weaker binding than the histones, as the case is with EB and DEMB, use of increasing concentrations of a drug-DNA complex of a given added ratio would result in wide variations of \( r_b \) within the range of DNA concentrations assayed for enzymatic activity. This point can readily be demonstrated with the use of equation 5 of section 8 which allows calculation of the values of \( r_b \) for any set of DNA and inhibitor concentrations based on the corresponding binding constants.

When the concentrations of DNA and actinomycin D in a constant ratio are varied over a ten fold range the values
of $r_b$ remain almost unchanged. On the other hand, variation of the concentrations of DNA and DEMB over the same range results in a three fold change in the values of $r_b$ due to the fact that the affinity of DEMB for DNA is approximately 40 times lower than that of actinomycin D.

In order to investigate the relevance of this observation to the methods of assaying RNA polymerase activity in the presence of increasing amounts of inhibitor-DNA complexes of a constant ratio, the concentrations of such complexes of DNA with actinomycin D and DEMB were varied over a ten fold range and assayed for enzymatic activity. The results shown in Figure 10 reveal the expected linear dependence between the reciprocal of enzymatic activity and the reciprocal of DNA concentration in the absence of inhibitor and in the presence of a constant added ratio of actinomycin D. On the other hand, severe deviations from linearity occur in the presence of a constant added ratio of DEMB which, as mentioned, has a much weaker affinity for the template than actinomycin D. In the case of DEMB, the values of $r_b$ change three fold over the DNA concentration range that was assayed for activity. This variation in $r_b$ produces a template of altered composition which, therefore, can support RNA synthesis at varying effectiveness.
Figure 10. Double reciprocal plots of the rate of RNA synthesis as a function of template concentration in the absence of inhibitor, -●-, and in the presence of a constant ratio of DEMB added per DNA nucleotide (0.35), -□-; or a constant ratio of actinomycin D added per DNA nucleotide (0.00115), -○-. Reaction conditions were identical with those in the legend to Figure 8.
b. Assaying RNA Polymerase in the Presence of a Constant Amount of Inhibitor

Further demonstration of the effect of template composition on enzymatic activity can be made by assaying RNA polymerase at increasing DNA concentrations in the presence of a constant amount of inhibitor. In experiments of this type, the $r_b$ of the inhibitor continuously decreases with increasing concentration of DNA as it can readily be calculated again with the use of equation 5, section 7 of Results. As a result of this gradual decrease in $r_b$, the double reciprocal plots shown in Figure 11 exhibit negative curvilinearity. The presence of curvilinearity in plots of this type has been reported previously although the reasons for this behaviour have not been discussed (Waring, 1965a).

It may parenthetically be noted that the curves of the double reciprocal plots shown in Figure 11 appear to converge at the same point of maximum velocity despite the fact that at least in the case of actinomycin D which is an inhibitor of the rate of chain elongation, the enzyme may have not been expected to achieve maximum rate of transcription. This unexpected behaviour however is apparently due to the fact that increasingly higher concentrations of DNA bind increasing amounts of drug and eventually "dilute" the inhibitor to the point that inhibitor-free stretches of DNA are created for the enzyme to use for transcription.
Figure 11. Double reciprocal plots of the rate of RNA synthesis as a function of template concentration in the absence of inhibitor, -○-, and in the presence of a constant amount of 15 μM DEMB, -□-, or 0.0382 μM actinomycin D, -■-. Reaction conditions were the same as those described in the legend to Figure 8.
Clearly then, experiments conducted in the presence of either a fixed added ratio of inhibitor per DNA nucleotide or in the presence of a constant amount of inhibitor are inappropriate for describing the properties of the template for transcription. Instead, measurements should be carried out under conditions that produce a template of constant composition, i.e., at a constant bound inhibitor to DNA ratio. Only under such conditions the results would be informative as to whether a given template inactivator inhibits RNA synthesis by interfering with the rate terms of the reaction or the number of sites that are available on the DNA for the binding of the enzyme in the manner described in section 7 of the Results.
DISCUSSION

The structural analogs of ethidium bromide were found to be strong inhibitors of RNA synthesis catalyzed by DNA-dependent RNA polymerase in vitro (sections 1-6 of Results). These compounds were shown to bind to the DNA-template and inhibit RNA synthesis linearly with their template-bound form within the limits of 10 to 90% inhibition. The inhibition of transcription by ethidium bromide and its structural analogs was then found to be the main result of a decrease in the number of RNA chains that can be initiated in the presence of inhibitor and, only to a minor extent, due to a reduction in the rate of elongation of the product chains (section 5). Subsequent examination of the effect of EB and DEMB, two of the inhibitors under study, on each step of the process of RNA chain initiation revealed that the rate constants of RNA polymerase progressing through these steps are not altered by the presence of inhibitor although at the same time enzymatic activity is drastically decreased (section 6).

Although the use of rifampicin in the elucidation of the mechanism of initiation was developed with T7 DNA as a template, initial work on the effect of this inhibitor on RNA polymerase involved T2 DNA as template and comparable results were obtained with both templates (Sippel and
Hartmann, 1971). Moreover, studies with T₄ DNA as template which, as stated in the Introduction, has identical properties with T₂ DNA, have shown that the mechanistic features revealed by the rifampicin method are the same when T₄ DNA is used as template (Hinkle, D. 1971. Ph.D. Thesis, University of California, Berkeley, cited by Chamberlin, 1974).

