raphy of the residue on grade II basic alumina with chloroform gave 690 mg (73%) of a pale yellow oil: λ_{max}^{KBr} 5.78 μ; λ_{max}^{EtOH} 223 (ϵ 20,900), 269 (10,720), 279 sh (9330), 283 (7940), 289 nm (5370); δ^{CDCl_3} 2.30 (s, 3 H), 3.33 (t, J = 5 Hz, 1 H), 3.51 (s, 3 H), 3.74 (s, 3 H), 8.57 (s, 1 H).

1,2,3,3a,4,5-Hexahydro-3-methyl-8-hydroxycanthin-6-one (5).[25] A mixture of methoxy-β-carboline **27** (500 mg, 0.0016 mol), polyphosphoric acid (5 g), and water (0.5 ml) was stirred at 100° for approximately 5 min. During this period, the reaction mixture foamed vigorously (methanol evolution) and then the foaming ceased completely. The reaction mixture was stirred an additional 2–3 min, poured into 500 ml of cold water, and treated with concentrated aqueous ammonia until the solution was basic to litmus paper. The reaction mixture was extracted several times with ether, and the combined ether extracts were dried, filtered, and evaporated to a dark oil. The residue was taken up in a small volume of chloroform and chromatographed on a column of grade I neutral alumina with chloroform. The green colored eluent was evaporated and the residue was dissolved in 5 ml of anhydrous methanol, and saturated ethanolic hydrochloric acid was added until the solution was acid to litmus paper. The crystalline material was collected by suction filtration and washed several times with cold methanol. Recrystallization from methanol gave 290 mg (62%) **5** as fine white needles: mp 299–300° dec; λ_{max}^{KBr} 6.10 μ; λ_{max}^{EtOH} 215 (ϵ 11,610), 243 (13,125), 327 nm (6050); δ^{CDCl_3} (free base) 2.33 (s. 3 H), 10.86 (s, 1 H); *m/e* 257 (14), 256 (68), 255 (32), 239 (15), 214 (20), 213 (100), 201 (7), 200 (30), 199 (20), 186 (5), 185 (40), 184 (41), 183 (4), 172 (5), 171 (10), 170 (8), 166 (4).

Canthiphytine (5) from Haplophytine. To a solution of haplophytine (132 mg) in dilute hydrochloric acid (3 ml, 12%) was added zinc dust (250 mg), and the mixture was refluxed under nitrogen for 1 hr. The mixture was left overnight. The clear pale brown solution was cooled in an ice bath, basified with ammonia, and extracted with ether. The washed and dried ether layer was concentrated and the dark residue (60 mg) was treated with methanolic hydrogen chloride. The gummy solid was crystallized from acetone–methanol to give 7 mg of canthiphytine hydrochloride: mp 302° dec; *m/e* 256.1211 (M^+ 256.1212); *m/e* 257 (14), 256 (82), 255 (37), 239 (12), 214 (17), 213 (100), 201 (5), 200 (30), 199 (20), 186 (5), 185 (30), 184 (32), 183 (4), 172 (4), 171 (9), 170 (5), 166 (3). This material was identical (mixture melting point and ir spectrum in KBr) with the hydrochloride of synthetic **5**.

Acknowledgments. We gratefully acknowledge the support of this work by the National Research Council of Canada and by the National Institutes of Health (Grant HE-12961).

Supplementary Material Available. Elemental analyses for the indicated compounds will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 20× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number JACS-73-7842.

Steric Effects on the Intercalation of Aromatic Cations to Deoxyribonucleic Acid[1,2]

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Abstract: In an attempt to define the three-dimensional size of the 10 possible intercalation sites on DNA, a series of substituted *N*-methylphenanthrolinium cations was synthesized. The interactions between nucleic acids of different base composition and the aromatic cations were studied by melting temperature (T_m), proton magnetic resonance (pmr), ultraviolet (uv) absorption, induced circular dichroism (CD), equilibrium dialysis, and viscometric techniques. The planar cations are found to intercalate between base pairs of DNA as evidenced by (1) total broadening of the pmr signals, (2) enhanced viscosity, (3) induced circular dichroism, and (4) dramatic stabilization of the DNA helix toward denaturation. In addition, selective interactions with DNA are observed as a function of the position and number of substituents on the *N*-methylphenanthrolinium ring. For example, the more highly substituted systems exhibit (i) higher affinity, (ii) greater stabilization, and (iii) higher viscosity upon binding to DNA. Enhanced binding to G-C sites (and/or a combined G-C/A-T site) by the more highly substituted aromatic cations is also indicated from the results.

The importance of the intercalation process,[3] *i.e.*, insertion of a planar aromatic ring between base pairs of DNA, in the formation of specific complexes between proteins and nucleic acids has recently been suggested by several investigators.[4–9] In particular, Brown[4] has proposed a "bookmark" hypothesis whereby the aromatic amino acid residue may serve to anchor and prevent slippage of the protein along the DNA helix. Helene and coworkers,[8,9] who studied the interactions of tryptamine, serotonin, and tyramine to nucleic acids and their components, find that the aromatic residues of the above systems are bound to DNA *via* an intercalation mechanism, and, therefore, they also suggested an "anchoring" role for the aromatic amino acids. Recent work from this laboratory[5–7] on the interactions of 70 different di-, tri-, and tetrapep-

(1) This work is part of XXIX, a series, "Topography of Nucleic Acid Helices in Solution." For the previous paper, see E. J. Gabbay, R. DeStefano, and C. S. Baxter, *Biochem. Biophys. Res. Commun.*, 51, 1083 (1973).

(2) The authors acknowledge the support of the U. S. Public Health Service Grants GM 17503 and GM 18643 and the National Science Foundation Grant GB 16044. Taken in part from R. E. S. thesis to be submitted in partial fulfillment for the Ph.D. degree, University of Florida, 1973.

