I. Bismethidium Intercalators: The Binding of Nucleic Acids

and

II. Experiments in the Design of Site Specific DNA Cleaving Agents

Thesis by

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To Mom and Dad

For Making Dreams Realities

ACKNOWLEDGMENTS

Sincerest thanks to my research advisor, Peter B. Dervan, for his constant support and direction throughout my graduate career. His enthusiasm and friendship will always be welcome.

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ABSTRACT

CHAPTER I

Part I

Bis(methidium)spermidine (BMSpd), an analog of ethidium bromide in which two phenanthridine chromophores are tethered by a 10 Å spermidine linker, has been synthesized and characterized in order to test the effect of linker length on DNA binding. Like bis(methidium)spermine (BMSp),[1] a dimer with a 15 Å tether, BMSpd unwinds DNA 36.5°. Unlike BMSp, the binding of BMSpd to calf thymus DNA, poly dG·poly dC, poly rA·poly dT, and poly dA·poly dT is complicated. At 0.4 M (M^+) and pH 7, the binding site sizes and binding constants of BMSpd range from 2.75 base pairs (calf thymus DNA) to 4.0 base pairs (rA·dT, dG·dC) and 5×10^5 M^{-1} (dA·dT) to 6×10^6 M^{-1} (rA·dT). These affinities are on the average, ten times higher than the analogous affinities of ethidium bromide and 300 times less than the binding constants of BMSp. The most dramatic change occurs in the binding specificity of BMSpd. Whereas, BMSp binds poly rA·dT 1440 times more tightly than poly dA·dT, the spermidine analog (BMSpd) binds poly rA·dT only 10 times more tightly than poly dA·dT.

Part II

The ethidium dimers, bis(methidium)spermine (BMSp) and bis(methidium)spermidine (BMSpd), were found to site selectively inhibit DNase I and a variety of restriction enzymes on the plasmid pBR322. In contrast to the dimers, the monomer, ethidium bromide (EB), shows no site selective action in competition with any of the enzymes tested. The sites preferentially inhibited by BMSp and BMSpd appear to be in the first third of the plasmid. These observations and the clustering of inhibited restriction sites then suggests that selective inhibition may be due to the recognition or perturbation of DNA secondary structure.

CHAPTER II

The compounds methidiumpropyl-EDTA, distamycin-EDTA, and penta-N-methylpyrrolecarboxamide-EDTA are three representative examples of a class of rationally designed DNA cleaving agents. By appending an iron chelator to different DNA binding vehicles certain properties of the resulting DNA cleaving agent can be controlled. Methidiumpropyl-EDTA randomly single strand cleaves DNA with high efficiency. Distamycin-EDTA nicks at 5 base pair recognition sites, and penta-N-methylpyrrolecarboxamide-EDTA double strand cleaves DNA at 6 base pair recognition sites. The success of these compounds led to the design of a controlled site cleaving agent, oligonucleotide-EDTA.

The construction of the molecule was approached in two ways, synthetically and enzymatically. Synthetically, the oligonucleotide-EDTA was constructed by known nucleotide triester methodology. Enzymatically, a modified uridine, EDTA-dUTP, was incorporated into a DNA fragment. Unfortunately, neither strategy yielded an active DNA cleaving agent. These failures, however, do not directly reflect on the viability of an oligonucleotide-EDTA. It is possible that an extended research effort will uncover the methods needed to create an active oligonucleotide-EDTA.

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CHAPTER I

Bismethidium Intercalators:

The Binding of Nucleic Acids

Part I

The Binding Properties of Polyintercalators

A. Intercalation

Acridines, phenanthridines and other small organic compounds characterized by a planar aromatic structure have been shown to bind to DNA (Fig. 1);[2-8] the binding of these compounds induces a distinctive set of changes in the macromolecular properties of the DNA. The sedimentation coefficient of the DNA decreases,[9] and the intrinsic viscosity increases.[10] The superhelicity of closed circular DNA increases,[11] and the microscopic contour of the nucleic acid helix lengthens.[12]

Observation of the bound chromophores by dichroism[9] and NMR[13] suggest that the molecules are bound perpendicularly to the helix axis of the DNA and that there is considerable electronic interaction between the bases of the DNA and the aromatic chromophore of the DNA bound effector. These data are consistent with binding by intercalation, the insertion of the aromatic molecule between the base pairs of the nucleic acid helix;[14] moreover, Sobell's dinucleotide crystal structures[15] lend structural evidence for this mode of binding (Fig. 2).

Ethidium bromide (EB)[16-20] is one of the best characterized intercalators. The association of EB with DNA is accompanied by an enhanced fluorescence yield from the chromophore and a red shift of the visible absorbance maximum;[9,12] consequently, the binding of EB to DNA is easily monitored by fluorescence and absorbance. Both equilibrium and kinetic studies reveal two bound species of EB: (1) an intercalated species and (2) an outside bound species.[16] The outside bound species is simply an electrostatic coupling of the EB cation to the DNA polyanion. Electrostatically bound EB saturates the available binding sites at a ratio of

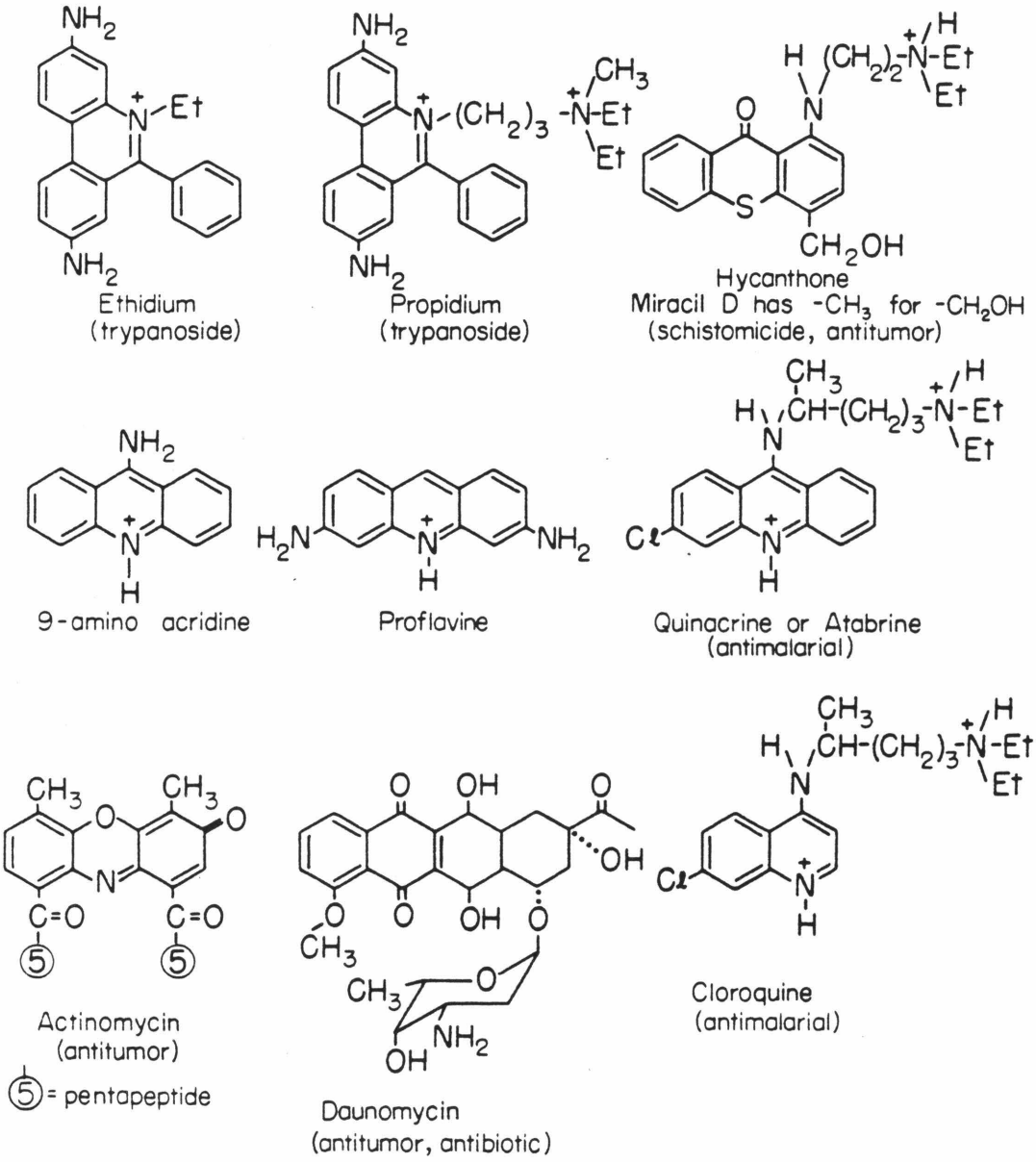
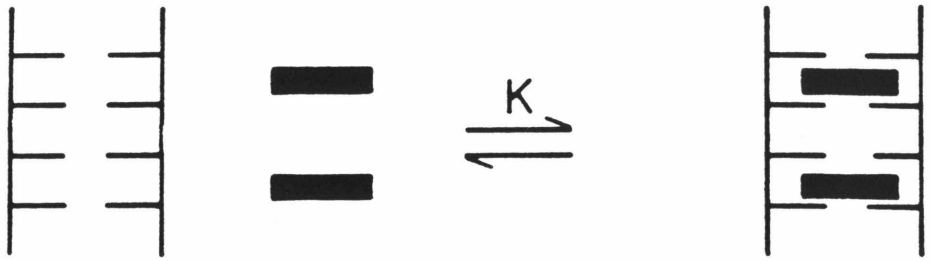

Ethidium
(trypanoside)
Propidium
(trypanoside)
Hycanthone
Miracil D has -CH3 for -CH2OH
(schistomicide, antitumor)
9-amino acridine
Proflavine
Quinacrine or Atabrine
(antimalarial)
Actinomycin
(antitumor)
⑤ = pentapeptide
Daunomycin
(antitumor, antibiotic)
Cloroquine
(antimalarial)

Figure 1

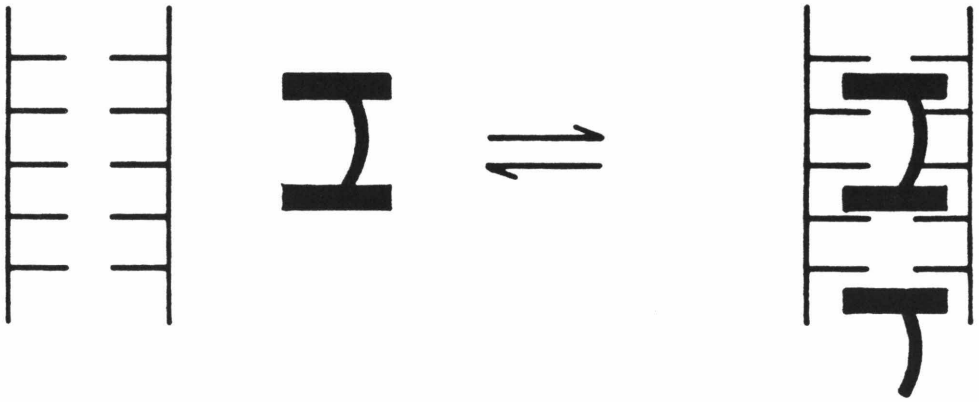
Intercalation



$K \sim 10^5 M^{-1}$

stoichiometry: 1 intercalator / 2 base pairs (nearest neighbor exclusion)

Polyintercalation



stoichiometry: 1 intercalator / 4 base pairs

Figure 2

1.0 EB per base pair of DNA and exhibits little enhancement in the fluorescence yield. Conversely, the intercalate saturates at levels less than or equal to 0.5 an EB per base pair and is distinguished by a 20 fold increase in the fluorescence yield.[9,21] It is this intercalative mode of binding that is of interest.

Investigations, that utilized both synthetic and natural DNA's as substrate, have shown that EB is not base specific[9] and have provided evidence that EB is instead "conformationally" selective.[1,7,16] EB binds the DNA/RNA hybrid rA·dT 100 times more tightly than dA·dT (Table 1);[16] furthermore, the RNA/DNA hybrid and the DNA saturate at different stoichiometries in 1 M Na^+ solutions. The DNA complex exhibits nearest neighbor exclusion (1 EB/2 bp), while the hybrid saturates at one EB per 3.0 base pairs.[1]

This behavior can then be used as a basis to speculate that differences in the binding of EB to defined synthetic DNA's are a reflection of the conformational variations of different DNA sequences and that EB can induce conformational changes in the receptor DNA to allow intercalation.

B. Polyintercalation

Whereas, the conformational selectivity of EB is remarkable, its inherent DNA binding affinity is unspectacular.[16] This problem of moderate DNA affinity characterizes many intercalators.[16,22] The natural product, echinomycin, intercalates two chromophores simultaneously.[23] Several researchers pursued nature's example and synthesized sets of acridine, phenanthridine, and mixed acridine-phenanthridine dimers.[24-27] Examination

of these compounds demonstrated that the DNA affinity of a dimer is substantially higher than the DNA affinity of the respective monomer. In many cases the dimer binds DNA so tightly that binding constants can only be estimated.

More importantly, the ability of a dimer to bisintercalate is dependent on the maximum separation of the tethered monomers, and the critical length is dependent on the nature of the linker connecting the chromophores. Polyamine linked dimers exhibit neighbor exclusion, while hydrocarbon linked dimers bisintercalate nearest neighbors.[8,24] This implies bisintercalative separations of 10 Å and 7 Å for the polyamine and hydrocarbon linkers, respectively. Consequently, these structural parameters were considered when the synthesis of a phenanthridine dimer, bis-(methidium)spermine, was conceived.[1]

The molecule synthesized was a spermine linked (15 Å) analog of ethidium bromide, designated bis(methidium)spermine (BMSp).[28] What became critical to any analysis was the realization that the interaction of the cationic BMSp with the anionic DNA could be treated as a polyelectrolyte problem.[29,30] By working in buffered 1 M Na^+ solutions the electrostatic contributions to the DNA binding affinity could be minimized thus allowing BMSp binding constants to be measured directly.[7]

The bisintercalation of BMSp is found to be 1.6 times more energetically favorable than the intercalation of EB. In addition, the rA·dT-dA·dT selectivity climbs from a ratio of 100 for ethidium to a ratio of 1440 for BMSp (Tables 1 and 2) where it has been suggested that this selectivity is due to conformational recognition.[7,16]

Table 1

Nucleic Acid	EB				BMSp			
	$[M^+]$	K	γ	Site Size	$[M^+]$	K	γ	Site Size
Calf Thymus DNA	1.0	4.5×10^4	0.1-0.5	2	1.0	1.5×10^7	0.1-0.2	4
Poly dG·dC	1.0	1×10^5	0.1-0.3	2	1.0	2.6×10^8	0.05-0.15	4
Poly rA·dT	1.0	2×10^5	0.1-0.3	3	1.0	2.3×10^8	0.05	6
Poly dA·dT	1.0	2×10^3	0.1-0.4	(1)	1.0	1.6×10^5 est.	0.05	(2)

Table 2

Nucleic Acids	EB (K_1/K_2)	BMSp (K_1/K_2)
$\frac{\text{r AdT}}{\text{dAdT}}$	100	1440

Whatever the molecular explanation, the data show three intercalated species: (1) A low affinity intercalation covering an undefined site, (2) a high affinity intercalation of DNA covering 2.0 base pairs per chromophore, and (3) a high affinity intercalation of DNA and RNA/DNA hybrids that excludes 3.0 base pairs per monomer. By exploiting these three intercalated forms the specificity of a potential bisintercalator might be predictably altered by simply changing the length and flexibility of the linker joining the two intercalating chromophores. As a result, a shortened dimer, bis(methidium)-spermidine, has been synthesized; the binding constants to a variety of nucleic acids have been determined, and the 10 Å spermidine linked bis-intercalator has been compared to the 15 Å spermine linked dimer.

C. Results and Discussion

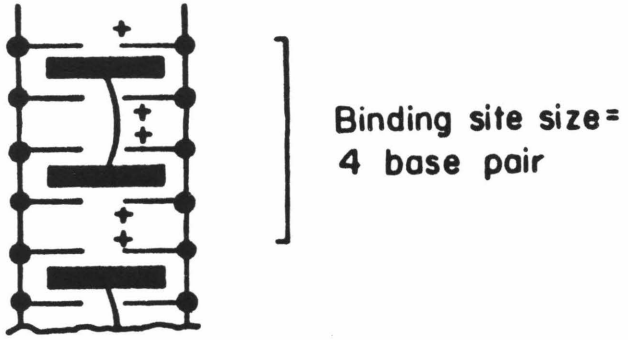
1. Synthesis. The rationale for the synthesis of an ethidium dimer with a 10 Å linker (spermidine) is based on the observations that linker length is important in determining the ability of a compound to bisintercalate and that at least two of the three intercalated species exhibit different binding stoichiometries. A linker long enough to allow bisintercalation at 1.0 molecule per 4.0 base pairs may not be long enough to allow bisintercalation of an RNA/DNA hybrid at 1.0 molecule per 6.0 base pairs (Fig. 3). This implies that one might be able to substantially alter the selectivity of an ethidium dimer by reducing the length of the linker.

The synthesis of the 10 Å dimer, bis(methidium)spermidine (BMSpd), is based on the synthesis of bis(methidium)spermine (Fig. 4).[1] Two equivalents of p-carboxylmethidium chloride[1] were coupled to the primary amines of one

equivalent of spermidine. The reaction was chromatographed twice on silica gel, and the isolated solids were repurified on AG50W-X8 cation exchange resin. The resulting sample proved to be analytically pure.

2. Physical Properties. BMSpd is a water soluble trication at physiological pH. The short, 10 Å, linker does not significantly change the solution behavior of the dimer. It is similar in many respects to BMSp. Like BMSp, BMSpd shows ultraviolet and visible absorptions characteristic of a phenanthridine chromophore. Both molecules exhibit intramolecular coupling of the tethered chromophores which leads to a visible absorbance that is red shifted with respect to EB and reduced in relative intensity. The visible maximum of BMSpd occurs at 501 nm (Fig. 5), 22 nm red shifted from the visible maximum of ethidium, and although the position of the visible maximum is independent of ionic strength the molar absorbtivity decreases with increasing ionic concentrations; BMSp also shows this same trend in extinction coefficient but the effect is more pronounced. This suggests that the orientation of the intramolecularly stacked chromophores is more constrained in the spermidine linked bisintercalator than in the spermine linked bisintercalator. As a consequence, the low salt (0.075 M (M^+)) spectrum of BMSpd most resembles the high salt (1.0 M (M^+)) spectrum of BMSp.

3. Intercalation and Helix Unwinding. Direct evidence for the simultaneous intercalation of both chromophores of BMSpd is provided by DNA unwinding experiments. Previous viscometric measurements at 0.075 M (M^+) had shown that the known bisintercalator, BMSp, unwound DNA 38°, twice the magnitude of the monointercalator, methidium spermine.



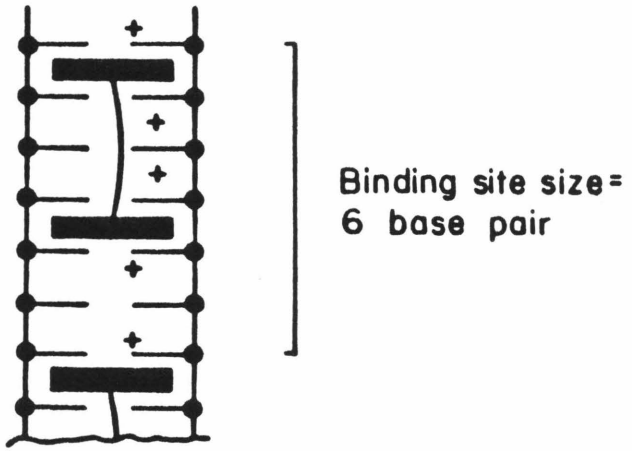
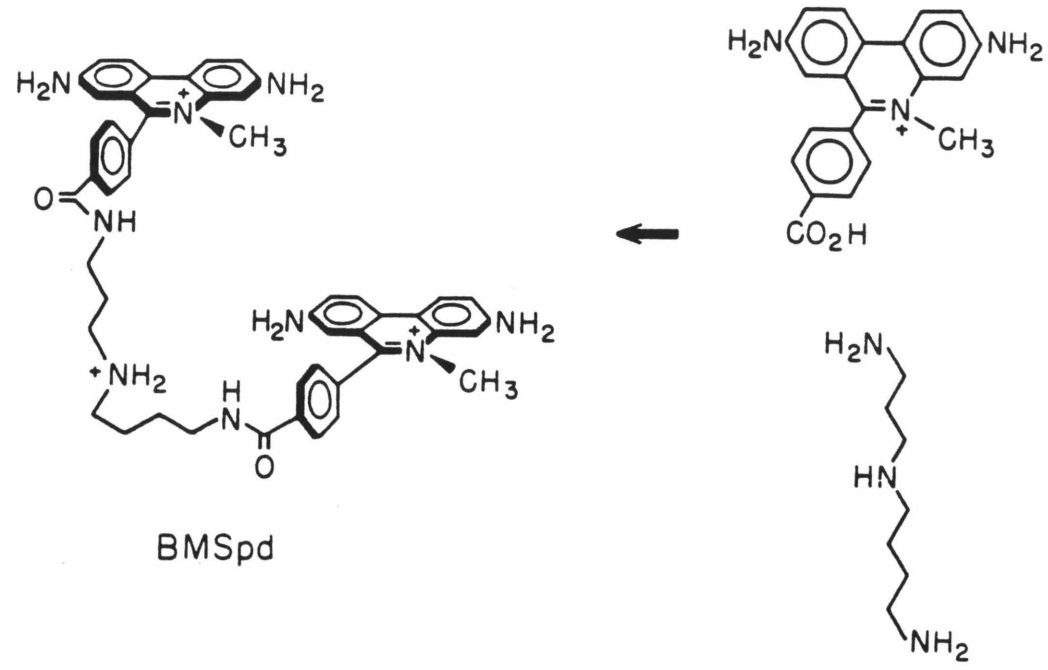
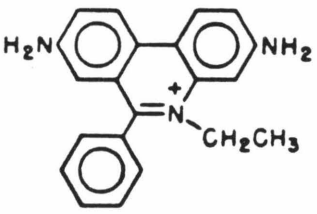


Figure 3





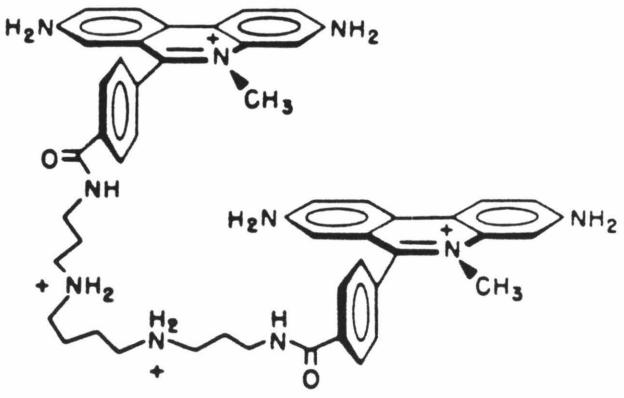


Figure 4

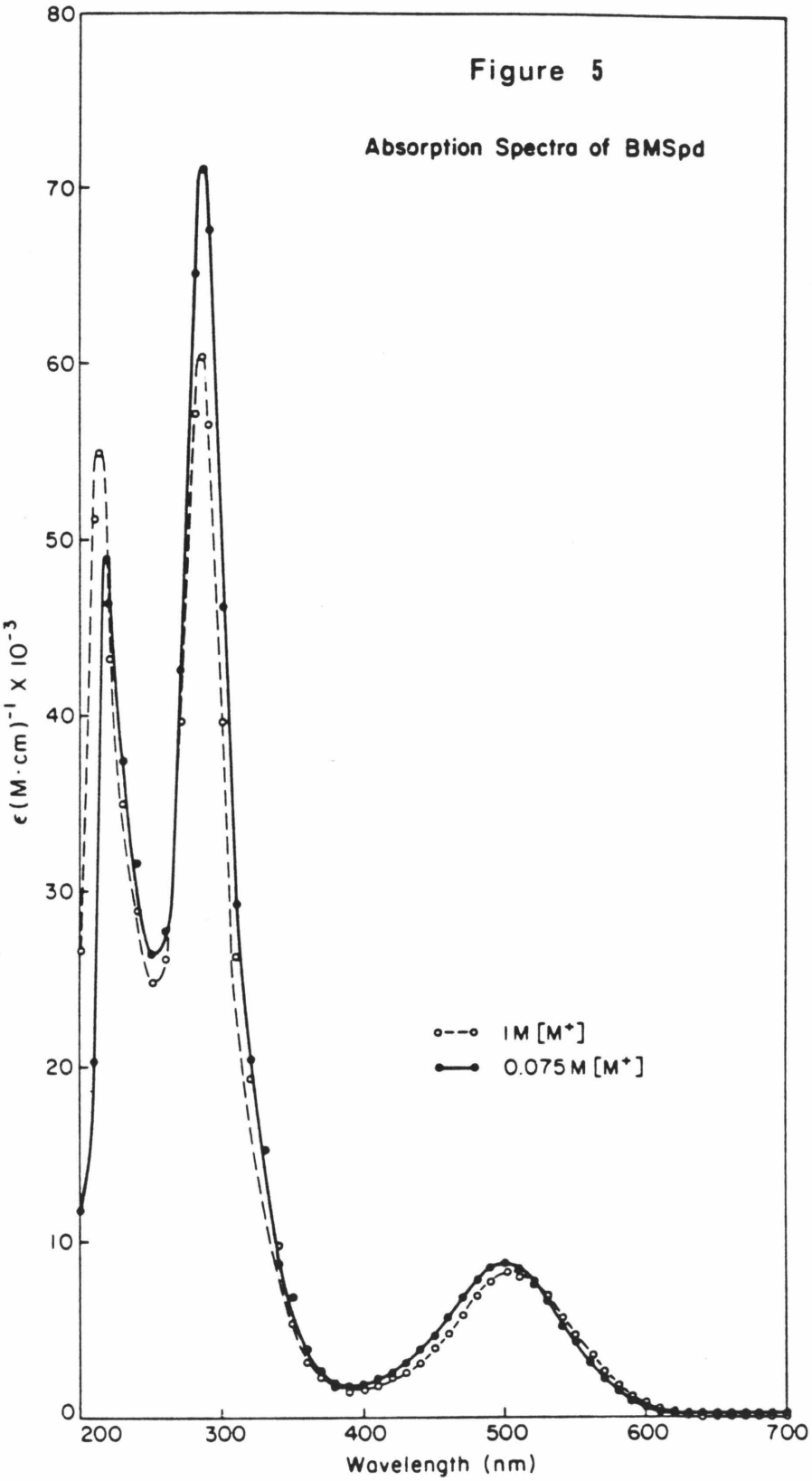


Figure 5
Absorption Spectra of BMSpd
80
70
60
50
40
30
20
10
0
200
300
400
500
600
700
Wavelength (nm)
$\epsilon (M \cdot cm)^{-1} \times 10^{-3}$
1 M [M+]
0.075 M [M+]

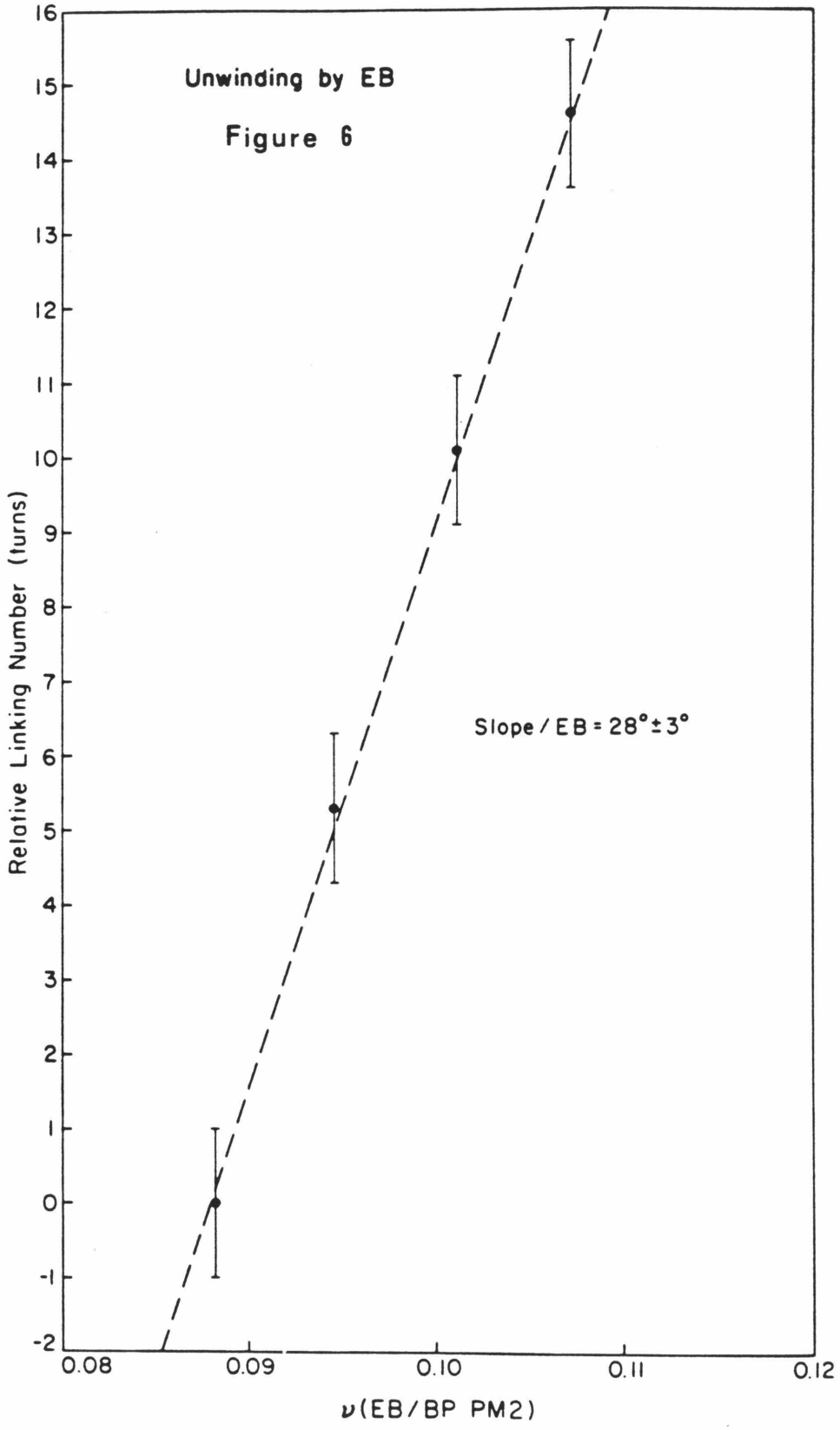
Since the time that the BMSp measurements were made, viscometry has been superseded by electrophoretic band counting.[31,32] One can deduce the unwinding angle of an intercalator from the number of supercoils induced in a closed circular plasmid.

$$\text{Unwinding Angle} = \frac{\text{Supercoils} \times 360}{\text{number of bound intercalators}}$$

Although viscometry is inherently more accurate, band counting is quicker and more versatile. Unwinding angles may be determined as a function of saturation with band counting, whereas, viscometry returns an average unwinding angle at a specific equivalence point.

Band counting gives unwinding angles for EB and BMSp of $28^{\circ} \pm 3^{\circ}$ (Fig. 6) and $36^{\circ} \pm 3^{\circ}$ (Fig. 7), respectively; while, BMSpd exhibits an unwinding angle of $36.5^{\circ} \pm 3^{\circ}$ (Figs. 8 and 9). The similarity of unwinding angles for BMSp and BMSpd suggest a common intercalated species. In the case of BMSp this intercalated species has been shown to be a "bisintercalation"; correspondingly, BMSpd appears to be capable of bisintercalating heterogeneous DNA.

4. Binding of BMSpd to Double Helical Nucleic Acids. Binding affinities of intercalators may be determined by spectrophotometric methods when the bound and free forms of an intercalator exhibit different absorption or fluorescence spectra. In the case of phenanthridine analogs the visible absorbance of the intercalated species is generally red shifted 30 nm from the absorption spectrum of the unbound intercalator. Since the visible spectrum


Unwinding by EB
Figure 6
Slope / EB = 28° ± 3°
Relative Linking Number (turns)
ν(EB/BP PM2)
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
-2
0.08
0.09
0.10
0.11
0.12