Based on these results, as well as the independent finding that EB does not inhibit the formation of non-specific complexes between RNA polymerase and DNA (see Introduction), it was concluded that EB and DEMB inhibit either the recognition step or one of the initiation steps by a mechanism which may not involve an effect of these compounds on the rate constants of the two initiation steps (section 6c). However, in order to identify further the primary site of inhibition, the effect of DEMB on the recognition step, i.e., the formation of the first productive complex I, would have to be studied with respect to the rate constants and the number of enzyme molecules forming I complexes in the presence and in the absence of inhibitor. Unfortunately, the formation of the I complex cannot, as yet, be studied directly. Nevertheless, DEMB was shown not to affect the rate constants of the other steps of transcription. Therefore, any method measuring the effect of the inhibitor on the maximum number and the maximum rate of
total product formation may be used to study the effect of this inhibitor on the formation of the I complex.

As already described in section 8 of the Results, the published methods for conducting experiments in the presence of a constant amount of inhibitor or in the presence of a fixed added ratio of inhibitor per DNA nucleotide are not suitable for describing quantitatively the properties of the template for transcription. Instead, measurements should be carried out under conditions that produce a template of constant composition; i.e., at a constant bound inhibitor to DNA ratio. Only under such conditions the results would be informative as to whether a given template inactivator inhibits RNA synthesis by interfering with the rate terms of the reaction or the number of sites that are available on the DNA for the binding of the enzyme.

The results of experiments conducted in the presence of a constant ratio of actinomycin D bound per DNA base (section 7a) revealed that this drug inhibits the rate of chain elongation by forming, with the propagating RNA polymerase, ternary ESI complexes which cannot yield further products causing termination of RNA chain growth. However, Hyman and Davidson (1970) have suggested that the decreased rate of RNA chain growth is due to the fact that actinomycin D slows down the enzyme without forcing it to terminate. A mechanism of inhibition for actinomycin D, in which
the transcribing enzyme can continue to synthesize product beyond an intercalated drug molecule at a reduced rate, would be kinetically equivalent to a model in which the ESI complex forms and breaks down to products at a rate different (slower) than that of the ES complex. Thus, if such a mechanism were correct, it would result, as shown in Table 4, in smaller values for both $V_{\text{int}}$ and $K_{\text{int}}$ in disagreement with the experimental data obtained with actinomycin D (Figure 8). Of course, the possibility still remains that $K_1 = K_3$ or that either mechanism may operate depending on the conditions under which RNA polymerase is assayed. Indeed, according to a proposal by Müller and Crothers (1968), an elongating enzyme molecule may proceed past an intercalated actinomycin D molecule only if that molecule dissociates from the template in the time period required for the addition of the subsequent nucleotide to the growing chain; if not, the enzyme would be forced to terminate. If this model adequately described the mechanism of inhibition of chain elongation by actinomycin D, then the enzyme in a ternary ESI complex would either terminate or slow down depending on the rate of chain elongation relative to that of actinomycin D dissociation from the template. Such rates depend on the temperature, the ionic strength and the type of the template. It is therefore possible that under the conditions of Hyman and
Davidson (0.4 M KCl, T\textsubscript{7} DNA) only a small fraction of RNA polymerase molecules would terminate when encountering an intercalated actinomycin D molecule while under the assay conditions of Figure 8 (0.2 M KCl, T\textsubscript{2} DNA) a major fraction of the transcribing enzyme molecules does so.

The results of the experiments conducted in the presence of a constant ratio of DEMB bound per DNA nucleotide are discussed below.
1. A Model for the Inhibition of RNA Synthesis by Structural Analogs of Ethidium Bromide

The derivation of equation 11' in section 7 of the Results which describes quantitatively the inhibition of RNA polymerase by EB, DEMB, DMEB and MAPEC was based on the following assumptions.

1. Binary complexes between RNA polymerase and inhibitor (EI) do not form.

2. RNA polymerase molecules participating in the formation of the first productive complex (ES) can transcribe without being affected by the presence of inhibitor.

3. Ternary complexes between the inhibitor (either free or template-bound) and a productive ES complex do not form.

4. Inhibition is the result of the limitations imposed on the number of ES complexes that can form. The inhibitor binds to and distorts the conformation of specific DNA recognition sites to the extent that the enzyme cannot recognize such sites and form with them the first productive ES complex.

5. The inhibitor prevents the formation of the first productive complex only, i.e., the formation of the recognition complex I. Inhibition of the formation of a subsequent complex, e.g., the RS or the OP complexes, would be equivalent to diverting some of the already formed ES
complexes into ESI complexes in violation of the previous assumption (3).

According to this mechanism, the primary site of inhibition by DEMB, EB, MAPEC and DMEB is the recognition site for RNA polymerase on the DNA template, and the active form of the inhibitor is the intercalated drug molecules at the recognition site. This conclusion is supported by the results of the experiments conducted with γ-32P-labelled nucleotides and rifampicin which demonstrated that neither the rate of chain elongation nor the rate constants of the two initiation steps are affected by the presence of the inhibitors. The fact that, in the presence of a constant \( r_b \) of DEMB, the maximum rate of the reaction remains identical to that of the uninhibited reaction (Table 4) indicates that inhibition is the result of a decrease in the number of chains that can be initiated, not the result of a decrease in the rate constants governing RNA chain initiation. Otherwise, if it were assumed for a moment that the reason for the observed inhibition is retarded initiation, then, at infinite template concentration carrying a given \( r_b \) of inhibitor, a fraction of the enzyme would still be retarded and the value of \( V_{\text{max}} \) would appear to be decreased.