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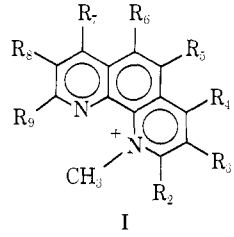
tides as well as di-, tri-, and tetrapeptides amides to DNA of various base compositions supports the above conclusions. In addition, the results of pmr, viscosity, CD, T_m, and equilibrium dialysis suggest that not only site-specific intercalation of the aromatic residue of the peptides but also a dependence on the primary structure are involved.[6,7] On this basis, a "selective bookmark" hypothesis was proposed whereby the "bookmarks," *i.e.*, the aromatic residues of the proteins, could recognize the "pages of the book," *i.e.*, the intercalating sites.[5]

The work reported in this paper attempts to probe the three-dimensional structure of the different intercalating sites in DNA *via* the use of substituted *N*-methylphenanthrolinium cations (I). It should be



I

	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
1	H	H	H	H	H	H	H	H
2	CH_3	H	H	H	H	H	H	CH_3
3	H	H	H	CH_3	CH_3	H	H	H
4	CH_3	H	H	CH_3	CH_3	H	H	CH_3
5	H	CH_3	H	H	H	H	CH_3	H
6	H	CH_3	H	CH_3	CH_3	H	CH_3	H
7	H	H	CH_3	H	H	CH_3	H	H
8	H	H	C_6H_5	H	H	C_6H_5	H	H

noted that as a consequence of the right-handed Watson–Crick–Wilkins double helix of DNA with the A-T and G-C base pairs, there are 10 distinctly different intercalating sites.[5] Each site may provide a different environment (steric as well as electronic) for the intercalating molecule. A series of substituted aromatic cations, I, has been synthesized and their interaction specificities with nucleic acids of various base content were examined. With one exception, *i.e.*, **8**, the planar cations are found to intercalate between base pairs of DNA as evidenced by (1) total broadening of the pmr signals, (2) enhanced viscosity, (3) induced circular dichroism, and (4) increased melting temperature (T_m) of the DNA helix. In addition, selective interactions with DNA are observed as a function of the position and number of methyl substitutents on the *N*-methylphenanthrolinium ring, I.

Experimental Section

Poly(dA-T)-poly(dA-T) (lot no. 30) and *M. luteus* DNA were purchased from Miles Laboratories. The *M. luteus* DNA was further purified to remove the 2–3% residual protein by a phenol-extraction procedure.[10] Salmon sperm and calf thymus DNA were obtained from Worthington Biochem. Corp. and found to be free of any detectable protein contaminants. Stock solutions of polymers were made in 10 m*M* 2-(*N*-morpholino)ethanesulfonic acid buffer (MES) (pH 6.2) (5 m*M* in Na^+), and stored at 0°. The stock solutions were removed and diluted in the various buffers as indicated in the tables and figures.

Equilibrium dialysis experiments were run in plexiglass blocks at ambient temperature according to the method of Wells, *et al.*[11] Time studies showed that equilibration was complete in 12 hr. The concentrations of the free cations, I, were determined spectrophotometrically at their respective λ_{max} (270–290 nm) using a Gilford 240 spectrometer (see Table I). T_m of the helix-coil transition, ultraviolet studies, circular dichroism, proton magnetic resonance, and viscometric studies were carried out according to previous published procedures.[6,12]

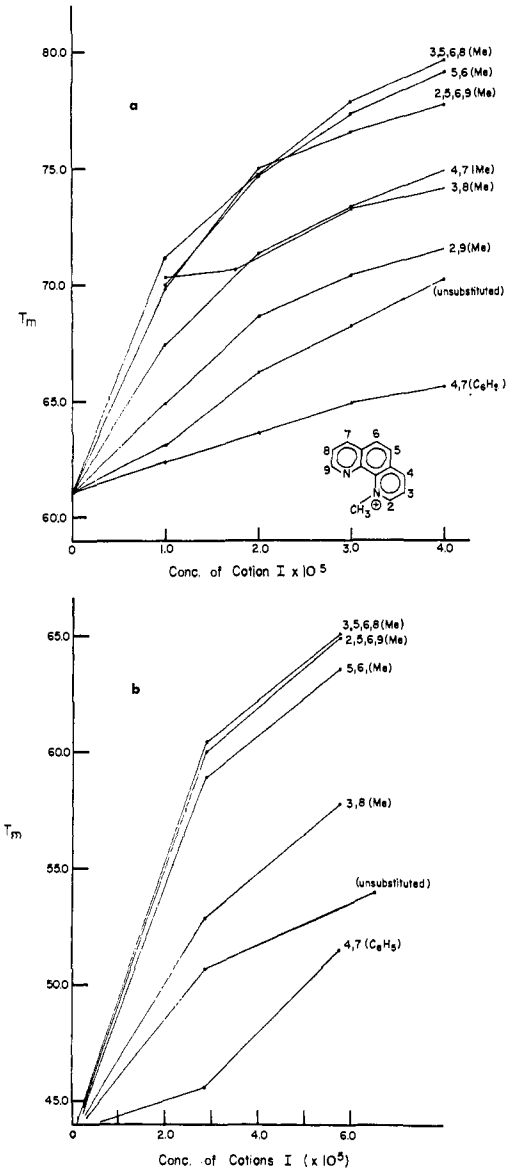


Figure 1. The effect of increasing concentrations of cations I on the T_m of the helix-coil transition of (a) salmon sperm DNA and (b) poly(dA-T)-poly(dA-T). The study was conducted in 0.01 *M* 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.2) (0.005 *M* in Na^+) using 8.40×10^{-5} *M* P/l. and 1.14×10^{-4} *M* P/l. of salmon sperm DNA and poly(dA-T)-poly(dA-T), respectively. The T_m in the absence of I are found to be 60.8 and 41.9° for salmon sperm DNA and poly(dA-T)-poly(dA-T), respectively.

Table I. Absorption Properties of *N*-Methylphenanthrolinium Cations, I, in 0.01 *M* MES buffer (pH 6.2) (0.005 *M* Na^+)

Compd	$\lambda_{sh}{}^{0-0}$	$\epsilon_{sh}{}^{0-0}$	λ_{max}	ϵ_{max}	λ_{max}	ϵ_{max}
1	300	6,810	271	35,500	218	38,600
2	305	7,350	282	31,600	220	36,300
3	310	6,250	283	30,900	223	29,300
4	318	7,150	292	34,700	229	32,800
5	317	7,000	274	33,000	226	33,800
6	320	6,410	288	38,300	223	35,400
7	302	5,140	273	35,800	~228	Shoulder
8	319	11,600	284	43,900	217	37,100

(10) D. Smith, A. M. Martinez, and R. L. Ratliff, *Anal. Biochem.*, 38, 85 (1970).