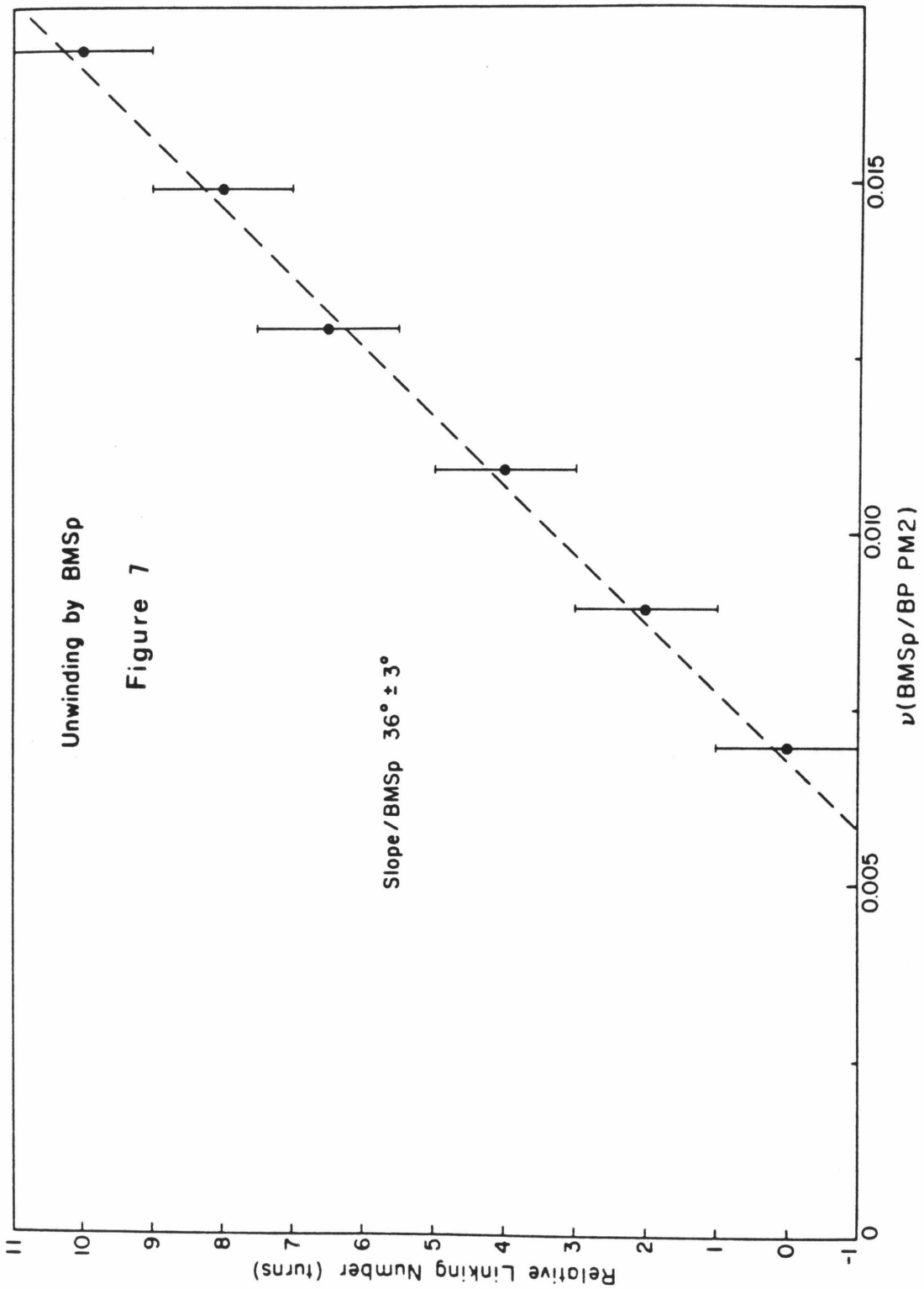
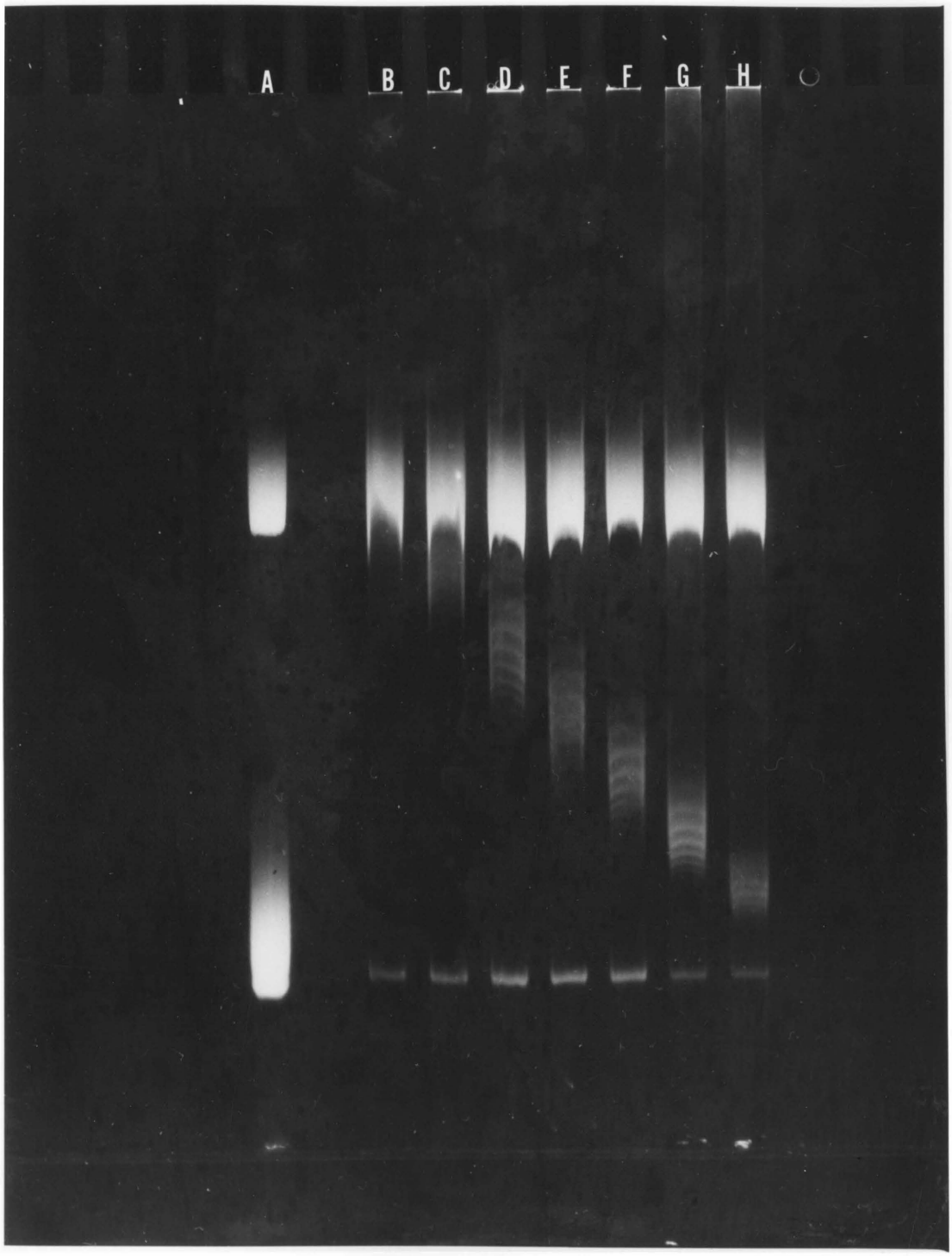
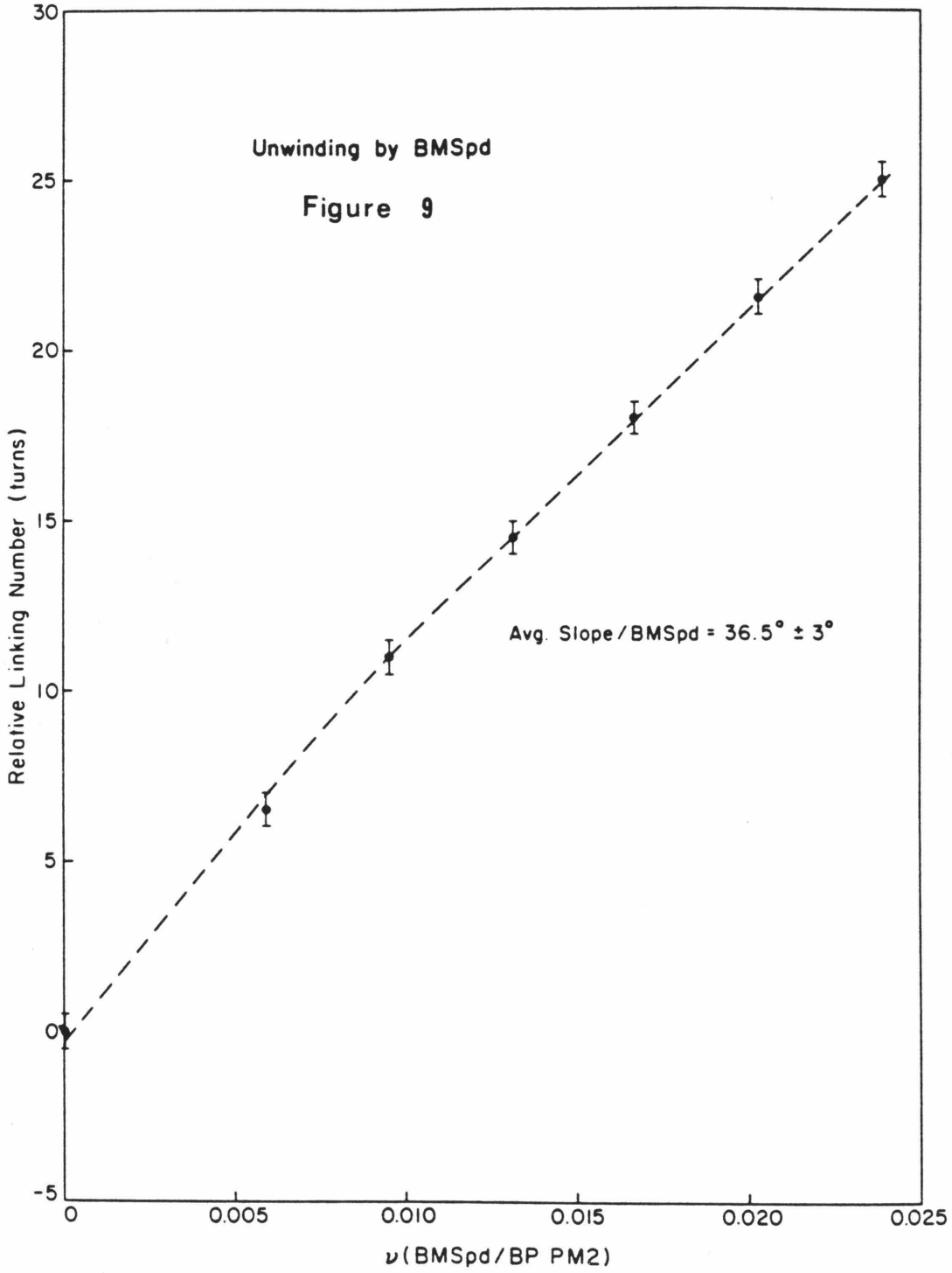

Unwinding by BMSp
Figure 7
Slope/BMSp 36° ± 3°
Relative Linking Number (turns)
ν(BMSp/BP PM2)
-1
0
1
2
3
4
5
6
7
8
9
10
11
0
0.005
0.010
0.015

Figure 8. BMSpd Unwinding.

Lane A: PM2, Lane B: BMSpd/PM2 (bp), $\nu = 0.0238$, Lane C: $\nu = 0.0202$, Lane D: $\nu = 0.0166$, Lane E: $\nu = 0.0131$, Lane F: $\nu = 0.0095$, Lane G: $\nu = 0.0059$, Lane H: $\nu = 0.0000$. Total DNA = 2 µg PM2, N/C enzyme = 9 units, incubation time: 24 h at 37°C. Electrophoresis: 18 h at 75 V/70 mA; the nicked/closed PM2 was run as positive supercoils in the presence of 5 µg/mL.


A
B
C
D
E
F
G
H


Unwinding by BMSpd
Figure 9
Avg. Slope / BMSpd = 36.5° ± 3°
Relative Linking Number (turns)
ν (BMSpd / BP PM2)
30
25
20
15
10
5
0
-5
0
0.005
0.010
0.015
0.020
0.025

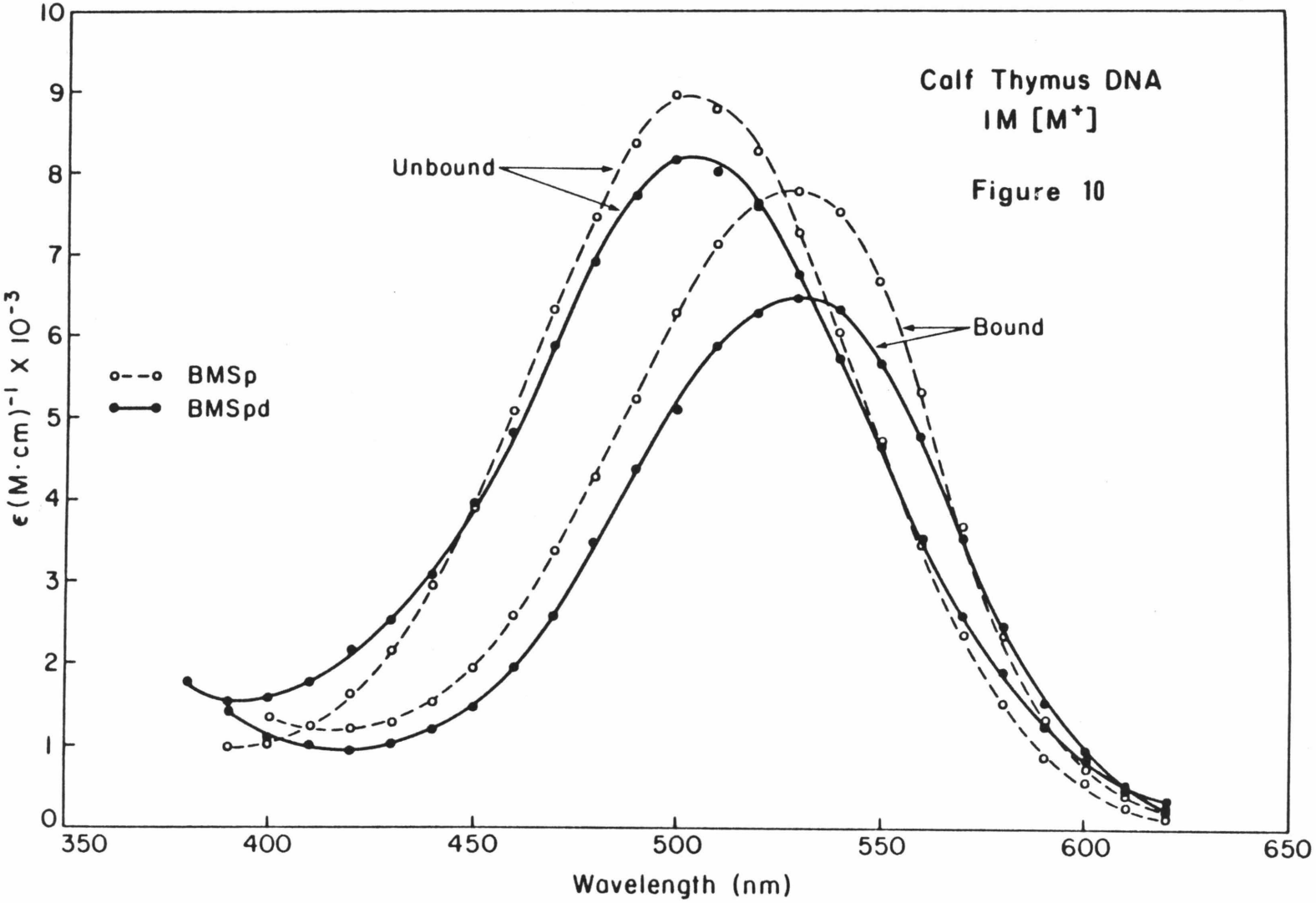
is also free of DNA absorptions, absorbance titrations are the preferred technique for the determination of phenanthridine binding constants. The titration data are most conveniently plotted in Scatchard form,[33] and the intrinsic DNA binding affinity of the intercalator is read off the experimental curve. Additional binding parameters may be extracted by comparing the data to the theoretical model proposed by von Hippel and McGhee,[34] where neighbor exclusion intercalation can be described by the equation

$$\frac{\nu}{L} = K(1-n\nu)\cdot\left[\frac{1-n\nu}{1-(n-1)\nu}\right]^{n-1}.$$

In this case ν is the ratio of bound intercalator to DNA concentration in base pairs, L is the concentration of free intercalator, K is the intrinsic binding constant of the intercalator, and n is the binding site size.

a. Binding of BMSpd to Sonicated Calf Thymus DNA at 1 M (M^+). The intercalation of an ethidium phenanthridine involves both electrostatic and non-electrostatic interactions. To minimize electrostatic contributions previous studies of BMSp and EB were conducted in 1 M (M^+) solutions. Thus, the binding of BMSpd to sonicated calf thymus DNA was first examined at an ionic strength of 1 M (M^+) to allow direct comparison to the calf thymus binding constants of EB and BMSp.

The visible absorbance spectra of BMSpd bound to calf thymus DNA in 1 M (M^+), pH 7, shows the expected metachromic shift of the absorption maximum, but the full width of the absorption band at half height is unusually wide for a bisintercalated species (Fig. 10).[28] The difficulty encountered in


Calf Thymus DNA
IM [M+]
Figure 10
Unbound
Bound
BMSp
BMSpd
ε (M·cm)⁻¹ X 10⁻³
Wavelength (nm)
0
1
2
3
4
5
6
7
8
9
10
350
400
450
500
550
600
650

determining this bound spectra also suggests that the binding affinity of BMSpd to calf thymus might be less than 10^6 M^{-1}.

The actual titrations confirmed a low binding constant. The data yielded Von Hipple/McGhee estimates of the binding affinity and binding site size of 3.5×10^5 M^{-1} and 2.5 base pairs (Fig. 11), respectively. The binding constant is 10 times higher than EB, but 50 times lower than BMSp; furthermore, the small, nonintegral binding site size suggests that mono-intercalation is competing with the bisintercalated forms. It may be possible that only a small number of sites are available to a short bisintercalator and monointercalation must occur along the remainder of the DNA helix.

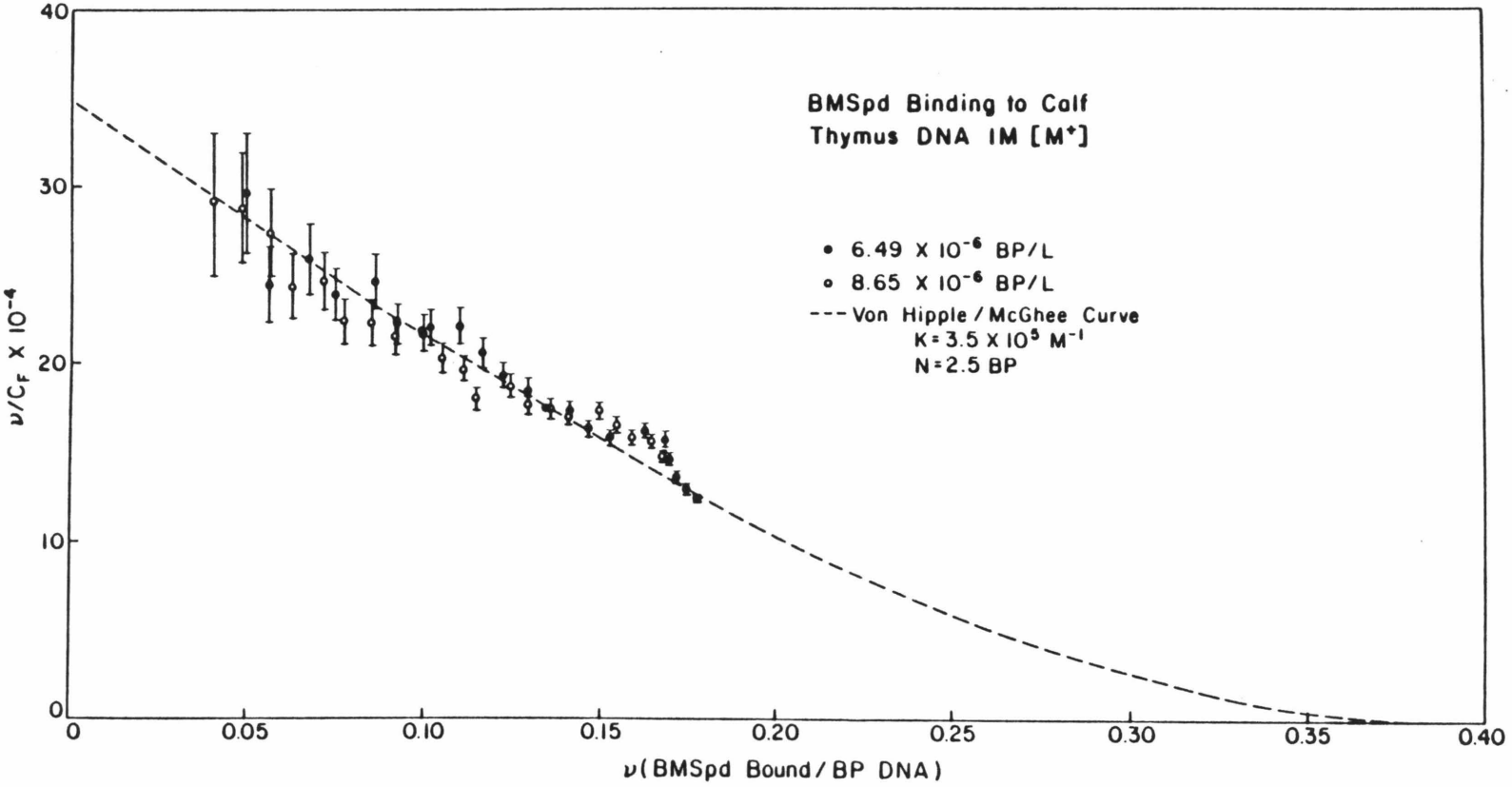
b. Determination of Binding Affinities at 0.4 M (M^+). The difficulty encountered in determining the spectrum of BMSpd bound to calf thymus at 1.0 M (M^+) lead to a reduction in the standard ionic strength. BMSpd binding constants for a variety of double helical nucleic acids were determined in 0.4 M (M^+) solutions in order to avoid the experimental problems associated with "low" binding affinities.

To compare binding constants at 0.4 M (M^+) and 1.0 M (M^+) the data may be extrapolated to one salt concentration using the equations of Manning[29] and Record.[30]

$$\frac{-\partial \log K_{obs}}{\partial \log (M^+)} = m \Psi,$$

where K is the binding constant, (M^+) is the salt concentration, m is the number of charges per binding molecule and Ψ is a shielding factor

Figure 11


BMSpd Binding to Calf
Thymus DNA IM [M+]
• 6.49 X 10-6 BP/L
○ 8.65 X 10-6 BP/L
--- Von Hipple / McGhee Curve
K = 3.5 X 10^5 M-1
N = 2.5 BP
ν/CF X 10-4
ν(BMSpd Bound / BP DNA)
0
10
20
30
40
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40

(0.88 for DNA). Assuming that the polyelectrolyte theory of Manning and Record applies to BMSpd as well as it has been shown to apply to EB, the BMSpd trication should bind DNA 10 to 15 times more tightly in 0.4 M (M^+) solutions than in 1.0 M (M^+) solutions.[7,25]

The spectra of BMSpd bound to sonicated calf thymus DNA, poly dG·poly dC, poly dA·poly dT, and poly rA·poly dT are generally similar and are characteristic of an intercalated phenanthridine (Fig. 12). The absorbance maxima are red shifted a minimum of 29 nm from the absorbance of BMSpd, and the intensity of the absorbance is slightly reduced. The widths of the bands at half height tend to be 10 nm narrower for the intercalated species than for the free dimer. An exception is poly dA·poly dT. The width of BMSpd bound to poly dA·poly dT is equal to the width of the free molecule; however, this was not unexpected. The red shifts, extinction coefficients and peak widths of BMSpd are comparable to BMSp (Table 3).

Scatchard analysis of the 0.4 M (M^+) titration data reveal BMSpd binding affinities and binding site sizes to calf thymus DNA, poly dG·poly dC and poly rA·poly dT of 5×10^6 M^{-1} and 2.75 base pairs, 5×10^6 M^{-1} and 4.0 base pairs, and 6×10^6 M^{-1} and 4.0 base pairs, respectively (Table 4, Figs. 13-16). These affinities are estimated to be 3 to 10 times greater than EB but 50-500 times less than BMSp, and although BMSpd excludes a larger site than EB the binding affinities are indicative of either a monointercalation or an unfavorable bisintercalation. Even the 4.0 base pair binding site sizes for poly dG·poly dC and poly rA·poly dT do not exclude the possibility of a monointercalated species. A monointercalated form could block a 4 base pair site by stacking the nonintercalated chromophore on the

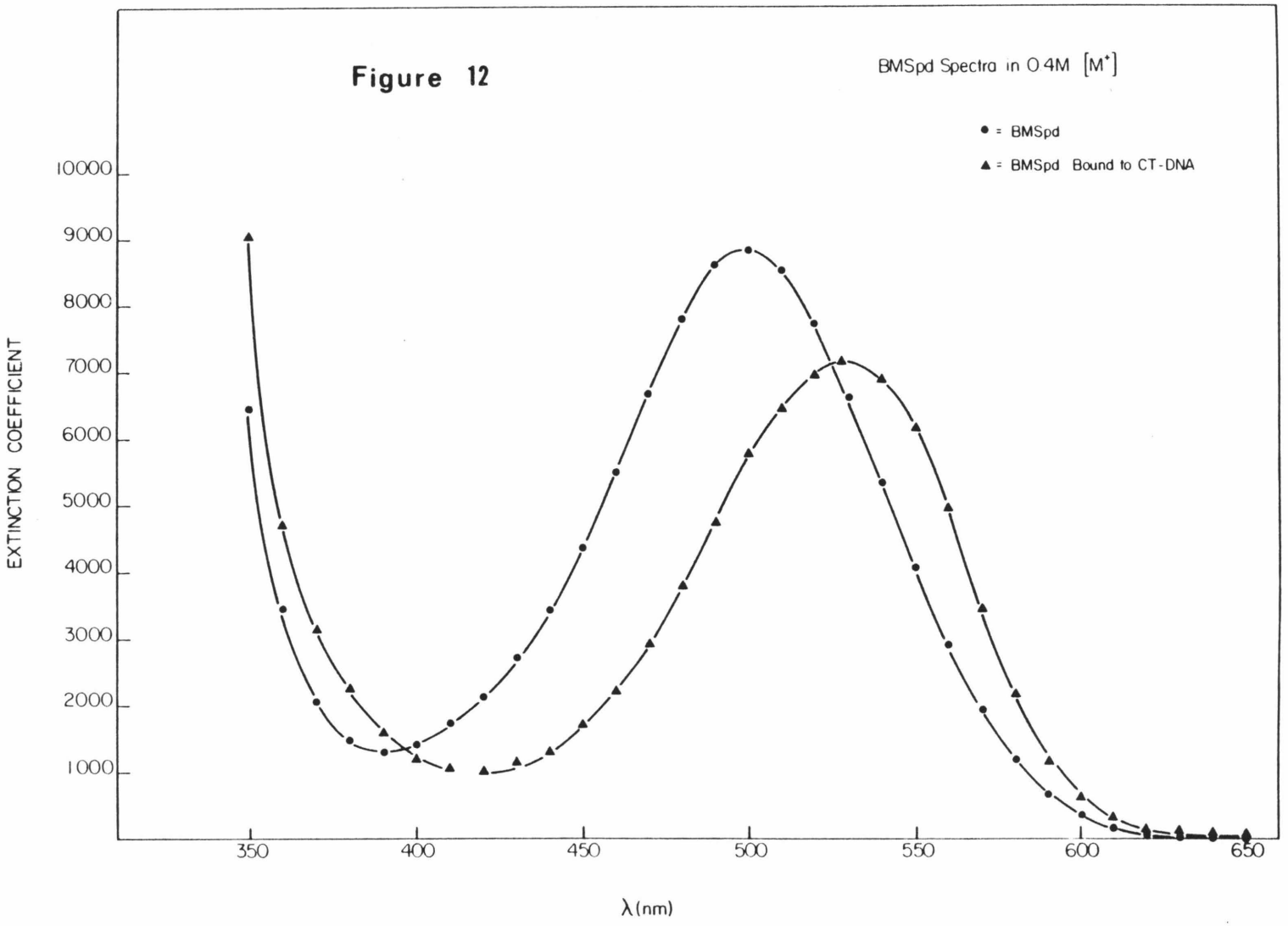


Figure 12
BMSpd Spectra in 0.4M [M+]
● = BMSpd
▲ = BMSpd Bound to CT-DNA
EXTINCTION COEFFICIENT
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
350
400
450
500
550
600
650
λ(nm)

Table 3

BMSpd	E(M^{-1} cm^{-1})	λ_{max}(nm)	1/2W (nm)
H_2O	9600	501	-
0.075 M^+	8835	501	101
0.40 M^+	8820	501	100
1.0 M^+	8520	501	101
C.T. 1.0 M^+	6450	532	95
C.T. 0.4 M^+	7140	530	90
dG·dC 0.4 M^+	7730	530	90
rA·dT 0.4 M^+	8160	531	89
dA·dT 0.4 M^+	8680	533	101
BMSp			
H_2O	10160	502	99
0.075 M^+	9850	502	98
1.0 M^+	8900	502	97
4.4 M^+	8400	502	98
C.T. 1.0 M^+	7800	530	92
dG·dC 1.0 M^+	7880	530	92
rA dT 1.0 M^+	8600	530	89
dA dT 1.0 M^+	7880	526	98
EB			
1.0 M^+	6060	480	87
C.T. 1.0 M^+	4000	520	85

		BMSpd	
Nucleic Acid	$[M^+]$	K	Site Size
Sonicated Calf Thymus	1.0	3.5×10^5	2.5
	0.4	5×10^6	
Poly dG·dC	0.4	5×10^6	4
Poly rA·dT	0.4	6×10^6	4
Poly dA·dT	0.4	5×10^5	—

Table 4

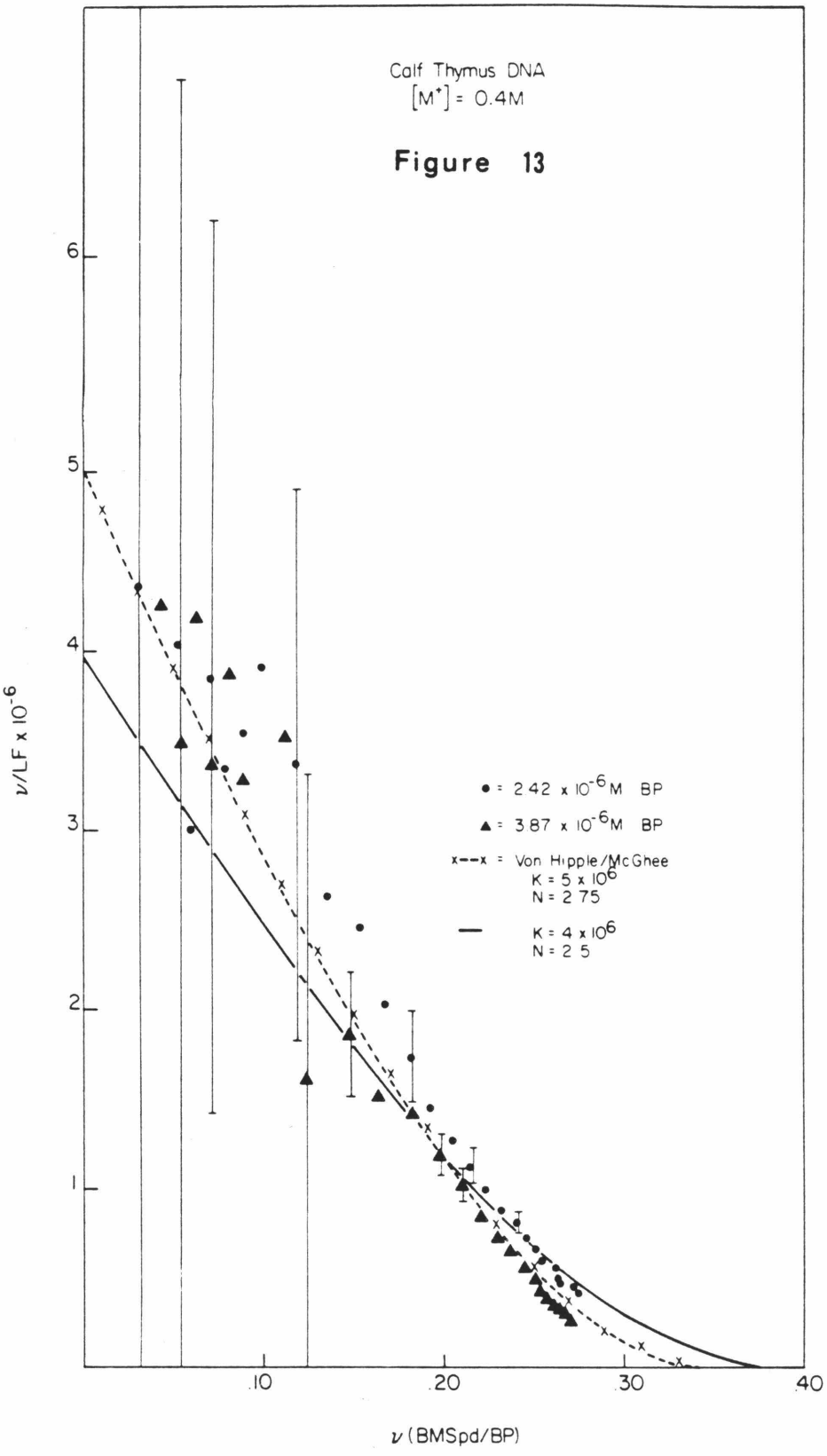

Calf Thymus DNA
[M+] = 0.4M
Figure 13
ν/LF x 10-6
ν (BMSpd/BP)
● = 2.42 x 10-6M BP
▲ = 3.87 x 10-6M BP
x--x = Von Hippel/McGhee
K = 5 x 10^6
N = 2.75
K = 4 x 10^6
N = 2.5
1
2
3
4
5
6
.10
.20
.30
.40

Figure 14

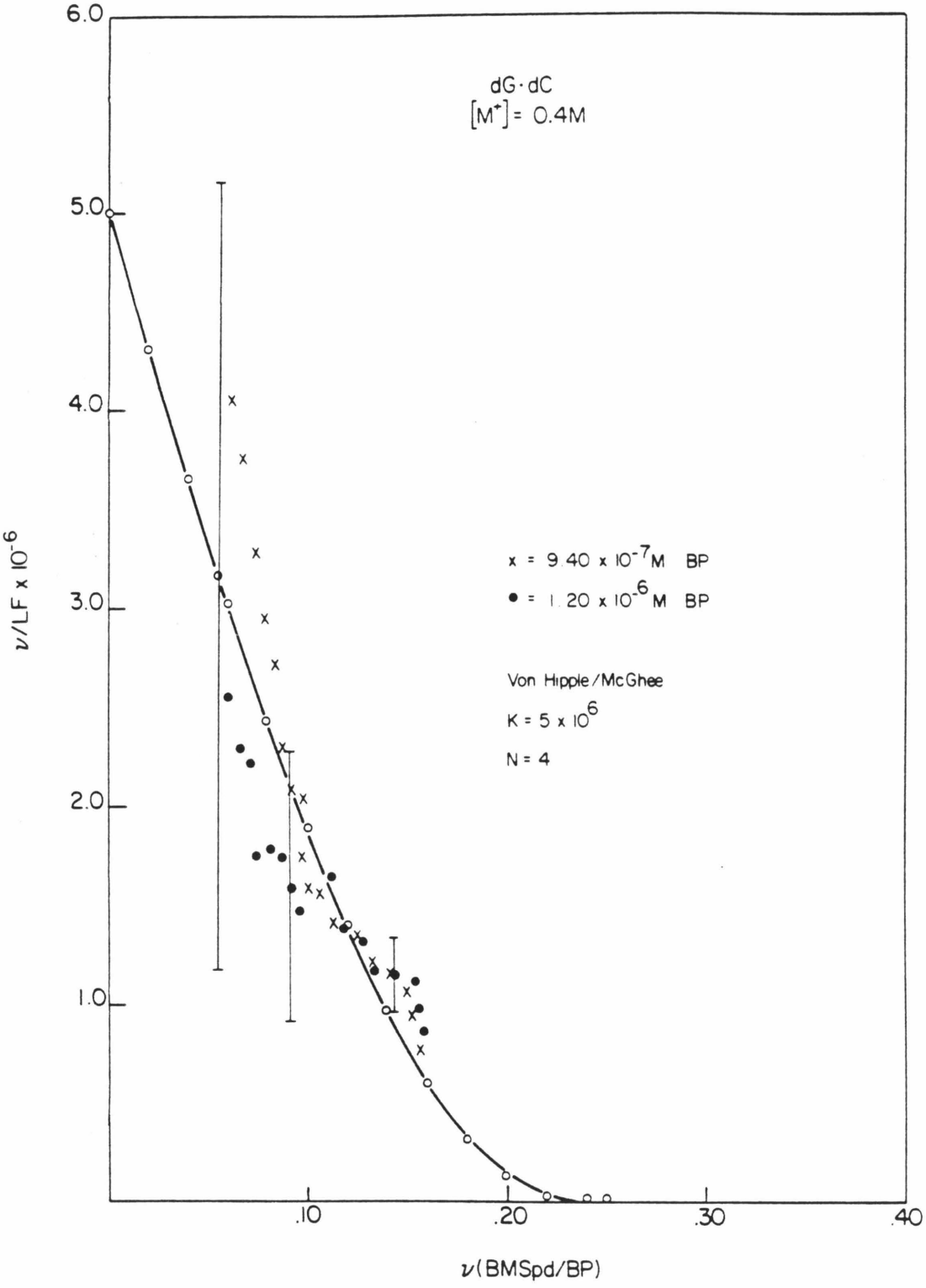

dG·dC
[M+] = 0.4M
x = 9.40 x 10-7M BP
● = 1.20 x 10-6M BP
Von Hipple/McGhee
K = 5 x 10^6
N = 4
6.0
5.0
4.0
3.0
2.0
1.0
.10
.20
.30
.40
ν/LF x 10-6
ν(BMSpd/BP)

Figure 15

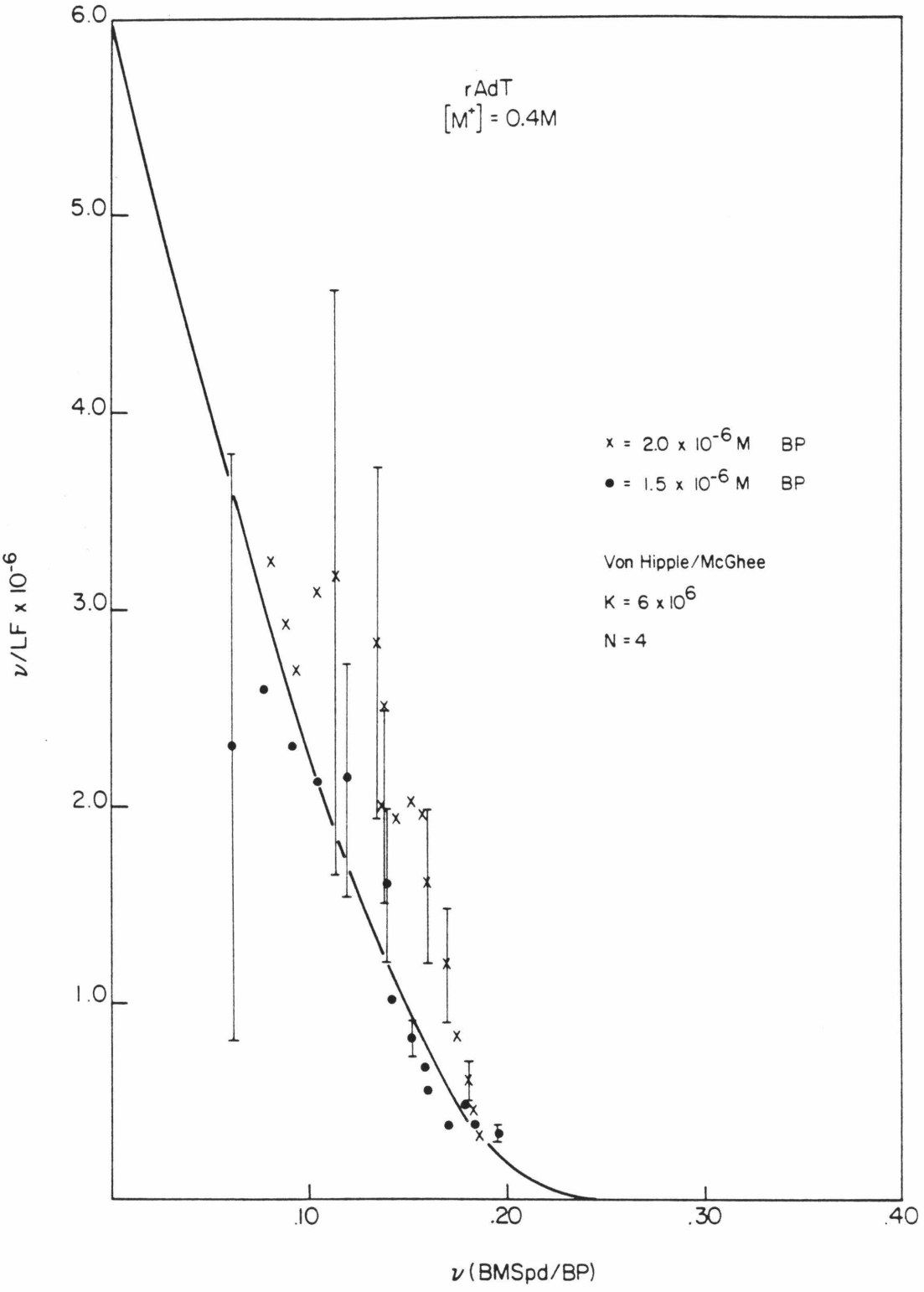
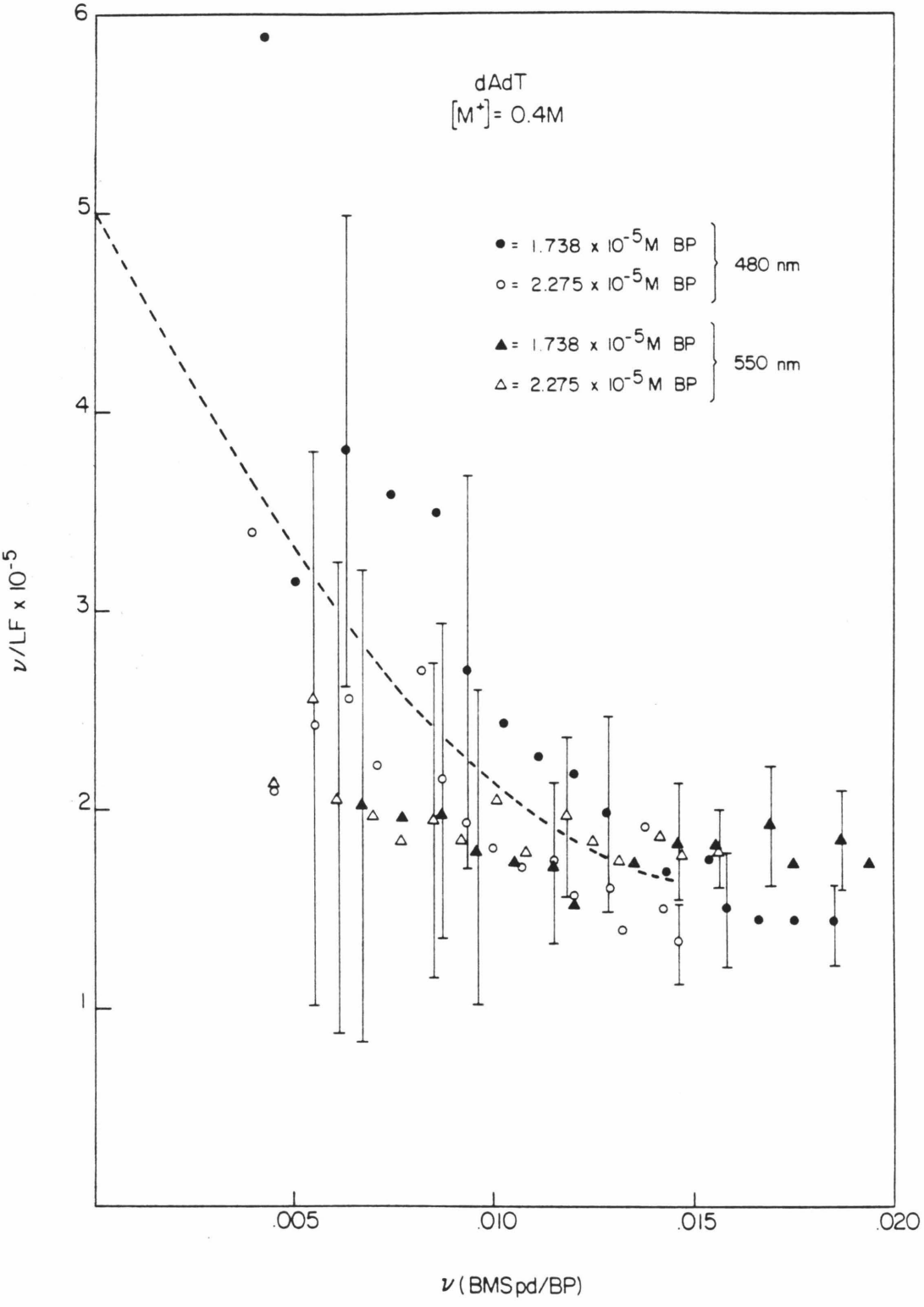

rAdT
[M+] = 0.4M
x = 2.0 x 10-6 M BP
● = 1.5 x 10-6 M BP
Von Hipple/McGhee
K = 6 x 10^6
N = 4
ν/LF x 10-6
6.0
5.0
4.0
3.0
2.0
1.0
.10
.20
.30
.40
ν (BMSpd/BP)

Figure 16


dAdT
[M+]= 0.4M
● = 1.738 x 10-5M BP
○ = 2.275 x 10-5M BP
480 nm
▲ = 1.738 x 10-5M BP
△ = 2.275 x 10-5M BP
550 nm
ν/LF x 10-5
ν (BMSpd/BP)
6
5
4
3
2
1
.005
.010
.015
.020

outside of the nucleic acid helix.[8-10]

In contrast to the previous nucleic acids, poly dA·poly dT binds BMSpd only 3 times less tightly than BMSp and 25 times more tightly than EB. The short dimer, BMSpd, mimics the documented bisintercalator, BMSp, on poly dA·poly dT.