However, a word of caution concerning a possible misinterpretation of the experimental evidence may be needed here. The results of the kinetic experiments in the
presence of a constant $r_b$ of DEMB were interpreted to indicate that inhibition by this compound is not the result of a decreased rate of recognition. Yet, DEMB may indeed alter the rate of recognition without contributing measurably to the overall inhibition, since the half-life for recognition ($\sim 20$ sec, Chamberlin, 1974) is much shorter relative to the time of pre-incubation of the enzyme-DNA complex (7.0 min) as well as the incubation time (7.0 min). Nevertheless, even if the rate of recognition is slower in the presence of DEMB, its magnitude is not altered enough for it to become an important contributor to the overall inhibition.

Inhibition by EB, DEMB, DMEB and MAPEC is a measure of the ratio of inhibitor-distorted vs. inhibitor-free recognition sites which, naturally, depends on the value of $r_b$ of each inhibitor. Therefore, other factors being equal, the inhibitory capacity of compounds obeying this mechanism should only be dependent upon their affinities for the recognition site on the template. In other words, the inhibitory capacity of the template-bound forms of this class of inhibitors should not vary among the individual compounds.

Yet, differences were observed among the inhibitory effectiveness of the intercalated forms of ethidium bromide and its structural analogs. Specifically, intercalated
molecules of EB inhibit RNA synthesis more effectively than intercalated molecules of DEMB, DMEB, or MAPEC (Table 3).

A reasonable explanation for the observed differences in the inhibitory effectiveness of the intercalated phenanthridines, which is compatible with the experimental evidence, is presented below.

a. **The Inhibitory Effectiveness of the Drug-DNA Complex may be a Function of its Dissociation Rate Constants**

Every intercalated inhibitor molecule must exist in a state of dynamic equilibrium with its free form in solution. At any concentration of DNA and inhibitor, the fraction of inhibitor molecules located within the intercalation site is naturally determined by the magnitude of the association constant, while the average life-time that an inhibitor molecule spends in the intercalation site is determined by the magnitude of the dissociation rate constant of the template-bound inhibitor. In other words, the dissociation rate constant, $k_{diss}$, of a given inhibitor will determine, to a large extent, the time period for which an inhibitor molecule intercalated at the recognition site will maintain this site distorted (see p. 111).

If within this time period, the distorted recognition site happens to be approached by an enzyme molecule,
recognition will not take place and a productive complex will not form. If, on the other hand, the inhibitor happens to dissociate at a time when an enzyme molecule is in the vicinity of the recognition site and within a distance permitting formation of the I complex, the inhibitor will be "substituted" by the enzyme on the recognition site. This is expected, because the latter has a much higher affinity for the template (5-6 orders of magnitude) than the inhibitor--mainly due to a $k_{diss}$ of the inhibitor 8-10 orders of magnitude smaller than the $k_{diss}$ of the enzyme (Bresloff and Crothers, 1975; Chamberlin, 1974).

Thus, intercalated inhibitor molecules which dissociate more slowly from the template would be stronger inhibitors of RNA synthesis since the enzyme is given less time for forming a productive complex at a site unoccupied by inhibitor. Therefore, the order of inhibitory effectiveness of the template-bound forms of compounds like EB, MAPEC, DEMB and DMEB which follow the mechanism of inhibition of RNA synthesis described quantitatively by equation 11', should be inversely related to the magnitude of their dissociation rate constants.

For any of these inhibitors and at any $r_b$ below $r_I$, two kinds of recognition sites exist in solution: a) inhibitor-free sites on which the enzyme can form productive complexes at its normal rate and b) inhibitor-carrying
sites which the enzyme cannot recognize except during the time the inhibitor momentarily dissociates from them. The inhibitor-free and the inhibitor-carrying sites are of course in dynamic equilibrium with one another (as well as with the rest of the DNA molecule) via the free form of the inhibitor and their relative population would be determined by the value of $r_b$.

When RNA polymerase is mixed with DNA at 0°C and then is incubated at 30°C for increasing periods of time before being assayed for RNA synthesis, a sharp increase in enzymatic activity at short pre-incubation times is observed followed by a much slower rise in activity at longer pre-incubation times (see section 6b of Results). This biphasic dependence of enzymatic activity on pre-incubation time may be interpreted as follows: The early sharp rise in activity may be associated with the formation of the recognition I complex on inhibitor-free sites and the much slower rise in activity may be attributed to the slow "displacement" of the inhibitor by the enzyme from the inhibitor-carrying recognition sites. The same interpretation could also be given to the biphasic character of the curves describing the formation of the cellulose-nitrate non-filterable complex reported by Richardson (1973a).