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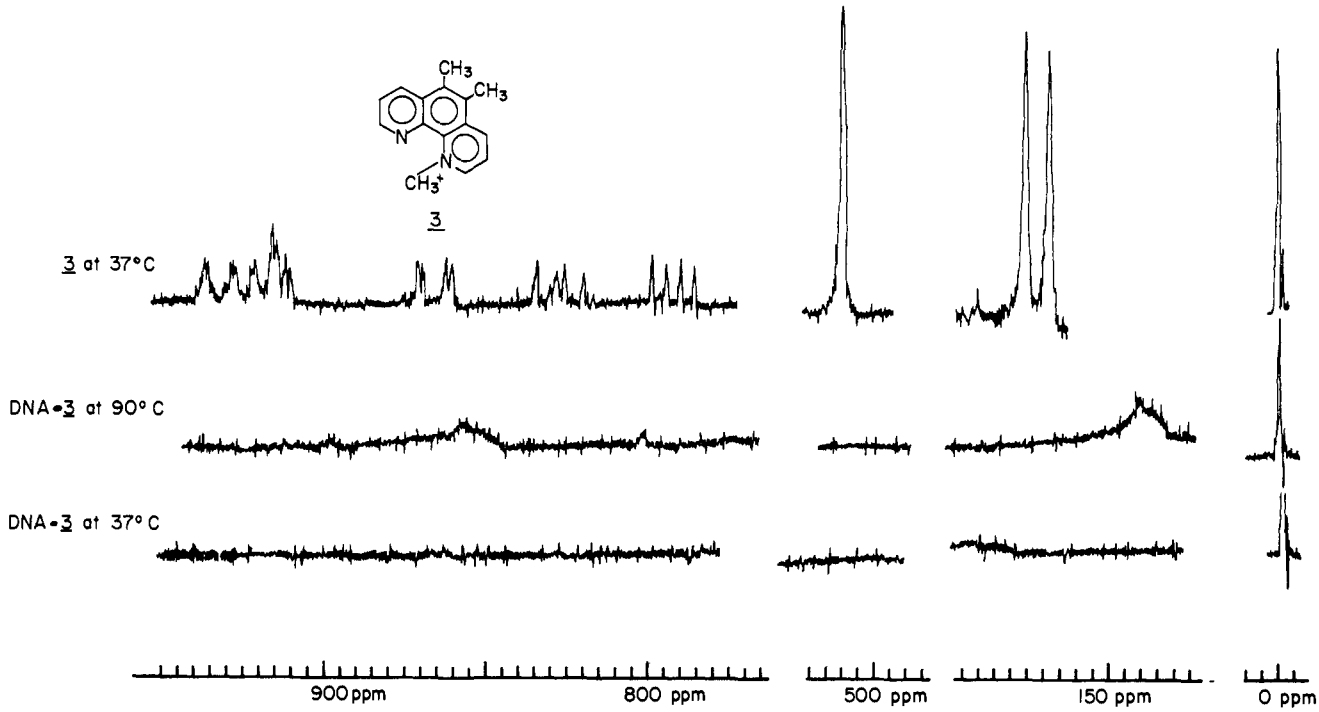

Figure 2. The temperature-dependent partial proton magnetic resonance spectra of **3** and salmon sperm DNA–**3** complex. Sonicated low molecular weight salmon sperm DNA was used at 0.16 mol of P/l. in D_2O in 10^{-4} *M* sodium phosphate buffer (pD 7.0 ± 0.2). The concentration of **3** was 0.02 *M*.

The *N*-methylphenanthrolinium cations (as the chloride salts) were synthesized from the respective parent phenanthroline system by alkylation with methyl iodide (Aldrich Chemical Co.), followed by conversion of the iodide to the chloride by stirring with freshly precipitated silver chloride. The various methyl-substituted phenanthrolines were made *via* the Skraup synthesis according to previous published procedures.[13,14] Melting points, ir, and pmr spectra of the intermediates agree with those reported in the literature. All new compounds, *i.e.*, **2–8**, were checked by melting point, ir, pmr, and elemental analysis and found to agree with the assigned structure.

Results and Discussion

T_m of Helix-Coil Transition. The effect of increasing concentrations of the phenanthrolinium cations, I, on the T_m of the helix-coil transition of salmon sperm DNA and poly(dA-T)-poly(dA-T) is shown in Figure 1. Several interesting observations may be made. (1) A large increase in the T_m of the helix-coil transition of nucleic acids is observed for all the phenanthrolinium cations even at very low concentrations (*i.e.*, 1×10^{-5} *M* and at a ratio of 4.2 base pairs per reporter molecule). (2) The greater the methyl substitution of the *N*-methylphenanthrolinium ring, the higher the stabilization of the DNA helix. (3) The degree of stabilization is dependent on the position of methyl substitution, *e.g.*, the following order is observed: poly(dA-T)-poly(dA-T), (3,5,6,8) ≥ (2,5,6,9) > (5,6) > (3,8) > unsubstituted > (4,7-diphenyl); salmon sperm DNA, (3,5,6,8) ≥ (5,6) ≥ (2,5,6,9) > (3,8) > (4,7) > (2,9) > unsubstituted > (4,7-diphenyl). (4) It is noted that the cation **8**, 4,7-diphenyl-*N*-methylphenanthrolinium chloride, is the least effective in stabilizing the DNA helices to heat denaturation (Figure 1). The binding mode of **8** to DNA is different than the cations **1–7**. The latter systems bind strongly to DNA *via* an intercalation mechanism (see below for further evidence) whereas **8** is not expected to intercalate between base pairs of DNA due to the presence of the bulky 4,7-diphenyl groups which are twisted out of plane of the *N*-methylphenanthrolinium ring. A similar effect, *i.e.*, steric hindrance to intercalation, has been observed with other types of substituted aromatic systems.[15] (5) Tetramethylammonium ion at the same concentration as that used for cations, **1–8**, has no significant effect on the T_m of the helix-coil transition. Clearly, therefore, the *N*-methylphenanthrolinium cations, I (with the exception of **8**), exhibit a high affinity to helical nucleic acids and stabilize the latter to heat denaturation.

Proton Magnetic Resonance Studies. It is well known that if the rate of molecular tumbling of molecules in solutions is lower than the typical Larmor frequencies W_0 (of the order of 10^8–10^9 radians sec^{-1} for protons in the conventional magnetic field), then T_2, the transverse relaxation time, is considerably diminished, leading to substantial line broadening of the pmr signal.[16] This situation is obtained if the proton is contained in a rigid macromolecule, *e.g.*, DNA,[17] or if the proton is contained in a slowly tumbling small molecule bound to a macromolecule. For instance, it has been shown by Gabbay and DePaolis[15] that the pmr signals of the aromatic protons of a molecule which intercalate between base pairs of DNA are extensively broadened and indistinguishable from base-line noise. The temperature dependent pmr spectra of the cations, **1–7**, in the presence and absence of DNA were taken at 37, 55, and 90°. In all cases, the pmr signals were indis-

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(17) C. D. McDonald, W. D. Phillips, and J. Lazar, *J. Amer. Chem. Soc.*, **89**, 4166 (1967).

tinguishable from base-line noise in the DNA–I complex at the lower temperatures. (Attempts to examine the DNA–**8** complex failed due to an insolubility problem.) Figure 2 illustrates this effect for the interaction of DNA with the 5,6-dimethyl-*N*-methylphenanthrolinium chloride (**3**). It is noted that at the higher temperatures broad pmr signals and large upfield chemical shifts are observed for the CH_3 group protons of **3**.