Becker and Dervan note that poly dA·poly dT is unique in its binding of intercalators. The intercalation constants are anomalously low, and the dimer, BMSp, exhibits an exceptionally wide bound spectra. This spectral deviation is proposed to reflect nearest neighbor bisintercalation since picosecond fluorescence decay shows that both chromophores are intercalated.[28] If this is the case, BMSpd probably bisintercalates poly dA·poly dT just as BMSp binds the homopolymer.

Whatever, the molecular phenomenon, however, the specificity of BMSpd is different from the specificity of BMSp. The alteration of specificity as a function of linker length is illustrated by the ratios of binding constants for the various nucleic acids and intercalators. Whereas, EB and BMSp prefer the rA·dT helix over the dA·dT helix by factors of 100 and 1440, respectively. BMSpd binds rA·dT only 10 times more tightly than dA·dT (Tables 5 and 6). BMSpd is characterized by a relatively reduced affinity for nucleic acids that are bound with high affinity by EB and BMSp and a relatively enhanced affinity for dA·dT. The shorter linker appears to have severely limited the sequences that are available for bisintercalation by BMSpd, but the unwinding angle of BMSpd proves that there are at least a small number of sites available for bisintercalation on a heterogeneous sequence. The low binding constants may be a consequence of a low affinity bisintercalation, a

Table 5

Nucleic Acid	$[M^+]$	EB		BMSp		BMSpd	
		K	Site Size	K	Site Size	K	Site Size
Sonicated Calf Thymus	1.0	4.5×10^4	2	1.5×10^7	4	3.5×10^5 5.0×10^5 *	2.5
Poly dG·dC	1.0	1×10^5	2	2.6×10^8	4	5×10^5 *	4
Poly rA·dT	1.0	2×10^5	3	2.3×10^8	6	6×10^5 *	4
Poly dA·dT	1.0	2×10^3	-	(1.6×10^5) est	2	5×10^4 *	(2)

* Extrapolated to 1 M $[M^+]$

Table 6

Nucleic Acids	EB (K_1/K_2)	BMSp (K_1/K_2)	BMSpd (K_1/K_2)
$\frac{C.T.}{rAdT}$	0.23	0.065	0.83
$\frac{dGdC}{rAdT}$	0.50	1.13	0.83
$\frac{rAdT}{dAdT}$	100	1440	12
$\frac{C.T.}{dAdT}$	22.5	94	10
$\frac{dGdC}{dAdT}$	50	1630	10

"hanging" monointercalation, or the over saturation of a small number of high affinity bisintercalation sites, and the predominence of a "hanging" monointercalator at the concentrations of BMSpd necessary for accurate optical measurements.

What possibly remains is the synthesis of a dimer tethered to select a 4.0 base pair bisintercalation but unable to reach a 6.0 base pair bisintercalation. Although such a dimer is very attractive, predictions based on the BMSp and BMSpd binding data suggest that such a molecule would only exhibit moderate selectivity. This compound would be expected to bind 4.0 base pair bisintercalation sites as well as BMSp and 6.0 base pair sites with the same affinity as BMSpd. In the best case one would predict poly dG·poly dC would be bound 400 times more favorably than poly rA·poly dT, and in the worst case calf thymus DNA would be preferred to rA·dT by only a factor of 20.

Although this moderate selectivity may be acceptable, problems would arise in the choice of a linker. X-ray crystallographic data[35,36] and molecular models suggest that the difference of linker length necessary to span a 4.0 base pair bisintercalation and a 6.0 base pair site is only 1.6 to 2.4 Å; consequently, there is little margin for error. A linker that would discriminate a 4.0 base pair site from a 6.0 base pair site must be chosen within a 1 Å window.

D. Summary

The spermidine linked phenanthridine dimer, BMSpd -- a shortened tether (10 Å <u>vs</u> 15 Å) analog of BMSp -- was designed to test the relationship

of linker length and binding specificity. Like BMSp, BMSpd unwinds DNA 36° per bisintercalator suggesting simultaneous intercalation of the two phenanthridine chromophores; however, in contrast to the unwinding angle determination, the optical titrations reveal binding affinities and binding site sizes characteristic of monointercalation. BMSpd binding constants and binding site sizes on sonicated calf thymus DNA in 1 M (M^+) solutions and sonicated calf thymus DNA, poly dG·poly dC, poly dA·poly dT, and poly rA·poly dT in 0.4 M (M^+) solution range from 3.5×10^5 M^{-1} and 2.5 base pairs to 6×10^6 M^{-1} and 4.0 base pairs. It is possible that the homopolymer sequences examined are incapable of accommodating a 10 Å bisintercalation, while BMSpd bisintercalates a small number of as yet undefined sites within a heterogeneous sequence and saturates the remainder of the "random" sequence with a hanging monointercalator. If this is true, BMSpd might be extremely selective in a small number of unpredictable cases.

With regard to the measured binding specificity, however, BMSpd is dramatically different from both EB and BMSp. Whereas, EB and BMSp prefer rA·dT to dA·dT, BMSpd is ambivalent; rA·dT is bound only 10 times more tightly than dA·dT. The nucleic acids that are bound with high affinity by EB and BMSp are bound by BMSpd with relatively reduced affinity, and the poorly bound homopolymer dA·dT is bound by BMSpd with a relatively enhanced affinity.

What is demonstrated is that linkers cannot only be used to create high affinity polyintercalators from monomers building blocks, but that linkers can be used to change the specificity of a particular intercalator.

Part II

Site Selective Inhibition of Restriction Enzymes

A. Introduction

Simple intercalators efficiently recognize and bind double helical nucleic acids. Acridines and phenanthridines exhibit binding constants of 10^4 M^{-1} to 10^6 M^{-1},[16,39,40] and in some cases these compounds are extremely biologically active; however, sequence specific DNA binding proteins binds DNA 10^5 to 10^7 times more effectively than a simple intercalator.[6,42] This led researchers to seek intercalators with higher binding affinities. The answer was to link two monointercalators with a molecular chain to produce a polyintercalator.[25-27,42] By choosing a sufficiently long linker, the affinity of the bisintercalator was buoyed to the affinity of the monomer raised to the power of 1.6.[7]

Unexpectedly, the early studies revealed that many of the small intercalators bind selectively. A number of the acridine derivatives are base specific,[39,40,43] while a phenanthridine, ethidium bromide, binds rA·dT 100 times more tightly than dA·dT.[16] Furthermore, the bisintercalators can be even more selective than their constituent monomers. The ethidium dimer, bis(methidium)spermine (BMSp), is 14 times more selective than EB itself.[7]

The base specificity of a few DNA groove binding molecules and the intercalator, actinomycin, has been probed with restriction enzymes.[44-47] The experiments, in which general inhibition can be distinguished from site specific inhibition, showed that different restriction sites are selectively inhibited by the base specific intercalator and the base specific groove binders. The selectivity can then be related back to the base specificity of the affector and the base sequence surrounding the restriction sites.

Interestingly, restriction enzymes appear to probe more than just

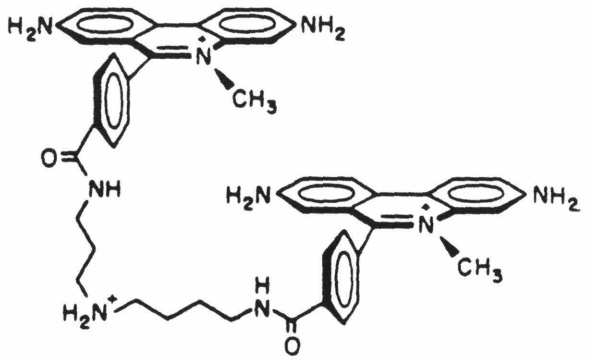
the sequence of the restriction site. Although the recognition sites of a given enzyme are identical, the rates of cleavage at each site are not equal,[48] because the recognition sites are uniquely located with the DNA sequence. By monitoring these differential restriction rates can a polyintercalator that has been shown to be structure specific with synthetic nucleic acid homopolymers and copolymers selectively bind heterogeneous sequences and report its locations by inhibiting DNA dependent enzymes directly or indirectly?

B. Results and Discussion

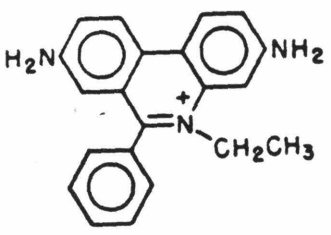
Complete cleavage of a covalently closed circular DNA by a specific endonuclease produces a unique set of DNA fragments that can be characterized by electrophoresis. Although the restriction sites of a given enzyme are identical, each site is uniquely located within the heterogeneous sequence of the DNA; consequently, the rates of cleavage at each site are not equal. The order of cleavage can be determined by observing the appearance of partial and final digestion fragments. If a DNA binding molecule can site selectively inhibit a restriction enzyme the partial digestion patterns may change; conversely, nonselective inhibition of a restriction enzyme will only affect the rate of digestion and will not change the order of cleavage. Using this logic, the binding of ethidium bromide (EB), bis(methidium)spermine (BMSp) and bis(methidium)spermidine (BMSpd), to a heterogeneous DNA sequence was probed with a variety of restriction enzymes (Fig. 17).

Initial experiments with EB and BMSp employed predominantly superhelical pBR322. Since such a circular substrate gives rise to a

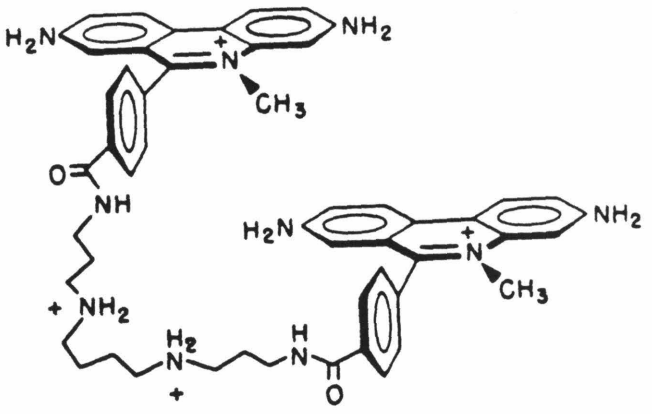
Figure 17



BMSpd



EB



BMSp

geometrically increasing progression of partial fragments with increasing numbers of restriction sites, the restriction enzymes -- Rsa I, Bgl I, and Mst I -- were chosen for their limited number of cleavage sites on pBR322. The simplicity of the restriction patterns then allows a direct interpretation of the changes in relative restriction rates.

Rsa I, Bgl I and Mst I cut pBR322 at 3, 3, and 4 sites, respectively. $^{5'}$GTAC$^{3'}$, the recognition sequence of Rsa I, is found at positions 165, 2282, and 3847; $^{5'}$GCCNNNNNNGGC$^{3'}$, the recognition sequence of Bgl I, is located at 934, 1168, and 3487; Mst I's $^{5'}$TGCGCA$^{3'}$ is situated at positions 262, 1357, 1455, and 3589. Complete digestion of pBR322 by Rsa I leads to 3 final fragments, 2117 bp, 1565 bp, and 680 bp, while Bgl I gives final fragments of 234 bp, 2319 bp, and 1809 bp, and Mst I produces 4 restriction fragments, 98 bp, 1035 bp, 1095, bp, and 2134 bp.

At intermediate digestion times partially restricted plasmid fragments will appear. Rsa I can produce 3 circularly permuted full length (4362 bp) fragments and smaller partial fragments of 3682 bp, 2797 bp, and 2245 bp. Likewise, Bgl I can give 3 permuted 4362 bp fragments and partials of 4128 bp, 2553 bp, and 2043 bp. Finally, Mst I, the most complicated of the three enzymes, can create 4 circular permutations of the 4362 bp plasmid, and 8 partial fragments, 4264 bp, 3327 bp, 3267 bp, 3169 bp, 2232 bp, 2228 bp, 2130 bp, and 1193 bp. The unperturbed restriction of pBR322 with each of the enzymes showed that Rsa I and Bgl I gave all the possible partial and final fragments that could be resolved, while Mst I failed to produce at least one partial fragment.

The order of appearance of these partial and final restriction

fragments reflects the relative restriction rates at the recognition sites of each enzyme. The unperturbed restriction pattern of each enzyme is shown in Figure 18. Interpretation of these cleavage patterns of covalently closed circular DNA hinges on the fact that the first scission of pBR322 forms a 4362 bp linear fragment that determines the possible partial fragments that may appear. The cleavage sites of an enzyme can then be ranked in order of their relative cleavage rates by identifying the bands that appear first. The relative restriction rates for the intercalator free digestions are: (1) Rsa I = 3847 > 165 > 2282; (2) Bgl I = 934 > 1168/3487,* and (3) Mst I = 1357 > 1455 > 262 > 3589. A flow chart example of the logic involved is presented in Figure 19.

The addition of EB at 30% saturation ([EB]/[BP] = 0.16) does not change the restriction patterns of Rsa I or Bgl I. There is only a reduction in the rate of appearance of all the restriction fragments. Conversely, the addition of BMSp at 30% saturation ([BMSp]/[BP] = 0.08) changes the restriction patterns of Rsa I, Bgl I, and Mst I. Rsa I does not produce a 2245 bp fragment. Bgl I reverses the order of appearance of the 2553 bp and 2043 bp partials; the 2043 bp fragment appears before the 2553 bp fragment. Finally, Mst I exhibits a totally different restriction pattern. Partial fragments of 2232/2228 bp and 2129 bp now appear in preference to fragments of 4264 bp, 3267 bp, and 3169 bp (Figure 18).

By analyzing the restriction patterns in the manner previously described for the intercalator free digestions, the relative order of site

---

*A slash punctuating two restriction sites designates insufficient data to distinguish the two sites.

Figure 18. Restriction Site Inhibition.

A) Rsa I - Lanes 1-8: Reaction volume = 80 µℓ, Total DNA = 4 µg pBR322, enzyme added = 0.5 units, BMSp/DNA (bp) = 0.08, Times (min) = 1) 45, 2)80, 3) 95, 4) 110, 5) 125, 6) 210, 7) 310, 8) 410. Lanes 9-16: Intercalator/DNA = 0.00, Times (min) = 9) 140, 10) 100, 11) 75, 12) 60, 13) 45, 14) 30, 15) 15, 16) 10. Electrophoresis = 80 V for 22 h. Gel = 15 x 14.5 x 0.4 cm.

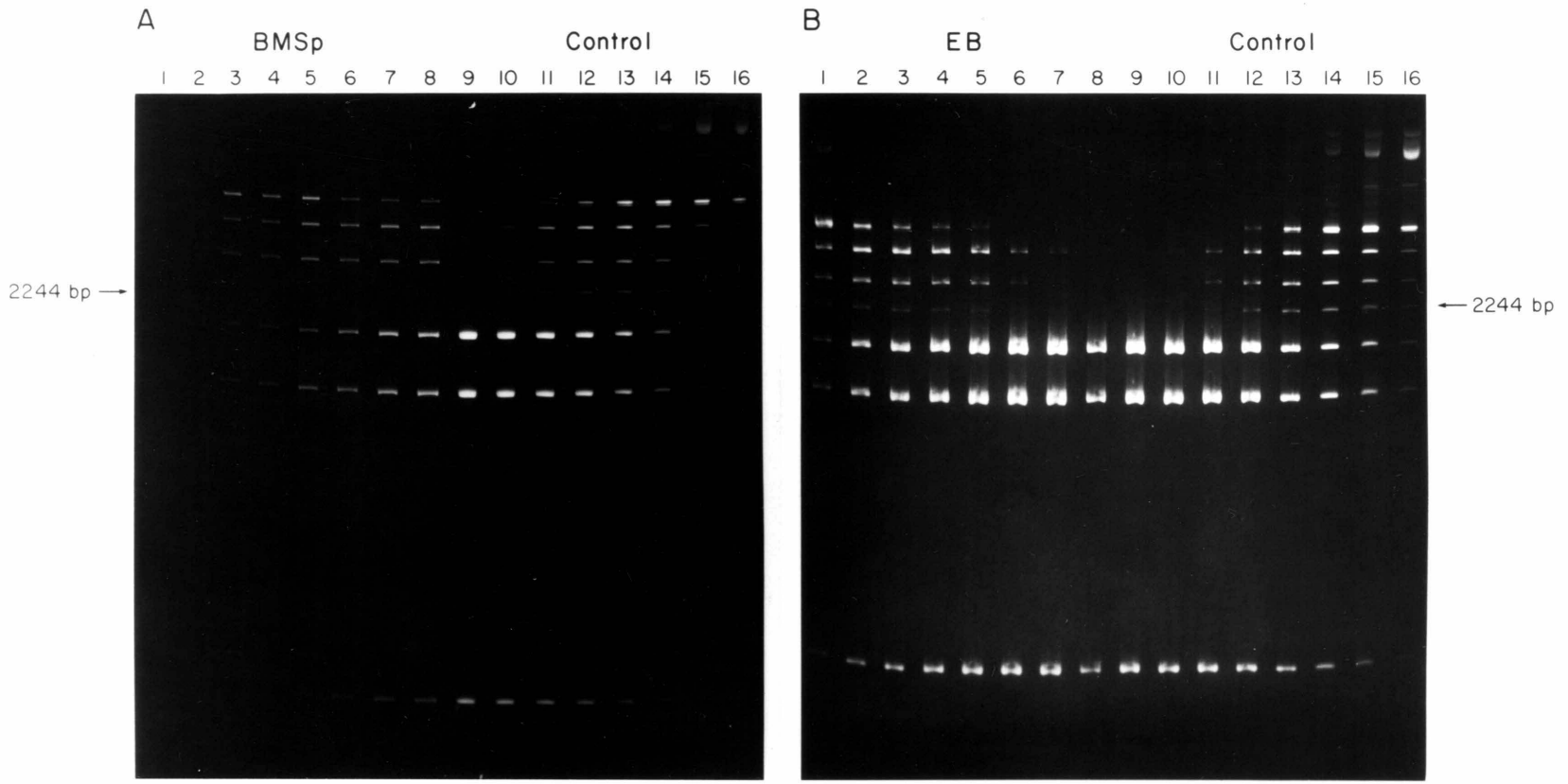
B) Rsa I - Lanes 1-8: Reaction volume = 80 µℓ, Total DNA = 3 µg pBR322, enzyme added = 0.375 units, EB/DNA (bp) = 0.16, Times (min) = 1) 30, 2) 60, 3) 75, 4) 90, 5) 105, 6) 150, 7) 190, 8) 270. Lanes 9-16: Intercalator/DNA = 0.00, Times (min) = 9) 160, 10) 130, 11) 100, 12) 75, 13) 55, 14) 40, 15) 25, 16) 10. Electrophoresis = 120 V for 12 h. Gel = 15 x 14.5 x 0.4 cm.

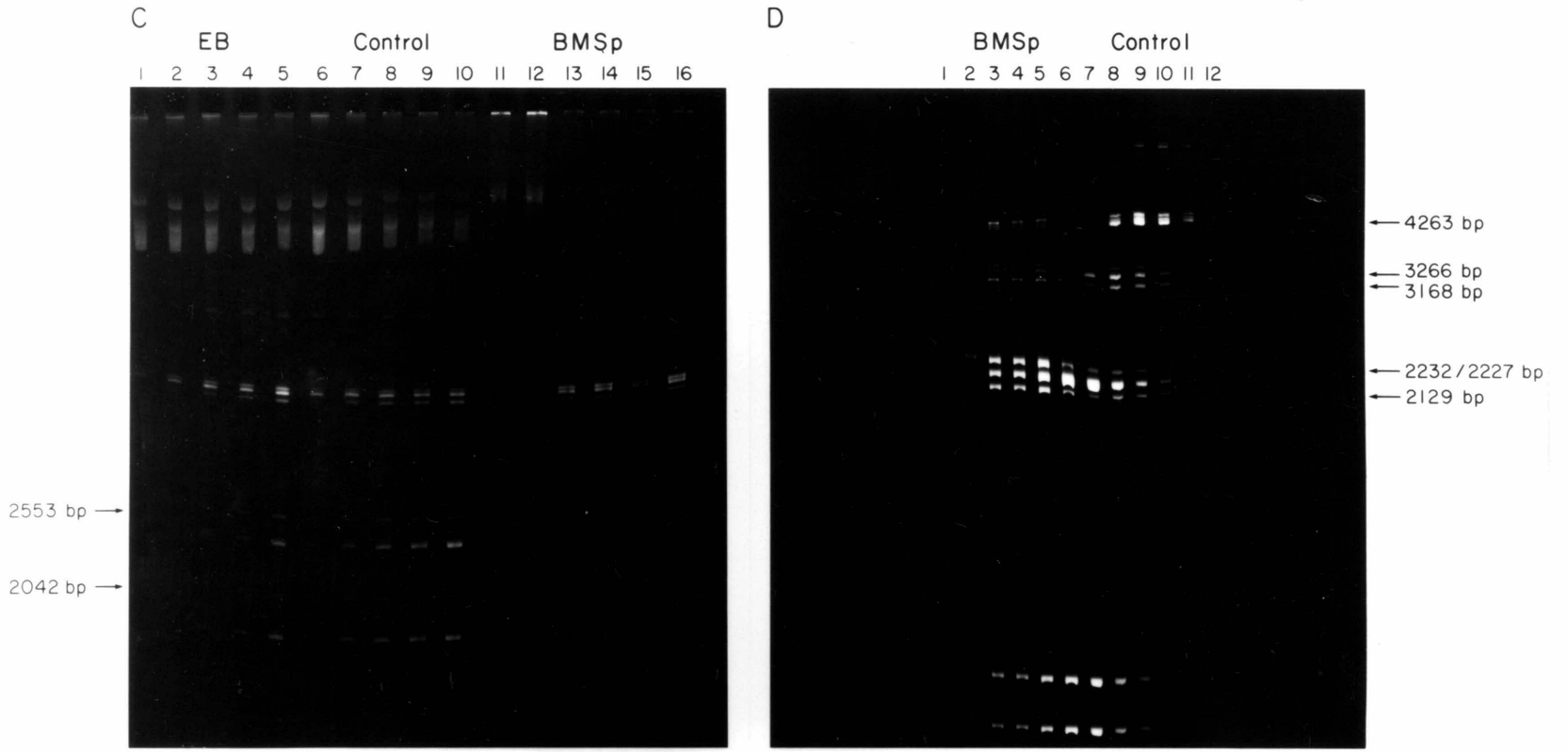
C) Bgl I - Lanes 1-10: Reaction volume = 50 µℓ, Total DNA = 2 µg pBR322, enzyme added = 2.8 units, Lanes 1-5 EB/DNA (bp) = 0.16, Times (min) = 1) 2, 2) 5, 3) 10, 4) 15, 5) 30. Lanes 6-10: Intercalator/DNA = 0.00, Times (min) = 6) 1, 7) 3, 8) 6, 9) 9, 10) 15. Lanes 11-16: Reaction volume = 50 µℓ, Total DNA = 3 µg pBR322, enzyme added = 4.2 units, BMSp/DNA (bp) = 0.08, Times (min) = 11) 85, 12) 90, 13) 98, 14) 106, 15) 115, 16) 150. Gel electrophoresis = 130 V for 19 h. Gel = 15 x 14.5 x 0.4 cm.

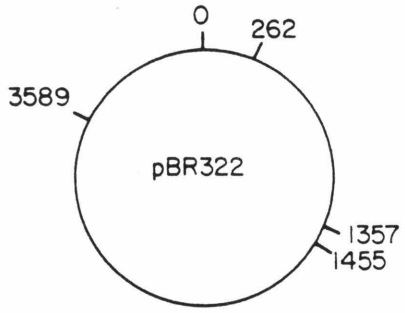
D) Mst I - Lanes 1-6: Reaction volume = 60 µℓ, Total DNA = 3.0 µg pBR322, enzyme added = 1.11 units, BMSp/DNA (bp) = 0.08, Times (min) = 1) 2, 2) 10, 3) 40, 4) 80, 5) 150, 6) 240. Lanes 7-12: Reaction volume = 50 µℓ, Total DNA = 2.4 µg pBR322, enzyme added = 0.89 units,

Figure 18. Continued

Intercalator/DNA = 0.00 Times (min) = 7) 540, 8) 300, 9) 270, 10) 240, 11) 210, 12) 180. Gel electrophoresis = 160 V for 41 h. Gel = 30 x 14.5 x 0.4 cm.


A
BMSp
Control
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
2244 bp
B
EB
Control
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
2244 bp


C
EB
Control
BMSp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
2553 bp
2042 bp
D
BMSp
Control
1 2 3 4 5 6 7 8 9 10 11 12
4263 bp
3266 bp
3168 bp
2232 / 2227 bp
2129 bp



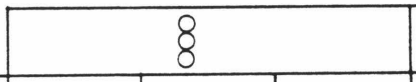
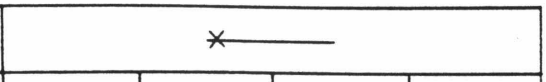
Possible Linear Permutations			
3589	1455	1357	262
Possible 3 Piece Fragments From 4 Piece Linears			
3327 bp 2228 bp	2228 bp 4264 bp	4264 bp 3267 bp	3267 bp 3327 bp
Possible 2 Piece Fragments From 4 Piece Linears			
2130 bp 2232 bp	3169 bp 2130 bp	2232 bp 2130 bp	1193 bp 3169 bp
1 Piece Fragments			
98 bp, 1035 bp, 1095 bp, 2134 bp			

Figure 19

cleavage may be determined for the intercalator doped digestions. Since EB does not change the restriction patterns of Rsa I and Bgl I, the analysis of these digestion patterns is identical to the intercalator free digestions. BMSp, however, alters the restriction patterns of Rsa I, Bgl I, and Mst I. The relative cleavage order in the presence of BMSp is now: (1) Rsa I = 3847 >> 165/2282; (2) Bgl I = 1168/3487 > 934, and (3) Mst I = 3589 > 1357/1455 > 262. The observed perturbations correspond to inhibition at 165 for Rsa I, 934 for Bgl I and 1357/1455 and 262 for Mst I (Table 7). Curiously, the span, 165 to 1455, is approximately the number of binding sites that BMSp would cover at 30% saturation (Fig. 20).

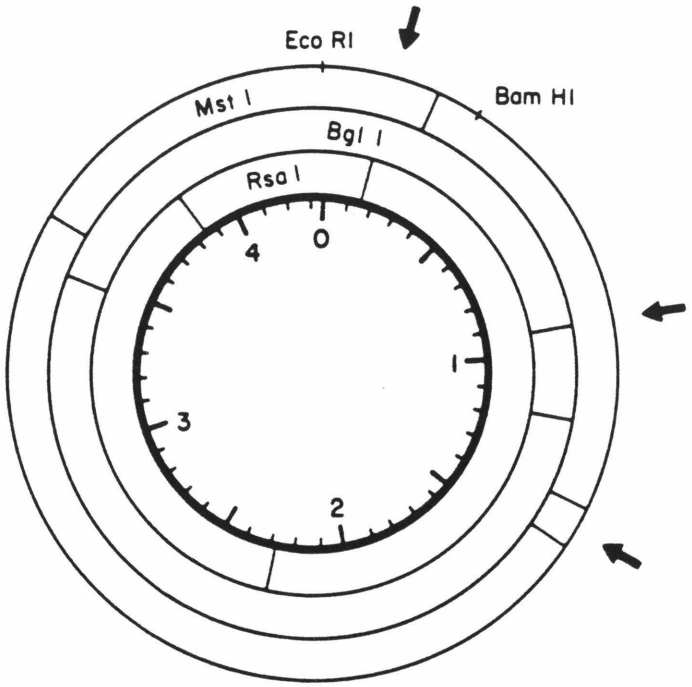
Unfortunately, the molecular interpretation of inhibition is obscured by the choice of substrate DNA. A topologically constrained molecule adds the factor of tertiary structure. Selective inhibition could be a result of (1) selective binding of BMSp and direct competition with the enzyme, (2) nonselective binding of BMSp and sequence selective alteration of the DNA helix to indirectly inhibit restriction activity or (3) alteration of the tertiary writhing of the plasmid. As a consequence, to address tertiary structure analogous restriction experiments were performed with <u>linear</u> pBR322 DNA that was end labeled at the Hind III site with α-^{32}P nucleotides. With this system Rsa I and Bgl I might produce four radioactive fragments: 4331 bp, 3818 bp, 2253 bp, 136 bp, and 4331 bp, 3458 bp, 1139 bp, 905 bp, respectively, while Mst I could show five fragments 4331 bp, 3560 bp, 1426 bp, 1328 bp, and 233 bp.

Interpretation of the linear restriction patterns is much simpler than the interpretation of the supercoiled restriction patterns. In the linear

							
Enzyme	Site Order	EB at 0.16	BMSp at 0.08	Site Order	EB at 0.16	BMSp at 0.08	BMSpd at 0.08
Rsa I	3847 > 165 > 2282	3847 > 165 > 2282	3847 ≫ 165 / 2282	3847 / 165 > 2282	3847 / 165 > 2282	3847 > 165 ~ 2282	3847 > 165 / 2282
Bgl I	934 > 1168 / 3487	934 > 1168 / 3487	1168 / 1387 > 934	934 > 1168 > 3487	934 > 1168 > 3487	934 ~ 1168 ~ 3487	934 ~ 1168 ~ 3487
Mst I	1357 > 1455 > 262 > 3589		3589 > 1357 / 1455 > 262	262 > 1357 > 1453 > 3589		3589 > 1357 ~ 262 > 1453	
Enzyme		EB Inhibition	BMSp Inhibition		EB Inhibition	BMSp Inhibition	BMSpd Inhibition
Rsa I		NSI	165		NSI	165	165
Bgl I		NSI	934		NSI	934 > 1168	934 > 1168
Mst I			1357 1455			1455 > 262 > 1357	

NSI = No Specific Inhibition

Table 7


Eco RI
Mst I
Bam HI
Bgl I
Rsa I
4
0
1
3
2

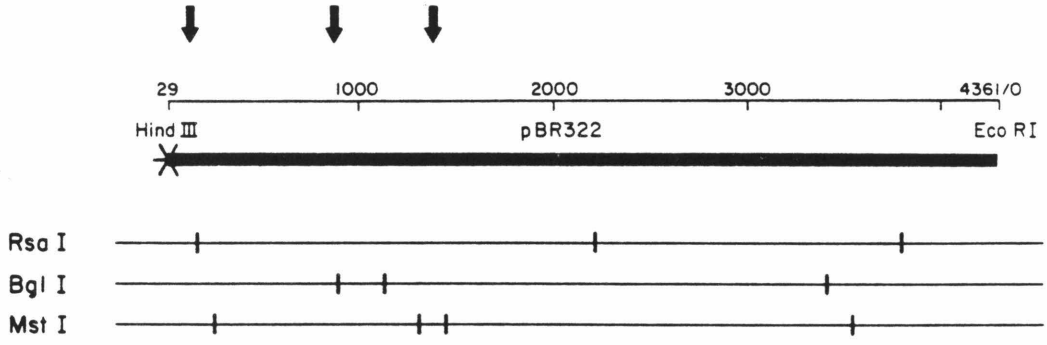

29
1000
2000
3000
4361/0
Hind III
pBR322
Eco RI
Rsa I
Bgl I
Mst I

Figure 20

Figure 21. Rsa I

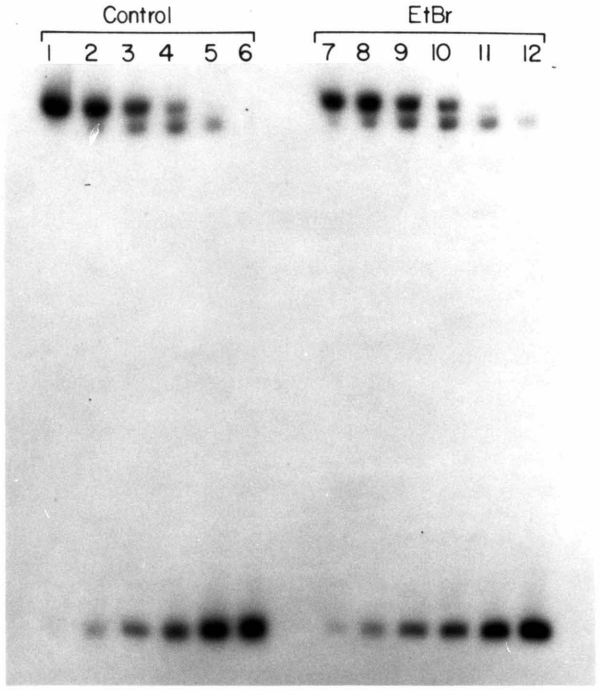
Control - Lanes 1-6: DNA = 2.5 μg, Rsa I = 0.5 un., No Intercalator, Times (min) = 0.5, 1.5, 3, 5, 9, 17 - from lane 1 to lane 6.

EtBr - Lanes 7-12: DNA = 2.5 μg, Rsa I = 0.5 un, EtBr/DNA = 0.16, Times (min) = 1, 2, 4, 6, 12, 22 - from lane 7 to lane 12.

BMSp - Lanes 13-18: DNA = 2.5 μg, Rsa I = 0.5 un, BMSp/DNA = 0.08, Times (min) = 1, 3, 7, 15, 25, 45 - from lane 13 to lane 18.

BMSpd - Lanes 19-24: DNA = 2.5 μg, Rsa I = 0.5 un, BMSpd/DNA = 0.08, Times (min) = 1, 3, 7, 15, 25, 45 - from lane 19 to lane 24.