According to this explanation, the displacement rate of the inhibitor from the DNA recognition site by RNA
polymerase would be of the order of several minutes. However, the two reactions determining the rate of displacement, i.e., the dissociation of the inhibitor from the DNA and the rate of recognition, are much faster processes ($k_{\text{dissociation}} = 10^2 \text{ s}^{-1}$ for EB; Bresloff and Crothers, 1975; $t_{1/2} \text{ recognition} \sim 20 \text{ sec}$, Chamberlin, 1974). Therefore, the rate of displacement should be a much faster process, having a half-life of the same order of magnitude as the recognition reaction. If, however, a recognition site is inactivated only when more than one inhibitor molecules are present, then, the period of time for which such a site would remain inhibitor-free, i.e., available for recognition, would be much smaller. In this case, the rate of substitution could easily be of the order of several minutes. In fact, the results shown in Figure 4 provide some indication that more than one inhibitor molecules are required for inactivating a recognition site. At least in the case of DEMB and DMEB, inhibition is not observed until approximately one inhibitor molecule is bound per 17 base pairs, a value close to the estimated size ($\sim 20 \text{ base pairs}$; Heyden et al., 1975) of the recognition site, while total inhibition is observed at an $r_b$ of 0.08 which corresponds to approximately three inhibitor molecules bound per recognition site.

As mentioned previously, the order of inhibitory effectiveness of the template-bound forms of EB, DEMB, DMEB
MAPEC should be inversely related to their dissociation rate constants. These parameters, though, have not been measured for the structural analogs of EB. Nevertheless, the magnitudes of the dissociation rate constants have been measured for the intercalated form of proflavine, another inhibitor of RNA synthesis which forms with DNA similar complexes like those of ethidium bromide. It was found that the magnitude of the dissociation rate constant of intercalated proflavine decreases as the temperature of the environment is decreased and as the ionic strength is increased (Li and Crothers, 1969). Accordingly, if the inhibitory effectiveness of the template-bound form of proflavine were indeed a function of the dissociation rate constant of its DNA complex, then, intercalated proflavine should be a stronger inhibitor at lower temperature and higher ionic strength. The inhibitory strength of template-bound proflavine has not been determined as a function of these two parameters. However, the intercalated form of ethidium bromide is indeed found to be a more effective inhibitor at lower temperatures and higher ionic strengths (Richardson and Parker, 1973). Due to the similarities of the DNA complexes of the two drugs, it may be assumed that the dependence of the $k_{diss}$ of the DNA-EB complex on ionic strength and temperature most probably parallels that of the proflavine-DNA complex. Therefore, the fact that the
template-bound form of EB is found to be a more effective inhibitor at low temperatures and higher ionic strengths, i.e., under conditions favoring slower dissociation of the drug-DNA complex, is consistent with the idea that the inhibitory effectiveness of the intercalated phenanthridines is a function of the magnitude of the dissociation rate constant of their template-bound forms.

However, due to the indirect nature of the evidence discussed above, alternative explanations for the observed differences in the inhibitory effectiveness of the template-bound phenanthridines must be considered.

A serious obstacle in the quantitative treatment of the system under study has been the lack of information concerning the exact stoichiometry between the inhibitor and the template, i.e., how many inhibitor molecules inactivate how much of the template. Related to this problem, also, are the lack of information concerning the exact nature of the DNA structural features involved in enzyme recognition, as well as the possibility that the structural analogs of EB possess some degree of preference (binding specificity) for interaction only with certain types of DNA bases. Thus, if the inhibitors under comparison exhibited binding specificity, a direct comparison of the inhibitory effectiveness of their bound forms would not be possible on the basis of plots of
activity vs. $r_b$ of the type shown in Figure 4, since the relationship $r_b = r'_b$ would not necessarily hold true ($r'_b$ is the ratio of inhibitor bound per recognition site as opposed to $r_b$, the ratio of inhibitor bound per deoxy-nucleotide). One, then, might postulate that the observed differences in the inhibitory effectiveness of the bound analogs of ethidium bromide are due to differences in their binding specificities for the template.

This possibility, however, seems unlikely since at least ethidium bromide exhibits no preference for promoter sites on DNA molecules (Giacomoni et al., 1974). The binding constants and the maximum number of binding sites for ethidium bromide of DNA molecules having base compositions varying between 35% and 72% in G + C content have been found to be almost identical and the small differences observed do not correlate to the G + C content of the DNA (Waring, 1965b). These results have, since, been interpreted to indicate lack of binding specificity for ethidium bromide. No evidence for binding specificity on the part of the analogs of ethidium bromide is available, except for the linearity of the Scatchard plots (Figure 1; Kindelis, 1976) which may be interpreted to indicate lack of significant binding preference for these compounds. The situation, however, appears to be different in complexes of ethidium bromide with dinucleotides, in which ethidium bromide exhibits some preference for interaction
with pyrimidine-purine sequence isomers (Krugh et al., 1975). Yet, the results of binding specificity or conformation studies of EB dinucleotide complexes may not be representative of the properties of the DNA ethidium bromide complex (Tsai et al., 1975).

