1976

The Interaction of Phenanthridinium Derivatives with Nucleic Acids

Antonis Kindelis

Loyola University Chicago

Recommended Citation
http://ecommons.luc.edu/luc_diss/1609

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 1976 Antonis Kindelis
THE INTERACTION OF
PHENANTHRIDINIUM DERIVATIVES
WITH NUCLEIC ACIDS

by
Antonis Kindelis

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

February
1976
ACKNOWLEDGEMENTS

The author would like to thank his advisor, Dr. Stelios Aktipis, for the encouragement and advice that he provided during the course of this work.

Thanks are also due to Laszlo Bodoni, Kenneth Micetich and Nikos Panayotatos for many hours of helpful discussion.

Finally, the help of Vern Arase, John Cheng and Andy Kowalczyk (Information Systems) in the preparation of this manuscript is gratefully acknowledged.
VITA

Antonis Kindelis was born in Athens, Greece. In 1970 he graduated from the University of Agricultural Science of Athens, specializing in Plant Pathology. He joined the Department of Biochemistry and Biophysics of Loyola University in the fall of 1970. He is presently a Research Associate in the Biophysics Laboratory of the University of Wisconsin.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1. Biological activity of phenanthridinium compounds</td>
<td>1</td>
</tr>
<tr>
<td>2. Physicochemical properties of the phenanthridinium-DNA complex</td>
<td>6</td>
</tr>
<tr>
<td>3. The scope of this investigation</td>
<td>12</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>14</td>
</tr>
<tr>
<td>I. Materials</td>
<td>14</td>
</tr>
<tr>
<td>II. Instruments</td>
<td>16</td>
</tr>
<tr>
<td>III. Methods</td>
<td>17</td>
</tr>
<tr>
<td>1. Preparation of stock solutions</td>
<td>17</td>
</tr>
<tr>
<td>2. Preparation of the complex</td>
<td>18</td>
</tr>
<tr>
<td>3. Binding determination</td>
<td>18</td>
</tr>
<tr>
<td>4. Circular dichroism measurements</td>
<td>20</td>
</tr>
<tr>
<td>5. Fluorescence measurements</td>
<td>21</td>
</tr>
<tr>
<td>6. Elevated temperature measurements</td>
<td>22</td>
</tr>
<tr>
<td>7. Deoxyribonuclease assay</td>
<td>23</td>
</tr>
<tr>
<td>8. DNA polymerase assay</td>
<td>26</td>
</tr>
<tr>
<td>9. Activation of DNA</td>
<td>28</td>
</tr>
<tr>
<td>RESULTS</td>
<td>31</td>
</tr>
<tr>
<td>1. The binding of the phenanthridinium drugs to DNA</td>
<td>31</td>
</tr>
<tr>
<td>1.1. Low ionic strength conditions</td>
<td>32</td>
</tr>
<tr>
<td>1.2. High ionic strength conditions</td>
<td>40</td>
</tr>
<tr>
<td>1.3. Binding the presence of magnesium</td>
<td>42</td>
</tr>
<tr>
<td>2. Circular dichroism studies</td>
<td>53</td>
</tr>
<tr>
<td>2.1. Diamino compounds</td>
<td>54</td>
</tr>
<tr>
<td>2.1.1. The circular dichroism at 500 nm</td>
<td>57</td>
</tr>
<tr>
<td>2.1.2. The circular dichroism at 310 nm</td>
<td>60</td>
</tr>
<tr>
<td>2.2. Monoamino compounds</td>
<td>63</td>
</tr>
<tr>
<td>2.2.1. The circular dichroism at 470 nm</td>
<td>68</td>
</tr>
<tr>
<td>2.2.2. The circular dichroism at the maximum near 315 nm</td>
<td>68</td>
</tr>
<tr>
<td>2.3. Circular dichroism of the EB-DNA complex at high ionic strength</td>
<td>76</td>
</tr>
<tr>
<td>3. Fluorescence studies</td>
<td>85</td>
</tr>
</tbody>
</table>
4. Temperature-optical density profiles.......................... 94
5. Inhibition of deoxyribonuclease................................. 106
6. Inhibition of DNA polymerase................................. 124

DISCUSSION.......................................................... 141
1. Factors influencing the stability of the complex.... 141
2. Information on the conformation of the complex obtained by physical methods..................... 145
   2.1. Differences in the origin of the CD bands of the complex............................................. 146
   2.2. The origin of the 310 nm CD band......................... 148
   2.3. Differences in the properties of DNA complexes with mono- and diamino phenanthridinium dyes. 152
       2.3.1. CD evidence.................................................. 152
       2.3.2. Fluorescence evidence................................. 154
3. The complex as an enzymic substrate.................... 158
   3.1. Modification of the DNA substrate as a result of dye binding...................................... 158
   3.2. Inhibition of DNase.......................................... 160
   3.3. Inhibition of DNA polymerase........................... 161
   3.4. Some biological implications of the inhibition of DNA polymerase................................. 164

SUMMARY.......................................................... 167

REFERENCES.................................................. 170
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Binding parameters for the interaction of certain phenanthridinium compounds with DNA in 0.04 M Tris buffer</td>
<td>39</td>
</tr>
<tr>
<td>2. Binding parameters for the interaction of certain phenanthridinium compounds with DNA in the presence of magnesium at room temperature and at 37°C</td>
<td>49</td>
</tr>
<tr>
<td>3. Circular dichroism of the EB-DNA complex at various salt concentrations</td>
<td>79</td>
</tr>
<tr>
<td>4. Dependence of molar ellipticity of the EB-DNA complex near 295 nm on r in 0.04 M Tris buffer</td>
<td>82</td>
</tr>
<tr>
<td>5. Dependence of molar ellipticity of the EB-DNA complex near 295 nm on r in 5.0 M NaCl</td>
<td>84</td>
</tr>
<tr>
<td>6. The effect of certain phenanthridinium compounds on the thermal stability of DNA</td>
<td>104</td>
</tr>
<tr>
<td>7. Association constants for the binding of certain phenanthridinium compounds to DNA</td>
<td>118</td>
</tr>
</tbody>
</table>
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. General formula of phenanthridinium compounds</td>
<td>2</td>
</tr>
<tr>
<td>2. Effect of DNase digestion on templating efficiency of calf thymus DNA</td>
<td>30</td>
</tr>
<tr>
<td>3. Spectrophotometric titration of EB with DNA in 0.04 M Tris buffer</td>
<td>33</td>
</tr>
<tr>
<td>4. Scatchard plot for the EB-DNA interaction in 0.04 M Tris buffer</td>
<td>34</td>
</tr>
<tr>
<td>5. Scatchard plot for the DDMB-DNA interaction in 0.04 M Tris buffer</td>
<td>35</td>
</tr>
<tr>
<td>6. Scatchard plot for the MAPEC-DNA interaction in 0.04 M Tris buffer</td>
<td>36</td>
</tr>
<tr>
<td>7. Scatchard plot for the MAPAC-DNA interaction in 0.04 M Tris buffer</td>
<td>37</td>
</tr>
<tr>
<td>8. Scatchard plot for the MMPB-DNA interaction in 0.04 M Tris buffer</td>
<td>38</td>
</tr>
<tr>
<td>9. Scatchard plot for the EB-DNA interaction in 5.0 M NaCl</td>
<td>41</td>
</tr>
<tr>
<td>10. Spectrophotometric titration of EB with DNA in the presence of magnesium at room temperature</td>
<td>43</td>
</tr>
<tr>
<td>11. Spectrophotometric titration of EB with DNA in the presence of magnesium at 37°C</td>
<td>44</td>
</tr>
<tr>
<td>12. Scatchard plots for the EB-DNA interaction in the presence of magnesium</td>
<td>45</td>
</tr>
<tr>
<td>13. Scatchard plots for the DDMB-DNA interaction in the presence of magnesium</td>
<td>46</td>
</tr>
<tr>
<td>14. Scatchard plots for the MAPEC-DNA interaction in the presence of magnesium</td>
<td>47</td>
</tr>
<tr>
<td>15. Scatchard plots for the MAPAC-DNA interaction in the presence of magnesium</td>
<td>48</td>
</tr>
<tr>
<td>16. CD spectra of the EB-DNA complex at three added EB/nucleotide ratios</td>
<td>55</td>
</tr>
<tr>
<td>17. CD spectra of the DDMB-DNA complex at three added DDMB/nucleotide ratios</td>
<td>56</td>
</tr>
<tr>
<td>18. Dependence of molar ellipticity of the EB-DNA complex near 500 nm on r</td>
<td>58</td>
</tr>
<tr>
<td>19. Dependence of molar ellipticity of the DDMB-DNA complex near 500 nm on r</td>
<td>59</td>
</tr>
<tr>
<td>20. Dependence of molar ellipticity of the EB-DNA complex near 310 nm on r</td>
<td>61</td>
</tr>
<tr>
<td>21. Dependence of molar ellipticity of the DDMB-DNA complex near 310 nm on r</td>
<td>62</td>
</tr>
<tr>
<td>22. CD spectra of the MAPEC-DNA complex at three added MAPEC/nucleotide ratios</td>
<td>64</td>
</tr>
<tr>
<td>23. CD spectra of the MAPAC-DNA complex at three added MAPAC/nucleotide ratios</td>
<td>65</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>24.</td>
<td>CD spectra of the MMPB-DNA complex at three added MMPB/nucleotide ratios</td>
</tr>
<tr>
<td>25.</td>
<td>Dependence of molar ellipticity of the MAPEC-DNA complex near 470 nm on r</td>
</tr>
<tr>
<td>26.</td>
<td>Dependence of molar ellipticity of the MAPAC-DNA complex near 470 nm on r</td>
</tr>
<tr>
<td>27.</td>
<td>Dependence of molar ellipticity of the MMPB-DNA complex near 470 nm on r</td>
</tr>
<tr>
<td>28.</td>
<td>Dependence of molar ellipticity of the MAPEC-DNA complex near 315 nm on r</td>
</tr>
<tr>
<td>29.</td>
<td>Dependence of molar ellipticity of the MAPAC-DNA complex near 315 nm on r</td>
</tr>
<tr>
<td>30.</td>
<td>Dependence of molar ellipticity of the MMPB-DNA complex near 315 nm on r</td>
</tr>
<tr>
<td>31.</td>
<td>CD spectrum of the EB-DNA complex in 5.0 M NaCl</td>
</tr>
<tr>
<td>32.</td>
<td>Dependence of molar ellipticity of the EB-DNA complex near 310 nm on r in 5.0 M NaCl</td>
</tr>
<tr>
<td>33.</td>
<td>Circular dichroism of the EB-DNA complex in 5.0 M NaCl</td>
</tr>
<tr>
<td>34.</td>
<td>Fluorescence emission spectrum of free and bound EB</td>
</tr>
<tr>
<td>35.</td>
<td>Dependence of molar fluorescence of the EB-DNA complex on r</td>
</tr>
<tr>
<td>36.</td>
<td>Dependence of molar fluorescence of the DDMB-DNA complex on r</td>
</tr>
<tr>
<td>37.</td>
<td>Fluorescence emission spectrum of free and bound MAPAC</td>
</tr>
<tr>
<td>38.</td>
<td>Dependence of molar fluorescence of the MAPEC-DNA complex on r</td>
</tr>
<tr>
<td>39.</td>
<td>Dependence of molar fluorescence of the MAPAC-DNA complex on r</td>
</tr>
<tr>
<td>40.</td>
<td>Dependence of molar fluorescence of the MMPB-DNA complex on r</td>
</tr>
<tr>
<td>41.</td>
<td>Temperature-optical density profile of calf thymus DNA</td>
</tr>
<tr>
<td>42.</td>
<td>Temperature-optical density profile of the MAPEC-DNA complex</td>
</tr>
<tr>
<td>43.</td>
<td>Temperature-optical density profile of the MAPAC-DNA complex</td>
</tr>
<tr>
<td>44.</td>
<td>Temperature-optical density profile of the MMPB-DNA complex</td>
</tr>
<tr>
<td>45.</td>
<td>Temperature-optical density profile of the EB-DNA complex</td>
</tr>
<tr>
<td>46.</td>
<td>Temperature-optical density profile of the DEMB-DNA complex</td>
</tr>
<tr>
<td>47.</td>
<td>Temperature-optical density profile of the DDMB-DNA complex</td>
</tr>
<tr>
<td>Figure</td>
<td>Inhibition of DNase by EB as a function of the added EB/nucleotide ratio</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------------------------------------------</td>
</tr>
<tr>
<td>48.</td>
<td>Inhibition of DNase by DDMB as a function of the added DDMB-nucleotide ratio</td>
</tr>
<tr>
<td>49.</td>
<td>Inhibition of DNase by MAPEC as a function of the added MAPEC/nucleotide ratio</td>
</tr>
<tr>
<td>50.</td>
<td>Inhibition of DNase by MAPEC as a function of the added MAPEC/nucleotide ratio</td>
</tr>
<tr>
<td>51.</td>
<td>Inhibition of DNase by MAPEC as a function of the added MAPEC/nucleotide ratio</td>
</tr>
<tr>
<td>52.</td>
<td>Lineweaver - Burk plot for the inhibition of DNase by EB</td>
</tr>
<tr>
<td>53.</td>
<td>Reiner plot for the inhibition of DNase by EB</td>
</tr>
<tr>
<td>54.</td>
<td>Reiner plot for the inhibition of DNase by DDMB</td>
</tr>
<tr>
<td>55.</td>
<td>Reiner plot for the inhibition of DNase by MAPEC</td>
</tr>
<tr>
<td>56.</td>
<td>Reiner plot for the inhibition of DNase by MAPAC</td>
</tr>
<tr>
<td>57.</td>
<td>Inhibition of DNase by EB as a function of ( r )</td>
</tr>
<tr>
<td>58.</td>
<td>Inhibition of DNase by DDMB as a function of ( r )</td>
</tr>
<tr>
<td>59.</td>
<td>Inhibition of DNase by MAPEC as a function of ( r )</td>
</tr>
<tr>
<td>60.</td>
<td>Inhibition of DNase by MAPAC as a function of ( r )</td>
</tr>
<tr>
<td>61.</td>
<td>Time dependence of DNA synthesis by <em>Micrococcus luteus</em> DNA polymerase</td>
</tr>
<tr>
<td>62.</td>
<td>Inhibition of DNA polymerase by EB as a function of the added EB/nucleotide ratio</td>
</tr>
<tr>
<td>63.</td>
<td>Inhibition of DNA polymerase by DEMB as a function of the added DEMB/nucleotide ratio</td>
</tr>
<tr>
<td>64.</td>
<td>Inhibition of DNA polymerase by MAPEC as a function of the added MAPEC/nucleotide ratio</td>
</tr>
<tr>
<td>65.</td>
<td>Inhibition of DNA polymerase by MAPAC as a function of the added MAPAC/nucleotide ratio</td>
</tr>
<tr>
<td>66.</td>
<td>Lineweaver - Burk plot for the inhibition of DNA polymerase by EB</td>
</tr>
<tr>
<td>67.</td>
<td>Lineweaver - Burk plot for the inhibition of DNA polymerase by DEMB</td>
</tr>
<tr>
<td>68.</td>
<td>Lineweaver - Burk plot for the inhibition of DNA polymerase by MAPEC</td>
</tr>
<tr>
<td>69.</td>
<td>Lineweaver - Burk plot for the inhibition of DNA polymerase by MAPAC</td>
</tr>
<tr>
<td>70.</td>
<td>Inhibition of DNA polymerase by EB as a function of ( r )</td>
</tr>
<tr>
<td>71.</td>
<td>Inhibition of DNA polymerase by DEMB as a function of ( r )</td>
</tr>
<tr>
<td>72.</td>
<td>Inhibition of DNA polymerase by MAPEC as a function of ( r )</td>
</tr>
<tr>
<td>73.</td>
<td>Inhibition of DNA polymerase by MAPAC as a function of ( r )</td>
</tr>
</tbody>
</table>
INTRODUCTION

The chemical structure of the phenanthridinium compounds is shown in Figure 1. The biological properties of these compounds, as well as the physicochemical effects of one of them, ethidium bromide (3.8-diamino-5-ethyl-6-phenyl phenanthridinium bromide), (usually abbreviated EB), on nucleic acids have been the subject of intense investigation in the past two decades.

Studies of the properties of phenanthridinium compounds have been carried out by two distinct approaches. Initially, the biological effects of these compounds were studied, occasionally as a function of their chemical structure. In contrast, during the past ten years, research in this subject has been increasingly oriented towards the physicochemical properties of the complexes of EB with nucleic acids.

1. Biological activity of phenanthridinium compounds

Phenanthridinium compounds were first found to possess antitypanosomal activity in 1938 (Browning, 1938). Later, they were also found to act as antibacterial (Seaman and Woodbine, 1954) and antiviral agents (Dickinson et al., 1953).

The in vivo antitypanosomal activity of the best studied phenanthridinium drug, ethidium bromide, is observed
Figure 1. General formula of the phenanthridinium compounds. The compounds used in this study have the following substituents:

EB: \( R_1 = R_4 = \text{NH}_2, \ R_2 = \text{ethyl}, \ R_3 = \text{phenyl} \).

DDMB: \( R_1 = R_4 = \text{NH}_2, \ R_2 = R_3 = \text{methyl} \).