Rsa I


Control
EtBr
1 2 3 4 5 6
7 8 9 10 11 12

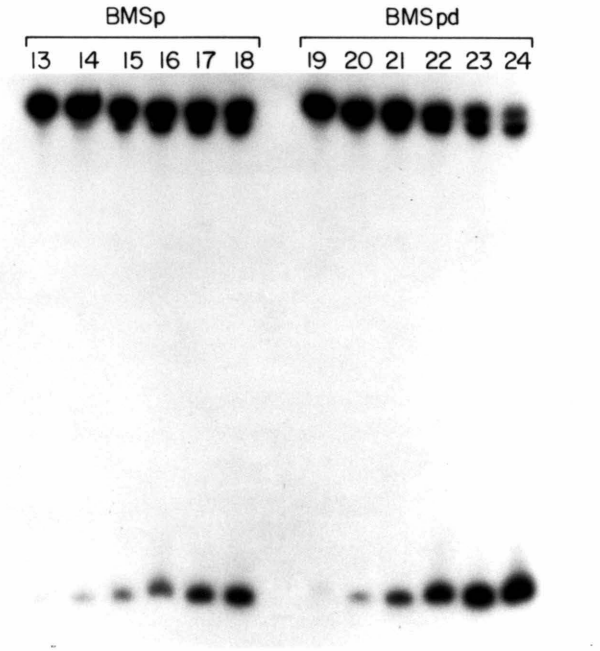

BMSp
BMSpd
13 14 15 16 17 18
19 20 21 22 23 24

Figure 22. Bgl I

Control - Lanes 1-6: DNA = 2.5 µg, Bgl I = 1.5 un., No Intercalator, Times (min) = 1, 2, 4, 7, 15, 25 - from lane 1 to lane 6.

EtBr - Lanes 7-12: DNA = µg, Bgl I = 1.5 un., EtBr/DNA = 0.16, Times (min) = 2, 3, 5, 8, 15, 25 - from lane 7 to lane 12.

BMSp - Lanes 13-18: DNA = 2.5 µg, Bgl I = 1.5 un., BMSp/DNA = 0.08, Times (min) = 10, 30, 60, 90, 130, 180 - from lane 13 to lane 18.

BMSpd - Lanes 19-23: DNA = 2.5 µg, Bgl I = 1.5 un., BMSpd/DNA = 0.08, Times (min) = 10, 30, 50, 80, 120 - from lane 19 to lane 23.

Bgl I

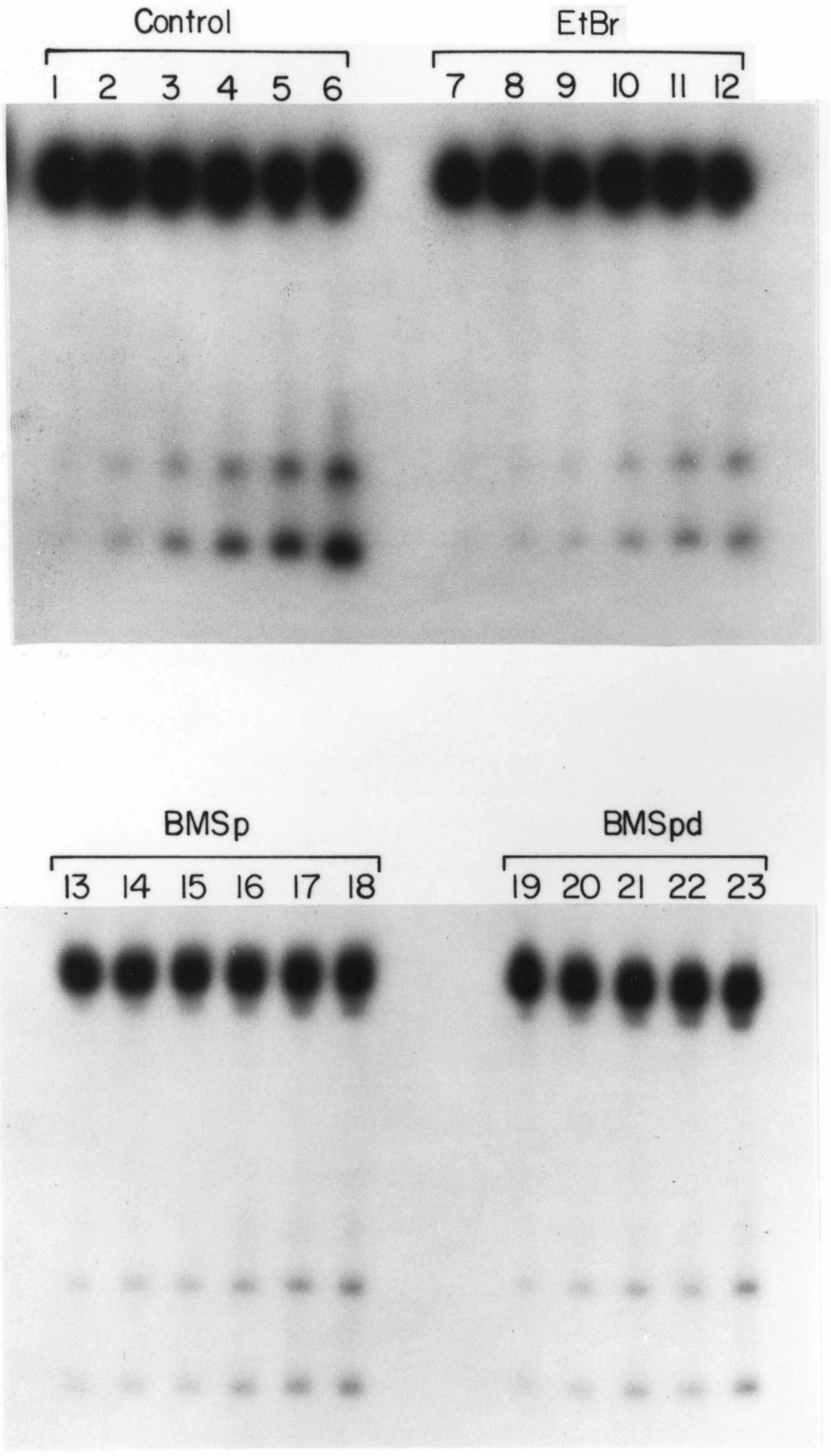

Control
EtBr
1 2 3 4 5 6
7 8 9 10 11 12
BMSp
BMSpd
13 14 15 16 17 18
19 20 21 22 23

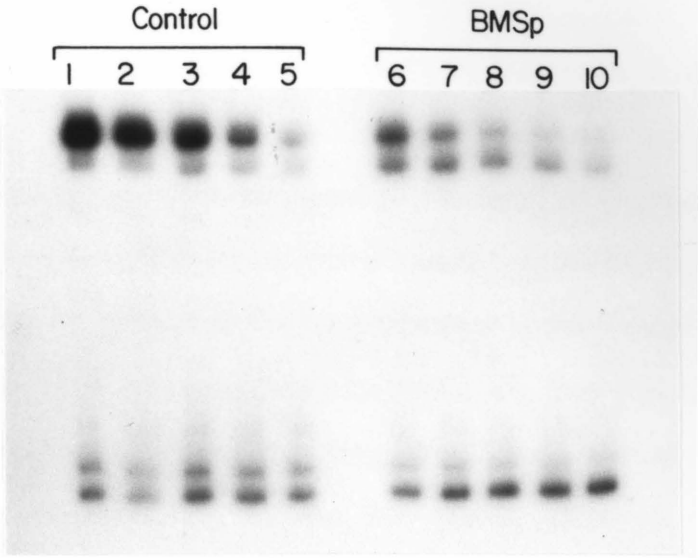
Figure 23. Mst I

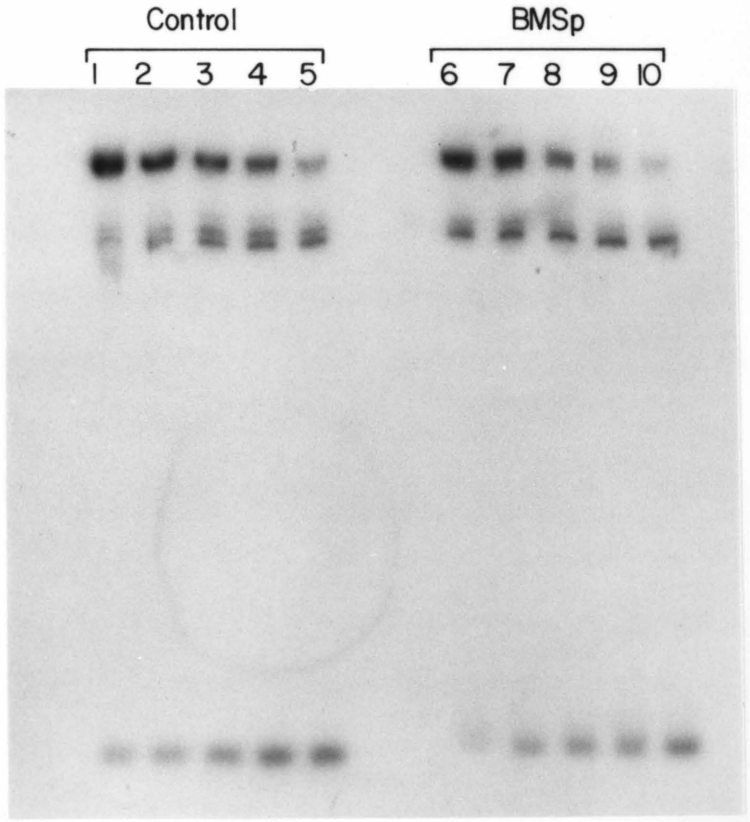
Control - Lanes 1-5: DNA = 2.3 μg, Mst I = 1.0 un., No Intercalator, Times (min) = 15, 20, 30, 45, 65 - from lane 1 to lane 5.

BMSp - Lanes 6-10: DNA = 2.3 μg, Mst I = 1.0 un., BMSp/DNA = 0.08, Times (min) = 45, 65, 90, 120, 165 - from lane 6 to lane 10.

Top set of lanes was electrophoresed for 24 h at 100 volts, while the bottom set of lanes was electrophoresed for 15 h at 30 volts.

Mst I





restrictions the order of appearance of the DNA fragments is exactly the order of site cleavage; however, the nature of the assay makes it more difficult to detect perturbations in cleavage rates. For example, specific inhibition would be seen as changes in the relative rates of appearance of specific fragments with respect to the fragment that appears most slowly.

Restrictions in intercalator free solutions show that Rsa I, Bgl I, and Mst I give all the resolvable restriction fragments. The order of appearance of these fragments (Figs. 21, 22 and 23) yields cleavage rankings of 3847/164 > 2282 for Rsa I, 934 > 1168 > 3487 for Bgl I, and 262 > 1357 > 1455 > 3589 for Mst I (Table 7).

Again, EB at 30% saturation induces no changes in the restriction patterns of Rsa I and Bgl I. There is only a general inhibition of all sites; no specific inhibition is apparent. The antithesis is once more BMSp. Even with linear pBR322, BMSp at 30% saturation ($([BMSp]/[BP] = 0.08)$ changes the restriction patterns of Rsa I, Bgl I, and Mst I. In the case of Rsa I, the appearance of the 136 bp fragment is slowed in relation to the appearance of the 2253 bp fragment. For Bgl I, the 905 bp fragment and the 1139 bp fragment appear in conjunction with the 3458 bp fragment instead of appearing ahead of the larger fragment. In addition, the two small fragment appear simultaneously; whereas the control digestion shows the 905 bp fragment appearing before the 1139 bp fragment. Finally, the Mst I 233 bp fragment and 1328 bp fragment are slow to appear in relation to the 3560 bp fragment, while the 2426 bp fragment is almost eliminated (Figs. 21 and 22).

These BMSp doped restriction patterns correspond to the cleavage orders (1) Rsa I = 3847 > 165 ~ 2282, (2) Bgl I = 934 ~ 1168 ~ 3487,

and (3) Mst I = 3589 > 1357 ~ 262 > 1455 which are different from the restriction orders of the intercalator free digestions. The changes induced by BMSp indicate specific inhibition at the same sites identified with the supercoiled substrate, Rsa I = 165, Bgl I = 934, and Mst I = 1455. Furthermore, it was possible to deduce that the sites at Bgl I = 1168, Mst I = 262, and Mst I = 1357 were also inhibited in the presence of BMSp (Table 7). Apparently, tertiary structure does not affect the restriction inhibition induced by a bisintercalator.

Since the two assays lead to the same conclusions a newly synthesized dimer, bis(methidium)spermidine (BMSpd), was added to the linear substrate assays. BMSpd differs from BMSp in the length of the linker tethering the chromophores. The spermine tether stretches to a length of 15 Å, while the spermidine tether only spans 10 Å. Biophysical measurements suggest that BMSpd may act like a dimer at very low saturations and resemble a monomer at high saturations. Consequently, it was not surprising to find that BMSpd at 30% saturation ([BMSpd]/[BP] = 0.08) is not as effective as BMSp, but does inhibit the same sites as the spermine dimer (Figs. 21 and 22, Table 7).

Having ascertained that methidium dimers inhibit restriction sites in the first third of the pBR322 plasmid, the restriction enzyme Taq I was chosen for a more detailed mapping of the inhibition induced by the two bisintercalators. With this system only the five smallest fragments were monitored and the data were interpreted as previously described. It was found that site selective inhibition by BMSp is saturation dependent. The number of Taq I sites inhibited decreases with decreasing concentrations of BMSp. At 0.03 molecules of BMSp per base pair of DNA only the Taq I site at 339 is

specifically inhibited. Moreover, EB again induces no selective inhibition, while BMSpd distinguishes itself from BMSp. At a saturation of 0.03 molecules of BMSpd per base pair of DNA, BMSpd inhibits three sites in the order 339 > 1126 > 651 (Table 8, Fig. 24). It might be that BMSpd is acting at two sites near positions 339 and 1126, while BMSp only sees the site at 339, or it is possible that BMSpd is simply less selective than BMSp.

To substantiate that the intercalators were acting on the DNA and not on the enzymes, a series of DNase protection experiments were attempted. In these studies DNase I was substituted for the restriction enzymes. If the first third of the pBR322 plasmid can be protected from DNase cleavage by the intercalators, a persistent "smear" should appear during the digestion of the linear plasmid. DNase nicking of pBR322 end labeled at the Hind III restriction site showed that an intercalator free digest was quickly and progressively reduced to small fragments. The same digestion pattern was observable in the presence of EB at 30% saturation; however, BMSp and BMSpd at 30% saturation cause the persistence of a smear between 500 bp and 1000 bp (Fig. 25). The opposite end label at Eco RI gives no smeared band as expected.

Direct physical corroboration of the relationship between restriction site inhibition and intercalator binding was addressed. A covalently binding analog of bis(methidium)spermine was designed and synthesized. The strategy was to radiolabel the bisintercalator, bis(monoazidomethidium)-spermine (BAMS) (Fig. 26), covalently bind BAMS to its equilibrium binding sites, and determine the location of the bound intercalator. It was found that the high specific activity required, and the lack of a dedicated radiosynthesis

Enzyme	Site Order	BMSp at 0.08	BMSp at 0.06	BMSp at 0.03	EB at 0.06	BMSpd at 0.03
Taq I	339 > 651 > 1126 > 2574 ≫ 1267	2574 > 1126 > 651 > 339 ≫ 1267	651 > 2574 > 1126 > 339 ≫ 1267	651 > 1126 > 2574 ~ 339 ≫ 1267	339 > 651 > 1126 > 2574 ≫ 1267	651 > 2574 > 339 > 1126 ≫ 1267

Inhibited Sites

Enzyme		BMSp at 0.08	BMSp at 0.06	BMSp at 0.03	EB at 0.06	BMSpd at 0.03
Taq I		339 ≫ 651 ≫ 1126	339 ≫ 651 > 1126	339		339 > 1126 > 651

NSI = No Specific Inhibition

Table 8

Figure 24. Taq I

Control - Lanes 1-5: DNA = 1.5 µg, Taq I = 0.5 un., No Intercalator, Times (min) = 0.5, 1, 2, 4, 8 - from lane 1 to lane 5.

BMSp 0.06 - Lanes 6-10: DNA = 1.5 µg, TaqI = 0.5 un., BMSp/DNA = 0.06, Times (min) = 2, 4, 8, 16, 32 - from lane 6 to lane 10.

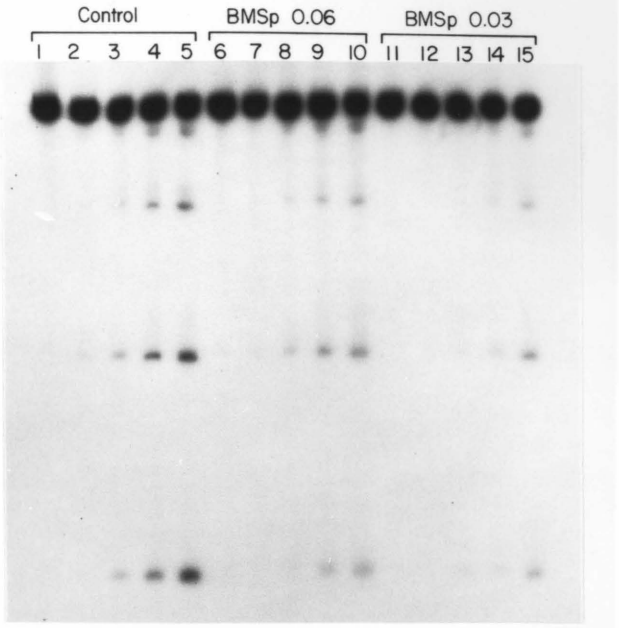
BMSp 0.03 - Lanes 11-15: DNA = 1.5 µg, Taq I = 0.5 un., BMSp/DNA = 0.03, Times (min) = 1, 2, 4, 8, 16 - from lane 11 to lane 15.

BMSp 0.08 - Lanes 16-20: DNA = 2.5 µg, Taq I = 2.0 un., BMSp/DNA = 0.08, Times (min) = 45, 65, 90, 120, 165 - from lane 16 to lane 20.

EtBr 0.06 - Lanes 21-25: DNA = 3.0 µg, TaqI = 3.0 un., EtBr/DNA = 0.06, Times (min) = 2, 4, 8, 16, 32 - from lane 21 to lane 25.

BMSpd 0.03 - Lanes 26-30: DNA = 3.0 µg, Taq I = 3.0 un., BMSpd/DNA = 0.03, Times (min) = 3, 6, 12, 24, 48 - from lane 26 to lane 30.

Taq I


Control
BMSp 0.06
BMSp 0.03
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

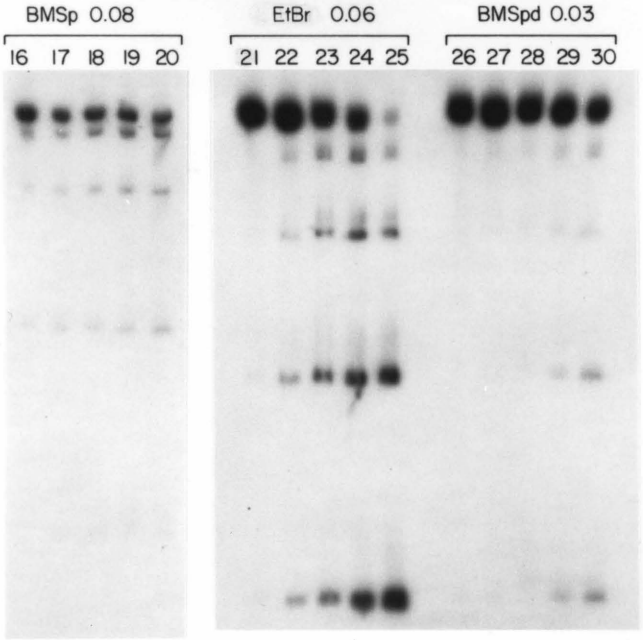

BMSp 0.08
EtBr 0.06
BMSpd 0.03
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

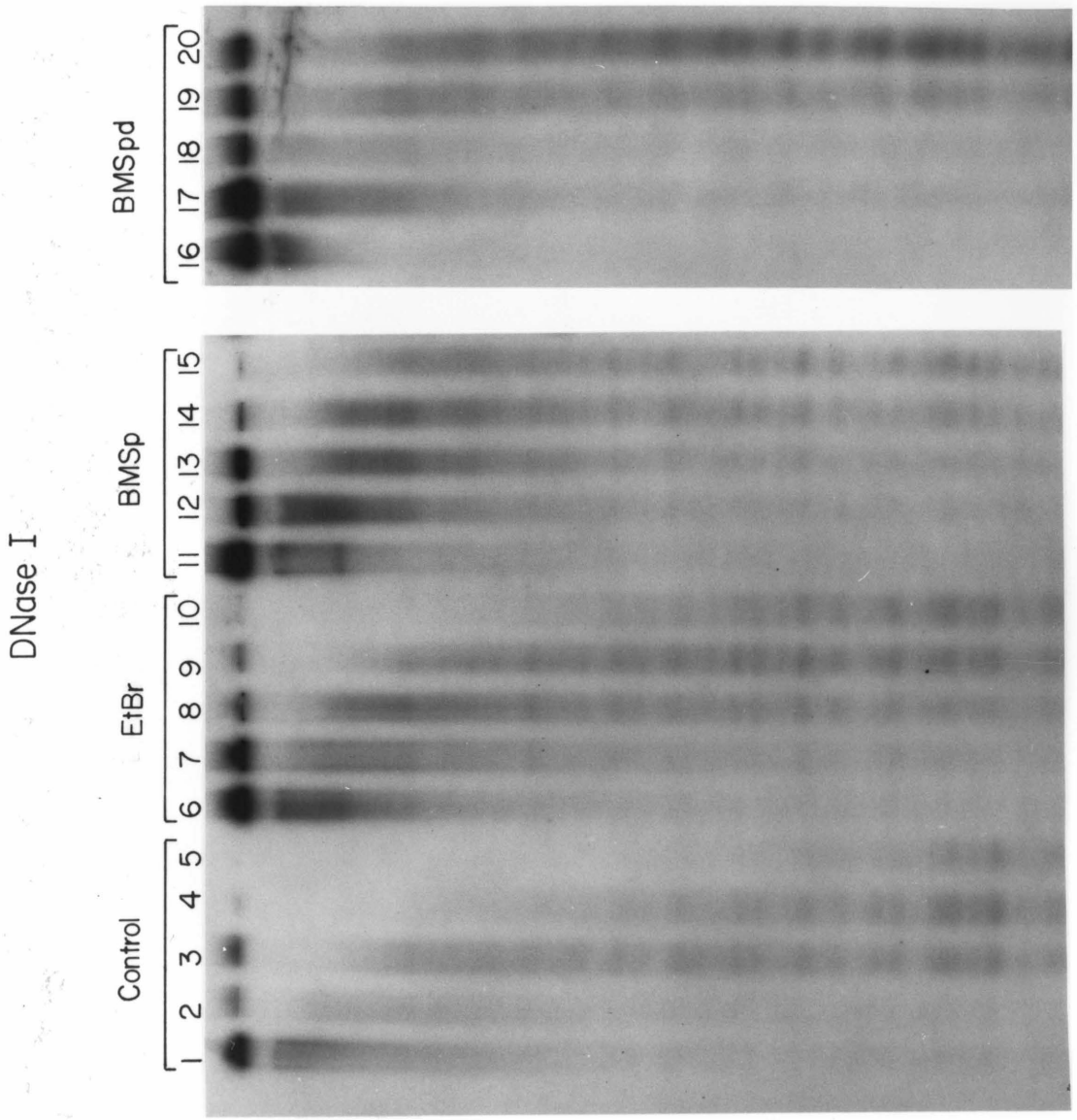
Figure 25. DNase I

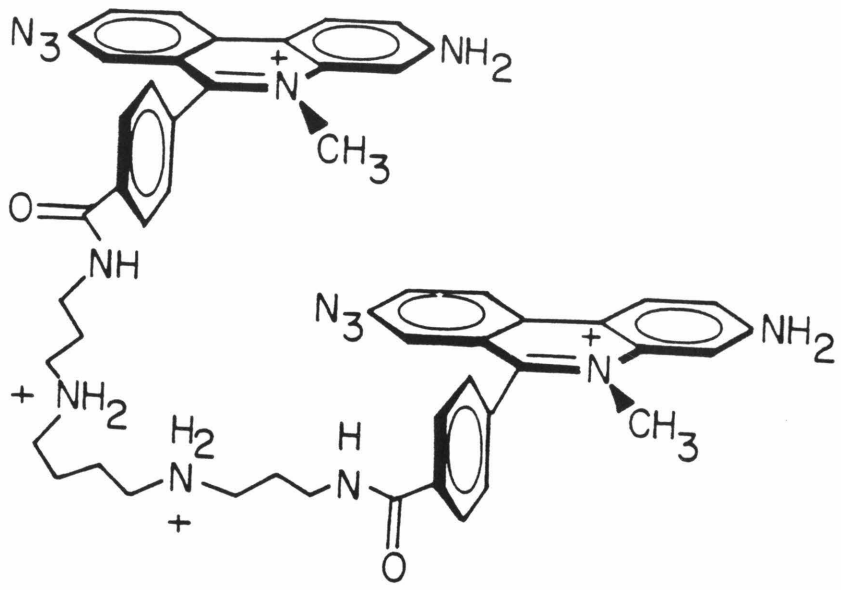
Control - Lanes 1-5: DNA = 1.5 μg, DNase = 20 ng/mL, No Intercalator, Times (min) = 1, 3, 6, 12, 24 - from lane 1 to lane 5.

EtBr - Lanes 6-10: DNA = 1.5 μg, DNase = 20 ng/mL, EtBr/DNA = 0.16, Times (min) = 1, 3, 6, 12, 24 - from lane 6 to lane 10.

BMSp - Lanes 11-15: DNA = 1.5 μg,DNase=20ng/mL,BMSp/DNA= 0.08, Times (min) = 1, 3, 6, 12, 24 - from lane 11 to lane 15.

BMSpd - Lanes 16-20: DNA = 1.5 μg, DNase = 20 ng/mL, BMSpd/DNA = 0.08, Times (min) = 1, 3, 6, 12, 24 - from lane 16 to lane 20.


DNase I
Control
EtBr
BMSp
BMSpd
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



BAMS

Figure 26

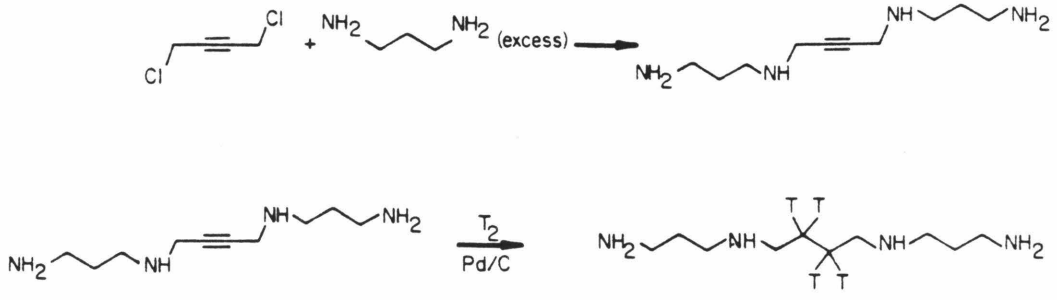


Figure 27

facility made the tritium labeled synthesis of BAMS too hazardous to be viable at Caltech (Fig. 27). The synthesis could even be unfit for radioisotope laboratories unless provisions were made for dark room illumination to protect the azide functionality of BAMS.

C. Summary

The bisintercalators, BMSp and BMSpd, can selectively inhibit restriction sites in a heterogeneous sequence of pBR322 DNA and can protect this same region from DNase cleavage. With pBR322, BMSp and BMSpd at 30% saturation inhibit DNA cleavage over the first third of the plasmid. Furthermore, the number of sites protected is proportional to the concentration of the bisintercalator. The greatest selectivity is observed with BMSp at low saturations. In this case, inhibition seems to predominate in the region of the Taq I-339 restriction site. Conversely, EB shows no selective inhibition. Why EB is not selective appears to be a consequence of both the low binding affinity of the monomers and the lack of other dimeric properties.

The molecular interpretation of the restriction inhibition by BMSp and BMSpd is a little more obscure. The "clustering" of inhibited restriction sites suggests a cooperative phenomenon. The bisintercalators may bind and physically protect the bound DNA, or they may induce a cooperative perturbation of the DNA secondary structure in the region of the inhibited restriction sites. In either case, tertiary structure is apparently not important. What may determine the region of inhibition is the nature of the primary sequence. Since methidium analogs are not base specific, and

secondary structure is not intimately specified by the identity of the bases, the selectivity would not be determined by the primary identity of the bases, but by the overall structure and conformational lability of the sequence. In short, selective DNA cleavage inhibition by BMSp and BMSpd might be a consequence of the recognition of DNA conformation by the intercalators, or the DNA dependent enzymes or both the intercalators and the enzymes.

Materials and Methods

Part I

Synthesis of Bis(methidium)spermidine Hydrochloride. A dry 200 mL, three-necked round-bottom flask was equipped with an argon inlet tube, a septum, and a stopper. A magnetic stirrer was placed in the flask, and the flask was flushed with argon for 1 min. 1.53 g of recrystallized paracarboxyl methidium chloride (PMCl, 100 mol %)[1] was placed in the flask, and the flask was sealed. 50 mL of dry dimethyl sulfoxide (Baker Analyzed reagent, DMSO) and 0.40 mL dry N-ethyl morpholine (Eastman Practical) were then syringed onto the PMCl, and the mixture was stirred till dissolution. 765 mg of freshly sublimed 1,1-dicarbonyl diimadazole (117 mol %, Aldrich, 98%) in 8.8 mL of dry DMSO were then syringed into the PMCl solution, and the mixture was allowed to stir. An hour after the addition of the dicarbonyl diimadazole, 285 mg of dry spermidine (97 mol %, Aldrich, 98%) in 2.85 mL of dry DMSO was added to the reaction mixture, and the reaction was followed by TLC (cellulose plates developed in 50/50 mixture of 0.025 M, pH 7, phosphate buffer, isopropanol). After 48 h of stirring the solvents were evaporated, and a purple solid was recovered.

Purification of BMSpd. During the synthetic reaction 750 g of silica gel 60 (EM reagents, 70-230 mesh) were prepared by repeatedly washing the material with eluent. The eluent consisted of dry methanol (Baker analyzed, low acetone) acidified with acetyl chloride (Mallinkrodt AR, bp, 49-53°C) at a level of 7 mL of acetyl chloride per 1000 mL methanol. A column was filled with the purified silica gel, and the column was pre-eluted with the methanol eluent. Approximately half the solids collected from the synthetic reaction were dissolved in 150 mL of acidic methanol and applied to the

column. The BMSpd fraction was collected, concentrated, and rechromatographed on fresh silica gel. This time, the BMSpd was concentrated and filtered through a fine glass frit and afterward evaporated to dryness. The solids were taken up in a minimum volume of 1 N HCl, and tetrahydrofuran was added to the solution to induce reprecipitation of the compound. The solids were collected, washed with THF, and dried at 100°C under reduced pressure. This yielded 290 mg of BMSpd contaminated with 3-5% uncombustible material.

Final purification of the BMSpd was accomplished by chromatography on Bio-Rad 70, hydrogen form, cation exchange resin (100-200 mesh). This was done by dissolving 19 mg of the 97% BMSpd in 15 mL of doubly distilled water. The solution was then applied to an 11 cm column of the cation exchange resin, and the column and BMSpd were washed with a liter of doubly distilled water. The BMSpd was then washed with 150 mL dry methanol and lastly, eluted with acidic methanol. The BMSpd fractions were collected, and the solution was evaporated to dryness. The BMSpd was then "recrystallized" out of 1 N HCl/THF and dried. 10 mg of analytically pure BMSpd were recovered. The overall yield was impossible to calculate since the reaction solids were split before the first chomatography. IR (KBr): Figure 28. UV/VIS: maxima = 214, 288, 501 nm (ε = 8835, 0.025 M, pH 7, phosphate buffer). ^{1}H NMR (D_6-DMS): Figure 29; ^{13}C NMR (D_6-DMSO): Figure 30.

Elemental Analysis: BMSpd·4 H_2O. Found: C, 59.82; H, 5.91, N, 12.94; Cl, 10.94. Calculated: C, 60.18; H, 6.18; N, 12.95; Cl, 10.88.

Buffers. All buffers are referred to by their total concentration of monovalent cations. Each was prepared by adding the appropriate amount of

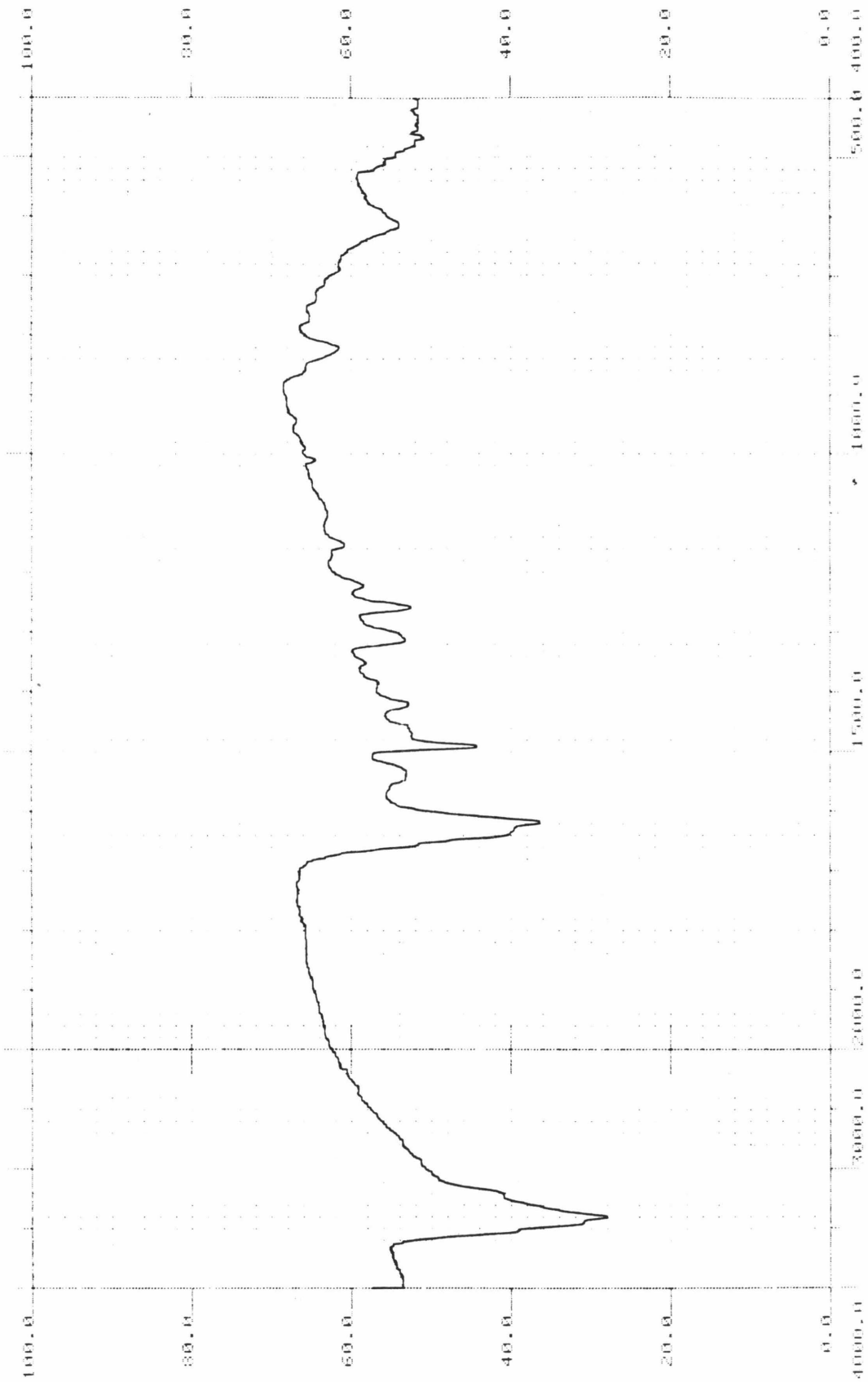


Figure 28

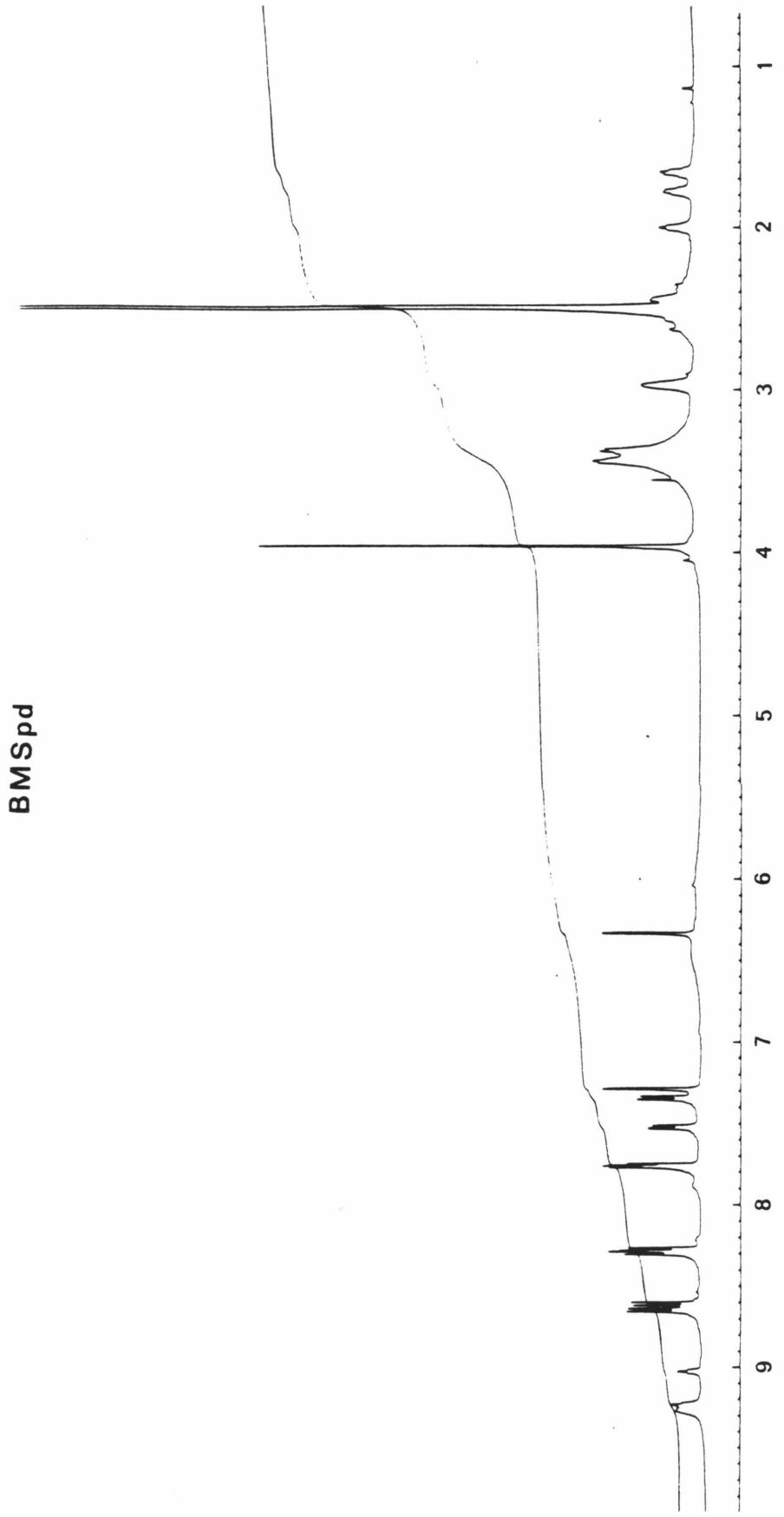
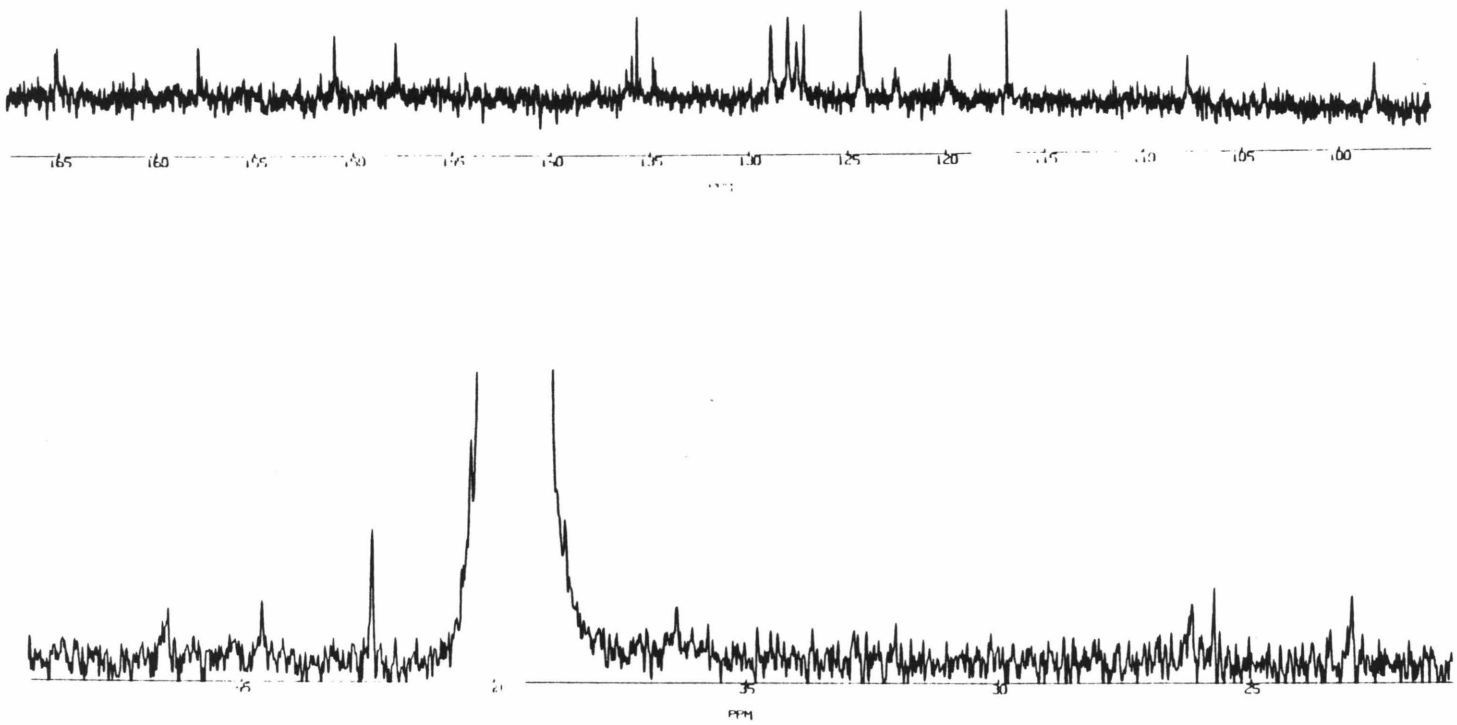


Figure 29

Figure 30

BMSpd



sodium chloride to a phosphate buffer containing 0.028 M Na_2PO_4 and 0.019 M $KHPO_4$: (1) (M^+) = 0.075 M, no NaCl added; (2) (M^+) = 0.4 M, (NaCl) = 0.31 M and; (3) (M^+) = 1.0 M, (NaCl) = 0.9 M. The pH of the solution was then adjusted to pH 7 with 1 M NaOH.