A second possible explanation for the observed differences in the inhibitory effectiveness among the bound forms of the structural analogs of ethidium bromide must be considered. One might be tempted to explain this phenomenon by a mechanism in which intercalation of one (or more) EB molecules distorts the conformation of a recognition site to a larger extent than DEMB so that, in the latter case, some degree of recognition with subsequent formation of productive complexes may take place. Alternatively, one might postulate that the presence of the bulkier phenyl group on the intercalated phenanthridinium ring of EB interferes with recognition more effectively than the smaller substituents on DEMB. Both of these mechanisms, however, should require partial recognition by the enzyme of a DNA site carrying one (or more) inhibitor molecules; i.e., a process equivalent to assuming formation of ternary ESI complexes. Such a mechanism would be in disagreement with the mechanism of inhibition by DEMB if the latter would occur through the inhibition of the formation of an ESI complex. Had the formation of a ternary complex been
responsible for the observed inhibition, the kinetic pattern in the presence of a constant $r_b$ of DEMB would resemble that of actinomycin D, in contradiction with the experimental findings. Therefore, although partial recognition by RNA polymerase of inhibitor-distorted sites on the DNA does probably take place, either its extent and/or its duration is presumably such that it does not constitute a major contribution to the overall inhibition by compounds of the same kinetic behavior as DEMB.

b. The Magnitude of the Dissociation Rate Constants May Distinguish Inhibitors of Chain Initiation from Inhibitors of Chain Elongation

A comparison of the dissociation rate constants of actinomycin D, proflavine and EB reveals that the dissociation rate constant of the "pure" elongation inhibitor actinomycin D is at least 3-4 orders of magnitude slower than that of proflavine (Li and Crothers, 1969; Müller and Crothers, 1968). The latter inhibits RNA chain initiation to the same extent as RNA chain elongation (Maitra et al., 1967). In turn, the dissociation rate constant for proflavine is half an order of magnitude lower than the dissociation rate constant for EB which inhibits RNA chain elongation to a smaller degree than proflavine (Bresloff
and Crothers, 1975). It thus appears that intercalators having small dissociation rate constants inhibit perhaps mainly RNA chain elongation (covalently bound intercalators would be the extreme class of elongation inhibitors). On the other hand, compounds having high dissociation rate constants from the template inhibit mainly RNA chain initiation. This differentiating effect of the magnitude of the dissociation rate constants on the mechanism of inhibition of RNA synthesis by DNA intercalators could perhaps be the result of two factors: a) the different mechanisms of chain recognition and chain elongation by RNA polymerase and b) the small target size of the recognition site (~20 base pairs; Heyden et al., 1975) relative to the size of the "elongation site" (up to 10,000 base pairs; Bremer, 1970). Because of these two factors a slowly dissociating intercalator at low values of $r_b$ would inhibit elongation. Under these conditions the majority of the recognition sites would remain inhibitor-free due to their small target size and, therefore, initiation would not be inhibited. In contrast, a fast dissociating intercalator at low values of $r_b$ would not affect elongation despite the fact that the elongation site would be loaded with inhibitor molecules. Also, at first, initiation would not be inhibited because of the small target size of the recognition site. In the latter system at higher values of
r_{b}, however, the recognition sites would be loaded with inhibitor molecules and the overall inhibition of RNA synthesis would parallel the inhibition of initiation.
2. The Molecular Basis of the Trypanocidal Activity of the Phenanthridines

It has been postulated that the trypanocidal activity of various phenanthridinium compounds resides in the capacity of these compounds to intercalate into the DNA templates and, as a result, interfere with the biological functions and/or the metabolism of these macromolecules (Waring, 1970).

In an attempt to determine the effect of the phenanthridines on particular functions of the DNA molecule and possibly identify the primary target of their pharmacological action, the inhibitory effectiveness of a number of phenanthridines against enzymes utilizing DNA as template or substrate was compared with the trypanocidal activity of these compounds (Kindelis, 1976). The results obtained with DNA polymerase I and pancreatic deoxyribonuclease suggested that the pharmacological action of the structural analogs of ethidium bromide may not parallel their effectiveness as inhibitors of these enzymes \textit{in vitro} (Kindelis, 1976). Therefore, the property of the structural analogs of EB to inhibit strongly at least one enzyme catalyzing DNA repair and one enzyme catalyzing DNA degradation does not appear to be directly related to the pharmacological action of these compounds.

In contrast, with the exception of DMNC, the order but not the magnitude of inhibition of RNA synthesis by a number
of structural analogs of EB is found to parallel their trypanocidal activity (Table 2). Consequently, the pharmacological activity of the phenanthridines appears to be directly related to the ability of these drugs to inhibit transcription by impairing the template properties of the DNA. According to the evidence discussed previously, one could propose that the capacity of at least some of the structural analogs of EB for template inactivation may be determined by the magnitude of the dissociation rate constants of their DNA complexes. According to this hypothesis, the variation in trypanocidal activity among these drugs could in turn be a function of the variation in the magnitude of the dissociation rate constants among the various phenanthridinium derivatives.

In the recent past substantial efforts have been directed at elucidating a possible relationship between the affinity for DNA of compounds believed to act by associating with this macromolecule and the carcinostatic properties of these compounds (Le Pecq et al., 1974). Some studies have also entertained the possibility of a close relationship between the extent of conformational distortion of the double helix upon drug binding and drug action (Wakelin and Waring, 1974). However, in view of the present findings with the structural analogs of ethidium bromide and actinomycin D, attention should be called to one
additional parameter, namely the dissociation rate constants of the drug-DNA complex, which might also play an important regulatory role in drug action. At least in the case of inhibition of transcription by intercalating compounds this parameter might turn out to be the factor that determines not only whether a given drug causes inhibition of RNA chain elongation or initiation but also, which one, among inhibitors having identical mechanisms of action, will exert the strongest overall effect on RNA synthesis.
SUMMARY

The mechanism of inhibition of DNA-dependent RNA polymerase of *E. coli* K-12 by structural analogs of ethidium bromide has been investigated in an in vitro system utilizing T₂ DNA as template.