In summary, the pmr results indicate a common mode of binding of the cations, **1–7**, to DNA. The total broadening of the pmr signals of the cations is indicative of an intercalation mode of binding (see below for additional evidence).

Ultraviolet Absorption and Circular Dichroism (CD) Spectral Studies. The interactions of **1–8** with native salmon sperm DNA were studied by uv absorption and CD techniques. Due to the overlapping uv absorption of DNA and λ_{max} of I, the effect of binding to nucleic acids on the oscillator strength of the electronic transition of I could not be determined. The cations (**1–8**) exhibit a λ_{max} in the 280-nm region (ϵ 34,000) with the lowest energy 0–0 band occurring above 300 nm with an extinction coefficient, ϵ 7000 (Table I). In all cases (not including **8**) it is observed that the 0–0 absorption band is shifted to the red upon binding to DNA. However, these effects cannot be quantitated due to the overlap of nearby vibrational bands in the absorption spectra of I. Studies of the circular dichroism induced in the electronic transition of I upon binding to salmon sperm DNA are also hampered by overlapping CD signal from the nucleic acid in the region below 300 nm. However, induced CD in the lowest energy 0–0 absorption band of I in the presence of DNA is noted in the region of 320–400 nm. For example, the addition of *N*-methylphenanthrolinium cation, **1**, to salmon sperm DNA leads to a positive CD band (between 320 and 360 nm) which increases with increasing concentration of **1** and finally levels off at a base pair (B.P.) to cation ratio of 2.49. With the exception of **8**, similar saturation effects are noted for all the cations, I. (Compound **8** *does not* exhibit an induced CD in the DNA complex presumably because it cannot intercalate between base pairs of DNA due to the presence of the bulky, and out-of-plane, 4,7-diphenyl substituents.) The above results strongly suggest that the induced CD observed in the absorption band of I in the DNA complex arise from an intercalation mode of binding. Moreover, the CD titration studies indicate that once all intercalation sites in DNA are filled by the cation I, further excess of the latter would not lead to further changes in the CD spectrum (supporting evidence from viscometric titration data are given below).

The results of the CD titration studies with cations **1–7** are given in Table II and a typical CD titration for cation **3** with salmon sperm DNA is shown in Figure 3. It is noted that the induced CD signal in the absorption band of **1–7** upon binding to DNA reaches a saturation limit that varies from a minimum of 1.87 B.P./cation (in the case of **7**, 4,7-dimethyl-*N*-methylphenanthrolinium cation) to a high of 2.51 B.P./cation (in the case of **5**, 3,8-dimethyl-*N*-methylphenanthrolinium cation). Similarly, viscometric titration studies of DNA intercalating sites by cations I (using the same DNA concentration and buffer conditions as the CD studies) show almost identical B.P./cation ratios at saturation as the

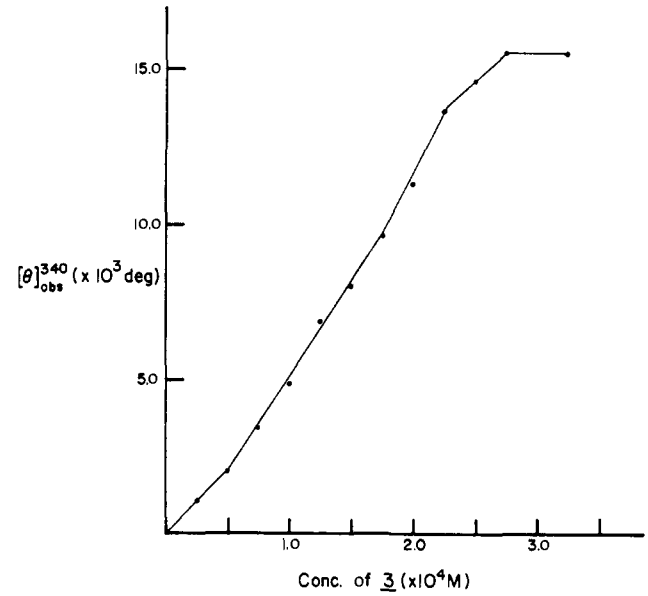


Figure 3. The effect of increasing concentration of **3** on the observed ellipticity, $(\theta)_{obsd}$, at 340 nm in the presence of 5.60×10^{-4} *M* P/l. of salmon sperm DNA.

Table II. Summary of the Circular Dichroism and Viscometric Titration Studies of Salmon Sperm DNA by Cations I[a]

Compd	CD studies[b] $(B.P./cation)_{sat}$[d]	Viscometric studies[c] $(B.P./cation)_{sat}$[d]
1	2.49	2.32
2	2.26	2.07
3	2.04	1.99
4	2.06	2.26
5	2.51	2.32
6	2.27	2.04
7	1.87	1.94

[a] All studies were carried out in 0.01 *M* MES buffer (pH 6.2) (0.005 *M* in Na^+). [b] CD titration studies employed a concentration of 5.6×10^{-4} *M* DNA P/l. using a 5-cm path length cells at 25°. [c] Viscometric titration studies employed a concentration of 5.6×10^{-4} *M* DNA P/l. and were carried out at 37°. [d] $(B.P./cation)_{sat}$, is the calculated value of the ratio of base pairs of DNA to *total* cation I present in solution, at saturation. It should be noted that these values do not represent the maximum number of binding sites for cations I to DNA since at saturation free cations in solution are also present.

CD results cited above (see Figure 6, Table II, and viscosity section below). It is therefore concluded (with a high degree of certainty) that the induced CD observed for the DNA–(**1–7**) complexes arise only from an *intercalation mode of binding* rather than from another type of mechanism, *e.g.*, external binding to the phosphate group. The results of the pmr and T_m studies (cited earlier) and the viscosity studies (discussed below) are consistent with this interpretation.