DEMB: \( R_1 = R_4 = \text{NH}_2, \ R_2 = \text{methyl}, \ R_3 = \text{ethyl} \).

MAPAC: \( R_1 = H, \ R_2 = \text{allyl}, \ R_3 = \text{p-aminophenyl}, \ R_4 = \text{NH}_2 \).

MAPEC: \( R_1 = H, \ R_2 = \text{ethyl}, \ R_3 = \text{p-aminophenyl}, \ R_4 = \text{NH}_2 \).

MMPB: \( R_1 = H, \ R_2 = \text{methyl}, \ R_3 = \text{phenyl}, \ R_4 = \text{NH}_2 \).
as a decrease in the number of trypanosomes in the blood of infected animals. This decrease is not immediate upon exposure to EB, but it is preceded by a lag phase of approximately 3 to 4 days (Hawking and Sen, 1960). As early as 4 hours after contact with the drug, however, basophilic granules, consisting mainly of nucleoprotein, are observed in the cytoplasm of the trypanosomes (Ormerod, 1951b). These granules do not immediately influence the development of the organisms, which, 24 hours after exposure to the drug, are still actively multiplying. The cell division rate, however, later declines and, after more prolonged contact with EB, an increased number of multinucleate and a decreased number of dividing forms are observed (Ormerod, 1951b; Ormerod, 1961).

Analogous morphological alterations in the kinetoplasts of trypanosomes grown in the presence of EB in vitro have been reported (Riou, 1967a).

Similar effects are also observed when Crithidia oncopelti are exposed to EB in vitro. Ribonucleoprotein granules appear in the cytoplasm but multiplication of the organisms is not inhibited until at least one cycle of division has been completed (Newton, 1957). It was also noted that transfer of the organisms to a drug-free medium, after their multiplication had been inhibited by EB, did not restore their ability to multiply. However, if the
organisms' exposure to the drug was terminated before their multiplication was completely inhibited, they continued to grow and multiply, albeit at decreased rate. It is interesting to note that the decrease in the multiplication rate is proportional to the organisms' exposure to the drug.

These findings, along with the observation that C. oncopelti maintained in a non growth-supporting medium, exposed to EB and later transferred to a drug-free growth-supporting medium grew at a normal rate (Newton, 1957), indicate that growth in the presence of EB is an essential condition for the lethal action of the drug.

Morphological and physiological alterations in the presence of EB, similar to those produced in trypanosomes, are also observed in other systems, such as algae; swelling of the chloroplasts and decomposition of the organelles' internal membrane systems is reported as a result of growth in the presence of EB (Heilporn and Limbosch, 1971). Similarly, EB causes morphological alterations of the smooth endoplasmic reticulum and the mitochondria in various lines of mammalian cells. These changes are accompanied by reduction and eventual disappearance of several respiratory enzymes (Nass, 1970; Naum and Pious, 1971; King et al., 1972).

On the molecular level, considerable evidence has been accumulated suggesting that EB interferes with the metabolism of nucleic acids. As early as 1957 it was reported
(Newton, 1957) that DNA synthesis is drastically inhibited in *S. oncopelti* grown *in vitro* in the presence of EB. More recently, EB was found to inhibit host cell mediated re-activation of UV-irradiated phage in bacteria (Shankel and Molholt, 1973) and to block RNA synthesis in mammalian cells (Watts, 1970).

An interesting aspect of the *in vivo* action of EB is that it is generally directed selectively against non-nuclear DNA. This selectivity is not limited to certain organisms only but appears to span a wide range of the phylogenetic spectrum. For example, in the presence of low concentrations of EB, mitochondrial DNA synthesis is selectively inhibited in both yeast and mammalian cells and the preexisting mitochondrial DNA of the yeast is degraded (Goldring *et al.*, 1970; Goldring *et al.*, 1971; Radsak *et al.*, 1971). Also, growth of trypanosomes in the presence of EB results in selective inhibition of kinetoplastic DNA synthesis (Riou, 1967b; Delain and Riou, 1969) accompanied by the appearance of abnormal oligomeric circular DNA molecules in the kinetoplast (Riou and Delain, 1969).

From these and other similar observations, the hypothesis was developed that the primary *in vivo* target of EB is the metabolism of nucleic acids. This hypothesis was further supported by *in vitro* experiments indicating that EB inhibits the action of pancreatic deoxyribonuclease (Eron and McAuslan, 1966), *E. coli* and mammalian DNA polymerases (Elliott,
A number of attempts have been made to establish a relationship between the trypanocidal activity and the molecular structure of phenanthridinium compounds. It appears that antitypanosomal activity requires at least one primary amino substituent on the phenanthridinium nucleus; a second amino substituent further enhances trypanocidal activity (Walls, 1945; Walls, 1947; Brownlee et al., 1950). The size of the quaternizing alkyl group at position 5 of the ring seems also to be important for activity; antitypanosomal activity increases with the length of the alkyl group for up to 3 carbon atoms and then decreases with increasing length of the chain (Woolfe, 1952; Woolfe, 1956).

2. Physicochemical properties of the phenanthridinium-DNA complex

Investigation of the physicochemical properties of the phenanthridinium-DNA complex has been, almost exclusively, limited to the study of the properties of the EB-DNA complex. Ethidium bromide forms complexes with double-stranded DNA and RNA (Waring, 1965b; Douthard et al., 1973) in which the dye is strongly bound to the polynucleotide. The nucleic acid-bound EB cannot be removed by dialysis (Waring, 1965b) but the complex is easily dissociated if passed
through an ion-exchange column (Fuller and Waring, 1964; Radloff et al., 1967).

The formation of a complex between EB and nucleic acids causes a metachromatic shift in the absorption maximum of the dye from 480 nm to approximately 518 nm (Elliott, 1963; Waring, 1965b). On the basis of this observation, the binding of EB to DNA can be studied and the equilibrium parameters determined by using the Scatchard relation

\[ r/c = K_n - K_r \] (Scatchard, 1949). The base composition of the nucleic acid does not seem to affect significantly the extent of binding or the properties of the dye (Waring, 1965b; LePecq and Paoletti, 1967).

Scatchard plots obtained at low ionic strength from spectrophotometric titration of EB with double-stranded nucleic acids consist of two intersecting straight lines (Waring, 1965b; Douthard et al., 1973) indicating that two distinct binding processes are taking place. These are usually referred to as primary and secondary binding, the primary being the stronger of the two.

It is generally accepted that the primary binding involves intercalation, i.e. positioning of the planar dye molecule between adjacent base pairs so that the plane of the ring is approximately perpendicular to the axis of the helix. The intercalation model was first proposed by Lerman (Lerman, 1961) to explain the binding of aminoacridines to DNA. In the case of EB, the forces stabilizing this
complex are mainly hydrophobic interactions between the DNA base-pairs and the phenanthridinium ring system, as suggested by the observation that ethanol dissociates the complex (Fuller and Waring, 1964). Also, since the binding of EB to DNA decreases with increasing ionic strength (LePecq and Paoletti, 1967), hydrogen bonds, which can be formed between ethidium amino groups and the negatively charged phosphates of the DNA backbone, may also contribute to the stabilization of the complex (Fuller and Waring, 1964).

The complex of ethidium bromide with double-stranded nucleic acids exhibits physicochemical properties which are consistent with intercalation, specifically:

1. The intercalation model requires that the plane of the bound drug molecules be parallel to that of the base pairs, i.e. perpendicular to the helix axis. Experimental results indicating such an orientation of the bound molecule were obtained by the use of flow dichroism and fluorescence polarization techniques (LePecq and Paoletti, 1967).

2. A second consequence of intercalation is the increase in the length of DNA as the helix unwinds to accommodate drug molecules. This increase has been noticed by electron microscopy (Freifelder, 1971). Indirect proof for this length increase is also provided by the observation that the intrinsic viscosity of double-stranded DNA or RNA
solutions increases significantly in the presence of EB (LePecq, 1965; Douthard et al., 1973).

3. The length increase in DNA due to intercalation is expected to result in decreased length-specific mass of the polynucleotide because the additional length due to each EB molecule is about the same as that of a nucleotide pair (mean molecular weight = 650), while the mass increment (molecular weight of the ethidium free base = 315) is less than one-half of that of a nucleotide pair. This decrease in length-specific mass is observed as a decrease in the sedimentation coefficient of the nucleic acid in the presence of the dye (LePecq, 1965; Douthard et al., 1973).

4. Upon binding to double-stranded DNA, the quantum efficiency of the EB fluorescence increases dramatically. No increase in fluorescence is noted, however, with single-stranded DNA (LePecq and Paoletti, 1967). Ethidium molecules intercalated in double-stranded DNA are placed in an environment of considerably lower dielectric constant (Gersch and Jordan, 1965) than the environment of the unbound dye, which may explain the observed fluorescence enhancement.

5. The effect of phenanthridinium compounds on covalently closed circular DNA constitutes perhaps the most convincing evidence in favor of the intercalation model. It was found that exposure of supercoiled DNA to increasing amounts of EB reduces its sedimentation velocity until it is equal to that of nicked circular DNA. Addition of larger
amounts of EB results in an increase of the sedimentation velocity of supercoiled DNA. In contrast, the sedimentation velocity of nicked circular DNA exhibits a monotonic decrease with increasing concentration of EB (Crawford and Waring, 1967; Bauer and Vinograd, 1968). These findings were interpreted as indicative of loss and subsequent reversal of the supercoiling turns of the DNA as a result of the local unwinding of the double helix, due to intercalation of the drug. Similar observations were also made with various EB analogues, indicating that the primary amino groups at positions 3 and 8 of the phenanthridinium ring are not mandatory for intercalation, although derivatives lacking the 3-amino substituent exhibit a slightly lower unwinding angle than the 3,8-diamino derivatives (Wakelin and Waring, 1974).

The angle of double-helix unwinding upon EB intercalation was estimated by measuring the fluorescence depolarization of the EB-DNA complex to be approximately 12° per intercalated EB molecule (Pigram et al., 1973). This value agrees with that originally proposed (Fuller and Waring, 1964) on the basis of model-building studies and X-ray crystallographic data, although, according to more recent evidence, the angle of unwinding may be as high as 26° per intercalated molecule (Wang, 1974).

Secondary binding between EB and double-stranded DNA is observed at relatively high added EB/DNA ratios and low ionic strengths. This binding is believed to arise mainly
from electrostatic interactions between the DNA phosphate residues and EB molecules attached to the outside of the helix. Increased ionic strength eliminates secondary binding, as evidenced from the straight-line Scatchard plots obtained under high ionic strength conditions (Waring, 1964; Waring, 1965b).

3. The scope of this investigation

As mentioned earlier in this Section, a number of attempts have been made to determine the relation between the exact chemical structure of phenanthridinium drugs and their pharmacological properties. The in vivo experiments performed so far have provided information as to the biological activity of these compounds. However, in such experiments a large number of parameters, such as in vivo stability of the drugs, permeability of the cell membranes to the drugs, selective binding of the drugs by various components of the blood, etc. are involved. Since in the in vivo experiments these parameters cannot be easily controlled, only empirical determinations of an overall structure-function relationship can, at best, be obtained by in vivo studies. Such experiments, however, provide little information about the existence and nature of any such relationship on the molecular level.

As previously indicated, biological properties of EB originate from the interaction of this compound with DNA (see, inter alia, Newton, 1957; Delain and Riou, 1969;
Goldring et al., 1970). It is therefore reasonable to assume that the specificity of action of the phenanthridinium drugs depends on the physical consequences of the DNA-drug binding. The interaction of EB with DNA influences both the conformation and the stability of double-stranded DNA and may, as a result, determine the function of DNA as template during replication and transcription.

In spite of accumulating information on the nature of the interactions between EB and DNA, no successful attempts has yet been made to account for the biochemical and pharmacological properties of the phenanthridinium drugs on the basis of the physicochemical properties of the DNA-phenanthridinium complex. This investigation was, therefore, undertaken as an attempt to answer two interrelated but distinct questions, namely:

A. Does the chemical structure of the intercalated drug molecule affect the overall conformation and stability of the drug-DNA complex and, if so, to what extent? and

B. If such a change in the conformation of the complex occurs, how does this change influence the function of the complex with respect to its role in nucleic acid metabolism. Specifically, how is the ability of the complex to function as a substrate or as a template for deoxyribonuclease I and DNA polymerase I affected by the specific three-dimensional structure of the complex?
MATERIAL AND METHODS

I. MATERIALS

Calf thymus deoxyribonucleic acid was purchased from Worthington Biochemical Corp., Freehold, New Jersey.

Salmon sperm deoxyribonucleic acid was obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

Deoxyribonuclease I from beef pancreas (Deoxyribonucleate oligonucleotide-hydrolase; E.C. 3.1.4.5.) was obtained from Sigma Chemical Co., Saint Louis, Missouri (Lot number 20C-1810, prepared chromatographically; supplied as a lyophilized powder, 2000 Kunitz units/mg; one Kunitz unit will cause the absorbance at 260 nm of a DNA solution to increase at a rate of 0.001 per ml at 25°C).

DNA polymerase I, from Micrococcus luteus (Deoxyribonucleosidetriphosphate: DNA deoxynucleotidyl transferase; E.C. 2.7.7.7.) was obtained from Sigma Chemical Co., Saint Louis, Missouri (Lot number 63C-2810, prepared by column chromatography on DEAE cellulose and gel filtration media; supplied as a lyophilized powder, 12.5 units per mg of protein; one unit will incorporate 10 nanomoles of total nucleotide into an acid insoluble product in 30 min at pH 7.0 at 37°C, using calf thymus DNA as template).
Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) was obtained from Calbiochem, Los Angeles, California, Lot number 100301.

Crystalline samples of the following phenanthridinium compounds were kindly provided by Dr. T. I. Watkins (Boots Pure Drug Co., Ltd., Great Britain):

- 3,8-diamino-5,6-dimethylphenanthridinium bromide (DDMB),
- 3,8-diamino-6-ethyl-5-methylphenanthridinium bromide (DEMB),
- 8-amino-6-p-aminophenyl-5-ethylphenanthridinium chloride (MAPEC),
- 5-allyl-8-amino-6-p-aminophenylphenanthridinium chloride (MAPAC), and
- 8-amino-5-methyl-6-phenylphenanthridinium bromide (MMPB).

These derivatives were used without further purification.

Sodium salts of deoxyribonucleotide triphosphates (TTP, dGTP, dCTP and dATP) were obtained from Worthington Biochemical Corp., Freehold, New Jersey.

Tritiated TTP was purchased from New England Nuclear, Boston, Massachusetts (tetrasodium salt, specific activity 40 Ci/mmole) and from Schwartz/Mann, Orangeburg, New York (tetralithium salt, specific activity 54 Ci/mmole).

Other chemicals used in these studies were of analytical reagent grade.
II. INSTRUMENTS

In these studies the following instruments were used:

Cary 15 recording spectrophotometer, Cary Instrument Corp.

Durrum-Jasco ORD/UV-5 recording spectropolarimeter, Durrum Instrument Co. The instrument was modified to a maximum sensitivity of 0.002 degrees of ellipticity per cm (SS-10 modification).

Aminco-Bowman spectrophotofluorimeter (Model 4-8202 SPF) with a ratio photometer (Model 4-8912), American Instrument Co., connected to an Omnigraphic 2000 X-Y recorder, Houston Instrument.

Tm Analyzer and Temperature Bridge, Beckman Instrument Inc., connected to a Moseley 7035B X-Y recorder, Hewlett Packard Corp.

Beckman LS-250 Liquid Scintillation Counter.

III. METHODS

1. Preparation of stock solutions

The buffer used in these studies, unless otherwise specified, was 0.04 M Tris-HCl, pH 7.9. For experiments carried out at higher ionic strengths, NaCl was added to the composition of this buffer so that the final salt concentration ranged between 0.025 and 5.0 M.

Calf thymus DNA was dissolved (4 mg/ml) in Tris-HCl buffer after slow stirring in the cold for approximately 24 hours and then dialyzed twice against 20 volumes of the same buffer for 48 hours. This stock solution of DNA was then kept in the refrigerator at 4°C.

Before every experiment, an amount of the DNA stock solution was diluted with buffer to give a working solution. The DNA phosphate concentration of this solution was determined spectrophotometrically using an extinction coefficient at 260 nm of 6600 (Mahler et al., 1964).

Ethidium bromide solutions were prepared by dissolving the salt into the buffer. The final concentration was determined spectrophotometrically using an extinction coefficient at 480 nm of 5600 (Waring, 1965a).

The working solutions of the other phenanthridinium compounds were prepared by dissolving a weighed amount of the corresponding salt into the appropriate volume of buffer.
The molar concentrations were calculated on the basis of the amount weighed and the formula weight of the compound. From these preparations, the following extinction coefficients were estimated: DDMB: 4140 at 463 nm; DEMB: 4740 at 465 nm; MAPAC: 4750 at 438 nm; MAPEC: 5100 at 430 nm; MMPB: 3255 at 430 nm.

All the phenanthridinium salt solutions were kept in the dark until they were used.