Calf thymus DNA: Highly polymerized calf thymus DNA was cleaned by extraction with phenol and ether. It was then sonicated at 0°C under argon[16] and fractionated on sepharose 4B. The fractions collected yielded double stranded DNA approximately 300 base pairs in length.

Synthetic polymers. Poly dA·poly dT, poly rA·poly dT, and poly dG·poly dC were obtained from P-L Biochemicals. Each was rehydrated and twice dialyzed against 0.4 M (M^+), pH 7, phosphate buffer. In addition, it was necessary to sonicate the poly dG·dC sample. Sonication and fractionation as previously described followed by pH annealing[37] yielded double stranded poly dG·dC approximately 500 base pairs long. The polymer solutions were then sterilized by filtration through an 0.45 μm millipore filter.

PM2 DNA. Covalently closed circular PM2 DNA was obtained from Boehringer Mannheim Biochemicals.

DNA Concentrations. All DNA concentrations were obtained spectrophotometrically assuming the following extinction coefficients: calf thymus and PM2 - 6600 nuc^{-1} cm^{-1} at 260 nm; poly dA·poly dT - 6000 nuc^{-1} cm^{-1} at 258 nm; and poly dG·poly dC - 7400 nuc^{-1} cm^{-1} at 255 nm.

Unwind Angle Determination. 50 μL of a 50 mM Tris/HCl (Sigma, reagent grade tris(hydroxymethyl) amino methane/tris(hydroxymethyl)amino methane hydrochloride) pH 7.8, 10 mM $MgCl_2$ (Baker, analyzed reagent,

99.5%) and 2 mM dithiothreitol (Calbiochem, 99%) buffer were measured into six bullet vials. 2 µg of closed circular PM2 DNA, 4 units of superhelical relaxing enzyme (BRL-purified from Agrobacterium tumefaciens strain 10135), and a progressively increasing amount of intercalator were added to each vial. The mixtures were incubated for 12 h at 37°C. Four units more superhelical relaxing enzyme were added and the reaction mixtures were incubated an additional 12 h. At the end of the incubation 10 µL of 10% sodium dodecyl sulfate (MC/B) were added to denature the enzyme, and 30 µg tRNA were added as carrier. The samples were phenol extracted and the intercalator was removed by chromatography on a microcolumn of cation exchange resin (Biorad AG50W-X8, Tris form). The DNA was recovered from the columns in 2-200 µL aliquots of 1 M sodium acetate (Mallinkrodt, AR) 1 mL isopropanol (Baker, analyzed reagent) was added and the alcoholic solution was frozen. The DNA was then pelleted by centrifugation at 15,000 rpm for 8 min. The pellet was washed with ethanol and reprecipitated. The washing was repeated, and the pellet was dried under vacuum. The dessicated DNA was then dissolved in 80 µL loading solution, 5% ficoll (Sigma, type 400), 0.025% bromophenol blue (Sigma), 0.025% xylene cyanol, and 1 mM ethylenediamine tetraacetic acid (Baker, analyzed reagent, 99.7%). Approximately 400 ng of DNA from each sample were loaded onto a 15 cm x 14 cm x 0.4 cm, 1% agarose (Sigma, electrophoretically pure) gel (buffer: 40 mM Tris/HCl, pH 7.8, 5 mM sodium acetate, 1 mM EDTA, and 1-25 µg/mL chloroquine -- a concentration appropriate to resolve the superhelical bands). The electrophoresis was allowed to run for 20 h at 70 volts and 60 milliamps. Electrophoresis buffers were circulated by peristaltic pump

and were replaced after 10 h of the electrophoresis. Upon completion of the electrophoresis, the gel was stained in 2 μg/mL ethidium bromide (Sigma) for up to 6 h and destained for 12 to 24 h. The gel was then recorded by photography under ultraviolet transillumination, and the unwinding angle was determined from the shift of the superhelical PM2 bands.

Spectrophotometric Titrations. Optical titrations were performed on a Cary 219 utilizing fixed block sample stages and 10 cm cells. Ambient temperature was controlled at 22°C ± 1°C to provide machine stability and thermodynamic constancy. The 10 cm cells were filled dust free with 25 mL of millipore filtered buffer, and clean DNA was syringed into the cells. Increasing amounts of BMSpd were added to the nucleic acid solution, and the absorbance was recorded for each addition of BMSpd. After the mixtures attained equilibrium, absorbances were reproducible to ±0.0002 Å.

Bound Spectra. The spectrum of BMSpd bound to a nucleic acid was determined by recording the spectrum of BMSpd in the presence of a high concentration of the appropriate DNA ([DNA] ~ 100/estimated BMSpd binding constant). Approximately, ten separate spectra were recorded at ten different ratios of intercalator to DNA. The bound spectrum of BMSpd was then extracted from the data by the methods of Bontemps and Fredericq.[38]

Part II

Enzymes. Rsa I, Bgl I, Mst I, Taq I, Eco RI, Hind III, large fragment DNA polymerase, and DNase I were purchased commercial.

Nucleic Acid.[49] Plasmid pBR322 was used as substrate DNA for the entire study. A portion of the pBR322 was purchased commercially. The remainder of the pBR322 was isolated from transformed E. Coli HB101 after amplification of the plasmid with chloramphenicol. The superhelical DNA was then purified by equilibrium sedimentation.

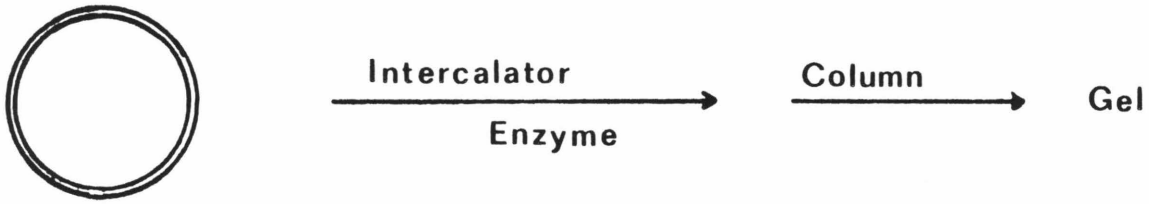
Concentrations were determined spectrophotometrically assuming an extinction coefficient at 260 nm of 6600 $(M\ nuc)^{-1}\ cm^{-1}$.

Intercalators. Ethidium bromide was obtained commercially, while bis(methidium)spermine[1] and bis(methidium)spermidine were synthesized.

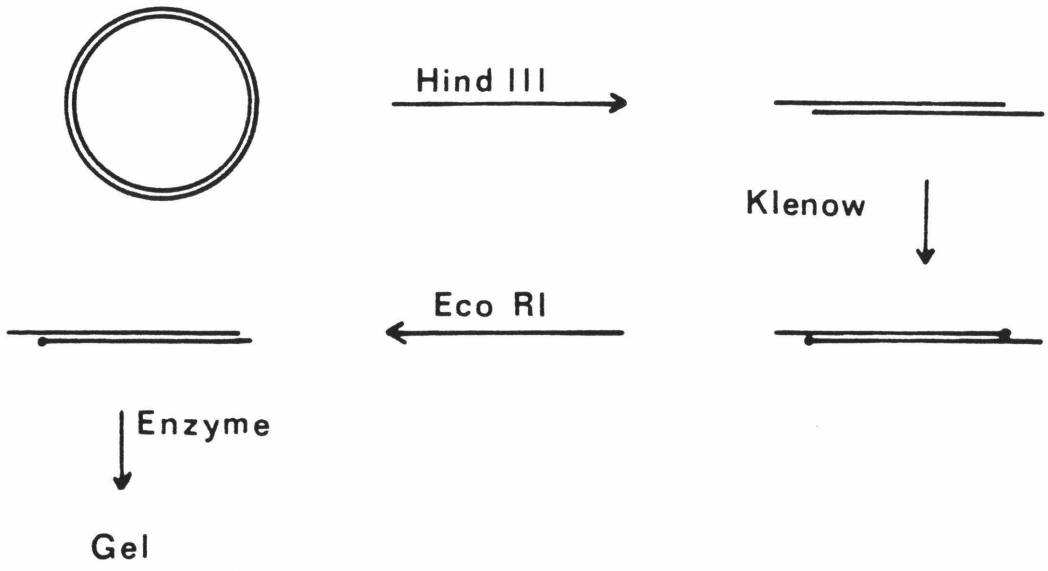
Intercalator concentrations were determined spectrophotometrically. Extinction coefficients - EB = 5700 $M^{-1}\ cm^{-1}$ at 480 nm in pH 7, 0.075 M phosphate buffer;[1] BMSp = 9850 $M^{-1}\ cm^{-1}$ at 501 nm in pH 7,[1], 0.075 M phosphate buffer; and BMSpd = 8835 $M^{-1}\ cm^{-1}$ at 501 nm in pH 7, 0.075 M phosphate buffer.

End Labeling.[49] Plasmid pBR322 cut with Hind III or Eco RI was labeled with an appropriate nucleoside 5'-(α-^{32}P) triphosphate and large fragment DNA polymerase. Recutting the products of the labeling reaction with Eco RI or Hind III yields an almost full length linear fragment labeled at one end.

Digestion Conditions. The amount of DNA (pBR322: 2-4 micrograms), the volume of the reaction buffer (60-100 microliters, 50 mM Tris/HCl - pH 7.4, 6 mM dithiothreitol, 6 mM $MgCl_2$ and 50 micrograms/mL bovine serum



Scheme 1



Scheme 2

albumin) and the amount of enzyme (0.5 to 2 units for restriction enzymes and 1.25 ng/mL DNase I) were set so that the relative concentrations of DNA and enzyme would remain near constant for the assays done with one enzyme and so that samples could be withdrawn from the reaction mixture at reasonable intervals over the entire digestion period. Since the method revolves about the relative kinetics of the restrictions, the digestion samples were mixed with the intercalator in the absence of the enzyme, and the system was allowed to equilibrate for 1 h. The samples were then brought to 37°C over 15 min, and the enzyme was added at time 0. Each sample was stopped with 5 to 15 microliters of 5% sodium dodecyl sulfate and was stored frozen until the sample could be purified.

Samples were extracted with phenol, washed with ether, and prepared for loading onto the gel. For gels where resolution was critical, the samples containing BMSp or BMSpd required an extra purification step, since either bisintercalator can seriously affect the mobility of DNA through a gel.

After phenol extraction of the dimer samples, each applied to a BioRad cation exchange (AG50W-X8, 100-200 mesh) column, and the DNA was eluted with 1 M sodium acetate. The DNA was then precipitated and prepared for loading onto the gel. Control experiments confirm that the cation exchange columns do not selectively remove any restriction fragments.

Electrophoresis.[49] The DNA samples cleaved by restriction enzymes were loaded onto 15 x 14 x 0.4 cm or 30 x 14 x 0.4 cm vertical gels of 5% polyacrylamide. The gels were then run in tris/borate buffer (90 mM Tris/borate - pH 8.3, 2.5 mM EDTA) for an appropriate period, and after-

wards the gels were visualized by either autoradiography or ethidium bromide staining and UV transillumination.

The DNase I digested samples were run on 15 x 14 x 0.1 cm 5% acrylamide, urea denaturing gels as described for the restricted samples. The gels were then visualized by autoradiography.

Synthesis

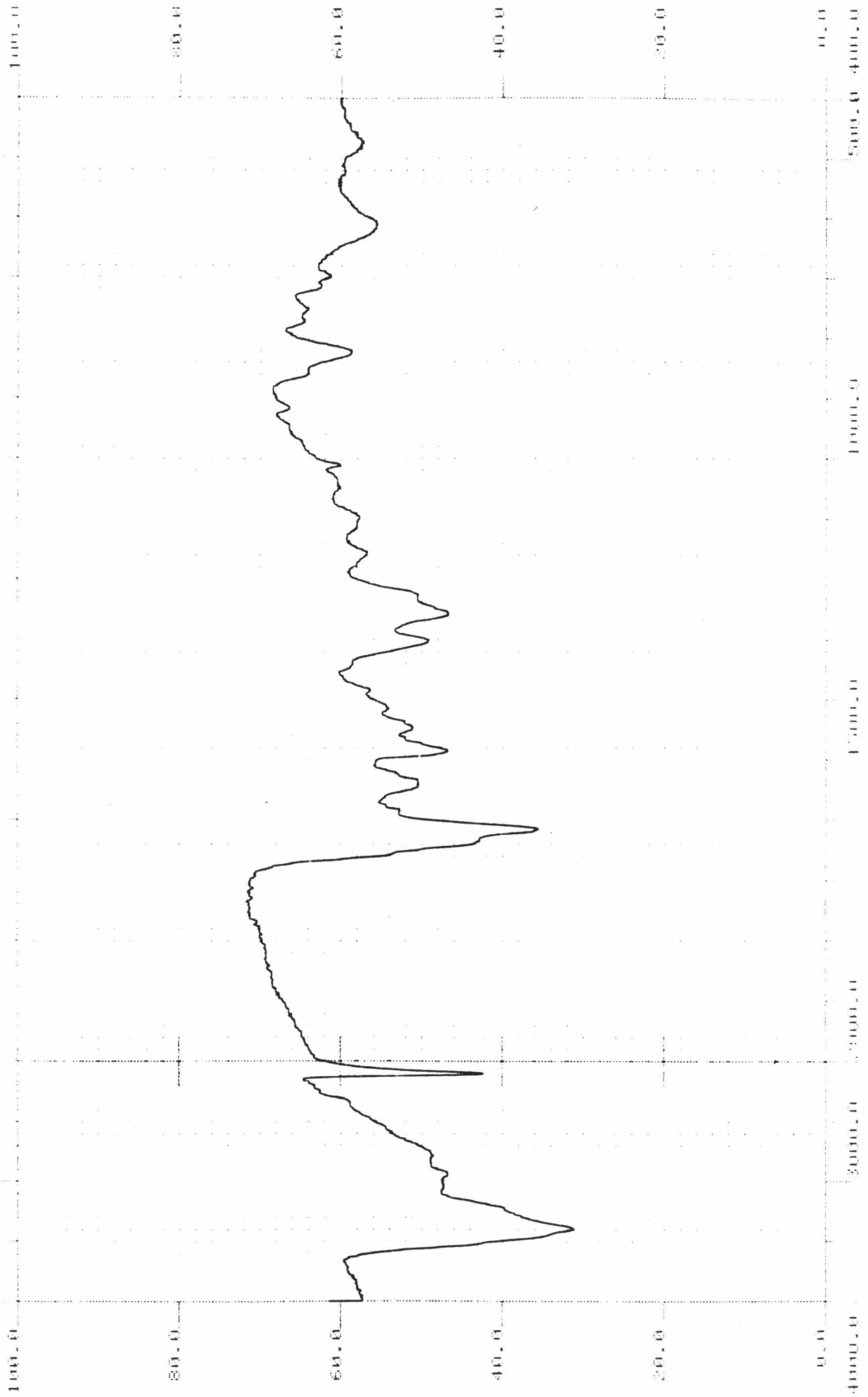
Bis(monoazidomethidium)Spermine. The following synthesis was performed under dark room conditions. Illumination was provided by a single safety lamp fitted with a Kodak Safelight Filter No. 2. A dry single neck 50 mL round-bottom flask was flushed with argon. A magnetic stirring bar, 212 mgs of para-carboxy(monoazidomethidium) chloride (PCAM),[50] and 20 mL dry dimethyl sulfoxide (DMSO) were placed in the flask, and the flask was sealed with a septum stopper. 85 mg 1,1-carbonyl diimidazole (Aldrich, mp 118-120°C, 105 mol %) were dissolved in 2 mL dry DMSO. This 2 mL solution was syringed into the reaction flask and the reaction was allowed to stir for 1 hour. 50 mg of spermine (Aldrich, 97% - 49 mol %) in 0.5 mL dry DMSO was then syringed into the reaction. The solution was allowed to stir for 48 h.

A hundred microliters of water were added to the reaction, and after a half hour the DMSO and water were evaporated at 60°C and 1 mm pressure. The solids were then dissolved in 100 mL methanol.

Silica gel 60, 70-230 mesh, was prepared by repeatedly washing the silica with dry methanol acidified with 4 mL of fresh acetyl chloride per 1000 mL of methanol. A column was filled with 650 g of the purified silica gel, and the column was pre-eluted with the acidic methanol. The methanolic

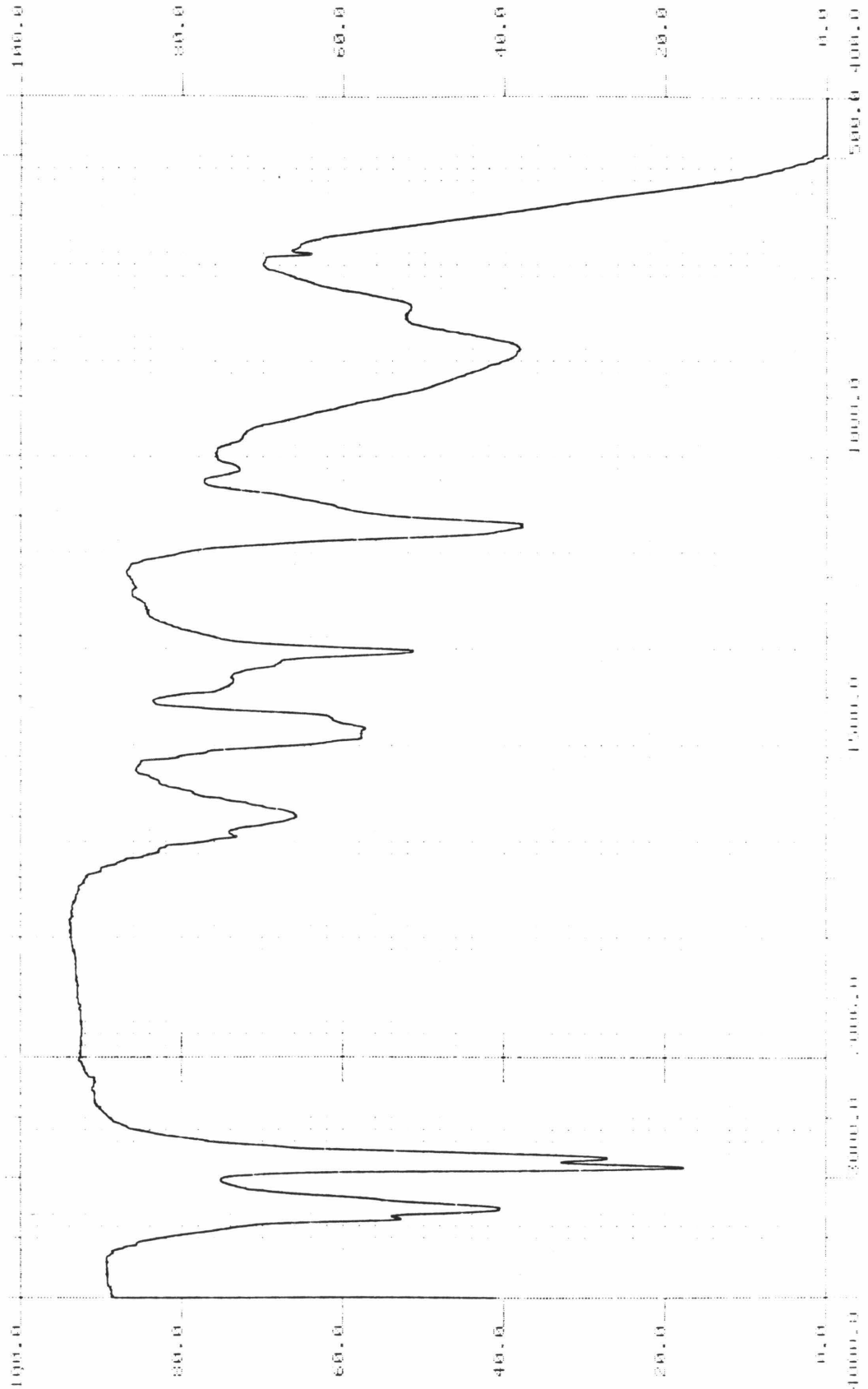
solution of the reaction mixture was then applied to the column and eluted with acidic methanol. The fractions containing the bis(monoazidomethidium) spermine were collected and concentrated. The concentrated sample was filtered through a fine glass frit, and afterwards the solution was evaporated to dryness. The solid BAMS was redissolved in a minimum volume of 1 N HCl, and the products were reprecipitated with tetrahydrofuran. The oily BAMS was collected by centrifugation and dried under reduced pressure. Overall, the synthesis yielded 100 mg of purified BAMS. IR (KBr): Figure 31; NMR (D_6-DMSO): δ 9.0-7.3 and 6.3 (m, 20H, aromatic), 3.98 (m, 6H, CH_3), 3.5 (m> 6H, NH and H_2O), 3.10 and 1.77 (m, 20H, CH_2). Secondary Ion Mass Spectroscopy: m/z = 879 (-N_2) and m/z = 850 (-2 N_2).

1,12-Diamino-4,9-diazodecyne-6.[51] The hood and reaction flask were covered with aluminum foil to protect the light sensitive starting material, 1,4-dichlorobutyne-2. A 100 mL, 3-neck round-bottom flask equipped with an argon inlet and sealed with two septum stoppers was filled with 50 mL of 1,3-diaminopropane (Aldrich, 98%). While the diamine was magnetically stirred, 6.1 g of 1,4-dichlorobutyne-2 (Aldrich, 99%) was added in increments of 0.2 mL over 12 h. The reaction was then allowed to stir an additional 12 h before 11 mL of 10 M KOH were added to the mixture. The precipitated salts were filtered out of the reaction, and the low boiling compounds were distilled from the product 1,12-diamino-4,9-diazadecyne-6. The product was redissolved in CH_2Cl_2, and the solution was filtered. The CH_2Cl_2 was evaporated and the viscous oil was Kugelrohr distilled. This yield 4.8 g of analytically pure 1,12-diamino-4,9-diazadecyne-6. IR (neat): Figure 32; NMR (CCl_4) δ 3.3 (s, 4H, CH_2), 2.6 (m, 8H, CH_2), 1.5 (q, 4H, J = 9 Hz, CH_2),



BAMS

Figure 31



1,12-Diamino-4,9-diazodecyne-6

Figure 32

1.1 (s, 6H, NH and HN_2); ^{13}C NMR (CCl_4) δ (TMS = 0ppm),33.20,38.21, 40.29, 46.34, 81.36 ppm.

Elemental analysis calcd. for $C_{10}H_{22}N_4$: C, 60.57; H, 11.18; N, 28.25. Found: C, 60.53; H, 10.95; N, 28.06.

(Tetramethylene-2,3-^{3}H) Spermine.[51] The following procedure was devised using H_2. The procedure and the polyamino-alkyne was then sent to New England Nuclear for conversion of the 1,12-diamino-4,9-diazadecyne-6 into tritium labeled spermine, (Tetramethylene-2,3-^{3}H) spermine. 200 mg of starting material yielded 4 Ci of ^{3}H activity.

104 mg of 1,12-diamino-4,9-diazadecyne-6 was dissolved in 0.8 mL of dry ethanol. 40 microliters of glacial acetic was added to the ethanol and, the solution was transferred to a microhydrogenator. The reaction mixture was degassed, and 30 mg 10% Pd/C were added to the mixture. The reaction was placed under 10 lbs (gauge) of H_2 and hydrogenated for 24 h.

After the hydrogenation was completed, the catalyst was filtered off the reaction and an excess of HCl was added to the ethanol. The reaction solvent was evaporated, and the salts were redissolved in distilled water. NaOH was added to the aqueous solution of spermine, and the water was evaporated. The solids were then triturated with CH_2Cl_2, and the CH_2Cl_2 was cleared of solids. Evaporation of the CH_2Cl_2 yielded 65 mg of spermine (62%). NMR ($CDCl_3$) δ 2.6 (m, 12H, CH_2), 1.5 (m, 8H, CH_2), 1.3 (s, 6H, NH and HN_2).

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CHAPTER II

Experiments in the Design of Site Specific DNA Cleaving Agents

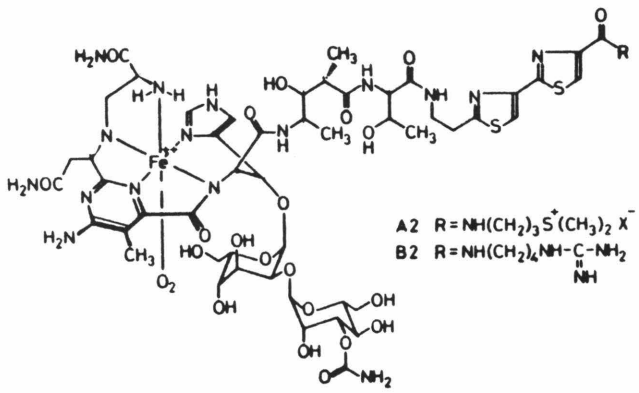
A. Introduction

It was recognized that the specificity and affinity of an intercalator[1-7] might be useful in delivering chemical functionality and/or chemical activity to the nucleic acid double helix,[18] the general strategy being the design of a synthetic route to piggyback a chemically reactive unit on a vehicle with nucleic acid destinations.[18]

The natural product bleomycin, has a chemically reactive unit tethered to a DNA specific vehicle. Bleomycin consists of a glycosylated pseudotetrapeptide that is capable of chelating a metal ion, and of tripeptide S vehicle that is thought to bind DNA at guanine residues[8-10] (Fig. 1).

In the presence of Fe(II) and O_2, bleomycin can mediate an efficient cleavage of DNA at $^{5'}GC^{3'}$ and $^{5'}GT^{3'}$ sites.[11-13] The oxygen dependent strand scission yields a 5' phosphate, a 3' glycolate, and a base propenal. In the absence of oxygen Fe(II)-bleomycin attack yields free base and base labile apyrimidinic sites[14-17] (Fig. 2). It is hypothesized that the antitumor properties of bleomycin may be due in part to the unrepairable double stranded cleavage of "host" DNA induced by activated bleomycin.

With bleomycin as a model, Hertzberg and Dervan designed a molecule to deliver a chemically reactive unit to the DNA double helix. The molecule synthesized, methidiumpropyl-EDTA (MPE) consists of a metal chelator, ethylenediamine tetraacetic acid, tethered by a diaminopropane linker to a nucleic acid intercalator, paracarboxylmethidium chloride (Fig. 3).[18] Like bleomycin, MPE cleaves DNA in the presence of Fe(II) and O_2, however, MPE is less efficient than bleomycin. A reducible co-factor (dithiothreitol-DTT) must be added to MPE reactions to obtain activity comparable to the natural



The proposed structure for bleomycin-Fe(II) complex

Figure 1

Reaction	①	②	③	④	⑤
$t_{1/2}$	n.d.	0.2s	6s	2min.	n.d.
EPR	SILENT		SILENT	g=2.26, 2.17, 1.94	g=2.45, 2.18, 1.89	g=4.3
λ_{max} (nm)	370, 476		385	365, 384	365, 384	

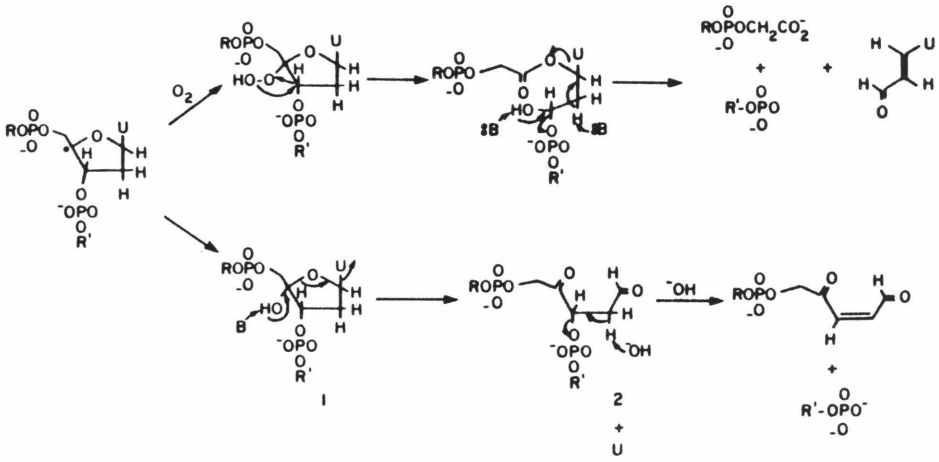


Figure 2

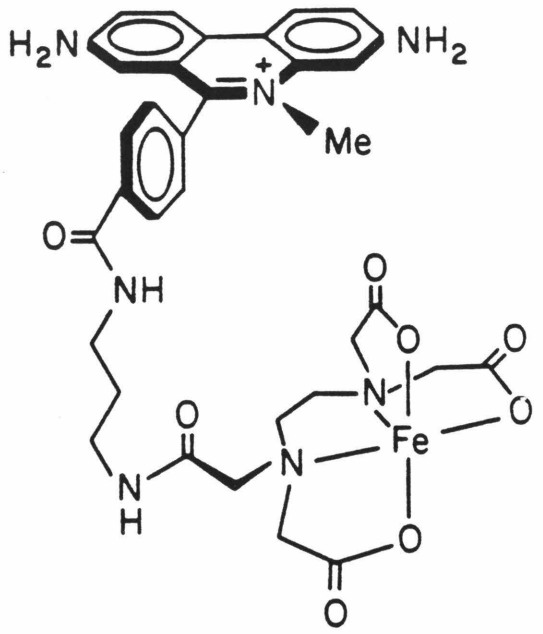
antibiotic.

Unlike bleomycin, MPE cleaves DNA randomly; it reflects the nonspecific intercalation of methidium.[19-21] As a nonspecific DNA cleaving agent, MPE may be considered the synthetic equivalent of DNase.

Although MPE like bleomycin uses Fe(II) as a co-factor for the cleavage of DNA, the mechanisms of the two compounds must diverge. In contrast to bleomycin, MPE·Fe(II) reactions exhibit a stoichiometric relationship of free base to strand scission, and a mixture of products at the 3' cleavage termini.[24] These characteristics are similar to the damage induced by X-ray irradiation of nucleic acid and suggest that the active intermediate in MPE·Fe(II) reactions is hydroxyl radical (Fig. 4).

Whatever else future experiments might intimate about the mechanism of MPE·Fe(II) cleavage they will not refute the observation that a properly delivered EDTA·Fe(II) can efficiently mediate the single strand cleavage of DNA. Having demonstrated that it is possible to piggyback a chemically reactive molecule on a DNA binding vehicle, the logical extension of this line of reasoning calls for the synthesis of a site selective cleaving agent.