Ethidium bromide analogs carrying large hydrophobic groups at position 6 of the phenanthridinium ring system are the strongest inhibitors of RNA synthesis.

All of the compounds examined appear to inhibit RNA synthesis as a result of their affinity for DNA by inactivating template molecules rather than enzyme molecules. The concentration of DNA-template bound drug, which is the active form of the inhibitor, is found directly related to the degree of inhibition of RNA polymerase. Furthermore, identical amounts of various template-bound inhibitors effect inhibition to different extents. These differences may be attributed to the individual structural characteristics of these drugs.

Measurements of the simultaneous incorporation of [³H]-uridine triphosphate and γ-[³²P]-adenosine and guanosine triphosphates revealed that phenanthridines inhibit mainly the initiation step of RNA synthesis with very little or no effect on the process of RNA chain elongation. It thus appears that ethidium bromide and its analogs inhibit differentially one or more of the steps recognized in RNA
chain initiation. Inhibition by these compounds might be the result of interference with the number of enzyme molecules attached to initiation sites or with the rate at which these molecules can initiate the synthesis of an RNA chain. Both mechanisms may also be involved. To test these alternatives, the rate and the number of enzyme molecules that proceed through individual initiation steps were measured in the presence and in the absence of inhibitor. The inhibitor was found to have no effect on the rates of either one of the initiation steps. The number of enzyme molecules that are proceeding through these steps, in the presence of ethidium bromide or its analogs was, however, dramatically reduced.

The reduction in the number of enzyme molecules which are able to proceed through the initiation events may be attributed either to an interference with the formation of the first recognition complex or to a decrease in the number of productive complexes that can form subsequent to recognition. These two alternatives were distinguished by determining the dependence of enzymatic activity on template concentration in the presence of a constant ratio of bound inhibitor to the DNA-template. The results are in good agreement with the theoretically predicted behaviour of inhibitors which act by preventing enzyme recognition of template binding sites. Such sites are apparently distorted as a result of intercalation of the drug.
The data also indicate that displacement of the inhibitor from the recognition site by direct interaction between RNA polymerase and the inhibitor-'recognition' site complex does not take place. Consequently, it appears that the enzyme may be able to recognize and bind productively and irreversibly only to recognition sites which are momentarily free of inhibitor. Therefore, the differential inhibitory effectiveness of the DNA complexes formed with structural analogs of ethidium bromide may be related to differences among the dissociation rate constants of these complexes.
REFERENCES


APPENDIX
APPENDIX

I. Explanation of Symbols

S : DNA
(s) : "Inhibitor-free" DNA concentration
I : Inhibitor
(i) : "Free" inhibitor concentration
(s) : DNA-inhibitor complex concentration, i.e. bound inhibitor concentration
r_b : Inhibitor bound per DNA nucleotide (molar ratio)
E : Enzyme
(e) : "Free" enzyme concentration
(es) : Enzyme-DNA complex concentration
(esi) : Enzyme-DNA-inhibitor complex concentration
K' : Enzyme-DNA dissociation constant
K_I : Apparent DNA-inhibitor association constant

II. The Relationship Between (s) and (s)

For an inhibitor interacting with a DNA site according to the expression

\[ S + I \rightleftharpoons SI \]

the following relationship may be derived:

\[ (s) = (s_0) - (si) \]

\[ \frac{(s)}{(s_0)} = 1 - \frac{(si)}{(s_0)} \]

\[ \frac{(s)}{(s_0)} = 1 - r_b \]

\[ (s) = (1-r_b)(s_0) \]  

(1)
III. Kinetics of Template Inactivation

a. Kinetic Scheme:

b. Assumptions:

1. ES does form and does yield product
2. SI does form
3. EI does not form
4. ESI does form and does yield product
5. On the time scale of the experiments described in this Thesis all steps are rapidly reversible with the exception of the formation of p from ES (or ESI).
6. Reinitiation does not alter significantly the rate of the reaction.
c. **Equations**

The following equilibrium expressions may be written to describe the reactions taking place under the scheme shown in Section IIIa of the Appendix.

\[ E_0 = E + ES + ESI \]
\[ (e_0) = (e) + (es) + (esi) \]
\[ (e) = (e_0) - (es) - (esi) \] (2)

\[ E + S = ES \]
\[ (es) = K_1(e)(s) = K_1{(e_0)-(es)-(esi)}(s) \] (3)

\[ S + I = SI \]
\[ (si) = K_I(s)(i) \] (4)

\[ E + SI = ESI \]
\[ (esi) = K_3(e)(si) = K_3{(e_0)-(es)-(esi)}(s) \] (5)

\[ ES + I = ESI \]
\[ (esi) = K_4(es)(i) \] (6)

\[ K_1 \cdot K_3 = K_1 \cdot K_4 \] (7)

\[ K_I(s) \cdot K_4(i) = K_3(si) \] (8)

d. **Derivations**

Based on the general kinetic scheme shown in Section IIIa of the Appendix, the expressions for the ES and the ESI complexes are obtained as shown below. These expressions are subsequently used in deriving the equations describing each particular mechanism of inhibition.
In order to obtain an expression for \((es)\), equation (6) is substituted into equation (3):

\[
(es) = K_1(s)(e_0) - K_1(s)(es) - K_1K_4(s)(es)(i)
\]

\[
(es)(1 + K_1(s) + K_1K_4(s)(i)) = K_1(e_0)(s)
\]

\[
(es) = \frac{K_1(e_0)(s)}{1 + K_1(s) + K_1K_4(s)(i)}
\]