2. Preparation of the complex

Complexes of various added dye/nucleotide molar ratios were prepared at a constant dye concentration and varying DNA concentrations. Typically, 2 ml of the dye solution were mixed with varying volumes of the DNA working solution and buffer was added to a final volume of 10 ml. In certain cases, however, as indicated, the reverse procedure was used, i.e. the DNA concentration was kept constant and the dye concentration was varied.

3. Binding determination

For the determination of the amount of dye bound to DNA, the spectrophotometric method of Peacocke and Skerett (Peacocke and Skerett, 1956) was used. The method is based on the bathochromic shift in the dye absorption spectrum upon binding to DNA.
According to this method, if:

1. DNA does not absorb in the wavelength region used;
2. Both free and bound dye obey Beer's law over the range of concentrations used for the binding studies;
3. The extinction coefficient of the bound dye is independent of the dye/DNA ratio;
4a. The bound dye is present as a single species so that the maximum number of species present, including the free dye, is two; or
4b. If more than one species with different spectra, apart from the free dye, are present, the relative proportion of these does not vary with the dye/DNA ratio, then, an isosbestic point, through which all spectra of samples containing a constant dye concentration and varying DNA concentrations would pass, is defined.

Under these conditions, the fraction, b, of dye bound to DNA can be calculated from the equation:

\[ b = \frac{A_f - A_x}{A_f - A_b} \] (Eqn. 1)

where \( A_f \) is the absorption of the free dye, \( A_b \) is the absorption of the bound dye, and \( A_x \) is the absorption of the unknown sample at any one wavelength. Equation 1 is valid at all wavelengths where the above mentioned conditions are satisfied. However, for increased accuracy, b is calculated at the wavelength where the difference \( A_f - A_b \) is maximum.
If the fraction, \( b \), of DNA-bound dye is known, then \( r \) is defined as the molar ratio of DNA-bound dye per DNA phosphate.

4. **Circular dichroism measurements**

Circular dichroism spectra were recorded using the Jasco spectropolarimeter. Measurements were carried out in cells with light paths ranging from 0.1 to 10.0 cm, so that absorbances remained below 2.0. This precaution was taken because optical artifacts may be observed at absorbance values above this limit.

Circular dichroism values are reported as molar ellipticities \((\varepsilon_1 - \varepsilon_r)\), which were calculated from the equation:

\[
\varepsilon_1 - \varepsilon_r = \frac{\text{Degrees of ellipticity}}{33 \times c \times l}
\]

where "Degrees of ellipticity" are obtained directly from the recorder chart, the concentration, \( c \), is expressed in moles per litre, and the light path of the cell, \( l \), is expressed in centimetres. In the calculation of the \( \varepsilon_1 - \varepsilon_r \), the concentration of DNA-bound rather than "total added" dye was used, except as otherwise specified.

The spectropolarimeter was standardized with a solution of d-10-camphosulphonic acid (1.0 mg/ml, supplied by Durrum Instrument Co.). The standard solution has an ellipticity of +0.313 degrees at 290 nm. The instrument
was calibrated so that the solution causes a 15.65 cm deflection on the 0.02 degrees/cm scale.

5. **Fluorescence measurements**

Fluorescence measurements were obtained using the Aminco-Bowman spectrophotofluorimeter. Cells with four optically polished windows and 1.0 cm rectangular cross section were used.

Excitation and emission fluorescence spectra were recorded on the Omnigraphic X-Y recorder with the X axis calibrated to read wavelength and the Y axis to correspond to the relative intensity reading of the Ratio Photometer.

The intensity values thus obtained were corrected for:

1. Fluorescence contribution of the free dye; fluorescence intensity proportional to the concentration of the free dye was subtracted.

2. Absorption of the excitation light by the sample; since fluorescence is measured at the middle of a 1.0 cm cell, the correction factor is the decimal antilogarithm of half the absorbance of the sample at the excitation wavelength.

No correction for absorption of the emission light was made, since neither the free nor the bound dye absorb appreciably at the emission wavelength.
Fluorescence values were plotted as "Net molar fluorescence", which was calculated from the Equation:

\[
\text{Net molar fluorescence} = \frac{I_{\text{obs}} \times \text{antilog } A}{\sum} - \frac{\text{Molar fluorescence}_f \times D_f}{D_b} \quad (\text{Eqn. 3})
\]

where: \(I_{\text{obs}}\) is the observed fluorescence; \(A\) is the absorbance of the sample at the excitation wavelength; \(\text{Molar fluorescence}_f\) is the fluorescence intensity per mole of free dye; \(D_f\) is the free dye concentration; and \(D_b\) is the bound dye concentration.

6. Elevated temperature measurements

For the study of the dependence of absorbance on temperature, a thermostated cell holder was used in connection with the Beckman Tm Analyzer and Temperature Bridge. With the use of these instruments the temperature of the sample can be linearly increased at a programmed rate. The rate used was 3°C/min unless otherwise specified. The temperature of the sample was monitored using a platinum resistance probe which produces a linear change in resistance with temperature. The probe is immersed in a reference cell which is being heated at the same rate as the sample-containing cell. The probe is connected to the Temperature Bridge, which converts the temperature-dependent resistance of the probe to a linear 10 mV output signal.
For the recording of absorbance at a given wavelength versus temperature, the Moseley X-Y recorder was used with the X axis calibrated to read temperature and the Y axis to correspond to the position of the pen of the spectrophotometer. The X signal was obtained directly from the Temperature Bridge. The Y signal was obtained from a potentiometer installed on the pen-drive shaft of the spectrophotometer. This signal was mediated by a custom-made calibration box (Martz and Aktipis, 1971) which provides for expansion and baseline adjustment of the scale.

Melting profiles of DNA and DNA-drug complexes were plotted as normalized hyperchromicity versus temperature. Normalized hyperchromicity is the hyperchromicity expressed as percent of the final hyperchromicity value observed at the end of the transition. Tm, then, is defined as the temperature at which a normalized hyperchromicity value of 50% is observed. $\Delta T_{30-70}$ is a measure of the width of the melting transition and represents the difference, in degrees, between the temperatures at which 30% and 70%, respectively, of hyperchromicity is observed.

7. Deoxyribonuclease assay

The assay for DNase activity used was essentially that of Lindberg (Lindberg, 1964) with minor modifications. The method is based on the measurement of DNA hyperchromicity at 260 nm as the polynucleotide is hydrolyzed.
The assay was performed, at room temperature, as follows:

A 3 ml aliquot of a DNA solution (0.15 mM) was added into a tube containing 1 ml of drug solution (or buffer, in the case of control). The contents of the tube were mixed and left standing for approximately 1 min. Subsequently, 0.03 ml of the enzyme solution (0.1 mg/ml) were added, the tube was covered with parafilm and inverted rapidly 3-4 times; the contents of the tube were then poured into a 1.0 cm cuvette and monitoring at 260 nm begun, generally within 15 - 20 seconds after the addition of the enzyme.

The assay mixture had the following composition:

0.04 M Tris-HCl, pH 7.9, 0.004 M MgSO₄, 0.0017 M CaCl₂,
0.00011 M calf thymus DNA, and 0.75 µg/ml enzyme.

The hyperchromicity at 260 nm exhibits an initial lag phase of approximately 20 sec., after which the absorbance increases linearly with time for at least 50 sec. This lag phase is due to the fact that the first few phosphate bonds to be hydrolyzed are statistically situated far apart from one another and, therefore, do not cause the release of any hyperchromic material.

For the calculation of the initial reaction rates, the linear part of the absorbance versus time plot, which follows the lag phase, was used.

Hyperchromicity at 260 nm during enzymatic degradation of the DNA-phenanthridinium complex is due to the
production of oligonucleotides, the extinction coefficient of which at 260 nm is higher than that of native DNA, and, also, to the release of intercalated molecules, which have a higher extinction coefficient at 260 nm in the free than in the bound state.

To correct for the latter type of hyperchromicity, the assay was performed as described above, but this time the absorption was monitored at a wavelength, \( \lambda \), at which DNA does not absorb and where the difference between the extinction coefficients of the free and the bound dye is maximum. For the various compounds studied, this wavelength was: EB: 460 nm; DDMB: 450 nm; MAPEC: 425 nm; MAPAC: 430 nm. The corrected hyperchromicity at 260 nm is:

\[
\Delta A_{260, \text{corr.}} = \Delta A_{260, \text{obs.}} - \Delta A \times \frac{\Delta \varepsilon}{\Delta \varepsilon_{260}}
\]  

(Eqn. 4)

where \( \Delta A_{260, \text{obs.}} \) is the observed hyperchromicity at 260 nm; \( \Delta A \) is the observed hyperchromicity at wavelength \( \lambda \); \( \Delta \varepsilon_{260} \) is the difference between the extinction coefficients of the free and bound dye at 260 nm; and \( \Delta \varepsilon \) is the difference between the extinction coefficients of the free and bound dye at wavelength \( \lambda \).

The corrected initial velocity obtained by this method was used to construct the inhibition plots. In these plots, the function \( i/(1 - i) \) (where \( i \) is the fractional inhibition) is plotted versus the total concentration of
inhibitor, $D_t$. It has been shown (Reiner, 1959) that when an inhibitor acts by decreasing the effective substrate concentration, a plot of $D_t$ versus $i/(1 - i)$ yields a curve that passes through the origin of the axes; the initial and final parts of this curve are straight lines with slopes of $Y_1 = \frac{K_3 + S_t}{1 + S_t/K_1}$ and $Y_2 = K_3(1 + \frac{S_t}{K_1})$, respectively, where:

- $K_1$ = Dissociation constant for the binding of the substrate to the enzyme.
- $K_3$ = Dissociation constant for the binding of the inhibitor to the substrate.
- $S_t$ = Total substrate concentration.

When the final linear portion of the curve is extrapolated to the $D_t$ axis, the intercept gives a value of $S_t$.

By measuring the slopes of these two linear portions of the curve and the extrapolative intercept on the $D_t$ axis, one can calculate the dissociation constants for the binding of the enzyme to the substrate and the binding of the inhibitor to the substrate.

8. **DNA polymerase assay**

The method used for determining polymerase activity was that of Bollum (Bollum, 1966). The procedure is based on the incorporation of radioactivity labeled deoxyribonucleotides into an acid insoluble product.
DNA polymerase I from Micrococcus luteus was dissolved (0.25 mg/ml) in 0.04 M potassium phosphate, 0.008 M MgCl₂, pH 7.0 containing 1 mg/ml BSA and 3 mmoles/l 2-mercaptoethanol. The mixture was subsequently divided into 1 ml portions that were kept frozen at -20°C until they were used.

The reaction mixture contained, in a final volume of 0.3 ml, the following components at the indicated concentrations: 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl₂; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 0.072 mg/ml activated calf thymus DNA (see below); 0.008 mg/ml enzyme; appropriate amounts of inhibitor (or buffer, in the case of control). The reaction was usually initiated with the addition of the nucleotide triphosphate (XTP) solution. On occasion, the enzyme solution was used to initiate the reaction. No significant difference was observed between the results of the two methods.

After incubation at 37°C for 30 min, the reaction was stopped by immersing the test tubes containing the reaction mixture in ice-water and adding 0.1 ml of ice-cold salmon sperm DNA solution (0.3 mg/ml) containing 0.1 M sodium pyrophosphate, followed by 0.4 ml of ice-cold 1 M perchloric acid containing 0.1 M sodium pyrophosphate.
After storing at 0°C for approximately 15 min, the tube contents were filtered through glass-fibre filters (Whatman GF/C, 2.4 cm in diameter) under mild suction. The tubes were subsequently washed three times with a total of 15 ml of 0.1 N HCl, 0.01 M sodium pyrophosphate and the washings were also filtered through the same filter. Finally, the filters themselves were washed twice with a total of 10 ml of the above solution and then with 10 ml of ethanol. The filters were dried for approximately 10 min at 120°C and counted in 10 ml of 0.4% PPO (2,5-diphenyloxazole) in toluene.

In every assay, one or two samples were included, containing buffer instead of enzyme, for the determination of background radioactivity. The radioactivity of these samples, typically less than 2% than that of the controls, was subtracted from the radioactivity of the rest of the assay samples.

9. Activation of DNA

The method used for partial digestion of DNA was that of Harwood et al. (Harwood et al., 1970a). The following solutions were used:

A. Calf thymus DNA (2 mg/ml) in 0.01 M Tris-HCl, 0.005 M MgCl₂, pH 7.8.

B. Pancreatic DNase (6.25 μg/ml) in 0.04 M Tris-HCl, 0.004 M MgSO₄, 0.00175 M CaCl₂, pH 7.8.
B. Pancreatic DNase (6.25 μg/ml) in 0.04 M Tris-HCl, 0.004 M MgSO₄, 0.00175 M CaCl₂, pH 7.8.

The enzyme was added to the DNA solution (0.012 μg protein per mg of DNA) and the mixture was incubated for 10 min at 37°C with gentle shaking. The period of 10 min was chosen because it coincided with maximum template activity of the product (Figure 2).

The reaction was stopped by immersing the tube containing the enzyme-DNA mixture in ice-water and adding ice-cold EDTA (0.5 M, pH 8.0) to a final concentration of 0.05 M. Subsequently, the DNA solution was extracted 4-5 times with equal volumes of a mixture of chloroform: isoamyl alcohol (24: 1, v/v) and then 4-5 times with ether. The evaporation of residual ether was accelerated by passing a stream of nitrogen over the solution.

After the extraction, the DNA solution (approximately 10 ml) was dialyzed for 24 hours versus 4 l of 0.01 M NaCl solution containing 10⁻⁴ M EDTA, pH 7.5. This solution was finally divided into portions (2 ml each) which were kept frozen at -20°C until they were used in the DNA polymerase assay.
Figure 2. Effect of DNase digestion on templating efficiency of calf thymus DNA. Abscissa: time of digestion of DNA by pancreatic DNase I. Ordinate: radioactivity incorporated into acid insoluble product after incubation with DNA polymerase I.
RESULTS

1. THE BINDING OF THE PHENANTHRIDINIUM DRUGS TO DNA

As already indicated in the Introduction, there is evidence today suggesting that the biological action of the phenanthridinium drugs is the result of complex formation between these compounds and DNA. Valuable information regarding the nature of forces stabilizing the complex may be obtained by examining the effect of various substituents of the phenanthridinium molecule, and especially the primary amino groups at positions 3 and 8, on the ability of these compounds to bind to DNA. Furthermore, the binding studies provide the necessary information for the calculation of DNA-bound drug concentration in subsequent experiments. For these reasons the binding of the phenanthridinium derivatives to DNA was studied under varying conditions of ionic strength and temperature.

The binding of the drugs to the DNA was determined from the metachromatic shift observed in the spectrum of the drug as a result of titration with calf thymus DNA, as described in Section 3 (Methods). In most instances, the absorption spectra showed good isosbestic points and provided little, if any, evidence of drug self-aggregation.
1.1. **Low ionic strength conditions**

Binding determinations at room temperature were performed in 0.04 M Tris-HCl, pH 7.9 buffer for five compounds: EB, DDMB, MAPEC, MAPAC and MMPB. A representative spectro-photometric titration of EB with calf thymus DNA is shown in Figure 3. Scatchard plots derived from these experiments are shown in Figures 4-8. The binding parameters are summarized in Table 1.

The Scatchard plots indicate the existence of two distinct processes of unequal strength for the binding of the phenanthridinium dyes to DNA. The intrinsic association constant calculated for EB (2 x 10^6 l/mole) is in good agreement with values obtained previously under similar conditions (Waring, 1965b; Angerer and Moudrianakis, 1972). Moreover, the association constants for the primary binding for all five compounds studied are in close agreement with each other. The diamino compounds, however, tend to exhibit somewhat higher association constants. A similar trend is also observed for the number of binding sites per DNA phosphate; again, the diamino compounds appear to have a higher number of binding sites than the monoamino derivatives, indicating that the primary amino substituent at position 3 of the ring may contribute to the overall stabilization of the complex.
Figure 3. Spectrophotometric titration of EB with calf thymus DNA in 0.04 M Tris-HCl, pH 7.9 buffer. Light path: 5.0 cm. (EB) = $4 \times 10^{-5}$ M. (DNA) = 0 (curve A), $0.2 \times 10^{-4}$ M (curve B), $0.4 \times 10^{-4}$ M (curve C), $0.8 \times 10^{-4}$ M (curve D), $13 \times 10^{-4}$ M (curve E), $3 \times 10^{-4}$ M (curve F).
Figure 4. Scatchard plot for the interaction of EB with DNA at room temperature. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 5. Scatchard plot for the interaction of DDMB with DNA at room temperature. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 6. Scatchard plot for the interaction of MAPEC with DNA at room temperature. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 7. Scatchard plot for the interaction of MAPAC with DNA at room temperature. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 8. Scatchard plot for the interaction of MMPB with DNA at room temperature. Buffer: 0.04 M Tris-HCl, pH 7.9.
**TABLE 1**

Binding parameters for the interaction of certain phenanthridinium compounds with DNA

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_{\text{ass.}} ) (1/mole)</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diamino:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EB</td>
<td>( 2.0 \times 10^6 )</td>
<td>0.27</td>
</tr>
<tr>
<td>DDMB</td>
<td>( 3.8 \times 10^6 )</td>
<td>0.27</td>
</tr>
<tr>
<td><strong>Monoamino:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPAC</td>
<td>( 1.3 \times 10^6 )</td>
<td>0.24</td>
</tr>
<tr>
<td>MAPEC</td>
<td>( 0.6 \times 10^6 )</td>
<td>0.24</td>
</tr>
<tr>
<td>MMPB</td>
<td>( 1.1 \times 10^6 )</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Buffer: 0.04 M Tris-HCl, pH 7.9. Temperature: 25°C.
1.2. High ionic strength conditions

In order to determine the extent of electrostatic force contribution to the overall binding energy of the EB-DNA complex, the binding of this compound to DNA was studied in the presence of 5.0 M NaCl. In addition, this binding study provided the necessary information for the calculation of the bound dye concentration in subsequent CD experiments that were performed under the same conditions (Results, Section 2.3).