The naturally occurring antibiotic distamycin[25,26] is known to preferentially bind in the minor groove of A-T double helices. The amide hydrogens of these polypyrrole molecules recognize A-T base pairs by hydrogen bonding to the O(2) of thymine or the N(3) of adenine[27-30] (Fig. 5). Schultz and Dervan recognized that an EDTA could be easily appended to either end of the distamycin tripyrrole and hoped that the EDTA would be selectively delivered to sequences specifically recognized by the distamycin



MPE

Figure 3

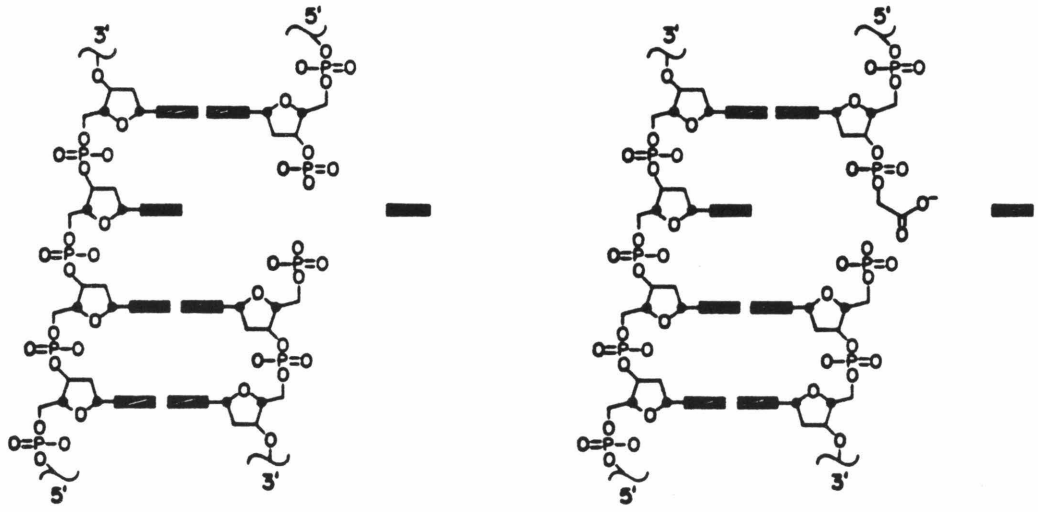
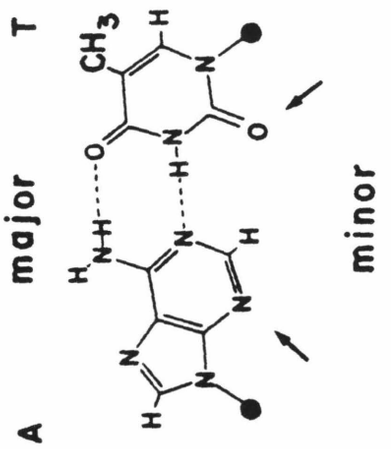


Figure 4



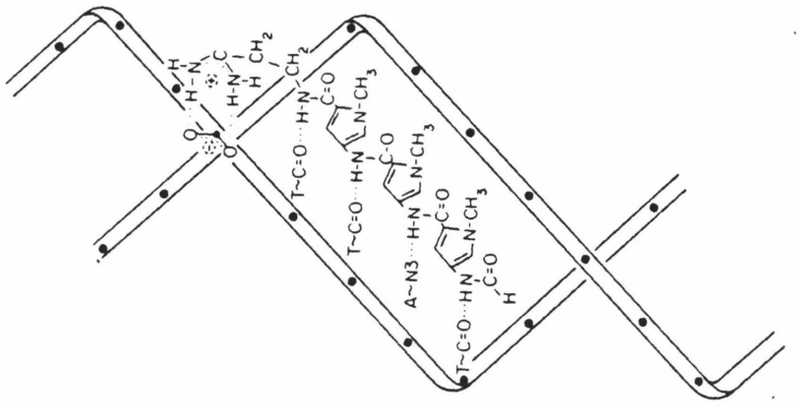
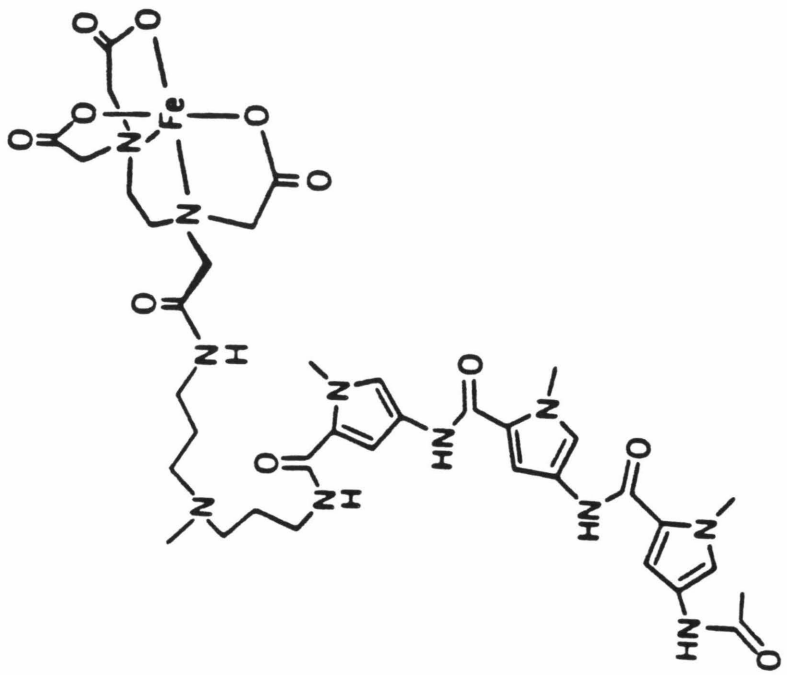


Figure 5

analog; subsequently, distamycin-EDTA (DE) and EDTA-distamycin (ED) were synthesized (Fig. 6).[31,32]

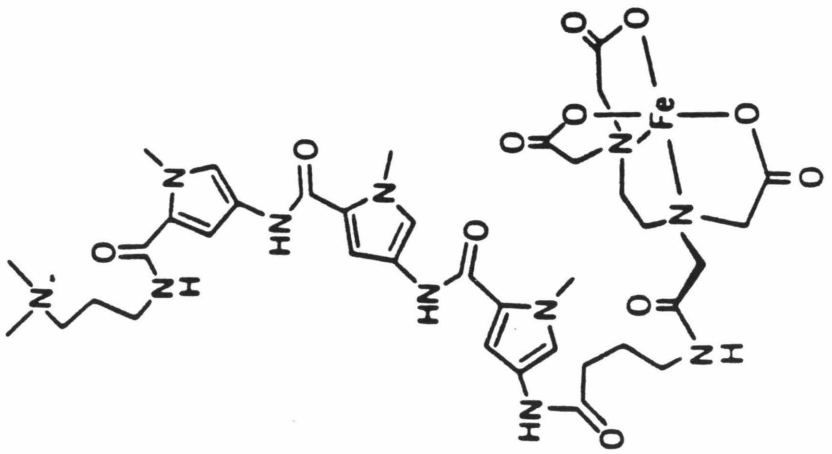
Analysis of the cleavage patterns of these two molecules revealed single strand scission at pentanucleotide recognition sites containing at least three adenines or three thymines, i.e., $^{5'}$AAATT$^{3'}$ and $^{3'}$AATAA$^{3'}$. Interestingly, nicking occurs at both ends of the recognition site, but with a consistent directional inequality; one end of the recognition site is preferentially cleaved. Since the directional inequality of DE and ED cleavage point in opposite directions, the tripyrrole must bind with a specific directional preference that the tethered EDTA does not significantly perturb.[32] Such information is an example of the advantages of affinity cleavage over footprinting in determining the binding characteristics of a small molecule.

The success of DE and ED lead to the synthesis of three new cleaving agents, penta-N-methylpyrrolecarboxamide-EDTA (P5E),[33] bis(EDTA-distamycin) (BED),[34] and EDTA-(bisdistamycin) (EBD).[34] The construction of these molecules was again based on the polypyrrole subunit of distamycin, but in this case the molecules were designed to recognize sequences longer than 5 base pairs (Fig. 7). Experimental analysis revealed that P5E·Fe(II) cleaves adjacent to sequences of six adenines and thymines (i.e., $^{5'}$TTTTTA$^{3'}$),[33] while BED·Fe(II) and EBD·Fe(II) fragment the DNA helix beside sequences of eight adenines and thymines (i.e., $^{5'}$TTTTTATA$^{3'}$).[34] Surprisingly, all three molecules were shown to cleave both strands of the DNA helix.[33,34] In short, P5E·Fe(II), BED·Fe(II) and EBD·Fe(II) can bind a specific recognition site and double strand cleave the DNA. In fact, digestion of pBR322 with any

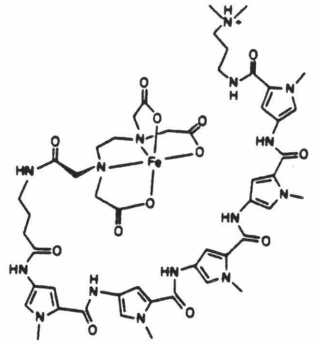


ED

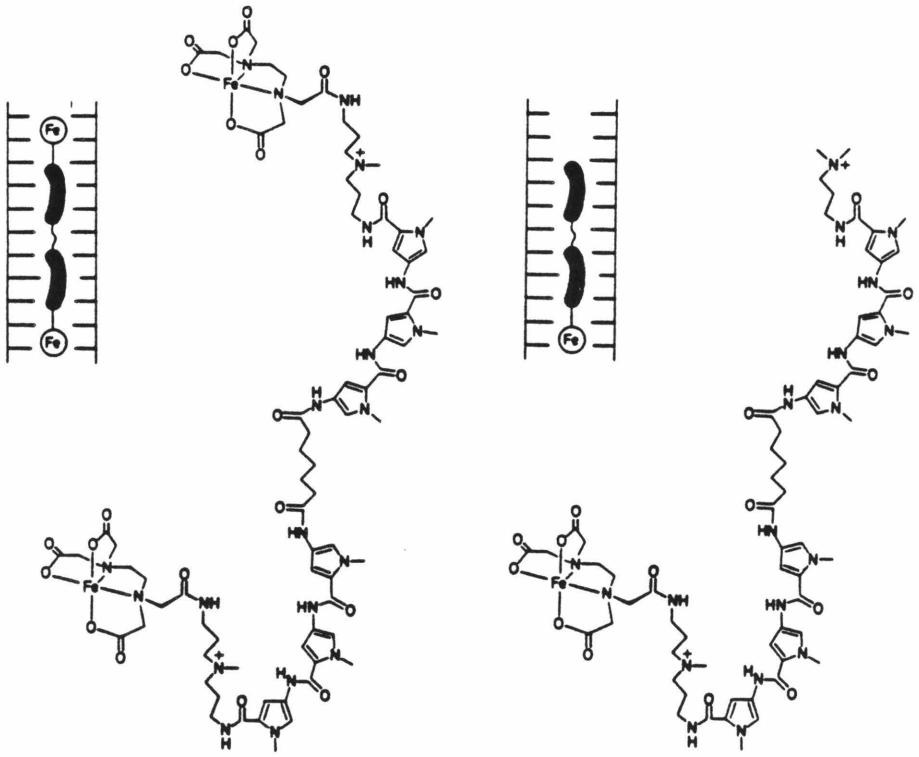
Figure 6



DE



P5E · Fe (II)



BED · Fe (II)

EBD · Fe (II)

Figure 7

one of these three reagents produces discrete fragments on a nondenaturing electrophoretic gel.

What began as a simple exercise in delivered chemical functionality has now evolved into the design of site specific DNA cleaving agents. Although P5E·Fe(II), BED·Fe(II), and EBD·Fe(II) are primitive, first generation molecules, their specificity and activity suggest that an efficient cleaving system may eventually become useful. In the interim, a problem that must be confronted is the design of specificity. How can a molecule be sculptured to recognize a defined sequence of any length?

It is well known that a small single stranded oligonucleotide will anneal to its complement to form a double helix. The renaturation reaction is highly specific and allows an oligonucleotide to locate a homologous sequence among a large excess of random sequences. If an EDTA·Fe(II) could be appended to a single stranded fragment of DNA, the cleaving agent could be delivered to any sequence of choice. As a consequence it might be possible to cleave DNA at experimentally convenient sites instead of at the limited number of recognition sites for known restriction enzymes.

B. Results and Discussion

1. Oligonucleotides. Chemical synthesis of DNA has progressed very rapidly over the past five years. Fully protected nucleotide precursors have emerged; coupling reactions and reagents have been optimized -- typical nucleotide condensations exhibit coupling yields of greater than 95% -- solid state synthesis technology has matured, and deprotection/purification schemes have been simplified.[35-39]

With the commercial availability of solid state supports, protected dinucleotides, and appropriate coupling agents, it is possible to synthesize and purify approximately one milligram of a 15 nucleotide sequence within a period of two weeks.[40] This ease and speed of solid state DNA synthesis makes the technology an attractive route for the synthesis of an oligonucleotide-EDTA. In fact, there are several obvious strategies for appending an EDTA to a synthetic oligonucleotide.

After careful consideration of the chemical details of solid state synthesis, it was decided that an 11 nucleotide sequence of thymines would be appropriate for a first attempt at synthesizing an an oligonucleotide-EDTA. The sequence, dT_{11}, minimizes chemically incompatible reactions and provides a simple substrate for the development of new chemistry. What remained was the solution for attaching EDTA to an oligonucleotide.

(a) EDTA-(triethyl ester)-(mono-N-(3-hydroxypropyl)amide) in the Design of an Oligonucleoltide-EDTA. Initially, it was thought that an EDTA could be introduced as a phosphorylated EDTA-(triethyl ester)-(mono-N-(3-hydroxypropyl)amide), **2.** With this unit the EDTA could be coupled to the oligonucleotide via known condensation technology; however, attempts to synthesize either the fully protected unit, **3,** or the corresponding acid, **2,** from the EDTA-triester-monoamide, **1,** were unsuccessful. The reactions produced a myriad of inseparable and unidentifiable products.

The next scheme involved the in situ coupling of EDTA-triester-monoamide, **1,** to dT_{11} via the chlorophosphate of **1.** After isolation of the nucleotide products and cleavage of the ethyl esters, the dT_{11}-EDTA was assayed for cleavage activity. Unfortunately, end labeled dA_{20} was not

degraded by the purported dT_{11}-EDTA·Fe(II). Since properly delivered EDTA·Fe(II) is known to nick DNA, it was concluded that the EDTA did not couple to the oligonucleotide.

(b) Phosphorylate Linkers in the Design of an Oligonucleotide-EDTA.[43] With the failure to introduce EDTA-linker as a complete unit, the design of the oligonucleotide-EDTA was modified to allow the linker and the EDTA to be appended to the oligomer in separate operations. For this purpose the phosphorylated linker, N-(4,4'-dimethoxytrityl)-5-aminopentyl-1-(0-2-chlorophenyl)phosphate (**4**) was synthesized. The linker is easily coupled to a supported oligonucleotide, and known condensation chemistry gives coupling yields of greater than 85%.

In a first attempt to couple an EDTA to the linker, the linker-dT_{11} was deprotected, and the solid supported free amine was treated with the N-hydroxysuccinimide ester of EDTA-triethyl ester. The oligonucleotide was then removed from the support, and the nucleotide fractions were treated with LiOH. Purified cleaving agent was then assayed with dA_{20}, but no appreciable activity was observed.

If the EDTA could be coupled to the linker after the oligonucleotide has been removed from the support, protected linker-dT_{11} may be separable from other nucleotide products. Unfortunately, the dimethoxytrityl group is extremely labile and is lost during the cleavage of the oligomer from the solid support. Without the trityl group, separation of the linker-oligomers from smaller oligonucleotides by reverse phase high pressure chromatography is no longer possible; consequently, the total mixture of purified oligomers was treated with the N-hydroxysuccinimide ester of EDTA-triethyl ester.

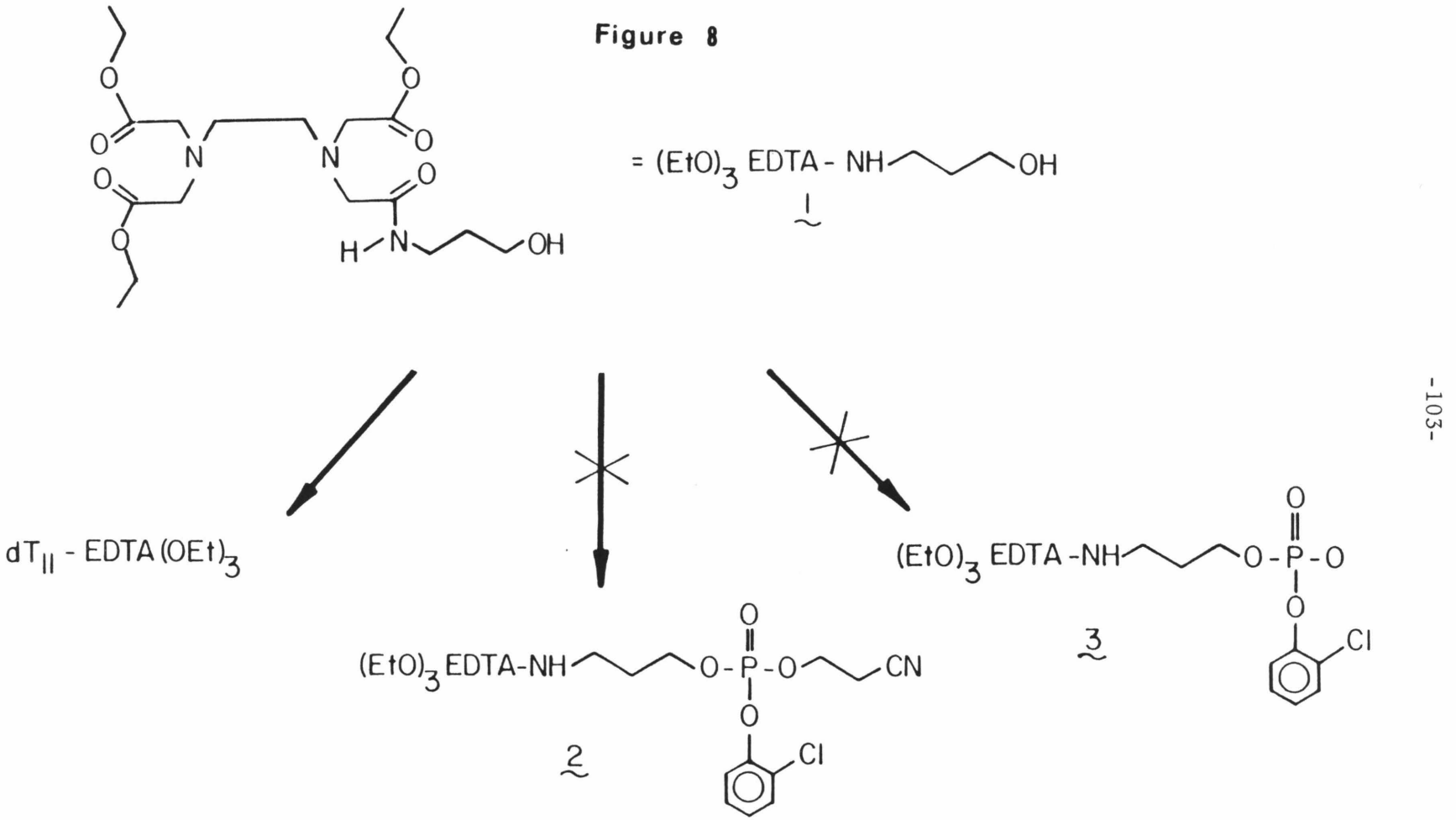
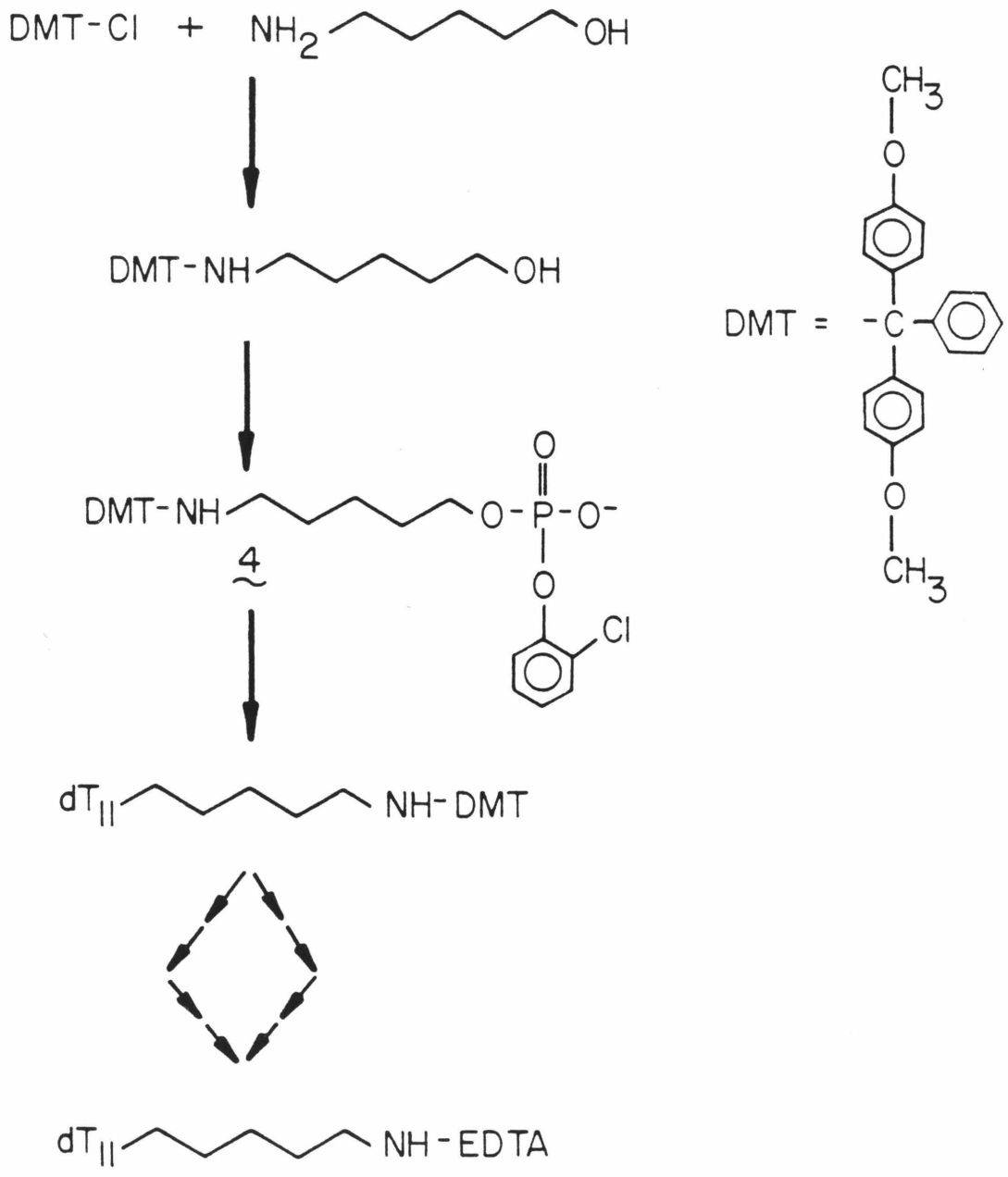

= $(EtO)_3$ EDTA - NH OH
1
dT_{II} - EDTA $(OEt)_3$
$(EtO)_3$ EDTA-NH O-P-O CN
O
Cl
2
$(EtO)_3$ EDTA -NH O-P-O
O
Cl
3

Figure 8

Figure 9


DMT-Cl + NH2 OH
DMT-NH OH
DMT-NH O-P-O-
4
O
Cl
dT11 NH-DMT
dT11 NH-EDTA
DMT =
CH3
O
O
CH3

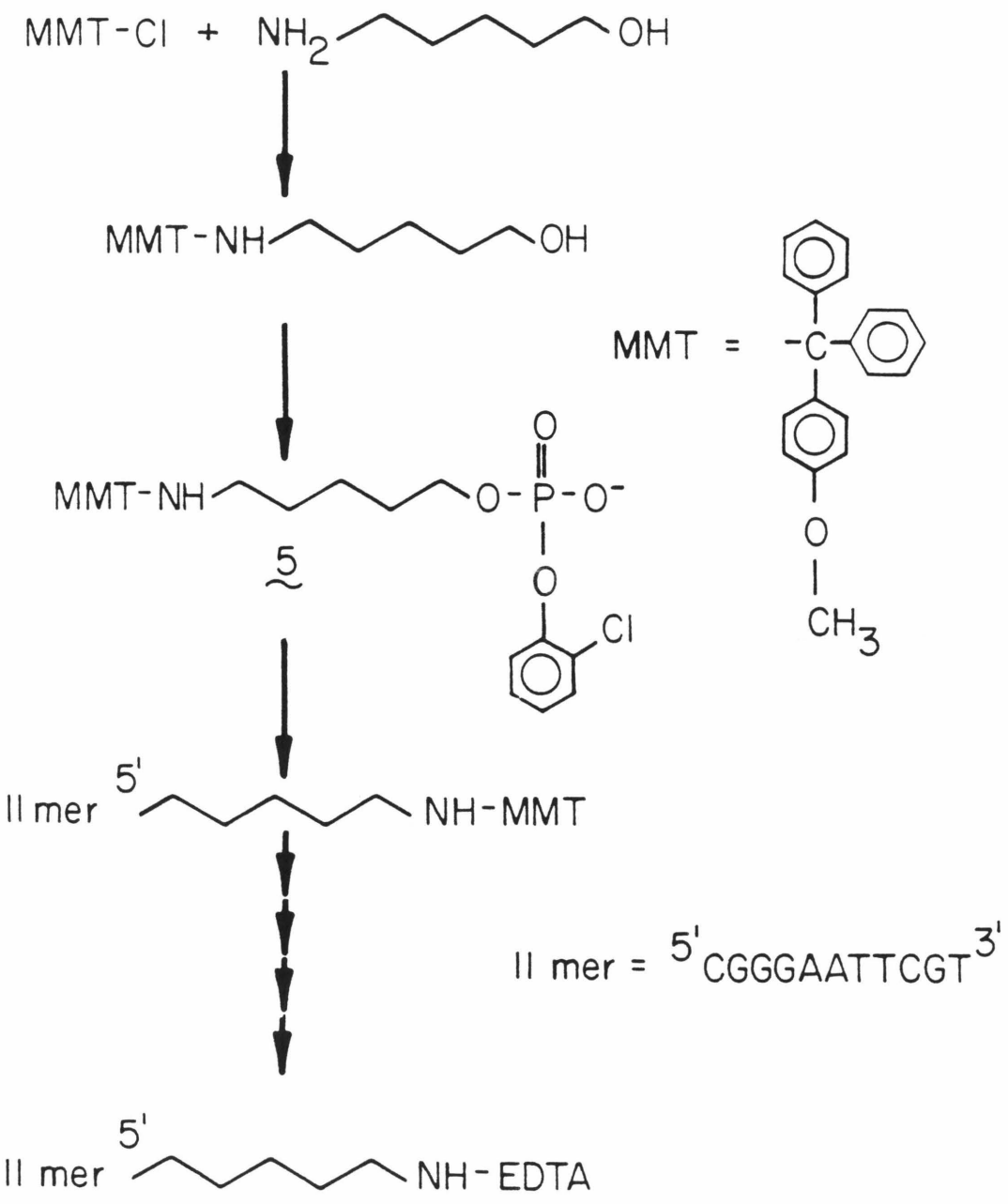
The nucleotide fractions recovered from the reaction were treated with LiOH and assayed for activity. In this case, a more sensitive assay was adapted from Sanger methodology and relied upon the truncation of DNA synthesis by runoff at the site of nicking on a single stranded DNA substrate.[42,43] The assay, however, was poorly chosen and gave ambiguous information.

To improve the cleavage assay it was necessary to switch to a substrate that was commercially available as both single stranded and double stranded DNA. M13 mp8 was the logical choice. As a consequence, dT_{11} was no longer useful, and the oligonucleotide, $^{5'}CGGGAATTCGT^{3'}$, was chosen to replace it.

The heterogeneous 11mer forms a more stable double helix than dT_{11}; as such, the 11mer might provide a more positive delivery of an appended EDTA·Fe(II). The disadvantage of the heterogeneous 11mer is the introduction of a reaction to deprotect the cytosines, guanines and adenines. Hot concentrated ammonium hydroxide is not compatible with an ester. The EDTA-triester must either be hydrolyzed before the sequence is deprotected or be appended to the oligomer after the basic hydrolysis. In either case, the latitude in introducing the EDTA chelator is seriously constricted.

Further improvements to the design of the experiment were confined to the linker. Monomethoxytrityl was substituted for the dimethoxytrityl protecting group. The linker, N-(4-monomethoxytrityl)-5-aminopentyl-1-(0-2-chlorophenyl)phosphate, **5**, could be coupled to the oligonucleotide in greater than 95% yield. Analysis of the products suggested that approximately 50% of the nucleotide fraction bore a linker, but even though the monomethoxytrityl group was stable to both the cleavage from the solid

Figure 10


MMT-Cl + NH2 OH
MMT-NH OH
MMT-NH O-P-O⁻ O Cl
≈5
11 mer 5' NH-MMT
11 mer 5' NH-EDTA
MMT = C O CH3
11 mer = 5' CGGGAATTCGT 3'

support and the basic deprotection reaction, the protected linker-oligomer still could not be purified by reverse phase high pressure chromatography. Apparently, the columns catalyzed the release of the trityl group.

Again, the total nucleotide fraction was treated with the N-hydroxy-succinimide ester of EDTA-triethyl ester, and the oligomers were purified from the reaction. The products were then subjected to 0.25 M LiOH and assayed for activity. The 11mer-EDTA was annealed to a single stranded end labeled fragment of M13 mp8, and Fe(II)/dithiothreitol were added to induce cleavage of the DNA. Specific strand scission would produce a discrete set of fragments on a high resolution polyacrylamide denaturing gel, but all that was observed was random nicking.

The failure of the 11mer-EDTA led to a modification of the EDTA coupling reaction. Instead of appending the EDTA in the last construction, the chelator would be attached to the oligonucleotide and deprotected before the reagent was cleaved from the solid support. For this purpose the N-hydroxysuccinimide ester of EDTA-triethyl ester was replaced with the dianhydride of EDTA. Further modifications included the shortening of the linker to bring the EDTA closer to the DNA helix.

The new linker N-(4-monomethoxytrityl)-2-aminoethyl-1-(0-2-chloro-phenyl)phosphate, **6**, could be coupled to the supported 11mer in greater than 90% yield. The linker was then deprotected with $ZnBr_2$, and the free amine was allowed to react with excess EDTA dianhydride. After hydrolysis of the substrate bound anhydride the reagent was cleaved from the support and deprotected with NH_4OH. Purified oligomer was finally assayed for activity, however, the M13 mp8 assay showed only random cleavage.

To determine where problems were arising, the synthesis was repeated with ^{14}C labeled EDTA dianhydride. Tracer analysis of the product fractions indicated that EDTA was incorporated in less than 5% yield. Fortunately, the tagged product could be enriched by gel permeation chromatography. The enriched fractions appeared to contain 50% 11mer-EDTA, but assays of these fractions again showed only random strand scission.

(c) Alternative Cleaving Agents in the Design of Functionalized Oligonucleotides. Frustration with the oligonucleotide-EDTA syntheses led to the examination of two alternative cleaving agents. The first alternative utilizes a phenanthroline chelator in place of EDTA, while the second alternative employs liganded ruthenium in place of iron.

The strategy for coupling a phenanthroline to an oligonucleotide is exactly analogous to the coupling of an EDTA to an oligomer, but in this case there are no protecting groups to interfere with the nucleotide synthesis. Diimidazole activated 2-carboxy-1,10-phenanthroline, 7, was allowed to react with the ethylamine linker of a heterogeneous 11mer. The oligonucleotides were isolated and assayed, but the purported 11mer-phenanthroline gave no specific cleavage of the end labeled DNA substrate.

The second strategy makes use of ruthenium(II) pentamine. Like Fe(II), Ru(II) is capable of nicking DNA, but unlike iron, ruthenium can be directly bound to a variety of nitrogen functional groups. In fact, ruthenium pentamine binds the N7 of guanine; consequently, ruthenium can be bound to DNA by simply soaking the nucleic acid in an aqueous solution of ruthenium pentamine. With this procedure, ruthenium was liganded to $^{5'}$CGGGAATTCG$^{3'}$. It was hoped that the oligonucleotide would

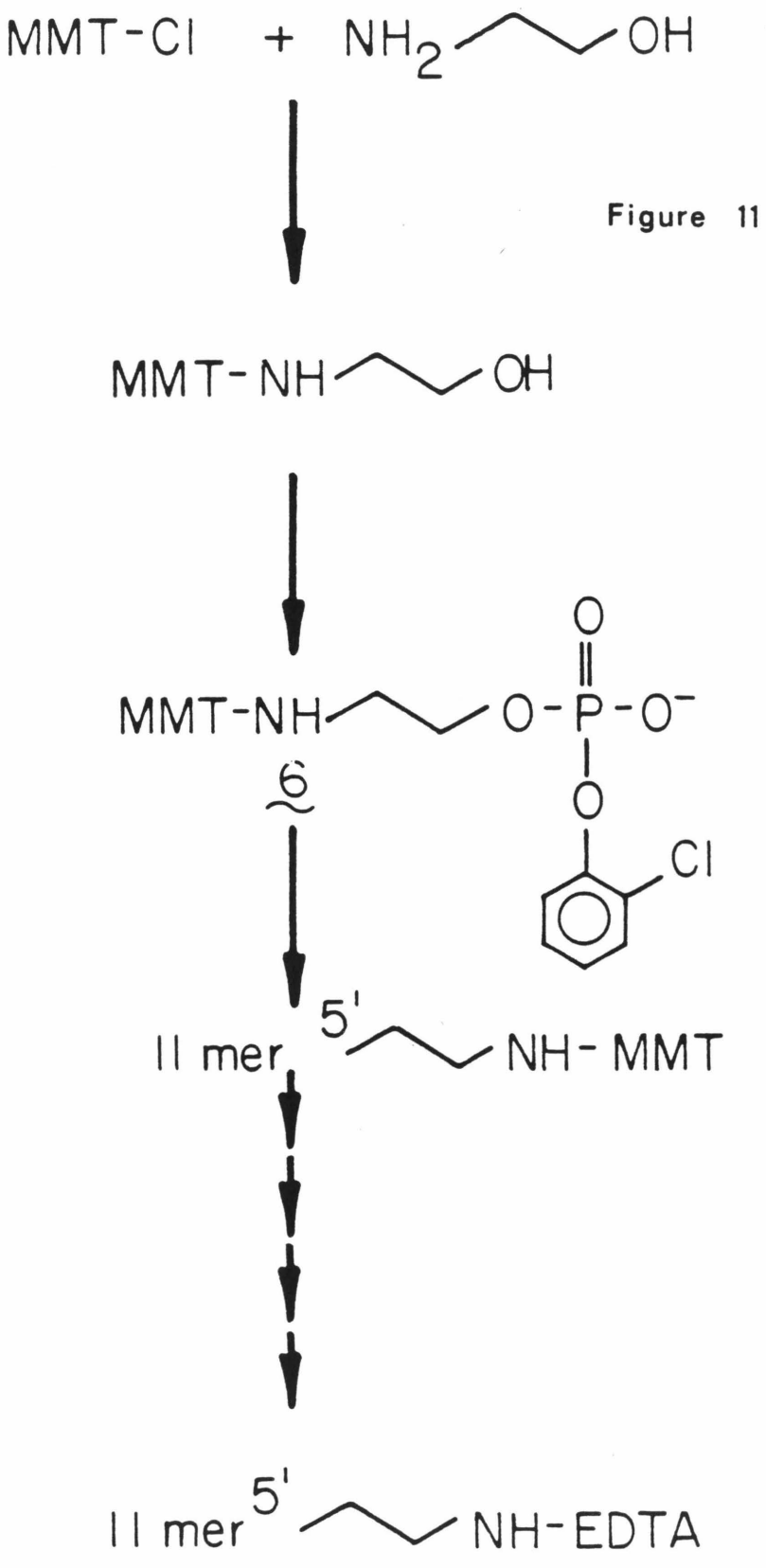

MMT-Cl + NH2 OH
MMT-NH OH
MMT-NH
O
O-P-O⁻
O
Cl
6
11 mer
5'
NH-MMT
11 mer
5'
NH-EDTA

Figure 11

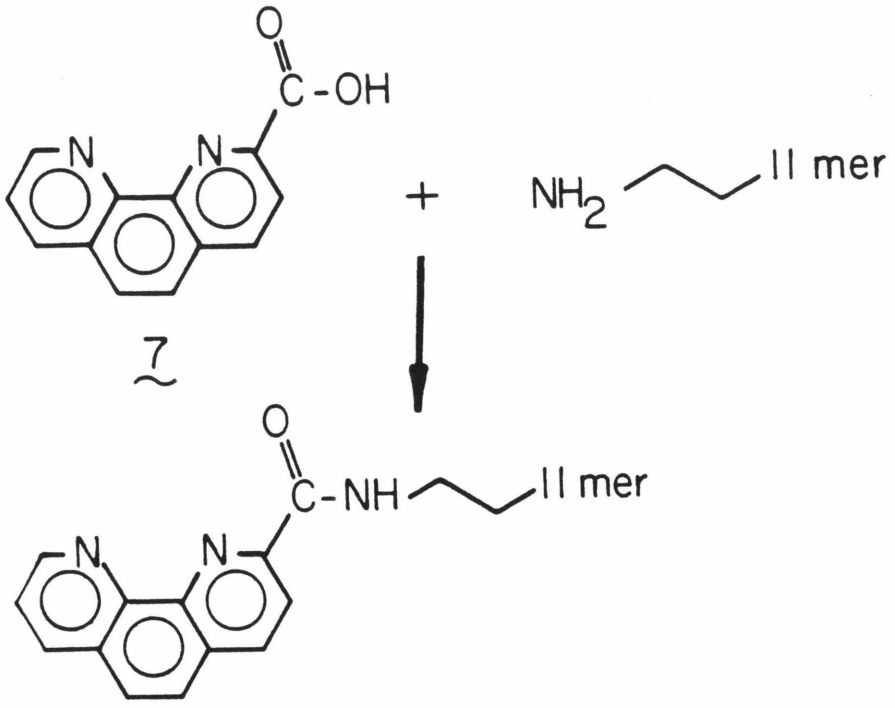


Figure 12

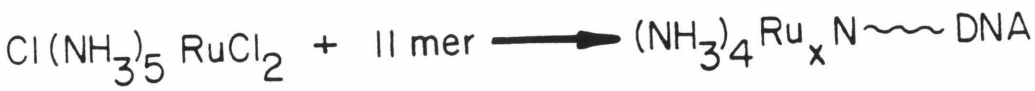


Figure 13

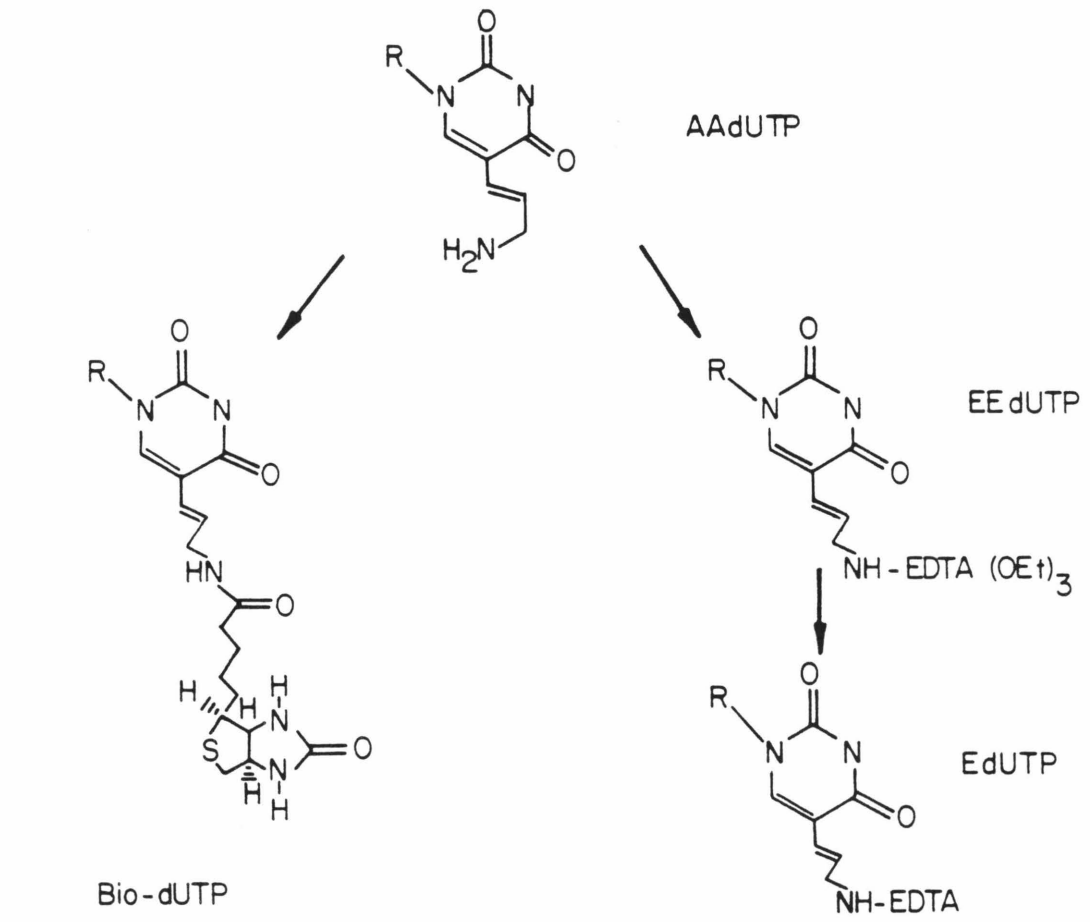
specifically deliver the ruthenium to the target substrate, but in actuality, it was found that the functionalized oligomer no longer formed a stable double helix and did not deliver the ruthenium to the target.