The induced circular dichroism in the absorption band of the cations, **1–7**, upon binding to salmon sperm DNA is shown in Figure 4. The spectra were obtained under conditions of saturation of the intercalation sites, *i.e.*, further addition of cations, I, to the DNA solution do not change the observed spectra. It should be noted that significant differences in the induced CD of DNA–(**1–7**) complexes are observed. In addition, two interesting observations can be made. (1) The cations which contain methyl groups at the 5,6 positions on the *N*-methylphenanthrolinium ring, *i.e.*, **3**,

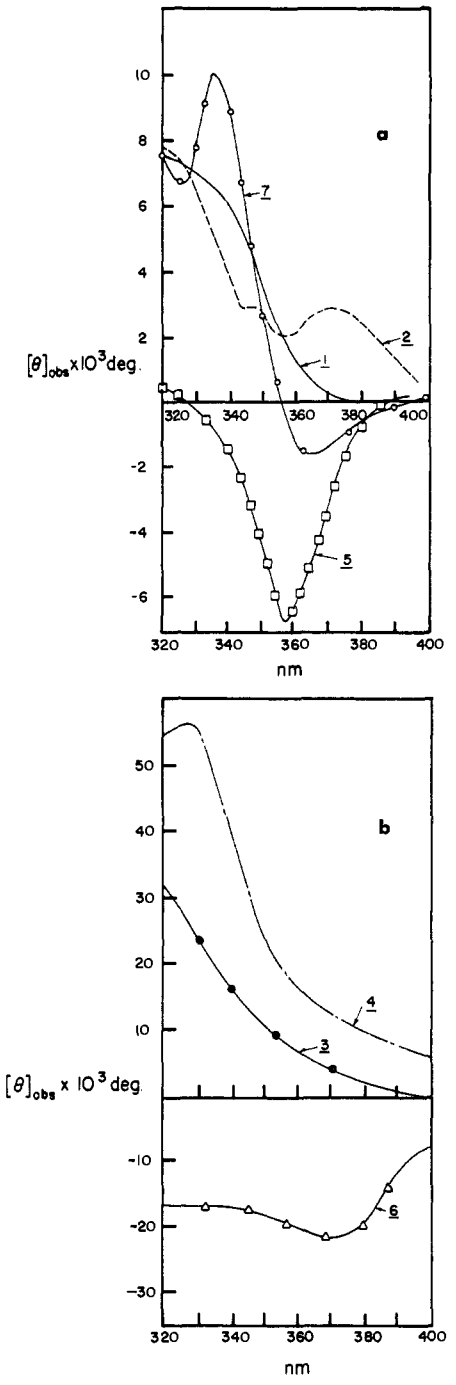
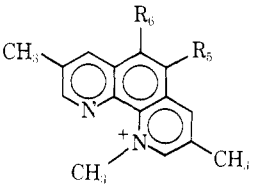


Figure 4. The induced circular dichroism spectra of salmon sperm DNA–I complexes at saturation: (a) **1**, **2**, **5**, and **7**; (b) **3**, **4**, and **6** (see text).

4, and **6**, exhibit an induced CD upon binding to salmon sperm DNA which is several magnitudes greater than the unsubstituted and dimethyl-substituted cations, **1**, **2**, and **7**. One possible explanation for this effect is the fact that the lowest energy 0–0 absorption band, λ^{0-0}, of **3**, **4**, and **6** (as the free cations) is red shifted by 10–20 nm as compared to **1**, **2**, and **7** (Table I). Therefore, the higher induced CD in the 320–400-nm region (Figure 3b) observed for the DNA–**3**, –**4**, and –**6** complexes may simply be due to the fact that the 0–0 absorption band of these systems is nearer to the region in which the CD spectra are being observed. (2) The cations which contain methyl groups at the 3,8 positions of the *N*-methylphenanthrolinium ring (**5** and **6**) ex-



5, $R_5 = R_6 = H$
6, $R_5 = R_6 = CH_3$

hibit a negative induced CD upon binding to salmon sperm DNA as opposed to positive induced CD observed for the other cations. One possible explanation is that the 3,8-dimethylated cations, **5** and **6**, cannot assume all possible geometries in the intercalating site due to steric restrictions. For example, the distance between CH_3 groups at the 3 and 8 positions of I is approximately 11.2 Å (including the van der Waal radius of the methyl groups); therefore unfavorable steric interactions with the deoxyribofuranoside rings (on opposite chains) may be expected to occur for certain geometries. Molecular framework model studies indicate that an intercalation geometry for the 3,8-dimethylated cations, I, whereby the long axis of the molecule is approximately parallel with respect to the H-bonds of the base pairs is highly unfavorable due to steric interactions with the sugar rings. A more reasonable intercalation geometry, for the above systems, is one in which the methyl groups at the 3 and 8 positions are pointing into opposite grooves. The oppositely induced CD observed for the DNA–**5** and –**6** complexes as compared to the other cations (**1**–**4** and **7**) can arise (on the basis of present theories[13,19]) from different intercalation geometries.

Viscometric Studies. It is well known that planar molecules such as acridine orange, ethidium bromide, and proflavine may intercalate between base pairs in DNA.[3,20,21] This phenomenon leads to an increase in the length of the helix and is usually accompanied by an increase in the viscosity of the solution. In order to determine the mode of binding of cations I to salmon sperm DNA, viscometric titration studies were carried out under the same concentration conditions as those employed for the circular dichroism studies, *i.e.*, 5.6 $\times$ 10^{-4} *M* DNA P/l. in 0.01 *M* MES buffer (pH 6.2). It is found that the specific viscosity, η_{sp}, of the solution increases with increasing concentration of I and finally reaches a saturation value. Figure 5 shows the effect of **1** on the relative specific viscosity, $\eta_{sp}^{complex}/\eta_{sp}^{DNA}$. It is noted that the relative specific viscosity increases with increasing concentrations of **1** and finally levels off at a B.P./cation ratio of 2.32. Similar saturation effects on the relative specific viscosity of DNA are observed for cations **2**–**7**. The values of B.P./cation ratio obtained by viscometric titrations are shown in Table II. It should be noted that these values are almost identical with those obtained by the CD titration technique, *i.e.*, $(B.P./cation)_{sat}^{CD} = (B.P./cation)_{sat}^{viscosity}$. The results are entirely consistent with an intercalation mode of binding for cations **1**–**7** to DNA. In support of this conclusion, the nonplanar compound **8** which contains 4,7-diphenyl substituents does not exhibit

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(19) J. A. Schellman, *Accounts Chem. Res.*, 1, 144 (1968).
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(21) G. Cohen and H. Eisenberg, *Biopolymers*, 8, 45 (1969).