The Scatchard plot for the interaction of EB with DNA in the presence of 5.0 M NaCl is shown in Figure 9. All points fell on a straight line, indicating that, under these conditions, secondary binding is eliminated. It is also interesting to note the resulting substantial decrease in the association constant (2.8 x 10^5 l/mole in 5.0 M buffer as compared with 2.0 x 10^6 l/mole in 0.04 M buffer) and in the number, n, of binding sites per DNA phosphate (0.19 in 5 M buffer as compared to 0.27 in 0.04 M buffer).

These changes in the binding characteristics of EB are not surprising, since, at neutral pH, electrostatic forces between the cationic dye and the negatively charged phosphates of the helix may comprise a considerable part of the forces stabilizing the dye-DNA complex (Gilbert and Claverie, 1968). Similar decreases in the association constant in the presence of increased sodium ion concentrations
Figure 9. Scatchard plot for the interaction of EB with DNA at room temperature. Buffer: 0.04 M Tris-HCl, 5 M NaCl, pH 7.9.
have also been reported previously (LePecq and Paoletti, 1967).

1.3. Binding in the presence of magnesium

The binding of four phenanthridinium compounds (EB, DEMB, MAPAC and MAPEC) was also studied in the presence of magnesium ions, both at room temperature and at 37°C. These experiments provided binding constant values which are needed for the estimation of the DNA-bound drug in conditions under which the subsequent enzymic studies were performed (Results, Sections 5 and 6).

The change in the absorption spectrum of EB upon addition of increasing amounts of calf thymus DNA is shown in Figures 10 and 11. The buffer used in these experiments was 0.04 M potassium phosphate, 0.008 M MgCl₂, pH 7.0. Similar absorption spectra were also recorded for the other three phenanthridinium compounds. The Scatchard plots for these experiments are shown in Figures 12-15 and the binding parameters are summarized in Table 2.

Examination of the Scatchard plots shows that all the experimental points fall on a straight line, indicating that, under these conditions, secondary binding is eliminated. This is an expected finding in view of a previous report (Waring, 1965b) that no secondary binding is observed in the interaction of EB with DNA in the presence of 0.004 M MgCl₂.
Figure 10. Spectrophotometric titration of EB with calf thymus DNA in 0.04 M potassium phosphate, 0.008 M MgCl₂, pH 7.0. Light path: 10.0 cm. Temperature: 25°C. (EB) = 2 × 10⁻⁵ M, (DNA) = 0 (curve A), 0.5 × 10⁻⁴ M (curve B), 0.7 × 10⁻⁴ M (curve C), 1.1 × 10⁻⁴ M (curve D), 2.3 × 10⁻⁴ M (curve E), 9.2 × 10⁻⁴ M (curve F).
Figure 11. Spectrophotometric titration of EB with calf thymus DNA in 0.04 M potassium phosphate, 0.008 M MgCl₂, pH 7.0. Light path: 10.0 cm. Temperature: 37°C. (EB) = 2 x 10⁻⁵ M. (DNA) = 0 (curve A), 0.7 x 10⁻⁴ (curve B), 1.2 x 10⁻⁴ M (curve C), 1.7 x 10⁻⁴ M (curve D), 15 x 10⁻⁴ M (curve E).
Figure 12. Scatchard plot for the interaction of EB with DNA at 25°C (○) and at 37°C (●). Buffer: 0.04 M potassium phosphate, 0.008 M MgCl₂, pH 7.0.
Figure 13. Scatchard plot for the interaction of DEMB with DNA at 25°C (○) and at 37°C (●). Buffer: 0.04 M potassium phosphate, 0.008 M MgCl₂, pH 7.0.
Figure 14. Scatchard plot for the interaction of MAPEC with DNA at 25°C (O) and at 37°C (●). Buffer: 0.04 M potassium phosphate, 0.008 M MgCl₂, pH 7.0.
Figure 15. Scatchard plot for the interaction of MAPAC with DNA at 25°C (○) and at 37°C (●). Buffer: 0.04 M potassium phosphate, 0.008 M MgCl₂, pH 7.0.
TABLE 2

Binding parameters for the interaction of certain phenanthridinium compounds with DNA

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{ass.}$ (1/mole)</th>
<th>n</th>
<th>$K_{ass.}$ (1/mole)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25°C</td>
<td></td>
<td>37°C</td>
<td></td>
</tr>
<tr>
<td>Diamino</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EB</td>
<td>$2.2 \times 10^5$</td>
<td>0.20</td>
<td>$1.6 \times 10^5$</td>
<td>0.20</td>
</tr>
<tr>
<td>DDMB</td>
<td>$1.9 \times 10^5$</td>
<td>0.23</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DEMB</td>
<td>$2.2 \times 10^5$</td>
<td>0.20</td>
<td>$1.0 \times 10^5$</td>
<td>0.20</td>
</tr>
<tr>
<td>Monoamino</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPAC</td>
<td>$1.7 \times 10^5$</td>
<td>0.17</td>
<td>$1.1 \times 10^5$</td>
<td>0.17</td>
</tr>
<tr>
<td>MAPEC</td>
<td>$2.0 \times 10^5$</td>
<td>0.16</td>
<td>$1.2 \times 10^5$</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Buffer: 0.04 M potassium phosphate, 8 mM MgCl$_2$, pH 7.0.
It is also evident that, under these conditions, as the case was with measurements made in 0.04 M Tris buffer (Results, Section 1.1), the association constants for the diamino compounds are slightly higher than those of the monoamino derivatives. These differences, however, are more pronounced at 25°C than at 37°C. The elimination of the differences between the binding constants of the mono- and diamino-derivatives at higher temperature suggests that any advantage, in terms of binding energy, conferred upon the diamino compounds by the primary amino group at position C_3, is almost totally eliminated by a 12-degree increase in temperature.

It is also interesting to note that an increase of the temperature from 25°C to 37°C does not affect appreciably, if at all, the number, n, of binding sites per DNA phosphate, although at the higher temperature the association constant decreases by a factor of, approximately, two (Table 2). A similar observation was previously reported in a fluorometric study of the binding of EB to DNA (LePecq and Paoletti, 1967).

In summary, the binding data presented above are in agreement with previous studies indicating that the interaction of phenanthridinium compounds with DNA consists of two binding processes: the primary binding, which is the stronger of the two and which predominates at low added
drug/nucleotide ratios, and the secondary binding, which is observed at high values of these ratios.

The secondary binding is extremely sensitive to changes in ionic strength and it is eliminated in the presence of 0.008 M MgCl₂ (Figures 12 - 15). In fact, it has been reported that no secondary binding could be detected in the interaction of EB with DNA in the presence of as little as 0.004 M MgCl₂ (Waring, 1965b). The increased sensitivity of this type of binding process to salt concentration indicates that secondary binding relies mainly, if not exclusively, on electrostatic forces. This observation further supports the idea, initially advanced by LePecq and Paoletti (LePecq and Paoletti, 1967) that electrostatic forces are primarily responsible for the binding of EB to the secondary sites of DNA. These electrostatic forces are exerted between the negatively charged phosphate residues on the outside of the helix and the cationic dye molecule, since, at neutral pH, the ethidium molecule carries a single positive charge due to the quaternized nitrogen at position 5 of the ring. This mechanism of binding may very well be true also for the other phenanthridinium derivatives studied.

The primary binding is also influenced by ionic strength, although much less extensively than the secondary binding. This is indicated in Tables 1 and 2. At relatively
low salt concentration (less than 0.1M), binding parameters were found to decrease with increasing salt concentration. However, at higher salt concentrations, the binding appears independent of ionic strength (Figures 9 and 12). Similar findings were reported previously for the EB-DNA interaction (LePecq et al., 1964). It was found in that instance that the affinity of EB for DNA becomes independent of the ionic strength above 0.2 M for divalent ions and above approximately 1 M for monovalent ions. These observations indicate that the stabilizing forces of the phenanthridinium-DNA complex consist mainly of two components, only one of which is sensitive to salt concentration.

This conclusion is consistent with the model of binding, initially proposed by Fuller and Waring (Fuller and Waring, 1964) and subsequently supported by the work of LePecq and Paoletti (LePecq and Paoletti, 1967). The model postulates that the DNA-EB complex is stabilized mainly by hydrophobic forces between the DNA base pairs and the phenanthridinium ring system. These forces are not expected to be affected by increased ionic strength and should be mainly, if not exclusively, responsible for the binding observed at high salt concentrations.

Additional stabilization of the complex is provided by hydrogen bonds formed between the primary amino groups at positions 3 and 8 of the ring and the negatively charged phosphate residues on both polynucleotide strands. These
interactions, in contrast with the hydrophobic forces mentioned in the previous paragraph, are sensitive to the salt concentration of the medium. Therefore, it appears that the decrease in binding strength, observed with increasing salt concentrations, is due to the elimination of these hydrogen bonds.

2. CIRCULAR DICHROISM STUDIES

The method of circular dichroism is especially well suited for the study of the conformation of the phenanthridinium-DNA complexes. CD is extremely sensitive to small changes in the conformation of the polymer under study; differences, therefore, in the conformation of the dye-DNA complexes may be conveniently detected.

In addition, evidence about the conformation of a dye-DNA complex may be obtained by relating the experimentally observed CD spectrum to that predicted by theoretical models.

The circular dichroism of the phenanthridinium-DNA complexes was studied in the wavelength region between 300 and 600 nm. Neither of the components alone exhibits any optical activity in this region. Several bands appear, however, in the CD spectrum when these dyes are complexed with DNA.
2.1. **Diamino compounds**

The CD spectra of the DNA complexes of two diamino phenanthridinium compounds (EB and DDMB) are shown in Figures 16 and 17 (note that the ellipticity scale must be multiplied by 0.1 for wavelengths above 350 nm). The spectra in each Figure are obtained from complexes containing increasing added dye/nucleotide ratios. The striking similarities between the two families of spectra should be noted. In both cases there are three main regions of optical activity: one major positive band with a maximum close to 310 nm and a shoulder at, approximately, 340 nm; a negative band with a maximum around 370 nm; and a weaker, wide negative band with a maximum close to 500 nm.

In addition, a small negative band, centered near 295 nm, may be observed in the spectrum of the EB-DNA complex. This band is clearly distinguishable only at the high added dye/nucleotide samples, i.e. those containing low DNA concentrations. It is likely, therefore, that this band is not observed in lower EB/nucleotide ratios merely because it is masked by the much stronger positive band at 280 nm of the DNA spectrum.

The significance of these bands, in respect to their possible asymmetric source(s), will be examined in subsequent sections by studying the dependence of molar ellipticity on the bound dye/nucleotide ratio.
Figure 16. CD spectra of the EB-DNA complex at three (indicated) added EB/nucleotide ratios. Ellipticity values for wavelength above 350 nm are multiplied by a factor of 10.
Figure 17. CD spectra of the DDMB-DNA complex at three (indicated) added DDMB/nucleotide ratios. Ellipticity values for wavelengths above 350 nm are multiplied by a factor of 10. Buffer: 0.04 M Tris-HCl, pH 7.9.
2.1.1. The circular dichroism at 500 nm

Examination of Figures 16 and 17 indicates that the molar ellipticity of the diaminophenanthridinium-DNA complexes at 500 nm is independent of complex concentration. This point is more clearly illustrated in Figure 18, in which the $\epsilon_1 - \epsilon_r$ at 500 nm of the EB-DNA complex is plotted as a function of $r$.

Allowing for experimental error, which, in this case, can be substantial because of the very low optical activity of the drug-DNA complex at this wavelength, a horizontal line can be drawn through all points. The molar ellipticity of the EB-DNA complex at 500 nm is approximately -0.4, irrespective of the concentration of the complex.

A similar behavior is exhibited by the DDMB-DNA complex. The value of $\epsilon_1 - \epsilon_r$ at 500 nm is, again, approximately -0.4 (Figure 19).

This independence of molar ellipticity of the complex on the concentration of DNA-bound dye is, of course, expected as a consequence of Beer's law. Indeed, since $\epsilon_1 - \epsilon_r$ is derived by, essentially, normalizing the observed ellipticity to the concentration of the complex (Methods, Section 4), it expresses the optical activity per mole of this complex, a parameter which should be independent of complex concentration.
Figure 18. Dependence of molar ellipticity of the EB-DNA complex at the band maximum near 500 nm on $r$.

Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 19. Dependence of molar ellipticity of the DDMB-DNA complex at the band maximum near 500 nm on r.
Buffer: 0.04 M Tris-HCl, pH 7.9.
2.1.2. The circular dichroism at 310 nm

As already indicated in Figures 16 and 17, quite different behavior is displayed by the molar ellipticity at 310 nm. The dependence of molar ellipticity of the EB-DNA complex at that wavelength on $r$ is depicted in Figure 20. It is evident from this Figure that, in contrast with the behavior of the molar ellipticity at 500 nm and in apparent contradiction of Beer's law, the molar ellipticity at 310 nm is not independent of complex concentration. In fact, $\epsilon_1 - \epsilon_r$ at 310 nm increases with $r$ reaching an apparent maximum of approximately 25 at an $r$ value between 0.30 and 0.35, a ratio which corresponds to the saturation of the DNA-binding sites by EB.

In the case of the DDMB-DNA complex, a very similar dependence of $\epsilon_1 - \epsilon_r$ on $r$ is observed, as shown in Figure 21; indeed, the molar ellipticity of the DDMB-DNA complex at the band maximum near 310 nm increases with increasing $r$. The dependence appears sigmoid in shape and the molar ellipticity value reaches an apparent maximum of 20 for an $r$ of approximately 0.30.

This variation of the molar ellipticity at 310 nm of the DNA-diaminophenanthridinium complexes suggests that the mere fact that a dye molecule is bound to DNA is not a sufficient requirement for optical activity at this wavelength. Rather, it appears that ellipticity at the band maximum
Figure 20. Dependence of molar ellipticity of the EB-DNA complex at the band maximum near 310 nm on r.
Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 21. Dependence of molar ellipticity of the DDMB-DNA complex at the band maximum near 310 nm on r.
Buffer: 0.04 M Tris-HCl, pH 7.9.
near 310 nm depends on the "density" of the bound molecule population within a given segment of the nucleic acid, indicating that this ellipticity results from interactions, either direct or indirect, between dye molecules bound at neighboring sites on the DNA.

2.2. Monoamino compounds

Compared with the CD spectra of the diaminophenanthridinium-DNA complexes just described, the spectra of the complexes of the monoamino substituted drugs with DNA show considerable differences both in terms of overall shape as well as ellipticity values. Three monoamino compounds were studied: MAPEC, MAPAC and MMPB. The CD spectra of the complexes of these compounds with DNA at three different added drug/nucleotide ratios are shown in Figures 22-24 (note that in this case, in contrast with the diamino dyes-DNA CD spectra, the ellipticity scale above 350 nm is not multiplied by 0.1).

Certain similarities, both qualitative and quantitative, may be noted between the spectra of the three families. Specifically, two main areas of optical activity are distinguished in each spectrum: one comprised of a negative band with a maximum at, approximately, 470 nm, and an area of positive optical activity which extends in the wavelength region between 300 and 360 nm and which consists of at least
Figure 22. CD spectra of the MAPEC-DNA complex at three (indicated) added MAPEC/nucleotide ratios. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 23. CD spectra of the MAPAC-DNA complex at three (indicated) added MAPAC/nucleotide ratios. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 24. CD spectra of the MMPB-DNA complex at three (indicated) added MMPB/nucleotide ratios. Buffer: 0.04 M Tris-HCl, pH 7.9.
three individual bands. A small, negative band centered around 390 nm may also be present. This latter band is not observed in the spectrum of the MMPB-DNA complex.

In quantitative terms, the spectra presented in Figures 22 - 24 indicate that the complexes of monoamino dyes with DNA exhibit substantially lower molar ellipticities at the short wavelength region (300 - 350 nm) as compared to the corresponding complexes of the diamino drugs. Exactly the opposite is observed at the long (470 - 500 nm) wavelength region, where the complexes of the monoamino drugs have ellipticity values that are approximately five times higher than those observed with the diamino compounds.

These differences between the two classes of the CD spectra examined indicate that the conformation of the DNA-monoamino phenanthridinium complexes is quite distinct from that of the complexes formed from diamino derivatives. This observation also suggests that, in general, the number of primary amino substituents on the phenanthridinium ring may have a significant effect on the conformation of the dye-DNA complex.