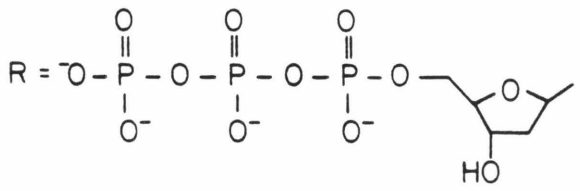
2. EDTA-dUTP. In a last effort to design a controlled site DNA cleaving agent, the synthetic routes were abandoned and a scheme exploiting commercially available enzymes and recombinant methodology was adopted. It is well known that DNA polymerases will accept a wide variety of nucleotide triphosphates for polymerization into DNA. Langer, Waldrop and Ward demonstrated that biotinylated-deoxyuridine-5'-triphosphate (Bio-dUTP) is an acceptable substrate for DNA polymerase I and is efficiently incorporated into nicks or gaps in double stranded DNA.[44] If an EDTA could be attached to deoxyuridine-5'-triphosphate (dUTP) the cleaving agent might be delivered to its substrate by direct polymerization of the modified nucleotide into DNA.

EDTA-dUTP (EdUTP) was synthesized by coupling the N-hydroxy-succinimide ester of EDTA-triethyl ester to 5-(3-aminoallyl) deoxyuridine-5'-triphosphate, triethylamine salt (AA-dUTP) in dimethylsulfoxide. $(EtO)_3$EDTA-dUTP (EEdUTP) could then be purified from the reaction mixture by gradient elution from a DEAE A25 Sephadex column. Analysis of the triphosphate fractions by high pressure reverse phase chromatography revealed that the compound was 90 to 95% pure. It was then shown that EEdUTP was an acceptable nucleotide triphosphate for large fragment DNA polymerase I.

To assay EEdUTP, the modified nucleotide was polymerased into the

Figure 14





Hind III site of M13 mp8, and the Hind III/Bgl I fragment was isolated. The fragment was then treated with pH 11 LiOH to cleave the esters of EEdUTP. After annealing the cleavage probe to an M13 mp8 Eco RI/Bgl I fragment that had been 5' labeled at the Eco RI restriction, Fe(II) and DTT were added to the reaction to induce cleavage of the DNA. Specific strand scission would show discrete fragments approximately 30 base pairs long; unfortunately, only random background cutting was observed.

To improve the sensitivity of the experiment the assay was repeated with T4 polymerase. The polymerase was allowed to cut back from the Hind III site of M13 mp8 and the single stranded "gap" was refilled. This procedure replaced 20 to 40 nucleotides. The Hind III/Bgl I fragment was again isolated and the probe was assayed against the labeled substrate. Once more, only random background cleavage was observed.

C. Summary

The compounds methidiumpropyl-EDTA, distamycin-EDTA, and penta-N-methylpyrrolecarboxamide-EDTA are three representative examples of a class of rationally designed DNA cleaving agents. By appending an iron chelator to different DNA binding vehicles the properties of a cleaving agent can be controlled. Methidium propyl-EDTA randomly single strand nicks DNA with high efficiency. Distamycin-EDTA nicks at 5 base pair recognition sites, and penta-N-methylpyrrolecarboxamide-EDTA double strand cleaves DNA at 6 base pair recognition sites. With the success of these compounds, a site controlled cleaving agent was envisioned. Maybe an EDTA could be

directly attached to a short length of DNA.

The first attempt at synthesizing an oligonucleotide-EDTA was designed around known nucleotide triester technology. A small length of single stranded DNA can be easily synthesized by solid state techniques. There are also a few obvious synthetic routes that might allow the sequence to be capped with an EDTA. The failure here to synthesize an active oligonucleotide-EDTA does not directly reflect on the viability of the cleaving agent, it simply suggests that known synthetic technology is inadequate for the synthesis of an oligonucleotide EDTA and that new chemistry must be developed to positively construct the nucleotide delivered cleaving agent.

The second approach to the problem of a controlled site cleaving agent is not totally synthetic. EEdUTP can be synthesized from EDTA triethyl ester and 5-(3-aminoallyl)deoxyuridine-5'-triphosphate. The EEdUTP can then be enzymatically incorporated into a short strand of DNA to produce a DNA-EDTA cutting probe. Immediate assays of the cleaving agent were again negative, but in the long term an extended analysis of the methodology for handling EEdUTP may eventually turn up a protocol for creating an active cutting probe.

In conclusion, this investigation has not closed the door on the synthesis of controlled site cleaving agents. The logic behind the designs of an oligonucleotide-EDTA or a DNA-EDTA still appears to be sound. What needs to be solved are the methods of the synthetic execution and the techniques for the direct physical characterization of the "synthesized" cleaving agents.

Materials and Methods

Oligonucleotides. The oligonucleotides, dT_{11} and $^{5'}CGGGAATTCGT^{3'}$, were synthesized *via* solid state triester technology as described by Tan *et. al.*[40] The solid support was obtain from Vega Biochemicals, while the protected dinucleotides and the coupling agent, 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole, were purchased from P-L Biochemicals. The remaining reagents and solvents were supplied by J. T. Baker, Aldrich or Fluka.

EDTA-(triethyl ester)-(mono-1-(3-hydroxypropyl)amide), 1. A solution of 5.7 g EDTA triethyl ester (100 mol %),[45] 4.2 g 1-hydroxybenzotriazole (Aldrich, mp 155-158°C, 200 mol %), and 2.3 g 3-amino-1-propanol (Fluka >97%, 200 mol %) in 40 mL of dry tetahydrofuran (THF) was cooled to 5°C in a three-neck 100 mL round-bottom flask that had been fitted with an argon inlet and sealed with two septum stoppers. 4.75 g of N,N'-dicyclohexylcarbodiimide (Aldrich 99%, 150 mol %) were dissolved in 20 mL THF, and the solution was syringed into the round-bottom flask. The reaction was stirred for 40 min at 5°C and was then allowed to warm to room temperature. After 24 h the reaction was filtered, and the THF was evaporated. The EDTA-(triethyl ester)-(mono-N-(3-hydroxypropyl)amide), was purified from the reaction mixture by step elution (5% to 55% THF in dichloromethane over 10% increments of THF) of the product from silica gel, 230 to 400 mesh (Sigma or Woelm Pharma). Repurification of **2** on silica gel (eluent = 7% ethanol in dichloromethane) yielded 5.3 g (80%) of pure material. IR (neat) Figure 15; NMR ($CDCl_3$) δ 4.2 (q, 6H, J = 7 Hz, CH_2), 3.9-3.3 (m, 12H, CH_2), 2.8 (s, 4H, CH_2), 1.7 (q, 2H, J = 6 Hz, CH_2), 1.2 (t, 9H, J = 8 Hz, CH_2).

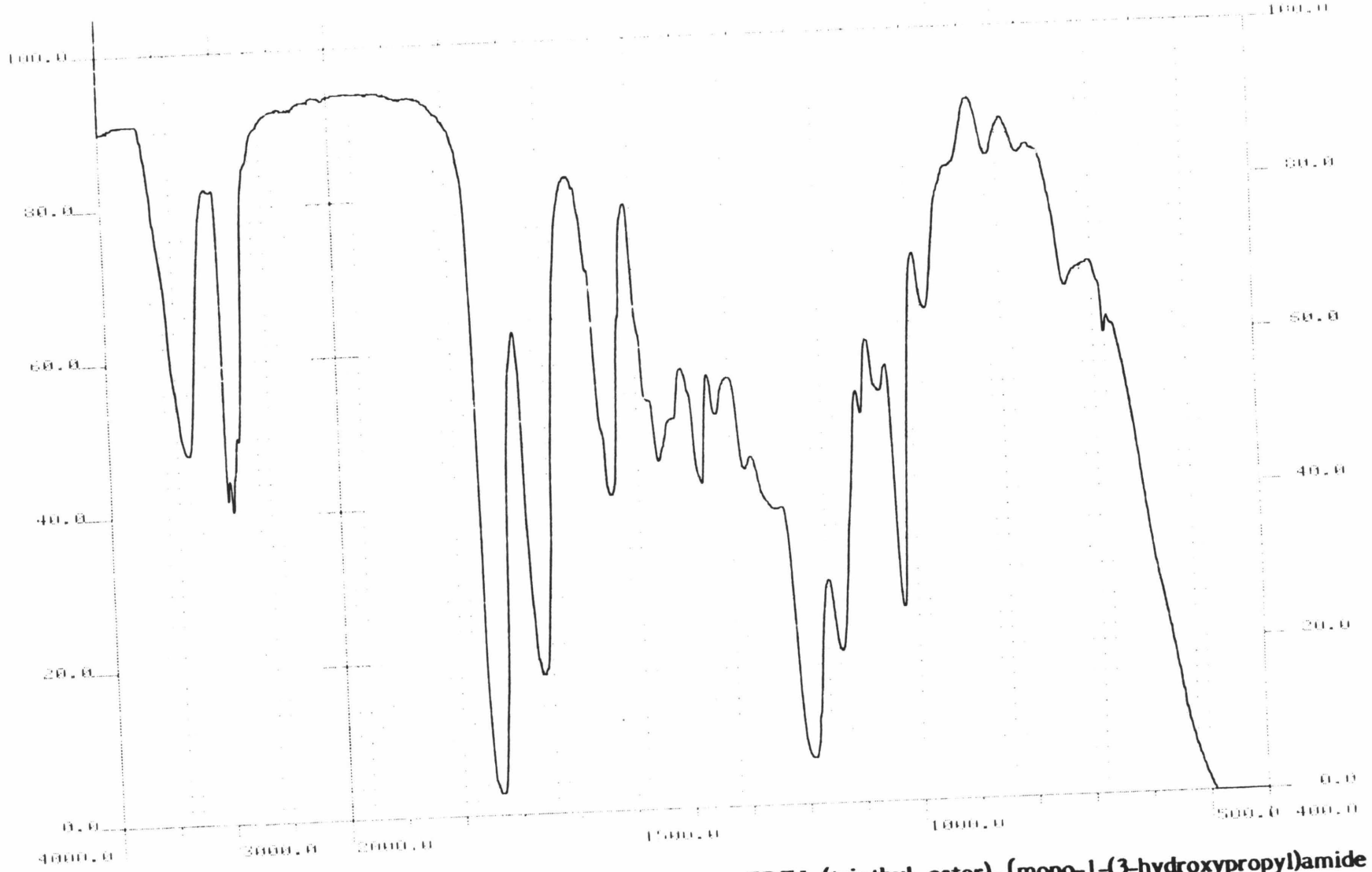


Figure 15

EDTA-(triethyl ester)-(mono-1-(3-hydroxypropyl)amide)

Elemental analysis calcd. for $C_{19}H_{35}N_3O_8$: C, 52.64; H, 8.14; N, 9.69. Found: C, 52.27; H, 7.96; N, 9.69.

EDTA-dT_{11}. Ten milligrams of polystyrene supported dT_{11} was deprotected with $ZnBr_2$ to expose the 5' hydroxyl function. The resin was washed with 1 mL of dry THF four separate times (4 x 1 mL THF), and after drying the resin under reduced pressure the reaction was covered with argon.

Meanwhile, 100 mg **2** were dissolved in 0.5 mL CH_2Cl_2. 67 mg 2-chlorophenyl dichlorophosphate (Fluka, 110 mol %) were added to the CH_2Cl_2 solution, and the reaction was stirred for 15 min.

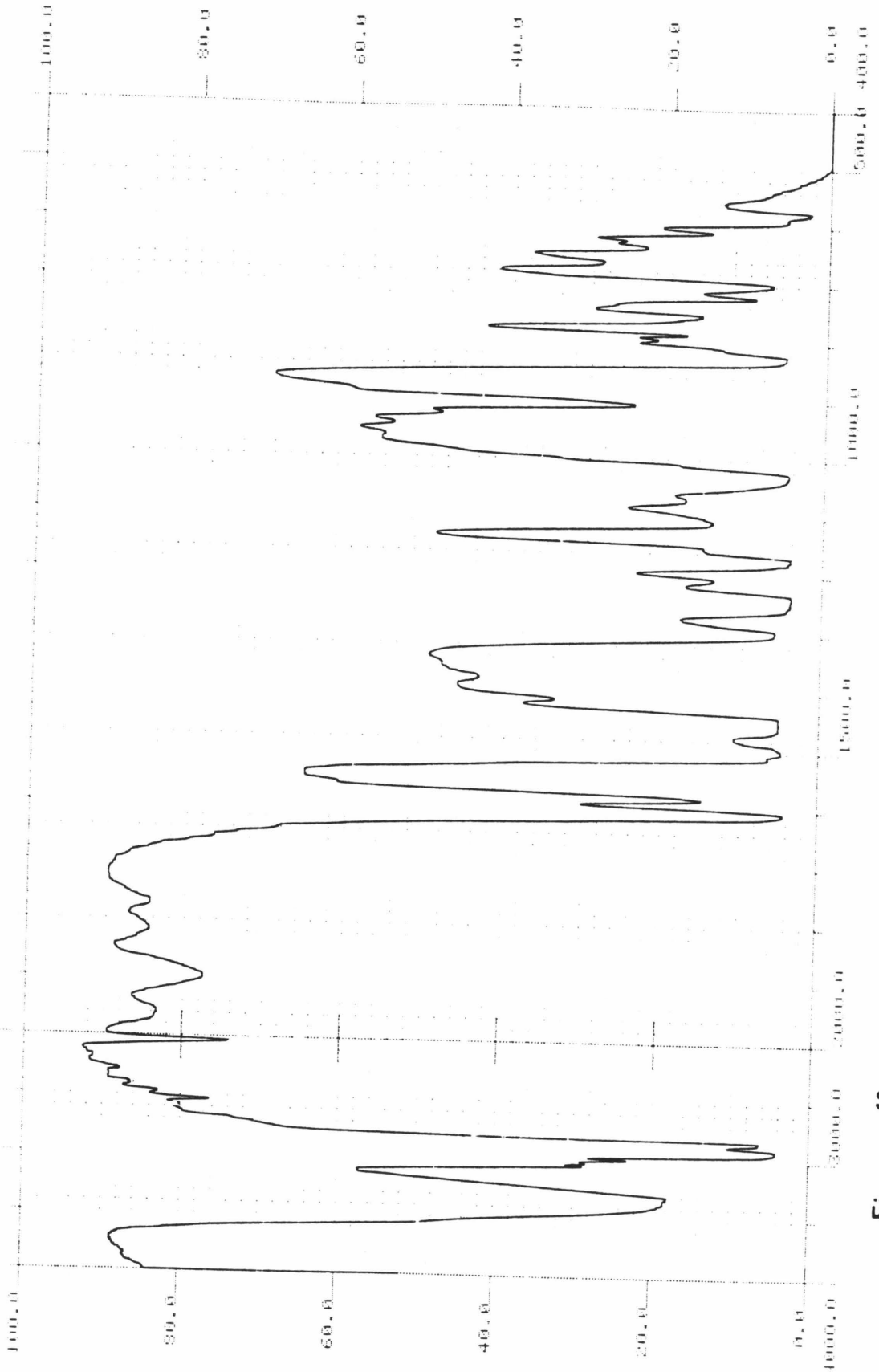
The phosphoprylated EDTA was syringed over the dT_{11} resin, and the mixture was allowed to stand for 1 h. After removing the coupling solution, the support products were washed with 3 x 1 mL CH_2Cl_2, 1 x 1 mL pyridine, 5 x 1 mL 15% isopropanol in CH_2Cl_2 and 4 x 1 mL THF. The nucleotide products were hydrolyzed off the support and the purported EDTA-llmer was precipitated from ethanol and was redissolved in 0.25 M LiOH. Twelve hours later the hydrolysis solution was neutralized and the oligomer was reprecipitated. The pellet yield 2 OD units of nucleotide oligomers (λ_{max} = 264 nm). The fraction was then assayed for specific cleaving activity on dA_{20}.

N-(4,4'-dimethoxytrityl)-5-amino-1-pentanol. In a 100 mL three-necked round-bottom flask equipped with an argon inlet and sealed with two septum stoppers, 1.0 g 5-amino-1-pentanol (Fluka 97%, 165 mol %) and 0.73 g triethylamine (Baker, 120 mol %) were dissolved in 50 mL isopropanol (Baker). 2.0 g 4,4-dimethoxytrityl chloride (Aldrich 98%, 100 mol %) were added to the flask, and the reaction was stirred for 10 h. The volume of the

solution was reduced to approximately 10 mL, and the reaction was poured into 250 mL of ethyl ether. The ethereal solution was extracted with 1 x 250 mL 1 N NaOH and 2 x 250 mL water. The organic layer was dried over Na_2SO_4, and the solvent was evaporated. Trityl-linker was purified from the reaction by column chromatography. Elution of the product from silica gel, 230-400 mesh, with 20% hexane in diethyl ether gave 1.84 g (77%) of pure protected linker. IR (neat) Figure 16; NMR ($CDCl_3$) δ 7.5-6.7 (m, 13H, aromatic), 3.6 (s, 6H, CH_3), 3.5 (t, 2H, J = 5 Hz, CH_2), 2.1 (t, 2H, J = 5 Hz, CH_2), 1.8 (s, 2H, OH and NH), 1.4 (m, 6H, CH_2).

Elemental analysis calcd. for $C_{26}H_{31}NO_3$: C, 77.00; H, 7.71; N, 3.45. Found: C, 77.02; H, 7.70; N, 3.51.

N-(4,4'-dimethoxytrityl)-5-aminopentyl-1-(0-2-chlorophenyl) Phosphate, 4. In a dry 50 mL three-neck round-bottom flask equipped with an argon inlet and two septum stoppers, 257 mg dry 1,2,4-triazole (Fluka >99%, 110 mol %) and 343 mg triethylamine (100 mol %) were dissolved in 20 mL dry THF. 416 mg 2-chlorophenyl dichlorophosphate (100 mol %) were syringed into the reaction flask. After vigorously stirring the reaction for 1 h, the solution was filtered, and the filtrate was transferred to a second three-neck reaction flask. A 2 mL solution of 491 mg N-(4,4'-dimethoxytrityl)-5-amino-1-pentanol in dry THF was added to the phosphorylation reaction, and the solution was again stirred for 1 h. 2 mL of 1 M triethylammonium carbonate buffer, pH 7.5, were then added to the reaction mixture, and after 5 min the solution was poured into 150 mL of dichloromethane. The dichloromethane was extracted with 3 x 150 mL of water and was, subsequently, dried over Na_2SO_4. The CH_2Cl_2 was stripped off the phosphates, and the solids were



N-(4,4'-dimethoxytrityl)-5-amino-1-pentanol

Figure 16

further dried by co-evaporation with pyridine. The product was dissolved in a minimal volume of dichloromethane. **4** was then precipitated from solution by the slow addition of the dichloromethane solution to 200 mL of 1% triethylamine in pentane. Typical reactions gave yields in excess of 90%. IR (KBr) Figure 17; NMR ($CDCl_3$) δ 7.7-6.7 (m, 17H, aromatic), 3.9 (q, 2H, J = 6 Hz, CH_2), 3.8 (s, 6H, CH_3), 2.1 (t, 2H, J = 6 Hz, CH_2), 1.4 (m, 6H, CH_2), triethyl amine signals occur at δ 3.0 and 1.2; M/e^- = 594 (**4**: free anion).

5-Aminopentyl-1-dT$_{11}$. N-(4,4'-dimethoxytrityl-5-aminopentyl-1-(0-2-chorophenyl) phosphate was coupled to the 5' hydroxyl of solid supported dT_{11} with the same procedures that Itakura uses to introduce a nucleotide. Itakura's methodology was not modified at all.

To obtain the solid supported free amine the dimethoxytrityl function was removed by treatment of the resin bound material with $ZnBr_2$. The nucleotide is not cleaved from the resin until the EDTA is coupled to the molecule.

In contrast the alternative solution chemistry dictates that the protected linker-dT_{11} be removed from the resin before the trityl group is cleaved. Protected linker dT_{11} can then be separated from contaminating oligomers by reverse phase chromatography, since the trityl function retards the elution of the dinucleotide; however, analysis of the nucleotide fractions on an Altex C-18 column, 1.0 cm x 25 cm, by a 20 min 20-30% acetonitrile gradient in 100 mM triethylammonium carbonate buffer, pH 7.5, at 1.5 mL per min indicated that the dimethoxytrityl protecting group was not present. The trityl group had been lost. It then became necessary to accept the 5-aminopentyl-1-dT_{11} without further purification. Nevertheless, the linker-

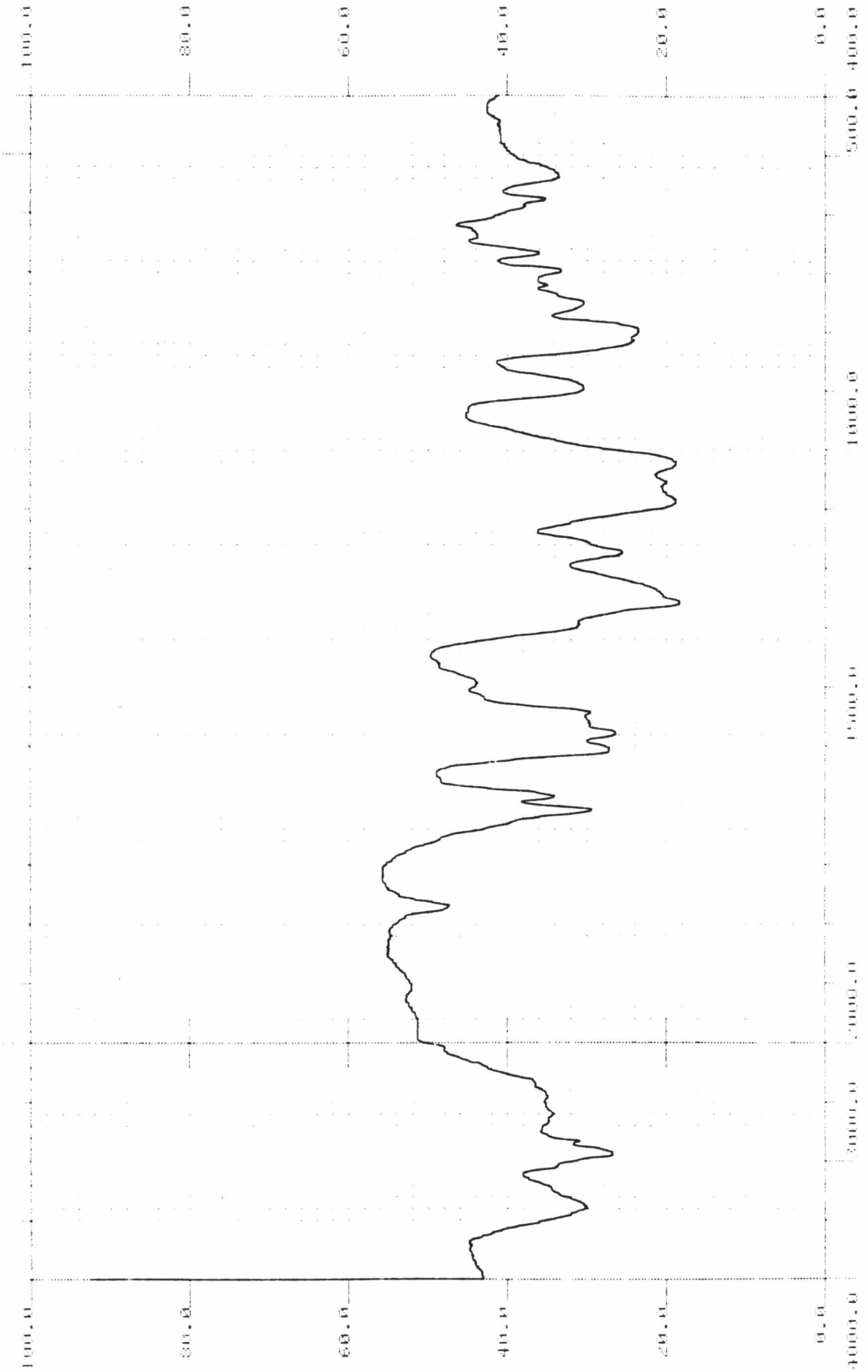


Figure 17 N-(4,4'-dimethoxytrityl)-5-aminopentyl-1-(0-2 chlorophenyl) Phosphate

dT_{11} was treated with acetic acid to insure the complete deprotection of the amine.

EDTA, Triethyl Ester, mono-N-Hydroxysuccinimide Ester (EDTA $(OEt)_3NHS$). In a septum stoppered vial, 200 mg EDTA, triethyl ester were dissolved in 2.5 mL THF or DMSO. 103 mg 1,1-carbonyldiimidazole (Aldrich, mp 118-120°C, 120 mol %) were added to the vial, and the reaction was allowed to stir for 1 h. 91 mg of dry N-hydroxysuccinimide (Aldrich 97%, 150 mol %) we then added to the reaction. After 12 h of stirring, the activated EDTA triester was then transferred into the coupling reaction. EDTA $(OEt)_3NHS$ cannot be isolated, the compound decomposes during chromatography.

EDTA-dT_{11}. Supported Substrate Coupling. A 2.5 mL solution of 130 mg of EDTA $(OEt)_3NHS$ was syringed onto supported amino-dT_{11}. After 24 h the resin was washed with 4 x 1 mL THF and the DNA was removed from the solid support. The oligonucleotides were precipitated, treated with 0.25 M LiOH, and reprecipitated. The purported EDTA-dT_{11} was then assayed with dA_{20}.

Solution Coupling. 5-Aminopentyl-dT_{11} was dissolved in pyridine, and 50 mg of EDTA $(OEt)_3NHS$ in 1.5 mL pyridine were added to the reaction. After 24 h the pyridine was evaporated, and the residue was redissolved in water. The DNA was precipitated from the aqueous solution, and the pellet was treated with 0.25 M LiOH. The hydrolysis solution was then neutralized, and the EDTA-dT_{11} was assayed by polymerase extension.

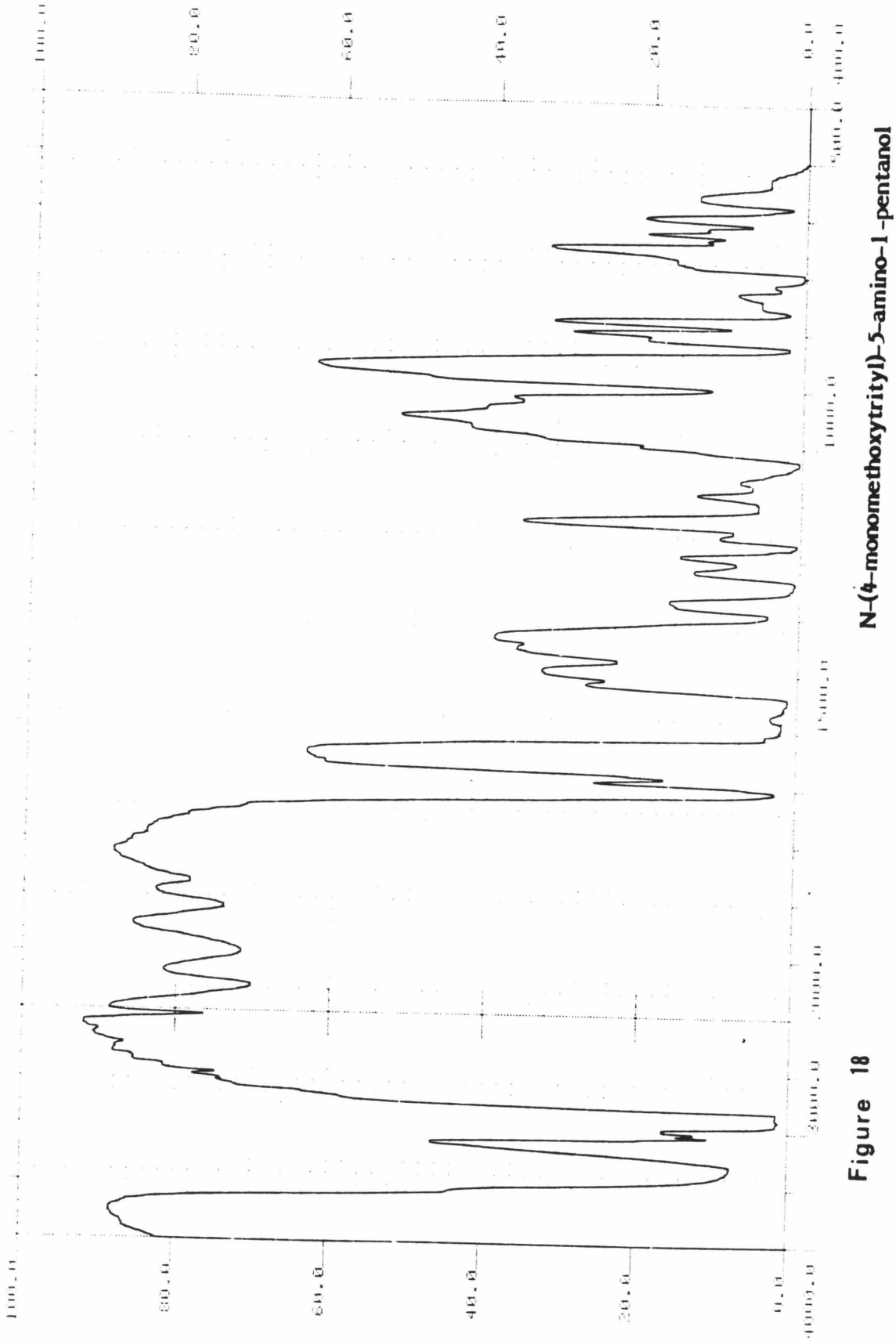
N-(4-monomethoxytrityl)-5-amino-1-pentanol. The synthesis of N-(4-monomethoxytrityl-5-amino-1-pentanol duplicates the synthesis of N-(4,4'-

dimethoxytrityl)-5-amino-1-pentanol. In the end, silica gel chromatography of the crude reaction mixture with 30% hexane in diethyl ether gave an 87% yield of pure material. Reagents: 4-monomethoxytrityl chloride (Aldrich 97%). IR (neat) Figure 18; NMR ($CDCl_3$) δ 7.6-6.7 (m, 4H, aromatic), 3.7 (s, 3H, CH_3), 3.6 (t, 2H, J = 6 Hz, CH_2), 2.1 (t, 2H, J = 6 Hz), 1.4 (m, 6H, CH_2).

Elemental analysis calcd. for $C_{25}H_{29}NO_2$: C, 79.97; H, 7.78; N, 3.73. Found: C, 79.62; H, 8.11; N, 3.56.

N-(4-monomethoxytrityl)-5-aminopentyl-1-(0-2-chlorophenyl) Phosphate, 5. Compound **5** was synthesized with the same procedure as was described for N-(4,4'-dimethoxytrityl)-5-aminopentyl-1-(0-2-chlorophenyl) phosphate. Yield = 91%. IR (neat) Figure 19; NMR ($CDCl_3$) δ 7.6-6.7 (m, 18H, aromatic) 3.9 (q, 2H, J = 6 Hz, CH_2), 3.7 (s, 3H, CH_3), 2.2 (t, 2H, J = 6 Hz, CH_2), 1.4 (m, 6H, CH_2), triethyl amine appears at δ 3.0 and 1.2; M/e^+ = 667 [$5 \cdot H^+ - N(Et)_3$].