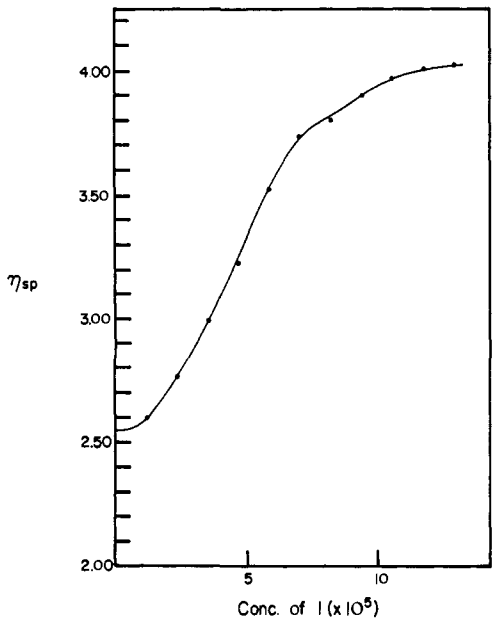


Figure 5. The effect of increasing concentrations of **1** on the relative specific viscosity, $\eta_{sp}^{complex}/\eta_{sp}^{DNA}$ in 0.01 *M* MES buffer (pH 6.2) (0.005 *M* Na^+). The study was conducted at 37.5° using 5.60 $\times$ 10^{-4} *M* P/l. of salmon sperm DNA.

an induced CD or an increase in viscosity upon binding to DNA.

In order to compare the effective increase in length of the DNA helix by cations **1–7**, attempts were made to determine the intrinsic viscosity, (η), of the complexes. However, the results are found to be uninformative since the value of the intrinsic viscosity at infinite dilution in the presence of other molecules will and does approach the value of the intrinsic viscosity of free DNA at infinite dilution, *i.e.*, since the binding constant of the small molecule to DNA is finite, the complex will be dissociated at the lower concentrations. Instead, the effect of increasing concentrations of **1–7** on the specific viscosity, η_{sp}, of DNA solution at low concentration of the latter was studied. For instance, at very low DNA concentration, the relative values of η_{sp} upon saturation of the intercalation sites by the cations **1–7** are a close approximation of the relative values of the intrinsic viscosity, (η), of the complexes (since by definition $(\eta) = (\eta_{sp/C})_{C\to 0}$ where C is the DNA concentration). The effect of increasing concentrations of cations I on the η_{sp} of a solution containing 1×10^{-4} *M* DNA P/l. is shown in Figure 6. A number of interesting observations can be made. (1) The limiting values of the η_{sp} of DNA solution upon saturation of the intercalating sites are dependent on the number and position of methyl group substituents on the aromatic ring of I. The order of increasing η_{sp} at saturation is found to be: 2,9- < unsubstituted < 3,8 < 4,7 < 5,6 < 3,5,6,8 < 2,5,6,9. (2) Since the study was carried out at near infinite dilution of the DNA, the relative values of η_{sp} at saturation are close approximations of the relative values of the intrinsic viscosity of DNA–I complexes. Moreover, the intrinsic viscosity of a rod-like polymer increases with increasing length.[21,22] Therefore, the order of increasing η_{sp} for the DNA–I complexes also reflects the order of increasing effective length of the helix. Hence, the helix length of salmon sperm DNA–**6** complex is found to be greater than that of the DNA–**1** complex when all the intercalating sites are filled (Figure 7). Such an effect may arise by two separate mechanisms. (i) There are more intercalation sites (on DNA) available for the binding of **6** as compared to **1**. The results of the equilibrium dialysis studies are consistent with this interpretation. For example, Scatchard-type treatment of the binding data shows one strong binding site per 4.35 and 2.96 base pairs of salmon sperm DNA for cations **1** and **6**, respectively (Table III). However, such an explanation does not account for the higher value of the η_{sp} at saturation obtained upon intercalation of **1** as compared to **2** since the number of strong binding sites per base pairs of DNA are found to be 4.35 and 2.86, respectively. Similar discrepancies between the values of η_{sp} at saturation and the maximum number of binding sites for cations I are also noted (Figure 6 and Table III). (ii) Differences in steric interaction between cations I and the base pairs of the intercalating site may also lead to differences in helical length. For example, the larger van der Waal radii of the four methyl group substituents on the aromatic ring of **6** as compared to the H-substituents of **1** can also account for the observed higher η_{sp} of DNA–**6** as compared to DNA–**1** complex. This argument, however, does not explain the lower η_{sp} observed for the DNA–**2** (**2** contains a 2,9-dimethyl substituent) as compared to the DNA–**1** complex nor does it account for the observed order of η_{sp} for the various DNA–I complexes at saturation.

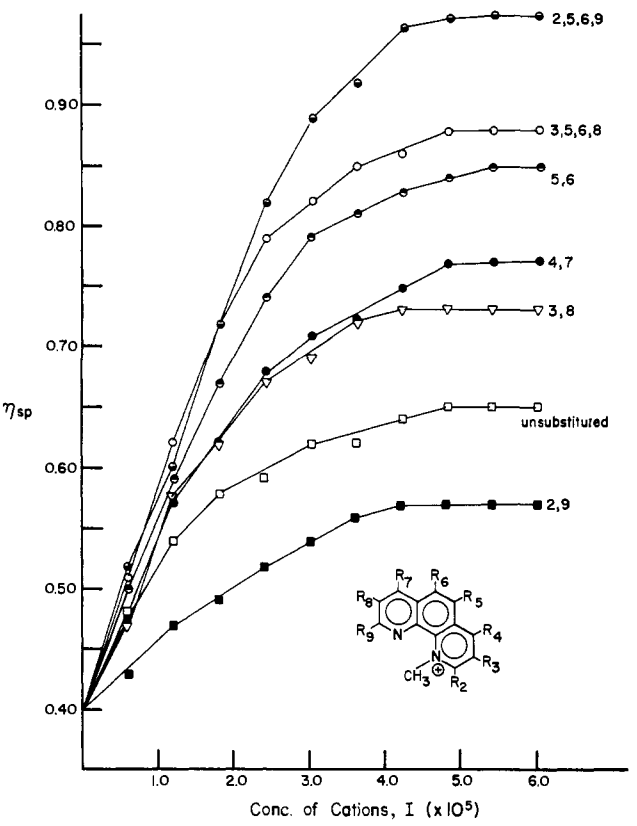


Figure 6. The effect of increasing concentrations of cations I on the specific viscosity, η_{sp}, of near infinitely dilute solution of salmon sperm DNA, *i.e.*, at 1×10^{-4} *M* P/l.