The conformation of the monoamino phenanthridinium-DNA complex was studied in more detail by examination of the dependence of the molar ellipticity at the band maximum near 315 nm and 470 nm on r.
2.2.1. **The circular dichroism at 470 nm**

As indicated in Figures 22 - 24, the negative band with the 470 nm extremum displays a behavior reminiscent of that of the 500 nm maximum of the diamino compounds, i.e. molar ellipticity at this wavelength appears independent of complex concentration. The dependence of molar CD at that wavelength on complex concentration is shown in Figures 25 - 27. Although the absolute magnitudes of molar ellipticities of -2.4 to -3.0 are higher than those noted with the diamino compounds, ellipticity values remain, as the case is with diamino derivatives, constant, irrespective of the concentration of the drug-DNA complex. These findings indicate that the nature of the interactions responsible for the long wavelength dichroic band are probably not affected significantly by the lack of the 3-amino substituent from the intercalated molecule.

2.2.2. **The circular dichroism at the maximum near 315 nm**

In contrast with the behavior of the molar CD at the 370 nm extremum, the molar CD at the short wavelength maximum is not independent of the concentration of the complex. The dependence of the molar ellipticity of the drug-DNA complex at the band maximum near 315 nm on the concentration of the complex is shown in Figures 28 - 30.
Figure 25. Dependence of molar ellipticity of the MAPEC-DNA complex at the band maximum near 470 nm on r.

Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 26. Dependence of molar ellipticity of the MAPAC-DNA complex at the band maximum near 470 nm on r.

Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 27. Dependence of molar ellipticity of the MMPB-DNA complex at the band maximum near 470 nm on $r$.
Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 28. Dependence of molar ellipticity of the MAPEC-DNA complex at the band maximum near 315 nm on \( r \).

Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 29. Dependence of molar ellipticity of the MAPAC-DNA complex at the band maximum near 315 nm on $r$.

Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 30. Dependence of molar ellipticity of the MMPB-DNA complex at the band maximum near 315 nm on r.
Buffer: 0.04 M Tris-HCl, pH 7.9.
Although the molar ellipticity increases somewhat with complex concentration, this increase is much less pronounced than the one observed with the diamino compounds and the final $\epsilon_1 - \epsilon_r$ values are less than one-third of those obtained with the diamino compounds for the same $r$ values. In addition, this modest increase in molar ellipticity is accompanied by significant changes in the shape of the spectrum itself, as indicated by the sign-reversal of the molar ellipticity near 315 nm. Indeed, Figures 28 - 30 indicate that, at low bound dye/nucleotide ratios, the ellipticity at 315 nm has negative values, although it becomes positive at higher $r$ values. This ellipticity sign change occurs at an $r$ of approximately 0.10.

A similar sign change in ellipticity has been reported for the interaction of proflavine with DNA. It was interpreted as the result of mutual cancelling or reinforcement between three circular dichroism bands (Li and Crothers, 1969). In the case of monoamino phenanthridinium compounds, at least three distinct bands may be observed in the 300 to 350 nm region of the CD spectrum of their complexes with DNA. Therefore, the sign reversal for the ellipticity at 315 nm mentioned above may reflect a mutually cancelling relationship between some or all of these bands.
2.3. **Circular dichroism of the EB-DNA complex at high ionic strength**

Under the conditions the present CD studies were performed (0.04 M Tris buffer, room temperature), two types of binding are observed: primary binding, involving intercalation of the drug molecules between successive base pairs, and secondary binding, representing drug molecules bound electrostatically to the outside of the helix. Thus the question arises whether the observed ellipticity is the result of only one type of binding or both. The study of the circular dichroism of the EB-DNA complex at high ionic strength (5.0 M NaCl) provides information helpful in answering this question.

In addition, high ionic strength conditions provide a means of studying in more detail the negative CD band observed near 295 nm in the EB-DNA spectrum. As mentioned earlier (Results, Section 2.1) the low ellipticity values associated with this maximum were attributed to an overlap of this band with the positive band of the DNA-CD spectrum. It was decided, therefore, to examine the behavior of the CD spectrum of the EB-DNA complex at that wavelength in conditions under which the positive CD contribution from nucleic acid is reduced. Such conditions exist in high salt concentrations; it has been reported that, in 6.0 M LiCl, the ellipticity of DNA in the 260 - 290 nm region is
substantially reduced, with an especially pronounced reduction above 290 nm (Studdert et al., 1972). We observed similar, although less drastic, changes in the CD of DNA in 5.0 M NaCl.

The general features of the CD of the EB-DNA complex are not substantially altered in 5.0 M NaCl solution (Figure 31). Apart from an increase in the ellipticity of the 375 nm band, both the ellipticities and the maxima of the other bands located at wavelengths higher than 310 nm remain unchanged. In fact, the results listed in Table 3 indicate that ellipticities at 310 nm as well as 500 nm remain remarkably constant for salt concentrations which vary between 0.04 and 5.0 M. Furthermore, examination of Figure 32 reveals that the dependence of the ellipticities near 310 nm on bound EB to DNA ratios at 5.0 M NaCl exhibits a close similarity to the results obtained in 0.04 M buffer. The only apparent qualitative difference between the spectra at 0.04 M buffer and 5.0 M NaCl is that in the latter the negative CD band located near 295 nm is present for all ethidium bromide to DNA ratios examined, while at the lower ionic strength this band is present only for high EB to DNA ratios.

These results suggest that contributions from negative CD in the vicinity of this band may in fact be present.
Figure 31. CD spectrum of the EB-DNA complex at an added EB/nucleotide ratio of 0.1. Buffer: 0.04 M Tris-HCl, 5.0 M NaCl, pH 7.9. Ellipticity values for wavelength above 360 nm are multiplied by a factor of 10.
TABLE 3

Circular dichroism of the EB - DNA complex at various salt concentrations

<table>
<thead>
<tr>
<th>NaCl, (M)</th>
<th>$\varepsilon_1 - \varepsilon_r$ near 310 nm</th>
<th>$\varepsilon_1 - \varepsilon_r$ near 500 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>5.2</td>
<td>-0.53</td>
</tr>
<tr>
<td>0.12</td>
<td>6.1</td>
<td>-0.41</td>
</tr>
<tr>
<td>0.20</td>
<td>6.0</td>
<td>-0.46</td>
</tr>
<tr>
<td>0.50</td>
<td>6.1</td>
<td>-0.48</td>
</tr>
<tr>
<td>0.60</td>
<td>5.4</td>
<td>-0.45</td>
</tr>
<tr>
<td>0.75</td>
<td>5.5</td>
<td>-0.41</td>
</tr>
<tr>
<td>1.0</td>
<td>6.0</td>
<td>-0.50</td>
</tr>
<tr>
<td>5.0</td>
<td>5.4</td>
<td>-0.46</td>
</tr>
</tbody>
</table>

Measurements were carried out for samples of added EB/nucleotide ratios between 0.085 and 0.28, with $r$ values ranging between 0.085 and 0.14. $\varepsilon_1 - \varepsilon_r$ values for the maximum near 310 nm are reported for an $r$ of 0.085 and were estimated from plots of $\varepsilon_1 - \varepsilon_r$ versus $r$. 
Figure 32. Dependence of molar ellipticity of the EB-DNA complex at the band maximum near 310 nm. Buffer: 0.04 M Tris-HCl, 5.0 M NaCl, pH 7.9.
for low EB to DNA ratios as well but a definite band near 295 nm can be distinguished only at higher ionic strengths or at higher ratios.

The increase in the CD and the shift in the apparent maximum of this band from 296 to 292 nm noted with increasing $r$ at constant concentration of EB and changing DNA concentrations (Table 4) may simply be interpreted as the result of two distinct trends: the increase in the negative CD originating from transitions characteristic of the interaction between DNA and EB and the increase in the overlap between the CD originating from this interaction with the positive CD of DNA present at wavelengths below 300 nm. This overlap would tend to produce the shift in the apparent maximum of the 295 nm band and would limit ellipticities even at the higher EB to DNA ratios. However, substantially higher ellipticities near 295 nm are noted in 5.0 M NaCl (Figure 33). This increase is, of course, consistent with the expected decrease in the contribution of the positive CD of DNA below 300 nm.

The variations in the ellipticities of the complex near 295 nm with $r$ noted both at 0.04 M buffer and at 5.0 M NaCl, when the CD is obtained at constant DNA concentration (Figure 33, Table 5) deserves some comment. The only apparent explanation for these results is that the negative CD
TABLE 4

Dependence of molar ellipticity of the EB-DNA complex near 295 nm on $\xi$

<table>
<thead>
<tr>
<th>Added EB/DNA ratio</th>
<th>$\xi$</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\varepsilon_1 - \varepsilon_r$ at $\lambda_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>0.15</td>
<td>296</td>
<td>-0.7</td>
</tr>
<tr>
<td>0.25</td>
<td>0.21</td>
<td>295</td>
<td>-1.7</td>
</tr>
<tr>
<td>1.00</td>
<td>0.24</td>
<td>292</td>
<td>-2.0</td>
</tr>
</tbody>
</table>

Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 33. The circular dichroism of EB-DNA complex in 0.04 M Tris-HCl, 5.0 M NaCl, pH 7.9 buffer at various \( r \) values. Upper curve, \( r = 0.086 \); middle curve, \( r = 0.162 \); lower curve, \( r = 0.180 \).
TABLE 5

Dependence of molar ellipticity of the EB-DNA complex near 295 nm on \( r \)

<table>
<thead>
<tr>
<th>Added EB/DNA ratio</th>
<th>( r )</th>
<th>( \lambda_{\text{max}} ) (nm)</th>
<th>( \epsilon_1 - \epsilon_r ) at ( \lambda_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>0.085</td>
<td>297</td>
<td>-3.2</td>
</tr>
<tr>
<td>0.20</td>
<td>0.133</td>
<td>296</td>
<td>-3.6</td>
</tr>
<tr>
<td>0.40</td>
<td>0.162</td>
<td>295</td>
<td>-5.4</td>
</tr>
<tr>
<td>0.50</td>
<td>0.164</td>
<td>295</td>
<td>-5.7</td>
</tr>
<tr>
<td>0.65</td>
<td>0.164</td>
<td>295</td>
<td>-6.3</td>
</tr>
<tr>
<td>0.80</td>
<td>0.165</td>
<td>294</td>
<td>-6.4</td>
</tr>
<tr>
<td>1.00</td>
<td>0.180</td>
<td>294</td>
<td>-7.2</td>
</tr>
</tbody>
</table>

Buffer: 0.04 M Tris-HCl, 5.0 M NaCl, pH 7.9.
near 295 nm exhibits an intrinsic dependence on the EB/DNA ratios, increasing as the ratio increases. The behavior of the 295 nm band appears therefore to be similar to that noted for the positive CD band near 310 nm and distinct from that of the band centered at 500 nm. This indicates that the 295- and the 310-nm bands may have similar origin. In fact, the relationship between these two bands in terms of both their neighboring positions and the dependence of their ellipticities on $r$ strongly suggests that these bands are manifestations of the positive and the negative CD components of the same conservative transition (Tinoco, 1964).

The generally lower ellipticities noted for the negative component and the somewhat asymmetric shape of the doublet are, of course, consistent with the overlap between this band and the CD of the DNA below 300 nm.

3. FLUORESCENCE STUDIES

Additional information regarding the conformation of the DNA-phenanthridinium complex may be obtained from the study of the effect of DNA binding on the fluorescence properties of these compounds. It is known that the fluorescence intensity of EB increases substantially upon binding to double-stranded DNA (LePecq et al., 1964). Fluorescence is a molecular property, the expression of which depends on such environmental factors as solvent pH,
viscosity, polarity etc., as well as the specific geometric orientation of the fluorescing molecules. Since the conformation of the DNA-phenanthridinium complex is influenced by the number of primary amino substituents of the intercalated molecule, as indicated by the CD studies presented above, it was thought interesting to examine the effect of these substituents on the fluorescence enhancement of the phenanthridinium compounds upon binding to DNA.

The effect of DNA on the fluorescence of EB is illustrated in Figure 34. The fluorescence increase in the presence of DNA is very similar to that reported previously (LePecq et al., 1964). The increase in fluorescence as a function of the bound dye/nucleotide ratio is shown in Figure 35. In this Figure, the net fluorescence per mole of bound drug, calculated as described in Section 5 (Methods), is plotted versus $r$.

As shown in this Figure, the net fluorescence per mole of bound drug increases approximately 30-fold when all the drug is intercalated to DNA (low $r$ values). The fluorescence values are independent of the concentration of the complex for low $r$ values, but later decrease as $r$ increases. This apparent decrease is probably due to the fact that the total concentration of bound ethidium is used in the calculation of fluorescence. However, a proportion of bound EB represents molecules which are attached via electrostatic
Figure 34. Fluorescence emission spectrum of free EB (curve A) and EB in the presence of $8.6 \times 10^{-4}$ M calf thymus DNA (curve B). (EB) = $4.6 \times 10^{-5}$ M in both samples. Excitation wavelength: 530 nm. The 530 nm band represents scattered excitation light. Ordinate: Fluorescence intensity in arbitrary units. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 35. Dependence of molar fluorescence of the EB-DNA complex on $r$. Excitation wavelength: 530 nm. Emission wavelength: 590 nm. Buffer: 0.04 M Tris-HCl, pH 7.9.
interactions on the outside of the helix and which have a fluorescence efficiency that is very similar to that of the free drug (LePecq and Paoletti, 1967). Since the proportion of these bound molecules increases with increasing r, it is reasonable to expect a decrease in the average fluorescence intensity per mole of bound drug.

Similar results were obtained with the DDMB-DNA complex (Figure 36). Again, an increase in fluorescence intensity of approximately 20-fold is observed upon binding of the drug to the nucleic acid.

A distinctly different picture, however, emerges from the interaction of monoamino compounds with DNA. A representative emission spectrum of MAPAC in the presence and absence of DNA is shown in Figure 37. As indicated in this Figure, the fluorescence of MAPAC does not increase very much upon binding to DNA.

The dependence of the fluorescence of MAPAC and MAPEC on the bound dye/nucleotide ratio is shown in Figures 38 and 39. Because of the very low fluorescence intensity of these compounds, the experimental error of the measurements presented in Figures 38 and 39 may be considerable. It is evident, nevertheless, that a fluorescence enhancement, similar to that observed in the case of the diamino derivatives, does not take place.
Figure 36. Dependence of molar fluorescence of the DDMB-DNA complex on $r$. Excitation wavelength: 517 nm. Emission wavelength: 580 nm. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 37. Fluorescence emission spectrum of free MAPAC (curve A) and MAPAC in the presence of $8.6 \times 10^{-4}$ M calf thymus DNA (curve B). (MAPAC) = $3.6 \times 10^{-5}$ M in both cases. Excitation wavelength: 470 nm. Ordinate: Fluorescence intensity in arbitrary units. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 38. Dependence of molar fluorescence of the MAPEC-DNA complex on $r$. Excitation wavelength: 470 nm. Emission wavelength: 555 nm. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 39. Dependence of molar fluorescence of the MAPAC-DNA complex on $r$. Excitation wavelength: 470 nm. Emission wavelength: 560 nm. Buffer: 0.04 M Tris-HCl, pH 7.9.
In the case of another monoamino phenanthridinium drug, MMPB (Figure 40), fluorescence per mole of DNA-bound drug does increase, approximately 5-fold, upon binding of the drug to DNA. This, however, is a relatively small increase, compared with the 20- to 30-fold increase exhibited by the diamino compounds, and, in any case, the resulting fluorescence intensity is less than 1/20 of the corresponding intensity observed with the diamino compounds.

It appears, therefore, that the effect of DNA binding on the fluorescence intensity of the phenanthridinium drugs is quite distinct for the monoamino derivatives than it is for the diamino compounds. This differential effect may be another reflection of the conformational differences, already suggested by the CD experiments, between the two classes of complexes. These conformational differences are expected to influence the interactions of the intercalated dyes with their molecular environment and, consequently, may be responsible for the observed differences in fluorescence intensity.

4. TEMPERATURE - OPTICAL DENSITY PROFILES

Several intercalating compounds, including EB, have been shown to increase the melting temperature of DNA (Kleinwachter and Koudelka, 1964; Gersch and Jordan, 1965; Waring, 1974). However, no systematic study has been
Figure 40. Dependence of molar fluorescence of the MMPB-DNA complex on $r$. Excitation wavelength: 475 nm. Emission wavelength: 560 nm. Buffer: 0.04 M Tris-HCl, pH 7.9.
undertaken to determine the relationship, if any, between the chemical structure of the intercalating molecule, as expressed in the conformation of the resulting complex, and the thermal stability of the complex. Such information is useful in understanding the nature of forces involved in stabilizing the dye-DNA complex, as well as evaluating the effect of the phenanthridinium dyes on the activity of enzymes, such as DNA polymerase, the action of which requires that DNA be in the single-stranded form.

The effect of the various substituents of the phenanthridinium ring on the stability of the DNA-phenanthridinium complexes was studied by means of the temperature-optical density profiles (Tm profiles) of these complexes. The phenanthridinium compounds used in this study were: EB, DEMB, DDMB, MAPEC, MAPAC and MMPB. The temperature-optical density profiles at 260 nm were obtained as described in Section 6 (Methods), using samples with an added dye to nucleotide ratio of 0.10.