Using equation (8), the above expression can be transformed to:

\[
(es) = \frac{K_1(e_0)(s)}{1 + K_1(s) + K_3(s)(i)} \tag{9}
\]

In order to obtain an expression for \((esi)\), the expression for \((es)\) from equation (6) is substituted into equation (5):

\[
(esi) = K_3(e_0)(si) - \frac{K_3(esi)(si)}{K_4(i)} - K_3(esi)(si)
\]

\[
(esi)\{1 + \frac{K_3(si)}{K_4(i)} + K_3(si)\} = K_3(e_0)(si)
\]

\[
(esi) = \frac{K_3(e_0)(si)}{1 + \frac{K_3(si)}{K_4(i)} + K_3(si)}
\]
Using equation (8), the above expression can be transformed to:

\[
(esi) = \frac{K_3(e_0)(si)}{1 + K_1(s) + K_3(si)}
\]  \hspace{1cm} (10)

1. **Binding Site Inactivation**

**Description:** It is assumed that ternary ESI complexes do not form. Therefore, \(K_3 = 0\). Only binary ES complexes yield product. Therefore, \(V_{\text{max}} = k(e_0)\) and the rate expression becomes

\[v = k(es)\]

Using equation (9), the general expression for \((es)\), the above expression can be transformed to:

\[v = \frac{K_3(e_0)K_1(s)}{1 + K_1(s) + K_3(si)}\]

but it is assumed here that \(K_3 = 0\). Therefore,

\[
v = \frac{V_{\text{max}}(s)}{1 + (s)} = \frac{V_{\text{max}}(s)}{K'_{1} + (s)}
\]

Substituting the expression for \((s)\) from equation (1):

\[v = \frac{V_{\text{max}}(1-r_b)(s_0)}{K'_1 + (1-r_b)(s_0)}\]
\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K'_{\text{1}}}{V_{\text{max}}} \cdot \frac{1}{(1-r_b)} \cdot \frac{1}{(s_0)}
\]
(11)

\[
\frac{V_{\text{max}}}{v} = 1 + \frac{K'_{\text{1}}}{(1-r_b)} \cdot \frac{1}{(s_0)}
\]
(12)

2. "Rate" Inhibition

Description: It is assumed that ternary ESI complexes form but do not yield product. The affinity of E for S is as strong as the affinity of E for SI; therefore, \( K_1 = K_3 \). Only the ES complex can form product. Therefore, \( V_{\text{max}} = k(e_0) \), and the rate expression becomes

\[ v = k(es) \]

Using equation (9), the general expression for (es), the above expression can be transformed to:

\[ v = \frac{k(e_0) \cdot K_1(s)}{1 + K_1(s) + K_3(si)} = \frac{V_{\text{max}}(s)}{K_1 + (s) + \frac{K_3(si)}{K_1}} \]

Substituting the expression for (s) from equation (1):

\[ v = \frac{V_{\text{max}}(1-r_b)(s_0)}{K'_{\text{1}} + (1-r_b)(s_0) + \frac{K_3}{K_1}(si)} \]
\[ v = \frac{V_{\text{max}}(1-r_b)}{K'_{1} + (1-r_b) + \frac{K_3}{K_1} \cdot r_b} \]

but, it is assumed here that \( K_3 = K_1 \). Therefore,

\[ v = \frac{V_{\text{max}}(1-r_b)}{1 + \frac{K'_{1}}{(s_0)}} = \frac{V_{\text{max}}(1-r_b)(s_o)}{K'_{1} + (s_o)} \]

\[ \frac{1}{v} = \frac{1}{V_{\text{max}}(1-r_b)} + \frac{K'_{1}}{V_{\text{max}}(1-r_b)} \cdot \frac{1}{(s_o)} \quad (13) \]

\[ \frac{V_{\text{max}}}{v} = \frac{1}{(1-r_b)} + \frac{K'_{1}}{(1-r_b)} \cdot \frac{1}{(s_o)} \quad (14) \]

3. Mixed Binding Site Inactivation

**Description:** It is assumed that ternary ESI complexes form and yield product at the same rate as the ES complexes, but the affinity of E for S is different than the affinity of E for SI. Therefore, \( K_1 \neq K_3 \), \( V_{\text{max}} = k(e_o) \) and the rate expression becomes

\[ v = k(es + esi) \]
Using equations (9) and (10), the general expressions for \((es)\) and \((esi)\), the rate expression becomes

\[
v = \frac{k\{K_1(e_0)(s) + K_3(e_0)(si)\}}{1 + K_1(s) + K_3(si)}
\]

\[
v = \frac{V_{\text{max}}\{K_1(s) + K_3(si)\}}{1 + K_1(s) + K_3(si)}
\]