(22) C. Tanford, "Physical Chemistry of Macromolecules," Wiley, New York, N. Y., 1961.

Table III. Summary of the Scatchard-Type Treatment of the Binding Studies of Cations I to Various Nucleic Acids[a]

Cation	Salmon sperm DNA			Poly(dA-T)-poly(dA-T)			*M. luteus* DNA		
	10^4K_a	$\bar{n}_{max}$	$1/2\bar{n}_{max}$[b]	10^4K_a	$\bar{n}_{max}$	$1/2\bar{n}_{max}$[b]	10^4K_a	$\bar{n}_{max}$	$1/2\bar{n}_{max}$[b]
1	4.03	0.123	4.05	2.83	0.122	4.10	3.85	0.174	2.87
2	4.94	0.173	2.89						
4	14.30	0.208	2.40						
6	18.5	0.165	3.03	16.0	0.180	2.78	22.7	0.235	2.12
7	8.17	0.192	2.60						

[a] Equilibrium dialysis studies were carried out in 0.01 *M* MES buffer (0.020 *M* in Na^+) (pH 6.2), using 5.53×10^{-4}, 5.26×10^{-4}, and 2.95×10^{-4} *M* P/l. of salmon sperm DNA, poly(dA-T)-poly(dA-T), and *M. luteus* DNA, respectively. Reporter concentrations were varied from 2×10^{-5} to 6×10^{-5} *M*. Duplicate measurements on each of the above systems were carried out and the average values are reported in the table. Deviations of not more than ±4 and ±7% of the $\bar{n}_{max}$ and K_a values are observed, respectively. [b] $1/2\bar{n}_{max}$ values represent the minimum number of base pairs per binding site.

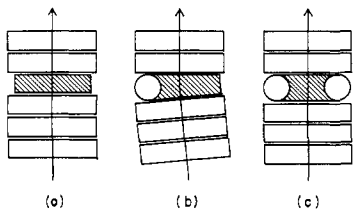


Figure 7. Schematic illustrations of the possible complexes of I to DNA showing lengthening (a) and (c) as well as bending of the helix (b) at the intercalation site.

Clearly, the observed order of increasing helical length of the various DNA–I complexes cannot be explained in terms of either mechanisms (i) or (ii) or a combination of the two. A model which fits the observed data is shown in Figure 7. It is based on the supposition that the "thickness" of the aromatic ring of I is not uniform and, moreover, is dependent on the position of substitution of the methyl groups. For example, the van der Waal contact distance of a CH_3 group is larger than the in-plane contact distance of an aromatic ring (*i.e.*, 2.1 and 1.7 Å for the former and latter, respectively).[23] Thus, the in-plane structure of *N*-methylphenanthrolinium cation **1** should be considered as a wedge of varying thickness. Therefore, intercalation of **1** between base pairs of DNA will not only lead to lengthening of the helix but also to slight bending of the rod at the point of intercalation (Figure 7). The first effect will lead to an increase in the η_{sp} while the second will lead to a decrease. Bending of the helix will be expected to occur to a larger extent if greater variation in thickness of the "wedge" from one end of the molecule to the other exists. Thus, the results shown in Figure 6 can be readily understood since the order of increasing η_{sp} at saturation by cations, I (*i.e.*, 2,9 < unsubstituted < 3,8 < 4,7 < 5,6 < 3,5,6,8 < 2,5,6,9), is also the expected order of decreasing *variation in thickness* of the aromatic ring along the short axis of the molecule. For example, substitution of methyl groups at the 5 and 6 positions will lower the difference in thickness across the short axis of the aromatic cation, I, and therefore bending of the helix will be minimal. However, bending of the helix will be expected to increase as substitution of the ring of I by dimethyl groups progressively gets nearer to the nitrogen atoms. This model, in conjunction with mechanisms (i) and (ii), adequately explains the viscometric data.

Equilibrium Dialysis Studies. The results of T_m (helix-coil transition), proton magnetic resonance (pmr), induced circular dichroism, and viscometric studies (cited earlier) strongly suggest *a common mode of binding of cations I* to DNA, *i.e., intercalation between base pairs*. In addition, the induced CD and viscometric data indicate that differences in the binding "geometry" of the intercalated cations, I, exist. In order to further understand the interaction specificities of *N*-methylphenanthrolinium cations, I, binding studies to nucleic acids of various base compositions were carried out using equilibrium dialysis techniques. In these studies, the nucleic acid concentration was kept constant and the concentrations of the cations were varied from 2×10^{-5} to 6×10^{-5} *M*. The concentration of free cations, I, was determined directly by uv absorption (Table I). The data obtained were analyzed by the Scatchard technique[24] according to the following equation

$$\bar{n} = \bar{n}_{max} - (1/K_a)(\bar{n}/R_f)$$

where $\bar{n}$ = the number of moles of I bound per mole of DNA phosphate, $\bar{n}_{max}$ represents maximal binding, K_a is the association constant for the DNA–I complex, and R_f is the concentration of unbound cation. A plot of $\bar{n}/R_f$ *vs.* $\bar{n}$ gives the values of $\bar{n}_{max}$ (*x* axis intercept) and $\bar{n}_{max}K_a$ (*y* axis intercept). The results of this study are shown in Figure 8 (for cations **1** and **6**) and the data are summarized in Table III. Several interesting observations may be made (Table III). (1) Differences in binding affinities, K_a, and maximum number of strong binding sites are noted for cations I with salmon sperm DNA. For example, the following order of increasing affinity is observed: unsubstituted (**1**) < 2,9-dimethyl (**2**) < 5,6-dimethyl (**7**) < 2,5,6,9-tetramethyl (**4**) < 3,5,6,8-tetramethyl (**6**). With one exception, a similar order of increasing number of strong binding sites is also noted, *i.e.*, **1** < **6** < **2** < **7** < **4**. Although the binding affinity results are consistent with the T_m data (which also show the same order of increasing stabilization of the helical structure), they are, nonetheless, surprising. For example, greater steric hindrance and hence lower affinity is expected for the DNA–**6** as compared to the DNA–**1** complex due to the presence of the 3,5,6,8-tetramethyl groups in the former. It is clear, however, that this is not the case. Moreover, the maximum number of strong binding sites for the methylated cations, I (*e.g.*, **2**, **4**, **6**, and **7**), is found to be higher than the unsubstituted *N*-methylphenanthrolinium cation, **1**. Therefore, it is concluded that adjacent base pairs of DNA may readily

(23) L. Pauling, "The Nature of the Chemical Bond," 3rd ed, Cornell University Press, Ithaca, N. Y., 1960.