The melting profiles of DNA alone and of DNA complexed with each of the above phenanthridinium compounds are illustrated in Figures 41-47 and the results are summarized in Table 6. The melting profiles are plotted as normalized (or total) hyperchromicity versus temperature.

The initial gradual increase in absorbance, observed for all drug-DNA complexes before the onset of the melting
Figure 41. Temperature-optical density profile at 260 nm of calf thymus DNA. \((\text{DNA}) = 1.5 \times 10^{-4} \text{ M}\). Heating rate: \(3^\circ\text{C}/\text{min}\). Buffer: \(0.04 \text{ M Tris-HCl}, \text{ pH 7.9}\).
Figure 42. Temperature-optical density profile at 260 nm of the MAPEC-DNA complex. (MAPEC) = 1.5 x 10^{-5} M. (DNA) = 1.5 x 10^{-4} M. Heating rate: 3°C/min. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 43. Temperature-optical density profile at 260 nm of the MAPAC-DNA complex. (MAPAC) = 1.5 x 10^{-5} M. (DNA) = 1.5 x 10^{-4} M. Heating rate: 3°C/min. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 44. Temperature-optical density profile at 260 nm of the MMPB-DNA complex. (MMPB) = 1.5 x 10^{-5} M. (DNA) = 1.5 x 10^{-4} M. Heating rate: 3°C/min. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 45. Temperature-optical density profile at 260 nm of the EB-DNA complex. (EB) = 1.5 x 10^{-5} M. (DNA) = 1.5 x 10^{-4} M. Heating rate: 3°C/min. Buffer: 0.04 M Tris-HCl, pH 7.9.
Figure 46. Temperature-optical density profile at 260 nm of the DEMB-DNA complex. (DEMB) = 1.5 \times 10^{-5} \text{ M}.
(DNA) = 1.5 \times 10^{-4} \text{ M}. Heating rate: 3^\circ\text{C/min}. Buffer: 0.04 \text{ M Tris-HCl, pH 7.9.}
Figure 47. Temperature-optical density profile at 260 nm of the DDMB-DNA complex. (DDMB) = 1.5 x 10^{-5} M. (DNA) = 1.5 x 10^{-4} M. Heating rate: 3°C/min. Buffer: 0.04 M Tris-HCl, pH 7.9.
TABLE 6

The effect of certain phenanthridinium compounds on the thermal stability of DNA

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tm (°C)</th>
<th>ΔT₃₀⁻₇₀(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>76.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Diamino:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EB</td>
<td>83.2</td>
<td>7.0</td>
</tr>
<tr>
<td>DEMB</td>
<td>83.8</td>
<td>7.0</td>
</tr>
<tr>
<td>DDMB</td>
<td>85.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Monoamino:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPEC</td>
<td>84.5</td>
<td>7.5</td>
</tr>
<tr>
<td>MAPAC</td>
<td>82.8</td>
<td>7.0</td>
</tr>
<tr>
<td>MMPB</td>
<td>82.8</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Buffer: 0.04 M Tris-HCl, pH 7.9. Light path: 1.0 cm. Heating rate: 3°C/min. (DNA) = 1.5 x 10⁻⁴ M.
transition, is probably due to the decrease in drug binding with increasing temperature (Results, Section 1.3). Since the binding of the dye to DNA is accompanied by a hypochromic effect, the release of bound drug would be expected to result in an increase in absorbance.

The effects of the phenanthridinium derivatives on the melting of DNA may be summarized as follows:

A. The phenanthridinium compounds tested increase the thermal stability of the double-helical form of the DNA. Similar observations have been made previously for a number of intercalating agents, including EB (Kleinwachter and Koudelka, 1964; Gersch and Jordan, 1965; LePecq and Paoletti, 1967).

B. All derivatives tested, whether mono- or diamino, increase the melting temperature of DNA to approximately the same extent, namely from 76.5°C to an average of 83.8°C. It has been reported that in the case of EB as well as of other intercalating compounds, such as acridines, the Tm is very sensitive to the added drug to nucleotide ratio at low values of this ratio (Kleinwachter and Koudelka, 1964; Gersch and Jordan, 1965; Waring, 1974). Therefore, the small differences observed in the presence of different phenanthridinium derivatives may simply be due to potential experimental error in determining the concentration of the drug.
C. The width of the melting transition, as measured by $\Delta T_{30-70}$, increases from $5.5^\circ C$, in the case of DNA, to approximately $7.0^\circ C$ for the dye-DNA complexes.

The significance of these findings, in terms of the stability and conformation of the phenanthridinium-DNA complexes, is examined in subsequent chapters.

5. INHIBITION OF DEOXYRIBONUCLEASE

A study of the inhibition of DNase by phenanthridinium compounds was undertaken in order to examine further the effect of the chemical structure of the intercalated drug on the conformation of the drug-DNA complex. Enzymes, in general, and DNase I, in particular, have specific conformational requirements for their substrates. Therefore, any factor affecting substrate conformation would also be expected to have an effect on the activity of the enzyme.

More specifically, the inhibition of DNase I activity on native DNA by EB, DDMB, MAPEC and MAPAC was studied. The dependence of the inhibition of the enzyme on the added drug/nucleotide ratio for these four drugs is shown in Figures 48 - 51. Since the amount of DNA present in all complexes is the same, this presentation of the data is equivalent to one where percent inhibition is plotted versus total amount of inhibitor present.
Figure 48. Inhibition of pancreatic DNase by EB as a function of the added EB/nucleotide ratio. Buffer: 0.04 M Tris-HCl, 0.004 M MgSO₄, 0.0017 M CaCl₂, pH 7.9. Room temperature.
Figure 49. Inhibition of pancreatic DNase by DDMB as a function of the added DDMB/nucleotide ratio. Buffer: 0.04 M Tris-HCl, 0.004 M MgSO$_4$, 0.0017 M CaCl$_2$, pH 7.9. Room temperature.
Figure 50. Inhibition of pancreatic DNase by MAPEC as a function of the added MAPEC/nucleotide ratio. Buffer: 0.04 M Tris-HCl, 0.004 M MgSO$_4$, 0.0017 M CaCl$_2$, pH 7.9. Room temperature.
Figure 51. Inhibition of pancreatic DNase by MAPAC as a function of the added MAPAC/nucleotide ratio. Buffer: 0.04 M Tris-HCl, 0.004 M MgSO₄, 0.0017 M CaCl₂, pH 7.9. Room temperature.
Examination of the dependence of DNase inhibition on the added dye/nucleotide ratio reveals the presence of some differences in the inhibition effects of the phenanthridinium derivatives used in this study. However, no definite conclusions can be drawn as to the differences in the effectiveness of these compounds as inhibitors without a knowledge of the mechanism whereby this inhibitory action is brought about. For this reason the question whether EB interferes with the substrate function of DNA or whether it affects the activity of the enzyme itself was investigated.

Figure 52 shows the results of an experiment, in which the enzyme activity was studied as a function of DNA concentration, either in the absence or in the presence of EB. The results are plotted according to Lineweaver and Burk (Lineweaver and Burk, 1934) as the inverse of the initial velocity versus the inverse of the substrate concentration. Experimental points obtained either in the presence or in the absence of inhibitor fall on straight lines intersecting each other on the ordinate. This observation indicates that the inhibition of DNase by these phenanthridinium compounds is related in a competitive fashion to the amount of DNA present and serves as strong evidence in support of the notion that EB has no effect on the enzyme itself, but rather interferes with the function of DNA as substrate.
Figure 52. Effect of the variation of the concentration of DNA substrate on the activity of pancreatic DNase in the absence (0) and in the presence of $2.7 \times 10^{-6}$ M EB (●). Buffer: 0.04 M Tris-HCl, 0.004 M MgSO$_4$, 0.0017 M CaCl$_2$, pH 7.9. Room temperature.
Further support for this mechanism of inhibition is provided by the observation that, when total inhibitor concentration, $D_t$, is plotted versus the inhibition function $i/(1-i)$, the experimental points can easily be fitted to a curve which passes through the origin of the axes and the initial and final parts of which are straight lines (Figures 53 - 56). This behaviour corresponds exactly to the case where the inhibitor binds to the substrate at the same site as the enzyme; the inhibition is the result of a diminution of the number of substrate molecules susceptible to an attack by the enzyme (Reiner, 1959). From the values of the slopes of the two linear portions and the extrapolative intercept on the $D_t$ axis, the association constants for the binding of the phenanthridinium drugs to DNA were calculated. These values are summarized in Table 7 along with the spectrophotometrically (Results, Section 1.3) determined values. There is remarkable agreement between the two sets of association constants determined by the two methods.

In view of the observation that DNase inhibition appears to be due to the binding of the phenanthridinium inhibitors to the DNA substrate, perhaps a more meaningful way of presenting the data is in the form of dependence of inhibition on the amount of drug bound to DNA or, equivalently, on $r$. The amount of drug bound to DNA was calculated on the basis of the binding constants obtained in 0.04 M potassium
Figure 53. Inhibition of pancreatic DNase by EB.
The data are plotted according to (Reiner, 1959). Buffer: 0.04 M Tris-HCl, 0.004 M MgSO₄, 0.0017 M CaCl₂, pH 7.9.
Room temperature.
Figure 54. Inhibition of pancreatic DNase by DDMB. The data are plotted according to (Reiner, 1959). Buffer: 0.04 M Tris-HCl, 0.004 M MgSO$_4$, 0.0017 M CaCl$_2$, pH 7.9. Room temperature.
Figure 55. Inhibition of pancreatic DNase by MAPEC. The data are plotted according to (Reiner, 1959). Buffer: 0.04 M Tris-HCl, 0.004 M MgSO$_4$, 0.0017 M CaCl$_2$, pH 7.9. Room temperature.
Figure 56. Inhibition of pancreatic DNase by MAPAC. The data are plotted according to (Reiner, 1959). Buffer: 0.04 M Tris-HCl, 0.004 M MgSO$_4$, 0.0017 M CaCl$_2$, pH 7.9. Room temperature.
TABLE 7

Association constants for the binding of certain phenanthridinium compounds to DNA

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reiner plot (^{(1)})</th>
<th>Scatchard plot (^{(2)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB</td>
<td>$2.2 \times 10^5$ 1/mole</td>
<td>$2.2 \times 10^5$ 1/mole</td>
</tr>
<tr>
<td>DDMB</td>
<td>$2.6 \times 10^5$ 1/mole</td>
<td>$1.9 \times 10^5$ 1/mole</td>
</tr>
<tr>
<td>MAPAC</td>
<td>$1.2 \times 10^5$ 1/mole</td>
<td>$1.7 \times 10^5$ 1/mole</td>
</tr>
<tr>
<td>MAPEC</td>
<td>$1.6 \times 10^5$ 1/mole</td>
<td>$2.0 \times 10^5$ 1/mole</td>
</tr>
</tbody>
</table>

\(^{(1)}\) Buffer: 0.04 M Tris-HCl, 4 mM MgSO\(_4\), 1.7 mM CaCl\(_2\), pH 7.9.

\(^{(2)}\) Buffer: 0.04 M potassium phosphate, 8 mM MgCl\(_2\), pH 7.0.
phosphate, 0.008 M MgCl$_2$, pH 7.0. Although these are not the exact conditions under which the DNase assay was performed, these binding constants are nevertheless very close to the values obtained under assay conditions (Table 7).

The percent inhibition of DNase by phenanthridinium dyes, as a function of $r$, is shown in Figures 57-60. Certain features of these graphs deserve comment.

The inhibition of DNase increases rapidly up to an $r$ of approximately 0.025 which corresponds to about 25% inhibition of the enzyme activity. Beyond that point, the inhibition increases linearly with the concentration of DNA-bound drug with slopes that are similar for the four drugs tested. Small differences, however, may exist and they seem to indicate that a phenyl substituent at position 6 (EB, MAPEC, MAPAC with slopes, respectively, or 548, 535, and 545) gives rise to more effective DNase inhibition than the 6-methyl substituent (DDMB with a slope of 498).

The number of amino substituents on the phenanthridinium ring apparently does not affect substantially the inhibitory effect of these compounds on the activity of DNase, as it is evident from a comparison of the slopes of the inhibition plots of a diamino compound (EB, slope = 548) and two monoamino compounds (MAPEC, slope = 535 and MAPAC, slope = 545).
Figure 57. Inhibition of pancreatic DNase by EB as a function of $r$. Buffer: 0.04 M Tris-HCl, 0.004 M MgSO$_4$, 0.0017 M CaCl$_2$, pH 7.9. Room temperature.
Figure 58. Inhibition of pancreatic DNase by DDMB as a function of $r$. Buffer: 0.04 M Tris-HCl, 0.004 M MgSO$_4$, 0.0017 M CaCl$_2$, pH 7.9. Room temperature.
Figure 59. Inhibition of pancreatic DNase by MAPEC as a function of r. Buffer: 0.04 M Tris-HCl, 0.004 M MgSO₄, 0.0017 M CaCl₂, pH 7.9. Room temperature.
Figure 60. Inhibition of pancreatic DNase by MAPAC as a function of $r$. Buffer: 0.04 M Tris-HCl, 0.004 M $\text{MgSO}_4$, 0.0017 M $\text{CaCl}_2$, pH 7.9. Room temperature.
6. **INHIBITION OF DNA POLYMERASE**

Additional information concerning the effect of the intercalated phenanthridinium drug on the conformation and stability of the DNA-drug complex can be obtained from the study of the effect of these compounds on the activity of DNA polymerase using this complex as template. Changes in the conformation of the DNA-dye complex due to intercalation of different phenanthridinium dyes are expected to alter the affinity of the enzyme for the template and result in altered overall enzymic activity. Furthermore, physical separation of the parent strands is known to be a pre-requisite for DNA replication. Agents stabilizing the double-stranded conformation of the DNA are expected to have an overall inhibitory effect on the action of DNA polymerase. Therefore, since phenanthridinium drugs preferentially stabilize the double-helical form of DNA (Results, Section 4), it appeared to be of interest to examine whether their DNA-stabilizing effect could be correlated with their inhibitory action on DNA polymerase.

The effect of four phenanthridinium compounds on the activity of the *Micrococcus luteus* DNA polymerase was studied. The compounds used were: EB, DEMB, MAPEC and MAPAC.

Initially, the amount of new DNA synthesis was studied as a function of the time of incubation of the assay
mixture at 37°C. As evidenced from the results shown in Figure 61, the amount of DNA synthesized is directly proportional to the incubation time, at least up to 40 min. In subsequent experiments, incubation time was limited to 30 min.

The percent inhibition of DNA polymerase as a function of the added dye/nucleotide ratio is shown in Figures 62 - 65. For all four drugs tested, the inhibition increases very rapidly for low added ratios, but appears to level off after a ratio of, about, 0.25. At this value, approximately 80% inhibition of the polymerase activity is observed.

Of course, no definite comparison between the inhibitory action of these phenanthridinium compounds on DNA polymerase activity is possible without some knowledge of the mechanism of their action and, specifically, without knowing whether the inhibition of the polymerase activity is due to the interaction of the inhibitor with the DNA template or with the enzyme itself. In order to answer this question, four experiments were carried out, in which the amount of new DNA synthesized was studied as a function of the concentration of the DNA template, both in the absence and in the presence of phenanthridinium inhibitors. The results were plotted according to Lineweaver and Burk as the inverse of the amount of newly synthesized DNA versus the inverse of the concentration of the template and are shown
Figure 61. Time dependence of DNA synthesis by *Micrococcus luteus* DNA polymerase I. The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl$_2$; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; temperature: 37°C.
Figure 62. Inhibition of DNA polymerase by EB as a function of the added EB/nucleotide ratio. The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl$_2$; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; 0.072 mg/ml activated calf thymus DNA. Temperature: 37°C.
Figure 63. Inhibition of DNA polymerase by DEMB as a function of the added DEMB/nucleotide ratio. The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl₂, 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; 0.072 mg/ml activated calf thymus DNA. Temperature: 37°C.
Figure 64. Inhibition of DNA polymerase by MAPEC as a function of the added MAPEC/nucleotide ratio. The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl₂; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; 0.072 mg/ml activated calf thymus DNA. Temperature: 37°C.
Figure 65. Inhibition of DNA polymerase by MAPAC as a function of the added MAPAC/nucleotide ratio. The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl₂; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; 0.072 mg/ml activated calf thymus DNA. Temperature: 37°C.
in Figures 66 - 69. With all four compounds studied, straight lines can be drawn through the experimental points obtained both in the presence and in the absence of inhibitor. These two lines intersect each other on the \(1/v\) axis, thus indicating that the inhibition of DNA polymerase by these phenanthridinium compounds is competitive with the concentration of the DNA template. This finding is in complete agreement with, and further supports, the notion that the inhibitory effect of these compounds on DNA polymerase is due to the formation of a complex between the dyes and the template. A similar observation was reported (Waring, 1965a) for the inhibition of the DNA-dependent RNA polymerase activity by EB, indicating a similar mode of inhibition of the two enzyme systems by EB.

Since the DNA polymerase inhibition seems to result from the interaction of the drug with the template, a more meaningful way of presenting the inhibition data is, perhaps, as a function of the template-bound inhibitor or, equivalently, as a function of \(r\) (Figures 70 - 73). The amount of template-bound drug is estimated by means of the binding constants obtained under assay conditions (Results, Section 1.3).