Substituting the expression for \((s)\) from equation (1):

\[
v = \frac{V_{\text{max}}\{K_1(1-r_b)(s_0) + K_3(si)\}}{1 + K_1(1-r_b)(s_0) + K_3(si)}
\]

and dividing by \((s_0)\):

\[
v = \frac{V_{\text{max}}\{K_1(1-r_b) + K_3\cdot r_b\}}{1 + K_1(1-r_b) + K_3\cdot r_b}
\]

\[
v = \frac{V_{\text{max}}\{K_1 - r_b(K_1-K_3)\}(s_0)}{1 + \{K_1-r_b(K_1-K_3)\}(s_0)}
\]

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{1}{V_{\text{max}}\{K_1 + r_b(K_3-K_1)\}(s_0)}
\]  

(15)

\[
\frac{V_{\text{max}}}{v} = 1 + \frac{1}{K_1 + r_b(K_3-K_1)} \cdot \frac{1}{(s_0)}
\]  

(16)
4. **Mixed Inhibition**

Description: It is assumed that ternary ESI complexes form and yield product at a different rate than binary ES complexes, but the affinity of E for S is different than the affinity of E for SI. Therefore, $K_1 \neq K_3$ and the rate expression becomes

$$v = k(es) + k(esi)$$

Using equations (9) and (10), the general expressions for (es) and (esi), the above equation becomes:

$$v = \frac{k \cdot K_1(e_0)(s) + k \cdot K_3(e_0)(si)}{1 + K_1(s) + K_3(si)}$$

Substituting the expression for (s) from equation (1):

$$v = \frac{(e_0)\{k \cdot K_1(1-r_b)(s_0) + k \cdot K_3(si)\}}{1 + K_1(1-r_b)(s_0) + K_3(si)}$$

Dividing by $(s_0)$:

$$v = \frac{(e_0)\{k \cdot K_1(1-r_b) + k \cdot K_3 \cdot r_b\}}{1 + K_1(1-r_b) + K_3 \cdot r_b}$$

$$v = \frac{V_{\text{max}} \cdot K_1(1-r_b) + V'_{\text{max}} \cdot K_3 \cdot r_b}{1 + \{K_1(1-r_b) + K_3 \cdot r_b\}(s_0)}$$
\[ \frac{1}{v} = \frac{K_1(1-r_b) + K_3 \cdot r_b}{V_{\text{max}} \cdot K_1(1-r_b) + V'_{\text{max}} \cdot K_3 \cdot r_b} \]

\[ + \frac{1}{V_{\text{max}} \cdot K_1(1-r_b) + V'_{\text{max}} \cdot K_3 \cdot r_b} \cdot \frac{1}{s_0} \] (17)
IV. Data for some figures

a. Data for Figure 1.

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<th>r$_b$ (M$\times$10$^4$)</th>
<th>IF (M$\times$10$^4$)</th>
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<td></td>
<td></td>
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<tr>
<td>satd</td>
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<tr>
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<td>0.1719</td>
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isosbestic points at: 390, 512 nm

| DEMB    |           |               |                  |
| satd    | 0.390     | --            | --              |
| 0.578   | 0.528     | 0.0225        | 0.0501          |
| 0.399   | 0.573     | 0.0285        | 0.0664          |
| 0.263   | 0.631     | 0.0353        | 0.0875          |
| 0.137   | 0.721     | 0.0439        | 0.1201          |
| 0.074   | 0.784     | 0.0504        | 0.1430          |
| --      | 0.886     | --            | 0.1800          |

isosbestic points at: 383, 496 nm

| MAPEC   |           |               |                  |
| satd    | 0.612     | --            | --              |
| 0.840   | 0.702     | 0.0198        | 0.0373          |
| 0.525   | 0.749     | 0.0280        | 0.0567          |
| 0.347   | 0.802     | 0.0361        | 0.0787          |
| 0.221   | 0.866     | 0.0447        | 0.1052          |
| 0.137   | 0.931     | 0.0525        | 0.1321          |
| 0.074   | 1.000     | 0.0586        | 0.1607          |
| --      | 1.104     | --            | 0.2038          |

isosbestic points at: 370, 460 nm

| DMEB    |           |               |                  |
| satd    | 0.485     | --            | --              |
| 0.840   | 0.560     | 0.0209        | 0.0357          |
| 0.525   | 0.601     | 0.0297        | 0.0552          |
| 0.347   | 0.647     | 0.0386        | 0.0772          |
| 0.221   | 0.703     | 0.0486        | 0.1038          |
| 0.137   | 0.764     | 0.0572        | 0.1329          |
| 0.074   | 0.825     | 0.0667        | 0.1619          |
| --      | 0.928     | --            | 0.2110          |

isosbestic points at: 380, 492 nm
b. Data for Figure 2.

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Plotted values for DEMB and DMEB have been multiplied by 12.5 and for the uninhibited reaction by 2.78 in order to normalize the various specific activities of XTP used in each reaction.
c. Data for Figure 3a.

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**DEMB**

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The dissertation submitted by Nikos Panayotatos has been read and approved by the following Committee:

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Loyola University

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Loyola University

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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 requirements for the degree of Doctor of Philosophy.

Date Dec 21, 1976
Director's Signature