(24) G. Scatchard, *Ann. N. Y. Acad. Sci.*, 51, 660 (1949).

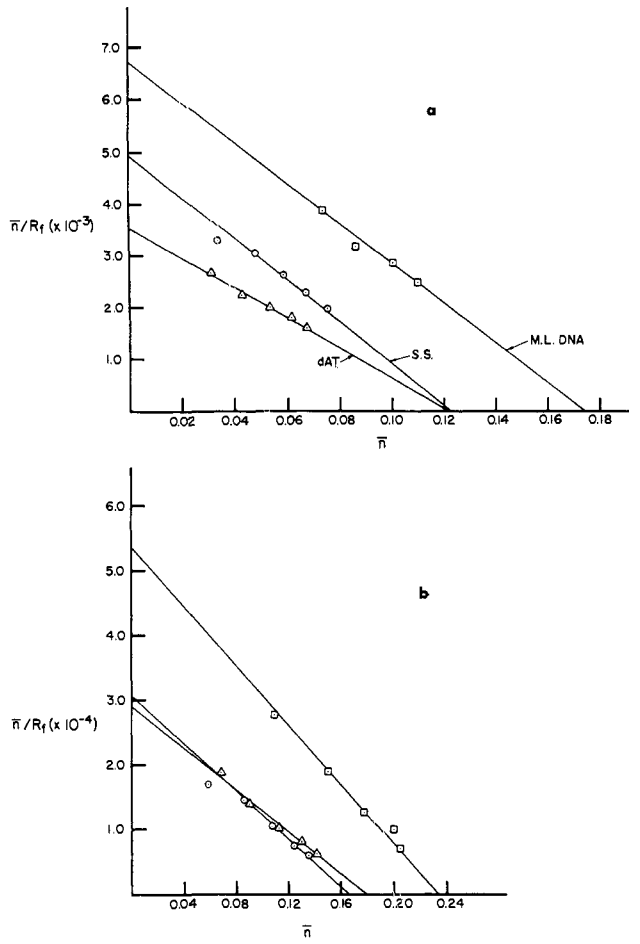


Figure 8. The Scatchard plots of the binding studies data obtained by equilibrium dialysis technique for interactions of cations **1** (a) and **6** (b) with poly(dA-T)-poly(dA-T) (Δ) salmon sperm DNA (O) and *Micrococcus luteus* DNA (□) (see Table III).

separate by distances greater than 6.8 Å in order to accommodate a bulkier intercalating cation, *e.g.*, **6**. In the latter case a separation distance of at least 7.6 Å is required. (2) The effect of nucleic acid base composition on the apparent binding constant and the maximum number of binding sites was studied for cations **1** and **6** (Table III). The results show the following order of increasing affinity of **1** to the nucleic acids, poly(dA-T)-poly(dA-T) (100% A-T) < *M. luteus* DNA (28% A-T) < salmon sperm DNA (58% A-T), and the following order of increasing affinity of **6** to the nucleic acids, poly(dA-T)-poly(dA-T) < salmon sperm DNA < *M. luteus* DNA. In addition, it is noted that G-C rich DNA, *i.e.*, *M. luteus* DNA, shows a higher maximum number of strong binding sites for **1** and **6** than the other nucleic acids. Steric hindrance to intercalation between A-T sites is one possible explanation for the above observation. For example, the separation distance required for intercalation of **6** between base pairs composed of A-T sites may be as high as 8.4 Å if the CH_3 group of thymine is in an eclipsed conformation with respect to a CH_3 substituent of the intercalating cation, **6**. Such effects are illustrated in Figure 9.

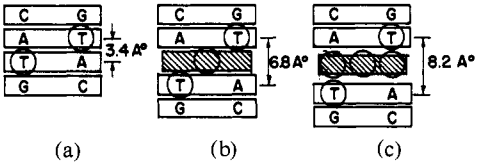


Figure 9. Schematic illustrations of the complexes of I to DNA showing the possible separation distances between base pairs required to accommodate unsubstituted (b) and methyl-substituted *N*-methylphenanthrolinium cation (c).

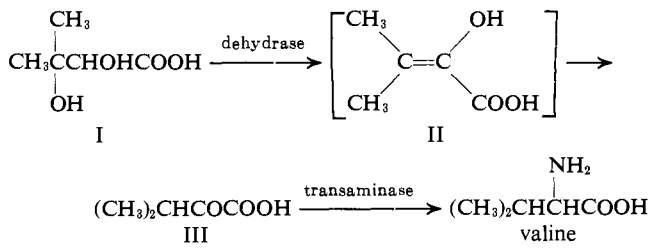
In summary, systematic studies of the interaction specificities of methyl-substituted *N*-methylphenanthrolinium cations, I, with nucleic acids of various base compositions have been carried out. In all cases, a common mode of binding is observed, *i.e.*, intercalation between base pairs of DNA. Selective interactions of I with DNA are noted as a function of the position and number of methyl substituents on the *N*-methylphenanthrolinium ring. For example, the more highly substituted systems exhibit (i) higher affinity, (ii) greater stabilization of the helix, and (iii) higher viscosity upon binding to DNA. Moreover, selective binding to G-C sites (and/or a combined G-C/A-T site) by the more highly substituted aromatic cations is observed. These as well as other effects are discussed and the results can be accounted for in terms of reasonable structural models.

Communications to the Editor

Enzymatic Discrimination between Diastereotopic Enol Faces in the Dehydrase Step of Valine Biosynthesis[1]

Sir:

The penultimate step of valine biosynthesis in bacteria[2] is the dehydration of (−)-α,β-dihydroxyisovalerate (I) to α-ketoisovalerate (III), catalyzed by an α,β-dihydroxy acid dehydrase. Arfin has shown conclusively by labeling experiments[3] that dehydration must proceed *via* an enol intermediate (II).



(1) Stereochemistry of Valine and Isoleucine Biosynthesis. III. For paper II in this series, see R. K. Hill and S. Yan, *Bioorg. Chem.*, **1**, 446 (1971).

(2) A. Meister, "Biochemistry of the Amino Acids," 2nd ed, Vol. 2, Academic Press, New York, N. Y., pp 729–739.

(3) S. M. Arfin, *J. Biol. Chem.*, **244**, 2250 (1969).