The inhibition of DNA polymerase by the four phenanthridinium drugs tested depends very strongly on the concentration of the DNA-drug complex and, at low \(r\) values,
Figure 66. Effect of the variation of the concentration of DNA template on the activity of DNA polymerase in the absence (▲) and in the presence of $4.3 \times 10^{-6}$ M EB (Δ). The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl$_2$; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; temperature: 37°C.
Figure 67. Effect of the variation of the concentration of DNA template on the activity of DNA polymerase in the absence (▲) and in the presence of $6 \times 10^{-6}$ M DEMB (Δ). The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl$_2$; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; temperature: 37°C.
Figure 68. Effect of the variation of the concentration of DNA template on the activity of DNA polymerase in the absence (▲) and in the presence of $4.5 \times 10^{-6} \text{M}$ MAPEC (▲). The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M $\text{MgCl}_2$; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; temperature: 37°C.
Figure 69. Effect of the variation of the concentration of DNA template on the activity of DNA polymerase in the absence (▲) and in the presence of $6 \times 10^{-6}$ M MAPAC (▲). The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl₂; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; temperature: 37°C.
Figure 70. Inhibition of DNA polymerase by EB as a function of $r$. The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl$_2$; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; 0.072 mg/ml activated calf thymus DNA. Temperature: 37°C.
Figure 71. Inhibition of DNA polymerase by DEMB as a function of $r$. The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl$_2$; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; 0.072 mg/ml activated calf thymus DNA. Temperature: 37°C.
Figure 72. Inhibition of DNA polymerase by MAPEC as a function of r. The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl$_2$; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; 0.072 mg/ml activated calf thymus DNA. Temperature: 37°C.
Figure 73. Inhibition of DNA polymerase by MAPAC as a function of $r$. The reaction mixture contained, in 0.3 ml, 0.04 M potassium phosphate, pH 7.0; 0.008 M MgCl$_2$; 0.33 mg/ml BSA; 0.001 M 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of tritiated TTP (approximately 15000 cpm/nmole); 8 µg/ml enzyme; 0.072 mg/ml activated calf thymus DNA. Temperature: 37°C.
it increases rapidly with $r$ so that the activity of the polymerase is already reduced by 50% at an $r$ value of approximately 0.05, i.e. when, on the average, one drug molecule is bound per 10 base pairs. However, at higher concentrations of the DNA-drug complex, the rate of inhibition gradually decreases and certain differences in the inhibitory effect of these drugs on DNA polymerase activity become apparent. Indeed, 90% inhibition with MAPEC is observed at an $r$ value of 0.13, with MAPAC at an $r$ of 0.14 and with EB at an $r$ of 0.15, whereas with DEMB, such level of inhibition is not observed until an $r$ of 0.18 is reached.

It is interesting to note that DEMB, the compound that appears as the weakest inhibitor, is the only one among the four derivatives studied in which the 6-phenyl substituent has been replaced by a relatively smaller ethyl group. This observation suggests that the size of the substituent at position 6 of the phenanthridinium ring plays some role in determining the extent of DNA polymerase inhibition by the phenanthridinium compounds.
DISCUSSION

1. FACTORS INFLUENCING THE STABILITY OF THE COMPLEX

It is generally accepted that intercalation of phenanthridinium compounds to DNA places the phenanthridinium ring between two successive base pairs in an orientation approximately perpendicular to the helix axis. Stabilization energy for the formation of this complex is provided mainly by hydrophobic forces between the phenanthridinium ring and the DNA bases. Additional stabilization is afforded by hydrogen bonds formed between the amino substituents at positions 3 and 8 of the intercalated molecule and the phosphate groups at the two DNA strands.

The above model, proposed initially for EB (Fuller and Waring, 1964), appears to be equally valid for the rest of the phenanthridinium compounds examined in this investigation. Indeed, measurements of the unwinding angle of supercoiled DNA in the presence of phenanthridinium derivatives suggest that the absence of one or even both amino groups at positions 3 and 8 of the ring does not affect appreciably the ability of these compounds to intercalate (Wakelin and Waring, 1974). The major component of the energy of formation of the phenanthridinium-DNA complex is derived from the hydrophobic interactions mentioned above, as indicated by the effect of the phenanthridinium derivatives
on the thermal stability of the DNA. On the basis of the evidence obtained from the binding studies, it appears that the extent of double-stranded DNA stabilization against thermal denaturation is the same for derivatives with the amino groups at both the C₃ and C₈ positions of the ring, as well as for those with only the 8-amino substituent. The absence of any differential stabilization of the complex by mono- or diamino-phenanthridinium derivatives indicates that a hydrogen bond between the 3-amino group of the intercalated molecule and a DNA phosphate residue does not contribute significantly to the overall stabilization of the complex, at least at melting temperatures. Indeed, it was estimated (Wakelin and Waring, 1974) that each of the two hydrogen bonds (involving the 3- and 8-amino groups of the molecule) contributes approximately 0.85 kcal/mole to the stability of the complex. A contribution of 0.7 kcal/mole for the hydrogen bond involving the 3-amino group may be calculated from the association constant values of EB and MAPEC (Table 1), with the assumption that the p-amino group of MAPEC does not affect the stability of the complex. Clearly, such small differences in the free energy of formation of the complex are not expected to result in any measurable differential effect on the melting temperature of the phenanthridinium-DNA complex.
Small differences in complex stabilizing energy are more easily detectable at lower temperatures. Indeed, such differences are expressed in the association constant values calculated for the binding of the phenanthridinium compounds to DNA. Although, at any one set of experimental conditions used, major differences in the binding properties of the drugs were not observed, small differences do exist, both in the intrinsic association constant and in the number of binding sites. Significantly, these differences are more pronounced under conditions of low temperature and low ionic strength, whereas at 37°C and moderately high ionic strength the association constants are almost the same for both mono- and diamino derivatives. These observations are consistent with the model of Fuller and Waring presented above and indicate that hydrogen bonds between the 3 and 8 amino groups of the phenanthridinium molecule and the DNA phosphates contribute a small but measurable fraction of the complex stabilizing energy. Therefore, the slightly weaker binding of the monoamino derivatives can be explained by their lack of the amino group at position C3 which would provide the opportunity for a second hydrogen bond with DNA.

Another interesting feature of the Tm profiles of the dye-DNA complexes is the observed increase in the width of the transition upon binding of the drugs to the nucleic acid. This widening of the melting transition is consistent
with the "reintercalation" mechanism proposed earlier (Aktipis et al., 1975). It was observed that, during thermal denaturation of the EB-DNA complex, the molar ellipticity at 310 nm increases sharply shortly after the onset of strand separation. This increase was interpreted as indicative of reintercalation of EB molecules that had been released from the already melted regions of the complex. It may be assumed that, at room temperature, the EB molecules are randomly and uniformly distributed over the length of the DNA molecule. As the temperature is increased, however, certain regions of DNA tend to melt first. EB molecules intercalated in these regions are released and reintercalate at more stable DNA regions which retain their double-stranded form at this temperature. This process of rearrangement of intercalated molecules results in a localized increase of the drug/nucleotide ratio. Since the thermal stability of the DNA increases with the increasing EB/nucleotide ratio (Martz, 1971; Waring, 1974), it is expected that the intercalation of these drug molecules to the as yet unmelted DNA regions will stabilize these regions further. The net result would be widening of the melting transition of the phenanthridinium-DNA complex as compared with the breadth of the transition of free DNA.

Alternatively, the widening of the melting transition of the DNA-dye complex may be considered as an indication of the distribution of the intercalated molecules over
the DNA helix. In the conditions under which the Tm experiments were performed there is, on the average, one dye molecule bound per five base pairs. The base pairs in the immediate vicinity of the intercalated molecule, i.e. the ones immediately above and below it, will be the most stabilized and, therefore, will melt at a temperature significantly higher than their respective melting temperature in free DNA.

However, because of the cooperative character of the melting of the DNA, the base pairs between, but not in the immediate vicinity of, two intercalated dye molecules will also be stabilized, although to a lesser degree. This differential stabilization would, of course, result in differences in the melting temperatures of the various groups of base pairs and these differences could be reflected in the widening of the overall melting transition of the dye-DNA complex.

2. INFORMATION ON THE CONFORMATION OF THE COMPLEX OBTAINED BY PHYSICAL METHODS

As already indicated in the Introduction, one of the major goals of this investigation concerns the relationship between the chemical structure of the intercalated phenanthridinium derivatives and the conformation of the
phenanthridinium-DNA complex. Specifically, the conformation of the complex is examined as a function of the primary amino groups at positions 3 and 8 of the dye molecule. Information concerning the conformation of the phenanthridinium-DNA complex presented in this dissertation is derived mainly from the results of the circular dichroism and fluorescence experiments.

2.1. Differences in the origin of the CD bands of the complex

Circular dichroism, as a phenomenon, expresses the differential absorption of right and left circularly polarized light by an optically active chromophore. Since circular dichroism is a result of differences in absorption of light, it is observed in the vicinity of the absorption bands of the compound under study. The wavelength, therefore, at which the optical activity of a chromophore is manifested, is determined inherently by the molecular structure of that chromophore. Optical activity may be inherent in the chromophore, because of its intrinsic configuration, or, alternatively, it may be induced by an asymmetric environment in a chromophore which is, by itself, optically inactive.

The circular dichroism observed in the 300-600 nm region when phenanthridinium drugs bind to DNA is of the second type of optical activity discussed above, namely induced optical activity. The DNA molecule alone, despite its
structural asymmetry, does not display any optical activity at wavelengths longer than 300 nm, because absorption of light by this macromolecule is insignificant in this region. Similarly, the phenanthridinium drugs studied, despite the presence of strong absorption bands in the wavelength region between 300 and 600 nm, are not intrinsically asymmetric and are, therefore, optically inactive.

Consequently, the ellipticity observed when phenanthridinium compounds bind to DNA may be the expression of optical activity induced in the optically inactive chromophore of the bound dye by the asymmetric environment of the nucleic acid (Yamaoka and Resnik, 1966). Alternatively, the possibility exists, especially at high bound drug/nucleotide ratios, that interactions among the intercalated dye molecules produce or contribute to the optical activity of the complex (Gardner and Mason, 1967).

Since the CD properties of the DNA-phenanthridinium complexes in the short (300 - 315 nm) wavelength region are radically different from those noted in the long (470 - 500 nm) wavelength region, bands with maxima in these two regions must be attributed to different sources of asymmetry.

Specifically, the characteristics of the long wavelength band, i.e. appearance of optical activity even at low bound drug/nucleotide ratios and independence of the molar ellipticity from this ratio, suggest that ellipticity at this
region is not influenced by the distance between intercalated molecules. It appears, therefore, that optical activity in the 470-500 nm region is the result of interactions of the first type described above, i.e. optical activity in this region results from the influence of the surrounding asymmetric polynucleotide structure on the intercalated molecule.

In contrast, the ellipticity at the short (300-315 nm) wavelength band exhibits a behavior which indicates a distinctly different origin. Specifically, the increase of molar ellipticity of the diamino drug-DNA complexes at 310 nm with increasing $r$ indicates that each bound dye molecule contributes more to the optical activity when the intercalated molecules are more densely distributed over the DNA helix. Therefore, ellipticity at 310 nm appears to originate from the second type of interaction described above, namely interactions between dye molecules intercalated at neighboring sites.

2.2. **The origin of the 310 nm CD band**

Because of this unusual behavior of the molar ellipticity at 310 nm, the origin of the 310 nm band was investigated in more detail.

A similar dependence of molar ellipticity on dye/nucleotide ratio was observed in the proflavine-DNA system and two possible mechanisms were proposed to explain the phenomenon:
1. The increase in molar ellipticity is the result of direct interactions between intercalated dye molecules. The possibility of such interaction, of course increases as the number of molecules bound within a given segment of the DNA increases (Blake and Peacocke, 1967).

2. Alternatively, the variation in optical activity may be due to an alteration of the conformation of the drug binding site, resulting from intercalation of additional dye molecules at neighboring sites (Dalgleish et al., 1969). In this case also, the probability of a dye molecule intercalating to a site adjacent to one already occupied by another molecule increases as the number of bound dye molecules per nucleotide increases.

All currently available experimental evidence is compatible with either one of the above models, so that elimination of one in favor of the other is not possible, at this time. In fact, the lack of theoretical predictions as to the characteristics of the CD resulting from the second type of interactions renders that model inaccessible to direct experimental verification. In contrast, according to Tinoco (Tinoco, 1964), direct interactions between the intercalated dye molecules which form a helical array are expected to give rise to a doublet of positive and negative peaks in the CD spectrum. In fact, there is an indication
of such a doublet centered at approximately 300 nm in the CD spectrum of the EB-DNA complex (Figures 16 and 31). The negative component of this probable doublet is not easily detectable at low ionic strength, probably because of a mutually cancelling effect between this band and the strong positive band of the DNA spectrum at 270 nm.

The existence of the conservative doublet in the CD spectrum of the EB-DNA complex lends support to the notion that direct interactions between EB molecules intercalated at neighboring sites are important contributors to the CD of the complex in the 300 nm region at high ionic strength. Furthermore, this conclusion may be expanded to low ionic strength conditions if it is assumed that the conformation of the complex is not significantly affected by increased salt concentration. There are indeed indications that, aside from a minor change in the average angle of rotation of the helix, the conformation of the DNA is not particularly sensitive to salt (Wang, 1969; Studdert et al., 1972). The similarities of the CD of the complex in 0.04 M Tris and in 5.0 M NaCl indicate that a similar condition may apply to the EB-DNA complex.

Another question which may be considered is whether these interactions between dye molecules intercalated at adjacent sites are transmitted through the intervening base
base pairs or whether they occur in some other more direct manner. According to the original intercalation model (Fuller and Waring, 1964), the phenanthridinium ring is completely immersed in the space between the base pairs and no part of it is accessible for direct interactions between intercalated molecules. The model, however, postulates that the 5- and 6-substituents of the drug are situated within the large groove of the helix. The possibility, therefore, exists that the interactions mentioned above could occur via these groups and especially the 6-phenyl substituent of the EB molecule. The behavior of the CD of the DDMB-DNA complex is an indication to the contrary. DDMB is the 5,6-dimethyl analogue of EB and the CD spectrum of its complex with DNA is both qualitatively and quantitatively very similar to that of the EB-DNA complex. This finding is an indication that, if interactions between phenyl groups of intercalated EB molecules occur to any significant degree, such interactions do not contribute appreciably to the CD of the complex near 300 nm.

It appears, therefore, that interactions between intercalated diamino phenanthridinium molecules are exerted from one drug molecule to the other mainly via the intervening base pairs.
2.3. Differences in the properties of DNA complexes of mono- and diamino phenanthridinium dyes

2.3.1. CD evidence

In comparison with the spectra of the complexes of the diamino phenanthridinium dyes with DNA, the CD spectra below 350 nm of the monoamino phenanthridinium-DNA complexes appear distinctly different, both in terms of shape as well as magnitude. Although molar ellipticity near 310 nm appears to depend on \( r \), the observed increase in \( \epsilon_1 - \epsilon_r \) is much weaker than that exhibited by the diamino phenanthridinium complexes. In addition, and perhaps more significantly, the shape of the CD spectrum itself changes with increasing dye/nucleotide ratio. Considering that changes in CD frequently reflect changes in the conformation of the macromolecule under study, this observation might be considered as indicative of differences in the conformation of the drug-DNA complex, depending on whether a mono- or a diamino phenanthridinium compound is intercalated.

Such differences in the circular dichroism spectra of the two classes of complexes are not unexpected, if certain conditions necessary for inducing optical activity are taken into consideration. Induction of optical activity requires that the intercalated molecules be maintained in a specific orientation with respect to one another (Yamaoka
and Resnik, 1966). Molecules, therefore, not properly oriented within their intercalation sites would not be expected to exhibit strong induced optical activity.

According to the model originally proposed by Fuller and Waring for the DNA-EB complex, the primary amino groups at positions 3 and 8 of the EB molecule contribute to the overall stabilization of the complex by forming hydrogen bonds with the phosphate groups on the backbone of the helix. Conceivably, these hydrogen bonds may also contribute in maintaining the intercalated molecule in a specific orientation within its binding site. The possibility of such a role for the C₃ and the C₈ amino groups was also considered recently (Wakelin and Waring, 1974) but a direct experimental testing of this possibility was not attempted. This orientation seems to be the most favorable for near neighbor interactions and it may be responsible for the increased optical activity observed with complexes formed between DNA and EB or DDMB.

Furthermore, this particular positioning may be difficult to maintain when the intercalated molecule lacks the amino group at the C₃ position. The absence of the hydrogen bond between that group and a DNA phosphate would be expected to increase the freedom of movement of the intercalated molecule within its binding site. Thus, the intercalated molecule would not be restricted to the specific
orientation associated with strong optical activity near 310 nm, which would result in the significantly reduced ellipticity in the 300-350 nm region observed for the complexes of DNA with MAPAC, MAPEC or MMPB.

2.3.2. Fluorescence evidence

The existence of differences in the conformation of the phenanthridinium-DNA complexes is further confirmed by the considerable increase in the fluorescence of the diamino compounds upon binding to DNA, whereas, under identical conditions, only a slight increase, if any, is observed with the monoamino derivatives.

Immersion of the intercalated molecule in the hydrophobic interior of DNA has been implicated as the reason for the increased fluorescence of intercalated EB molecules (LePecq and Paoletti, 1967). The main argument supporting this conclusion was the observed increase in the quantum efficiency of EB in nonpolar solvents. It was reported that, when EB is dissolved in ethylene glycol, methanol, ethanol, propanol, butanol or octanol, its quantum efficiency increases from ethylene glycol to ethanol. The quantum efficiency in ethanol is about six times greater than in water, but it decreases slightly from ethanol to octanol (LePecq and Paoletti, 1967). Similar observations were reported for various other aromatic hydrocarbons, the fluorescence
intensity of which increases with decreasing solvent polarity (Majumdar and Basu, 1960; Van Duuren, 1963; Stryer, 1965).

The differences, therefore, observed in the fluorescence enhancement between the mono- and the diamino phenanthridinium compounds may be an indication that these two classes of compounds interact to different extents with the hydrophobic environment of the nucleic acid.

By studying the decay of the emission anisotropy of the EB-DNA complex, Wahl and his co-workers (Wahl et al., 1970) obtained evidence that the intercalated drug molecules are subject to internal Brownian motion. This motion consists of an oscillation of the bound molecules within their plane of intercalation. The angle of the oscillation was estimated to approximately 35°. This angle is probably limited by the two hydrogen bonds formed between the two amino groups of the EB molecule and the phosphate groups on the two DNA strands. The intercalated molecule is, therefore, only following the transient local unwinding ("breathing") of the helix (Wahl et al., 1970).

In contrast, such a topological restriction is probably not imposed upon the monoamino compounds. These molecules lack the amino group at the C₃ position. Consequently, they can only form one hydrogen bond, namely that between their 8-amino group and a DNA phosphate group.
Therefore, the freedom of oscillation of monoamino phenanthridinium compounds within their intercalation plane is expected to be higher than that of the diamino compounds and the field of movement of the monoamino derivatives is not restricted to the hydrophobic volume between successive base pairs, but may extend into the aqueous environment surrounding the nucleic acid as well. Put in another way, if two types of environment are considered, i.e. a hydrophobic one, between the DNA base pairs, and an aqueous, surrounding the DNA-drug complex, then the distribution of the diamino compounds in the hydrophobic medium at any given moment is expected to be higher than the corresponding distribution of the monoamino derivatives.

However, as it was mentioned earlier, the polarity of the environment has a profound effect on the quantum yield of aromatic compounds. It has been reported that the quantum yield of at least one such compound, 1-anilino-8-napthalene sulfonate (ANS), decreases dramatically with increasing water content of the solvent (Stryer, 1965). Therefore, the, even partial, immersion of the monoamino phenanthridinium dyes into an aqueous environment would be expected to have a considerable quenching effect on their fluorescence intensity. On the other hand, the quantum efficiency of the intercalated diamino derivatives would be expected to increase considerably over that of the free dye,
since these compounds are almost always located within the less polar environment of the interior of the nucleic acid.

The above model of differential distribution of mono- and diamino compounds into the hydrophobic environment of the DNA base pairs also provides an explanation for the modest increase in fluorescence of MMPB upon binding to DNA. As indicated in the Results (Section 3), the fluorescence of MMPB increases approximately 5-fold upon binding to DNA, whereas the fluorescence of the other two monoamino drugs studied, MAPAC and MAPEC, shows no significant changes. This difference on fluorescence enhancement may be an indication that the exposure of MAPAC and MAPEC to the aqueous environment of the solution is higher than that of MMPB. The reason for this may lie in the differences in the substituents at the C₆ position between MMPB and the other two monoamino phenanthridinium derivatives. The C₆ substituents, according to the original intercalation model (Fuller and Waring, 1964), are situated within the major groove of the DNA helix and are in contact with the medium surrounding the polynucleotide. It is expected, therefore, that the relatively more polar p-aminophenyl group at position C₆ of MAPAC and MAPEC will tend to increase the exposure of the phenanthridinium ring to the aqueous environment more than the relatively more hydrophobic 6-phenyl substituent of MMPB.
3. **THE COMPLEX AS AN ENZYMIC SUBSTRATE**

The conformation and stability of the phenanthridinium-DNA complex are expected to affect the ability of this complex to serve as template or substrate for DNA polymerase or DNase respectively. Both DNA polymerase and DNase have well defined requirements regarding the conformation of the nucleic acid that serves as their respective template or substrate (Zimmermann, 1966; Harwood *et al.*, 1970a; Junowicz and Spencer, 1973; Ehrlich *et al.*, 1973). Therefore, agents that affect the DNA conformation will very likely influence the affinity of these enzymes for the nucleic acid and this influence will be reflected in the rate of the enzymic reaction.

3.1. **Modification of the DNA substrate as a result of dye binding**

It has been well established that binding of phenanthridinium compounds alters the topological characteristics of DNA (Wakelin and Waring, 1974); it has been estimated that each intercalated molecule unwinds the DNA helix by $8 - 12^\circ$ (Pigram *et al.*, 1973; Wakelin and Waring, 1974) and possibly by as much as $26^\circ$ (Wang, 1974). Even if it is assumed that the change in the conformation of the DNA induced by dye binding is not being transmitted more than a few base pairs
along the helix, the overall conformational change will be proportional to the number of drug molecules bound to it. Since the ability of an enzyme to recognize its substrate and bind effectively to it depends on the conformation of the substrate, it should be expected that the activity of the enzyme be inversely related to the concentration of the bound dye.

A possible alternative to the mechanism of inhibition just described involves the substituents of the phenanthridinium molecules at positions 5 and 6 of the ring. According to the original intercalative model of Fuller and Waring (1964), these groups are not inserted between the base pairs but lie in the large groove of the helix. Therefore, they probably do not affect directly the DNA conformation (Results, Section 2), but they may reduce the affinity of the enzyme for its substrate by sterically preventing the binding of the enzyme to DNA. Specifically, molecules with bulky groups at the 5 and/or 6 positions of the ring would be expected to be more effective inhibitors of the enzyme activity than the derivatives with small alkyl groups at these positions.

Finally, enzymic inhibition may result from the stabilization of the double-stranded form of DNA. It was shown that the phenanthridinium compounds preferentially stabilize that form (Results, Section 4) and that this stabilization increases with increasing added dye/nucleotide
ratio (Martz, 1971; Waring, 1974). Therefore, if the enzyme requires a single-stranded substrate (or template), this preferential stabilization of double-helical DNA will result in decreased enzymic activity.

The three possible mechanisms of inhibition described above appear to participate, albeit to different extent in each case, to the inhibition of DNase and DNA polymerase by the phenanthridinium compounds.

3.2. Inhibition of DNase

The first two mechanisms, namely those involving conformational change of the substrate and physical prevention of binding, appear to play a major role in the inhibition of DNase. On the contrary, stabilization of the DNA double-helix is not expected to be a significant factor in the inhibition of this enzyme, since DNase I is, in fact, known to hydrolyze native DNA faster than denatured DNA (Dirksen and Dekker, 1958).

The effect of the dye-DNA complex conformation on the activity of DNase is indicated by the linear dependence of the inhibition of this enzyme on the bound dye/nucleotide ratio, as suggested by the inhibition mechanism (Discussion, Section 3.1). In addition, the different rates of DNase inhibition observed with different phenanthridinium derivatives indicate that the groups at positions 5 and/or 6 of
the ring contribute to the inhibition of DNase by these compounds. Indeed, as judged from the slopes in the linear portion of the dependence of enzyme inhibition on $r$, it appears that drugs with a 6-phenyl substituent are somewhat stronger inhibitors than the 5,6-dimethyl derivative.

3.3. Inhibition of DNA polymerase

In contrast, in the case of DNA polymerase, the above two factors affecting inhibition, i.e. conformational change of the template and physical prevention of binding, are not expected to affect significantly the activity of the enzyme, at least as far as its initial binding to the template is concerned, under the experimental conditions used. This is because the template used for the assay was "activated", i.e. the DNA was pretreated briefly with DNase so that single-stranded nicks were introduced to it. It has been shown (Kornberg, 1969) that DNA polymerase binds preferentially and quantitatively to single stranded nicks of the template. However, it is in the immediate vicinity of the nicks that the double-helical structure of the DNA is the less stable. Since EB is known to bind preferentially to the double-stranded structure, the density of the intercalated molecules population close to the nicks would be significantly lower than the average density for the remaining DNA molecule. It follows, therefore, that, since nicks
constitute major polymerase binding sites, the intercalation of phenanthridinium compounds to DNA will not affect significantly the binding of the polymerase to its template.

Although the initial binding of the polymerase to its template may not be substantially inhibited by the intercalated phenanthridinium dyes, the propagation of the enzyme along the DNA molecule could be hindered by the presence of these compounds. Undoubtedly, a major part of this inhibition of propagation is due to the stabilization of the double-helical form of the DNA by the intercalated dye. The physical separation of the two parent strands during DNA replication was established with the classical experiments of Meselson and Stahl (Meselson and Stahl, 1958), as well as with subsequent studies with similar aims (Cairns, 1962; Baldwin and Shooter, 1963). Agents, therefore, inhibiting this strand separation would effectively inhibit replication of DNA.

Since phenanthridinium dyes preferentially stabilize the helical form of DNA, as evidenced by the increased Tm values of the dye-DNA complexes (Results, Section 4), it is not surprising that replication of DNA is substantially inhibited in the presence of these compounds. Evidence supporting such a relationship between stabilization of the DNA double-helix and inhibition of DNA polymerase is provided by the similarity of the dependence of these two properties.
on the added dye/nucleotide ratio. Indeed, the dependence of inhibition of DNA polymerase versus added EB/DNA ratio (Figure 62) is very similar to that of the Tm of calf thymus DNA versus the same ratio (Waring, 1974). Furthermore, the same similarity is observed for another intercalative compound, acridine orange, which also increases the melting temperature of DNA (Kleinwachter and Koudelka, 1964) and inhibits the activity of DNA polymerase (Eberhard and Herrmann, 1972).

In addition, it appears that the size of the substituent at position 6 of the phenanthridinium ring may also play a role in determining the extent of polymerase inhibition by the phenanthridinium derivatives. Indeed, small differences are observed in the inhibitory effect of these compounds on the activity of DNA polymerase; these differences suggest that 6-phenyl-substituted drugs are slightly more effective inhibitors than the derivative with a relatively small ethyl group at position 6. Since no difference in the stabilization of DNA was observed between those compounds, the slightly stronger inhibitory effect of the 6-phenyl derivatives may be related to the presence of the bulky phenyl substituent, which projects into the major groove of DNA and which may impede further the propagation of the enzyme along the template.
3.4. Some biological implications of the inhibition of DNA polymerase

As early as 1957, it was observed that DNA synthesis in certain organisms is drastically inhibited in the presence of EB (Newton, 1957). In addition, inhibition of mitochondrial DNA synthesis by EB has been reported in a variety of organisms (Riou, 1967b; Goldring et al., 1970; Radsak et al., 1971). In many cases, inhibition of DNA synthesis is accompanied by alterations in the morphology and physiology of the organism under study (Riou, 1967a; Nass, 1970; Heilporn and Limbosch, 1971; Goldring et al., 1971). It is likely, therefore, that inhibition of DNA polymerase may be a major factor influencing the biological activity of the phenanthridinium compounds on the molecular level. However, the role of DNA polymerase I in DNA replication has not been unequivocally established. It has been suggested (Okazaki et al., 1971) that this enzyme serves to close the gaps in the daughter strand of the DNA which result from the discontinuous DNA synthesis (Okazaki et al., 1968). Also, it is believed that DNA polymerase I participates in the repair of UV-induced damage to the nucleic acid (Harwood and Wells, 1970; Hamilton et al., 1974).

Evidence compatible with both of these hypotheses was obtained recently. The mitochondrial DNA of HeLa cells grown in the presence of EB is fragmented into pieces which
are approximately one-tenth the size of the original DNA molecule (Koch, 1973). Since, unlike nuclear DNA, the mitochondrial DNA of mammalian cells is continuously degraded and replaced by newly synthesized mitDNA (Gross et al., 1969; Koch, 1972), two interpretations were advanced to account for this fragmentation of mitDNA in the presence of EB. It may be assumed that the mitDNA is normally subject to single stranded nicks which are subsequently closed by a DNA polymerase I-like enzyme. According to this mechanism, EB inhibition of this enzyme results in the accumulation of mitDNA fragments. Alternatively, it may be assumed that nicking constitutes part of the normal degradation process for mitochondrial DNA; EB then is believed to act by inhibiting a DNA polymerase that is responsible for the de novo synthesis of mitDNA (Koch, 1973).

Furthermore, EB was found to inhibit the host cell mediated reactivation of UV-irradiated bacteriophage T1 as well as to enhance UV-induced mutation rate and lethality in E. coli possessing the dark repair mechanism (Shankel and Molholt, 1973). Host cell reactivating ability and resistance to UV radiation were repeatedly associated with the presence of active DNA polymerase I (DeLucia and Cairns, 1969; Kato and Koudo, 1970; McPhee, 1974) indicating that one of the biological functions of this enzyme is the repair of
UV-induced DNA damage. It is conceivable, therefore, that the observed increase in UV sensitivity of *E. coli* and bacteriophage T1 in the presence of EB is due to inhibition by the dye of the activity of DNA polymerase I.
SUMMARY

Ethidium bromide (EB) and some of its analogs are known to bind to DNA by intercalation and preferentially stabilize its double-stranded form, as indicated by the increased $T_m$ values noted for the resulting complexes. Hydrophobic forces between the phenanthridinium ring and the DNA bases are major contributors to the binding. Binding parameters for the 3,8-diamino derivatives are slightly higher than those for the 3-monoamino compounds, indicating that hydrogen bonds between the 3- and 8-amino groups and the DNA phosphate residues confer additional stabilization to the complex. This conclusion is further reinforced by the observed decrease in binding parameters at high ionic strength.

Examination of the CD spectra of the phenanthridinium-DNA complexes reveals several bands in the 300 - 600 nm region. Molar ellipticities in the long (470 - 500 nm) band are independent of complex concentration; therefore, optical activity in this region may be ascribed to the influence of the asymmetric environment of the binding site on the intercalated molecule.

The molar ellipticity near 310 nm increases with increasing bound dye/nucleotide ratio ($r$), indicating that
optical activity at this wavelength depends on the "population density" of bound molecules. Since the CD of the EB-DNA complex near 310 nm exhibits, at least at high ionic strength, an exciton doublet, the optical activity of the diamino phenanthridinium-DNA complexes at this wavelength may be attributed to direct interactions between dye molecules intercalated at neighboring sites.

In the case of the monoamino dye-DNA complexes, a significant and gradual modification of the overall shape of the CD spectrum takes place with increasing r. The distinctly different behavior of the CD of the monoamino dye-DNA complexes suggests a different orientation of these compounds within their binding sites. This may result from the inability of the monoamino derivatives to form a second hydrogen bond with DNA residues.

Differences in the positioning of the intercalated molecules are also indicated by the differences in the enhancement of the fluorescence intensity of the mono- and diamino derivatives upon binding to DNA. The fluorescence intensity of the diamino derivatives increases 20- to 30-fold upon complexing with DNA, whereas a small, if any, increase is observed with the monoamino compounds. The increase in fluorescence intensity of the dye is attributed to the influence of the hydrophobic environment of the interior of the DNA on the intercalated molecule. The much smaller increase in fluorescence of the monoamino dyes indicates
that the exposure of these compounds to the aqueous medium is more pronounced than that of the diamino derivatives. This assumption is consistent with the inability of the monoamino dyes to form a second hydrogen bond with DNA phosphate residues.

Differences in the conformation of the complexes of DNA with the phenanthridinium dyes are also consistent with the pattern of inhibition of DNase I and DNA polymerase I by these compounds. The finding that, in both cases, the inhibition if overcome by increased DNA concentration, suggests that inhibition results from the binding of the drugs to DNA rather than from direct drug-enzyme interactions. The inhibition of DNase by the phenanthridinium dyes is probably due to an alteration in the conformation of the substrate leading to a decrease in the affinity of the enzyme for it. In contrast, a stabilization of the double-stranded form of the template may be an important factor in the inhibition of DNA polymerase, since strand separation is a prerequisite for the action of this enzyme.

For both enzymes, 6-phenyl compounds are slightly more effective inhibitors than the 6-methyl or 6-ethyl substituted derivatives. It appears that a bulky group at position 6 of the phenanthridinium ring increases the effectiveness of the inhibitor by sterically preventing the binding and/or the propagation of the enzyme on the substrate or the template.
REFERENCES


170


of aminoacridines and ethidium bromide bound to DNA.", Biopolymers 10, 1853-1863.


Pigram, W. J., Fuller, W., and Davies, M. E. (1973), "Unwinding the DNA helix by intercalation.", J. Mol. Biol. 80, 361-365.


Waring, M. J. (1966), "Structural requirements for the binding of ethidium to nucleic acids.", Biochim. Biophys. Acta 114, 234-244.


The dissertation submitted by Antonis Kindelis has been read and approved by a committee from the faculty of the Graduate School.

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 with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Dec 3, 1975
Date

Signature of Advisor