EDTA-pentyl-$^{5'}$CGGGAATTCGT$^{3'}$ (EDTA-11mer). N-(4-monomethoxytrityl)-5-aminopentyl-1-(0-2-chlorophenyl) phosphate was coupled to the solid supported oligonucleotide, $^{5'}$HO-CGGGAATTCGT$^{3'}$, by typical nucleotide triester chemistry. The nucleotide products were cleaved from the solid support and treated with concentrated NH_4OH. The NH_4OH was evaporated, and the residue was redissolved in 0.3 mL H_2O. One-third of the oligonucleotide solution was purified by elution of the DNA from a G50 Sephadex column with 100 mM triethylammonium carbonate buffer, pH 8.0. A 10 microliter aliquot of the chromatographed oligomers was dissolved in 0.2 mL H_2O, and the optical density of the solution was measured at 260 nm. A second 10



N-(4-monomethoxytrityl)-5-amino-1-pentanol

Figure 18

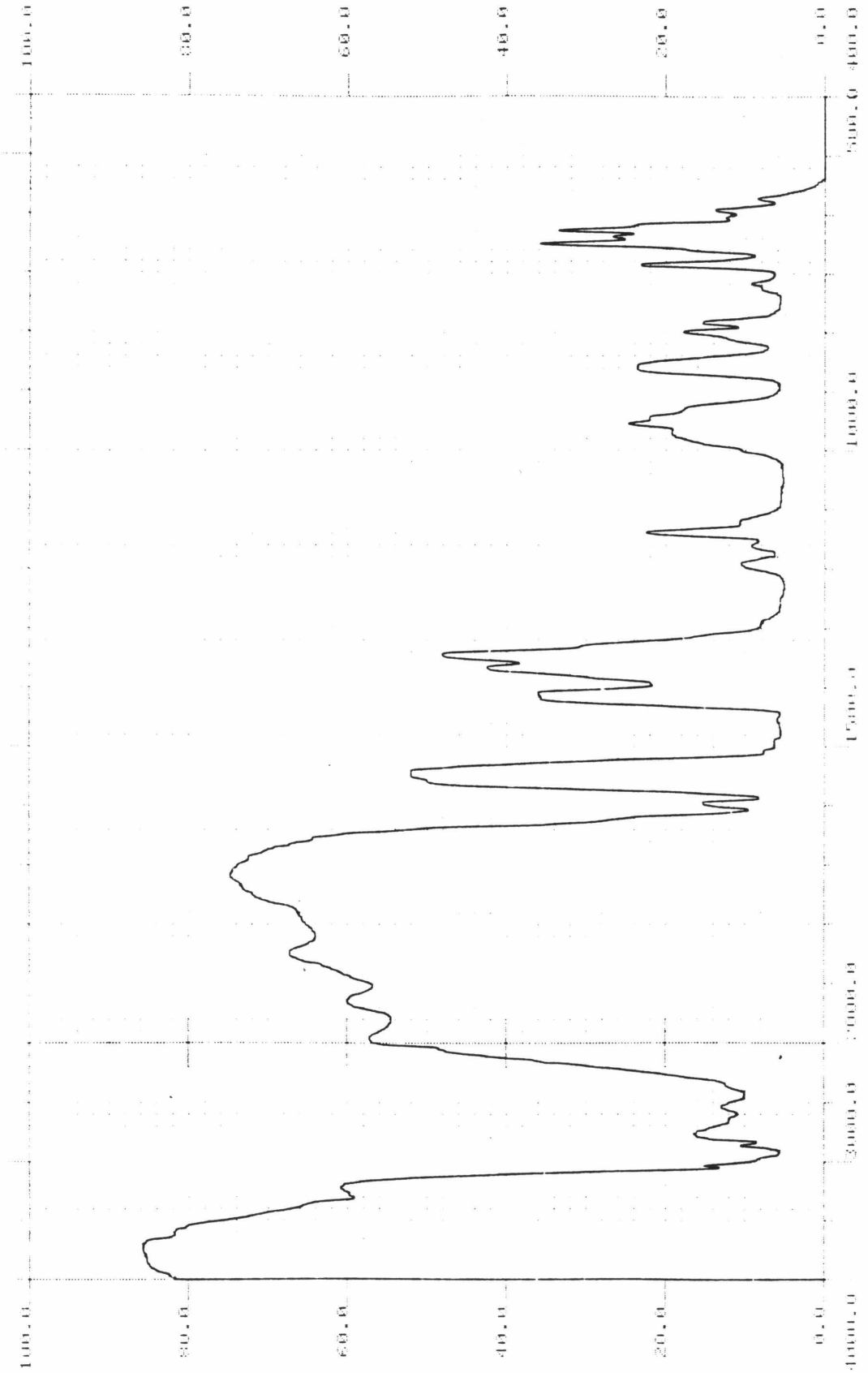
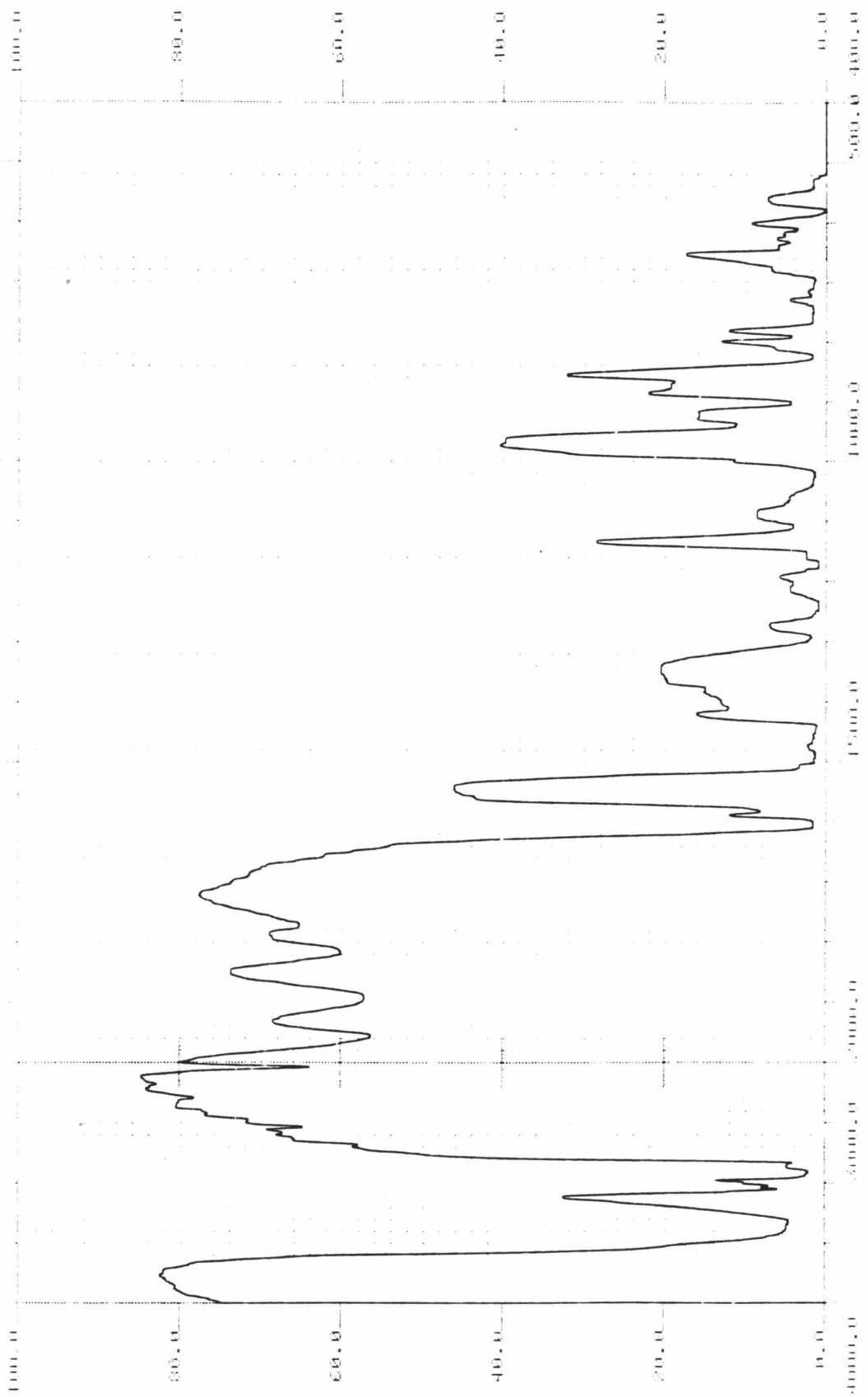


Figure 19 N-(4-monomethoxytrityl)-5-aminopentyl-1-(0-2-chlorophenyl) Phosphate

microliter aliquot was dissolved in 60% $HClO_4$ in ethanol and the optical density of the solution was measured at 470 nm. Comparison of the OD_{260} against the OD_{470} showed that approximately 55% of the oligonucleotide fraction carried a trityl function; however, further purification of protected linker-limer was impossible. The trityl function was not stable to aqueous chromatography on a high pressure reverse phase column.

The sephadexed fractions were then treated with 80% acetic acid to remove the monomethoxytrityl protecting group. The acetic acid was evaporated, and the DNA was redissolved in triethylammonium carbonate buffer, pH 7.8. The aqueous solution was extracted with 3 x 1 mL diethyl ether, and the water was evaporated. The DNA was dissolved in 50 microliters H_2O, and was added to a 0.25 mL solution of 50 mg $EDTA(OEt)_3NHS$ in DMSO. 10 microliters triethylamine were than added to the reaction to scavenge excess acid. After 24 h the $EDTA(OEt)_3$-limer was isolated from the reaction by G50 sephadex chromatography. The isolated nucleotide fractions were treated with 0.25 M LiOH for 12 h. The hydrolysis solution was subsequently neutralized, and the EDTA-limer was assayed for activity after hybridization to an end labeled M13 mp 8 fragment.

N-(4-monomethoxytrityl)-2-aminoethanol. N-(4-monomethoxytrityl)-2-aminoethanol was synthesized by the procedure described under N-(4,4'-dimethoxytrityl)-5-amino-1-pentanol; however, the product was isolated from the reaction mixture by chromatography on silica gel, 230-400 mesh, with a different solvent then described previously. Elution with 70% hexane/25% diethyl ether/5% ethanol gave an 83% yield of protected aminoethanol. Reagents: 2-aminoethanol (Sigma). IR (neat) Figure 20;



N-(4-monomethoxytrityl)-2-aminoethanol

Figure 20

NMR ($CDCl_3$) δ 7.6-6.7 (m, 14H, aromatic), 3.8 (s, 3H, CH_3), 3.7 (t, 2H, J = 6 Hz, CH_2), 2.3 (t, 2H, J = 6 Hz, CH_2), 1.9 (s, 2H, NH and OH).

Elemental analysis calcd. for $C_{22}H_{23}NO_2$: C, 79.25; H, 6.95; N, 4.20. Found: C, 79.09; H, 7.25; N, 4.14.

N-(4-methoxytrityl)-2-amino-1-ethyl-(0-2-chlorophenyl) Phosphate 6. The synthesis of compound **6** was an exact duplication of the synthesis of N-(4,4'-dimethoxytrityl)-5-amino-1-pentyl-(0-2-chlorophenyl) phosphate **4**. Yield: 75%; IR (KBr) Figure 21; NMR ($CDCl_3$) δ 7.8-6.7 (m, 18H, aromatic), 4.1 (q, 2H, J = 6 Hz, CH_2), 3.7 (s, 3H, CH_3), 2.3 (t, 2H, J = 6 H, CH_2), triethylamine appears at δ 2.9 and 1.2; M/e^+ = 625 ($6 \cdot H^+ - N(Et)_3$).

EDTA-ethyl-$^{5'}$CGGGAATTCGT$^{3'}$ (EDTA-e-linker). N-(4-monomethoxytrityl)2-aminoethyl-1-(0-2-chlorophenyl) phosphate was coupled to supported $^{5'}$HOCGGGAATTCGT$^{3'}$ by conventional nucleotide triester chemistry. The monomethoxytrityl group was then removed with $ZnBr_2$, and the resin was dried. In a bullet vial, 20 mg of supported linker-linker was suspended in a solution of 40 mg EDTA dianhydride[46] in 0.8 mL DMSO. 50 microliters triethylamine were added to the reaction, and the vial was heated to 37°C. After 24 h, the coupling solution was filtered off, and the resin was washed with 2 x 1 mL pyridine and 5 x 1 mL THF. The supported product was then resuspended in aqueous DMSO and the reaction was incubated at 37°C for 15 h. The DMSO was filtered off and the resin was rewashed with 3 x 1 mL THF. The EDTA-e-linker was then removed from the solid support, and the oligomers were treated with NH_4OH. Oligonucleotides were then isolated by G50 sephadex chromatography, and the fractions were assayed for

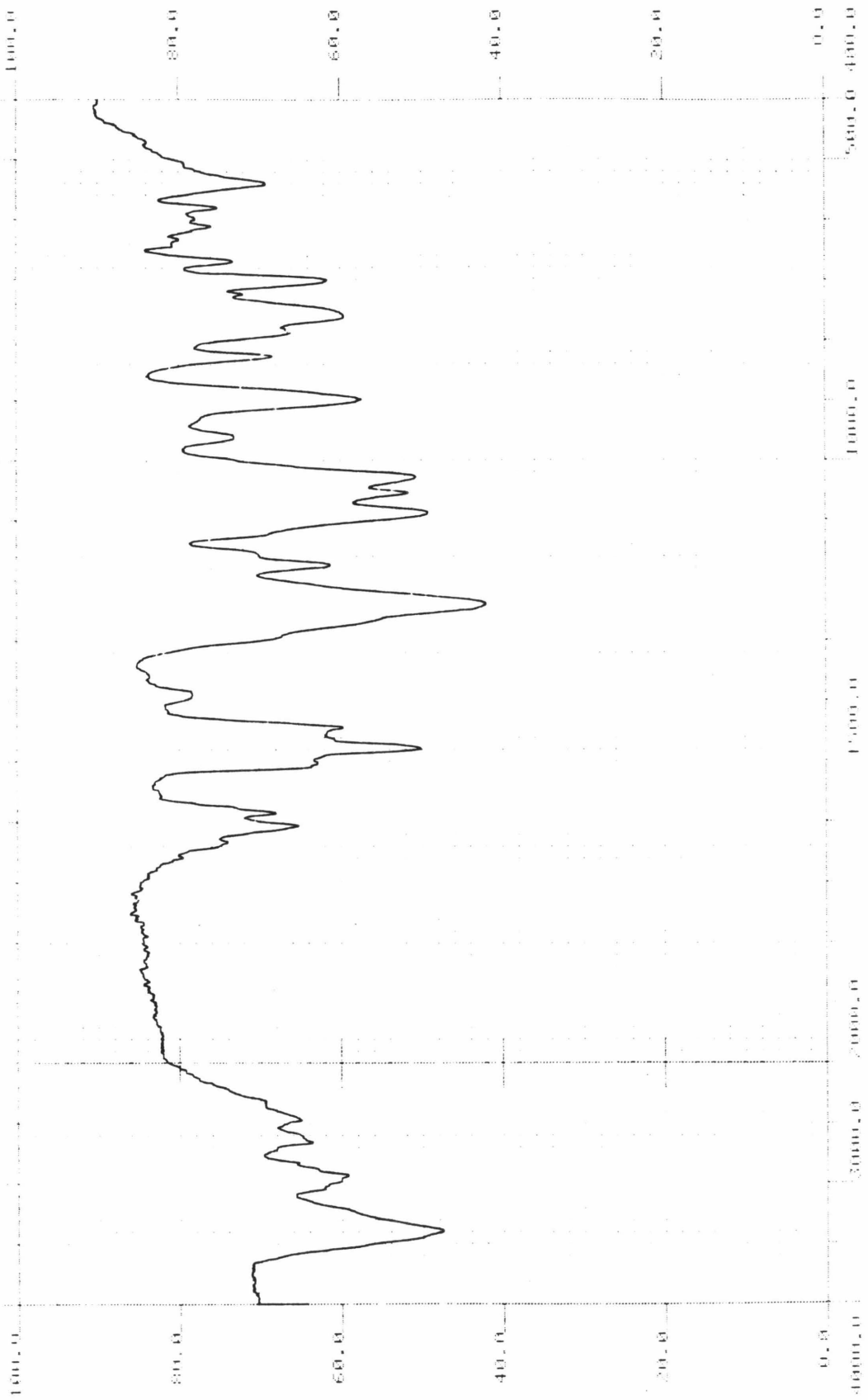


Figure 21

N-(4-methoxytrityl)-2-amino-1-ethyl-(0-2-chlorophenyl) Phosphate

cleaving activity against M13 mp8 substrate.

This synthesis was repeated with ^{14}C labeled EDTA dianhydride, and the G50 sephadex column was monitored by ^{14}C activity and optical density. Total ^{14}C activity indicated that the reaction yielded less than 5% coupling; however, the labeled EDTA-e-linker was enriched in early column fractions. ^{14}C to optical density ratios suggested that one fraction carried an EDTA on 50% of the oligonucleotides. This fraction was subsequently assayed against M13 mp8 substrate.

^{14}C-EDTA Dianhydride.[46] In a 3 mL vial, 50 microcuries EDTA-(acetic-2-^{14}C) and 245 mg EDTA were suspended in a solution of 0.25 mL acetic anhydride (Mallinkrodt) and 1.25 mL pyridine. The reaction was heated to 65°C and was left stirring for 24 h. The solids were then filtered away from the reaction solution and were washed with 2 x 1 mL pyridine. The labeled EDTA dianhydride was then dried under reduced pressure. Analysis of the labeled dianhydride yielded a specific activity of 78 μCi/mmol.

1,10-Phenanthroline-ethyl-$^{5'}$CGGGAATTCGT$^{3'}$. N-(4-monomethoxytrityl)-2-aminoethyl-1-(0-2-chlorophenyl) phosphate was coupled to supported $^{5'}$HOCGGGAATTCGT$^{3'}$ by conventional nucleotide triester chemistry. The monomethoxytrityl function was then removed, and the supported linker-linker was dried.

Meanwhile, 50 mg 2-carboxy-1,10-phenanthroline[47] and 36 mg 1,1-carbonyldiimidazole were dissolved in 1.5 mL pyridine. The reaction was allowed to stir for 1 h, and was subsequently syringed over the supported linker-linker. After 24 h, the reaction solution was withdrawn, and the resin

was washed with 4 x 1 mL THF. The oligonucleotides were cleaved off the resin and were deprotected in NH_4OH. The NH_4OH was evaporated and the residue was redissolved in water. The reaction mixture was then fractionated by G50 sephadex chromatography, and the purported phenanthroline-e-llmer was assayed against M13 mp 8 substrate.

$5'$CGGGAATTCGT$3'$-ruthenium Pentamine. The oligonucleotide was synthesized and purified according to the procedures of Tan et al.[40] 1.2 OD units of llmer were dissolved in 0.4 mL water. The sample was then dialyzed against pH 3 HCl in 2-200 microliter dialysis buttons. The buttons were then suspended in a 100 mL three-neck round-bottom flask. The flask was equipped with a glass stopper and suspension hook, an argon inlet, a gas outlet and a magnetic stirring bar. 50 mL pH 3 HCl were added to the round-bottom flask and the solution was deoxygenated by bubbling argon through the solution for 30 min. 0.2 g of fresh Hg-Zn amalgam were added to the flask, and argon was bubbled through the flask for an additional 30 min. 10 mg $Cl(NH_3)_5RuCl_2$ were added to the flask, and the reaction was stirred under positive argon pressure for 22 h. The dialysis buttons were then removed from the round-bottom flask and were successively dialyzed against pH 3 HCl and 40 mM Trizma/HCl pH 7.8. The llmer-Ru oligonucleotide[48,49] was then assayed against M13 mp 8 substrate. Additional analyses demonstrated that the llmer-Ru would not serve as a primer for large fragment polymerase, presumably, the oligonucleotide does not form a stable DNA double helix. As such the oligonucleotide cannot deliver the chemically active ruthenium.

5-(3-Amino)allyldeoxyuridine-5'-triphosphate (AAdUTP).[44] 700 mg 5-

mercurideoxyuridine-5'-triphosphate[50] were dissolved in 50 mL 0.1 M NaOAc buffer, pH 5. The solution was transferred into 100 mL three-neck round-bottom flask. The flask was equipped with an argon inlet, addition funnel, glass stopper, and magnetic stirring bar. The 50 mL solution was cooled to 5°C in an ice bath, and 6 mL of freshly neutralized 2 M allylamine was added to the reaction flask -- allylamine is neutralized by adding 3 mL allyamine to 17 mL 4 M acetic acid. 280 mg of K_2PdCl_4 were dissolved into 8 mL water, and the solution was slowly dripped into the stirred solution of HgdUTP and allylamine. The ice bath was then allowed to melt. The reaction was then left to stir at room temperature for 36 h. Afterward, the reaction was filtered through a 0.5 micron filter, and the filtrate was diluted to 300 mL with water. The reaction solution was loaded on a 150 mL DEAE A-25 Sephadex column that had been previously equilibrated with 0.1 M, pH 5, NaOAc. The column was then flushed with one volume of 0.1 M pH 5, NaOAc and was subsequently eluted with a 1.1 liter linear gradient (0.1-0.7 M) of triethylammonium carbonate (TEAB), pH 7.8. The AAdUTP eluted at approximately 0.3 M TEAB and fractions with absorption maxima greater than 285 nm were collected. The TEAB was evaporated, and the residue was redissolved in 10 mL H_2O. The AAdUTP was then collected by ethanol precipitation from 0.4 M NaOAC, pH 7. The synthesis yielded ~250 mg of pure AAdUTP (~30%).

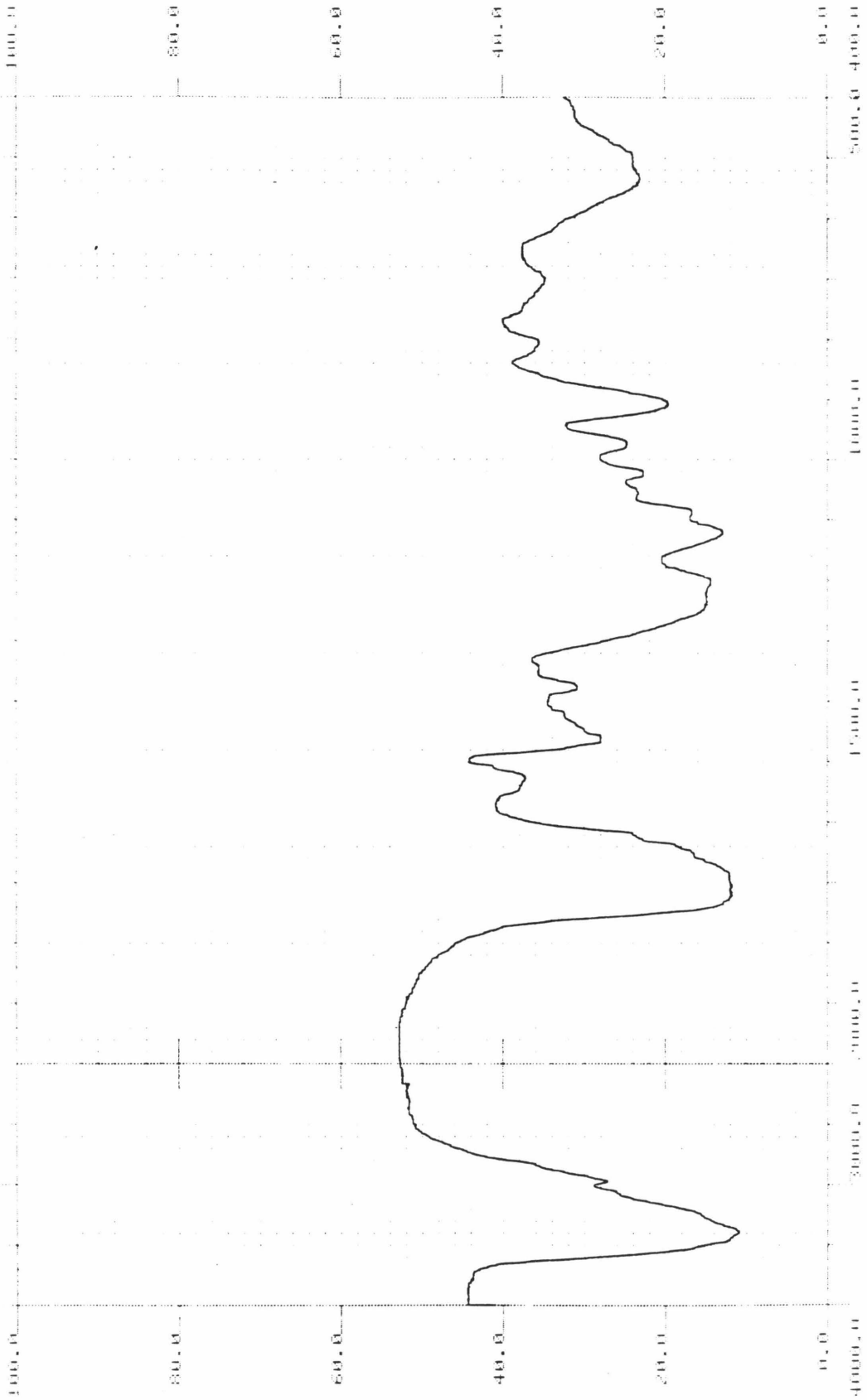
EDTA(OEt)$_3$dUTP (EEdUTP). 660 mg EDTA-triethylester were dissolved in 6.0 mL dry DMSO, and the solution was transferred into a 25 mL round-bottom flask. 490 mg 1,1-carbonyldiimidazole (170 mol %) were added to the flask, and the reaction was stirred for 1 h. 450 mg N-hyroxy

succinimide (220 mol %) were then added, and the reaction was stirred overnight.

85 mg AAdUTP, triethylamine salt, were dissolved in 5.0 mL DMSO, and the solution was pipetted into the reaction flask. 0.5 mL triethylamine were syringed into the solution, and the reaction was stirred for 24 h. Afterward, the reaction was diluted with water to a volume of 100 mL, and the solution was loaded on a 100 mL DEAE A-25 sephadex column. The column was washed with one column volume of 0.1 M TEAB, pH 7.6, and eluted with an 800 mL linear gradient (0.1-0.7 M) of TEAB. The major fraction eluted at approximately 0.4 M TEAB. Analysis of the fraction by high pressure liquid chromatography (column: Whatman ODS-2; solvent: 0.5 M $AcO^{-}HN^{+}Et_3$, pH 4.3) showed that no starting material was present, and that the EEdUTP was 95% pure. The compound was then assayed for cleaving activity. IR (KBr) Figure 22; NMR (D_6-DMSO) Figure 23; M/e^{+} = 906 (EEdUTP·Na·5 H^{+} = fully protonated).

dA_{20} Cleavage Assay. dA_{20} was 5' end labeled with (γ-^{32}P)-ATP. The molecule to be assayed and an aliquot of dA_{20} were dissolved in 10 microliters of 20 mM Trizma/HCl, pH 7.4, 50 mM NaCl (TN). The nucleic acid was then annealed by slowly cooling the samples from 90°C to 4°C over 2 h. One microliter 2 x 10^{-4} M $Fe(NH_4)_2(SO_4)_2$ was added to the samples and the reactions were equilibrated for 1 h. One microliter 50 mM dithiothreitol (DTT) was then pipetted into the samples, and the cleavage reaction was run for 2 h.

The samples were then denatured with formamide at 90°C, and the fragments were analyzed by electrophoresis on 20% acrylamide denaturing



EEdUTP

Figure 22

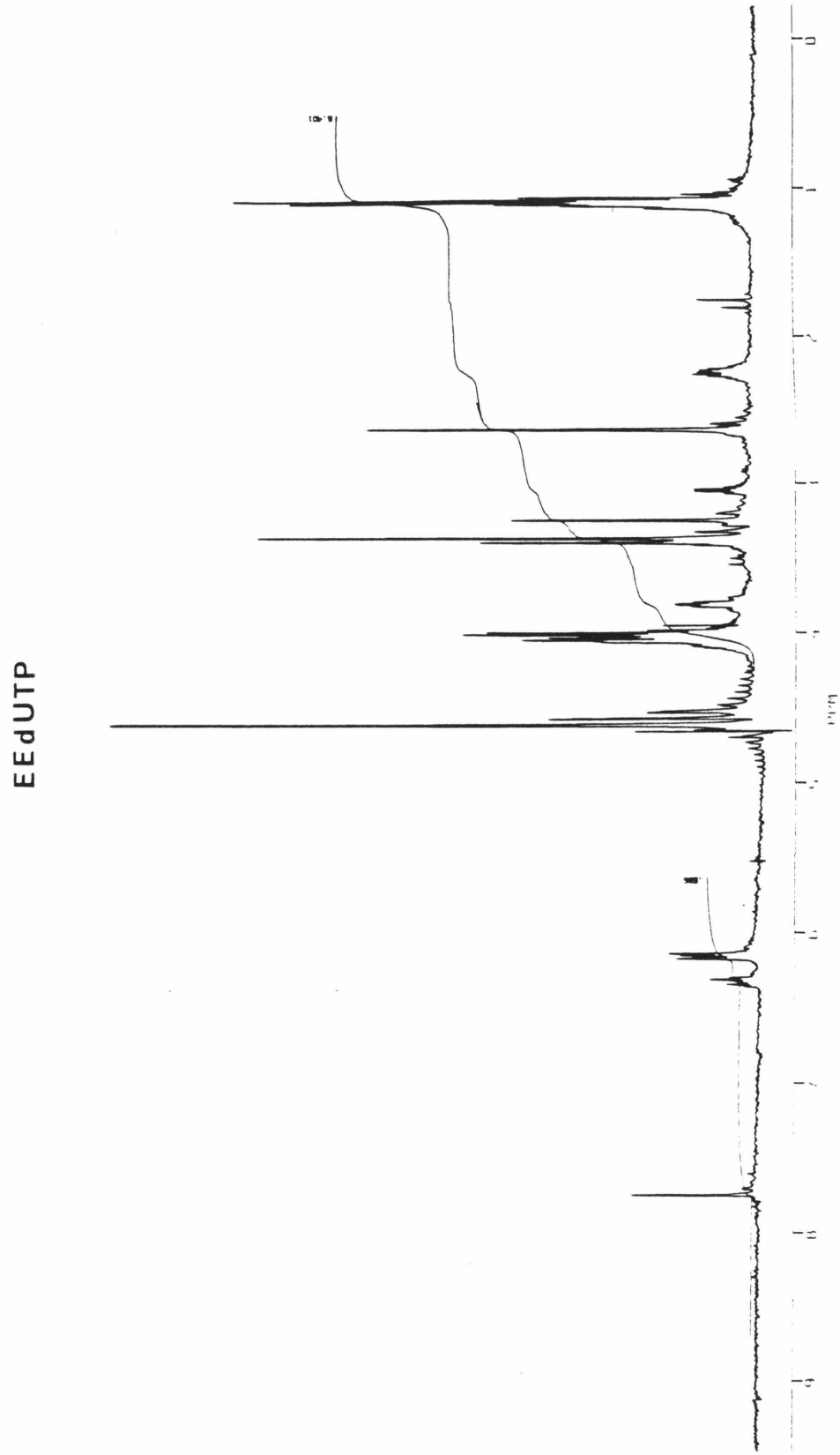


Figure 23

Figure 24

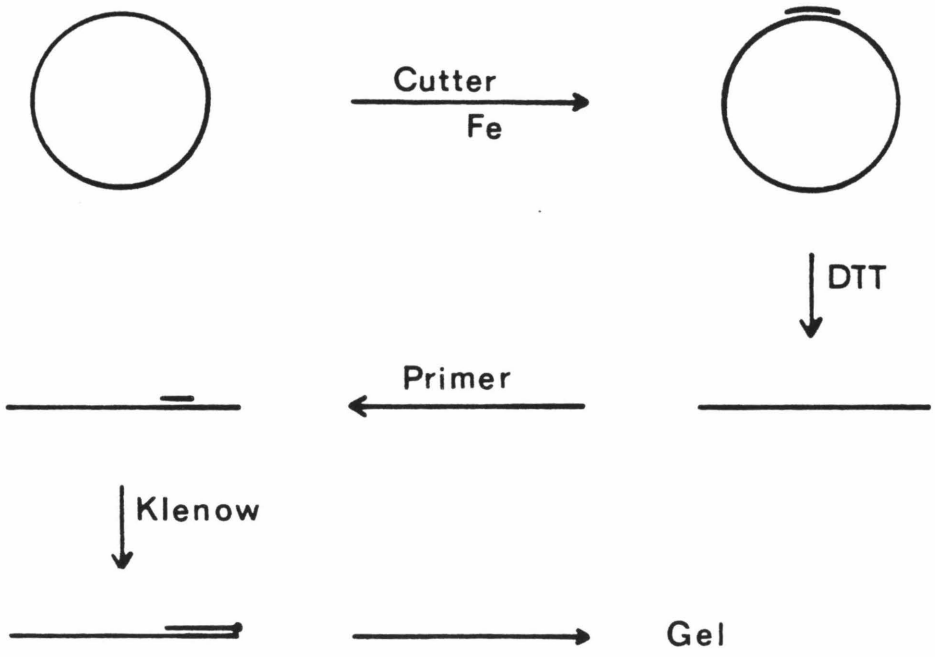


Figure 25

gels. Specific activity would be characterized by efficient cleavage of the dA_{20}.

Polymerase Extension Cleavage Assay. An M13 mp9 clone obtained from Richard Firtel contained the Eco RI/Hind III fragment of the discoidin IA gene. This sequence harbors a string of 30 adenines approximately 180 nucleotides from the primer site for Sanger sequencing. If a cut is made in the string of 30 adenines, polymerase extension of Sanger sequencing primer should truncate at the cleavage site that ends the sequence.

An aliquot of dT_{11}-EDTA was annealed to the M13 mp9 clone in 10 microliters of TN buffer. A microliter of 1×10^{-4} M $Fe(NH_4)_2(SO_4)_2$ was added to the vials, and the samples were equilibrated for 1 h. Finally, one microliter of 50 mM DTT was added to the mixtures, and the samples were allowed to stand for 2 h.

Afterwards, the samples were ethanol precipitated and redissolved in polymerase buffer. A Sanger sequencing primer (New England Biolabs) was then annealed to the clone, and (α-^{32}P) dATP, dGTP, dCTP, and dTTP were added to the sample. One unit of large fragments polymerase was allowed to extend the Sanger primer for 20 min. In the last 5 min of the polymerase reaction , cold dATP was added to the mixtures to complete DNA synthesis.

The samples were denatured with formamide at 90°C, and were electrophoresed on a 8% acrylamide denaturing gel. The gel was visualized by autoradiography. Specific cleavage should produce a discrete 180 nucleotide fragment; unfortunately, complications and background make this assay useless.

M13 Cleavage Assay. Sixteen micrograms of M 13 mp8 RF were restricted with Hind III and the plasmid was 3' end labeled with large fragment polymerase and (α-^{32}P) dATP. The M13 was re-restricted with Pvu II and the DNA was electrophoresed down an 8% acrylamide gel. The 208 bp fragment was cut from the gel, and the labeled fragment was eluted from the gel slice with 1 mL 0.5 M NH_4OAc, 10 mM Mg^{2+}, 2 mM EDTA and 0.1% sodium dodecyl sulfate. The DNA was then purified by millipore filtration and precipitation.

Four milliroentgen labeled DNA, 0.5 micrograms single stranded M13 mp 8, and 5 equivalents of cutting agent were annealed from 90°C in 15 microliters 20 mM Trizma/HCl (pH 7.4), 50 mM NaCl and 1 x 10^{-5} M $Fe(NH_4)_2(SO_4)_2$. One microliter 50 mM DTT was added to the vials after the solution had cooled to room temperature (~1.5 h), and the reactions were then allowed to stand for 2 h at room temperature. The samples were then denatured with formamide at 90°C, and the DNA was analyzed by electrophoresis on a 20% acrylamide denaturing gel. The "sequencing gel" was then visualized by autoradiography. Specific cleavage would produce fragments approximately 25 nucleotides long.

EDTA-dUTP Cleavage Assay. (a) EEdUTP was initially tested with large fragment polymerase to determine if the modified triphosphate could be incorporated by the polymerase. Two micrograms pBR322 were restricted with Sal I. The DNA was split into two samples. The first vial was doped with dUTP, (α-^{32}P) dCTP, dGTP and dATP, while the second vial was laced with EEdUTP, (α-^{32}P) dCTP, dGTP and dATP. Large fragment polymerase was added to both tubes, and the polymerase was allowed to fill the sticky

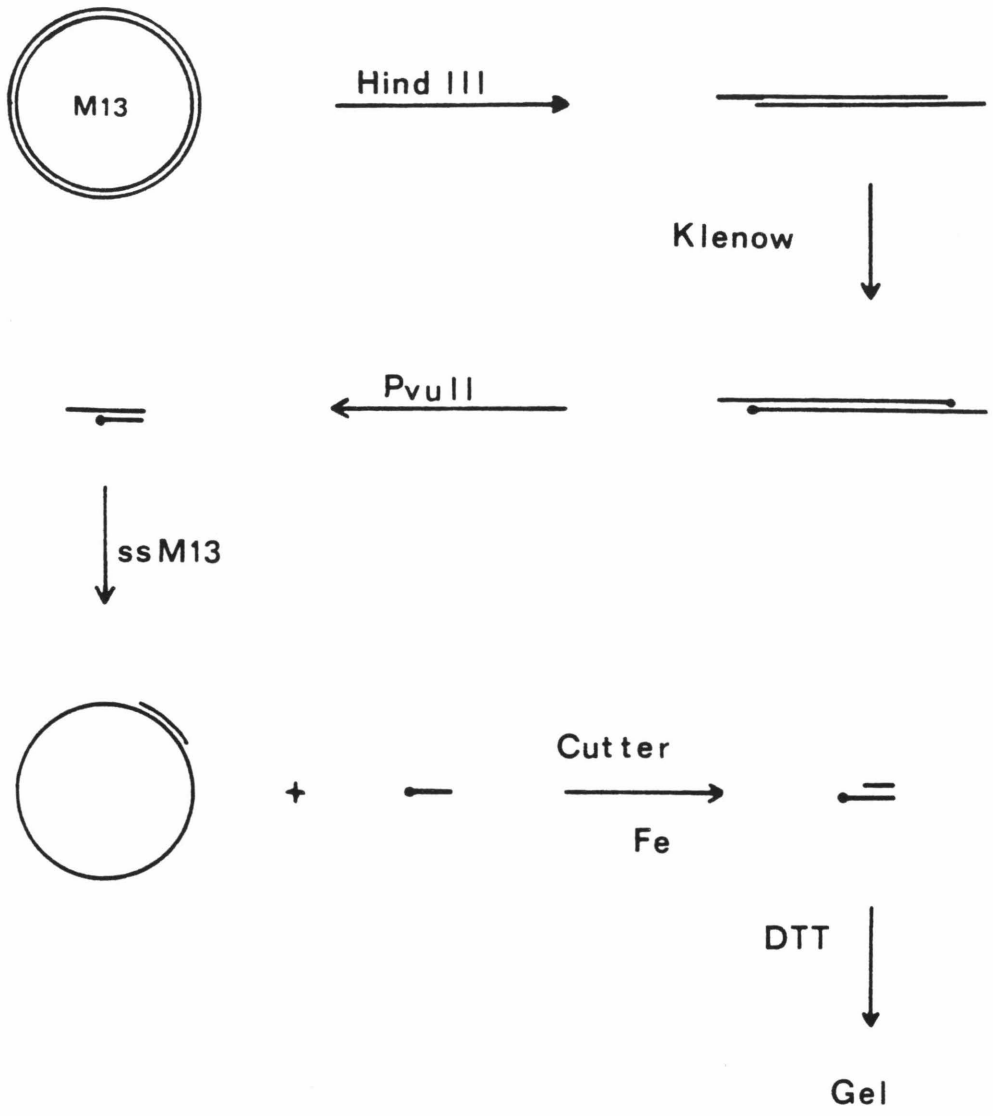


Figure 26

ends of the restriction. If EEdUTP is not a substrate for large fragment polymerase, radiolabeled dCTP will not be incorporated. Autoradiography of a 5% polyacrylamide gel showed that the two samples that had been run on a gel contained approximately equal amounts of radiolabel in the pBR322. EEdUTP is apparently a legitimate nucleotide precursor for large fragment polymerase.

(b) Cleavage Assay.[51] Ten micrograms M13 mp8 RF were restricted with Eco RI and 5' end labeled with (γ-^{32}P) ATP. The plasmid was restricted with Bgl I, and the labeled 179 nucleotide strand was isolated from an 8% acrylamide denaturing gel. After precipitation, the DNA was dissolved in 0.1 mL TN buffer.

A second lot of 10 micrograms of M13 mp8 RF was restricted with Hind III. The sticky ends of the plasmid were filled by polymerase in the presence of EEdUTP. The DNA was re-restricted with Bgl I, and the sample was electrophoresed through an 8% acrylamide gel containing 0.5 micrograms/mL ethidium bromide. The 149 base pair fragment was isolated from the gel and treated with pH 11 LiOH for 4 h. After precipitation, the DNA was redissolved in 0.1 mL TN.

Five microliters of labeled fragment and five microliters of "cutting probe" in 30 microliters TN were annealed from 90°C over 1.5 h. Three microliters of 2.5×10^{-4} M $Fe(NH_4)_2(SO_4)_2$ were added to the samples, and the reactions were allowed to equilibrate for 1 h. DTT, 3.0 microliters of a 50 mM solution, was added to the vials, and the solutions were incubated at 37°C for 2 h. The samples were lyophilized and afterward were redissolved in 60% formamide loading solution. The DNA was then denatured at 90°C

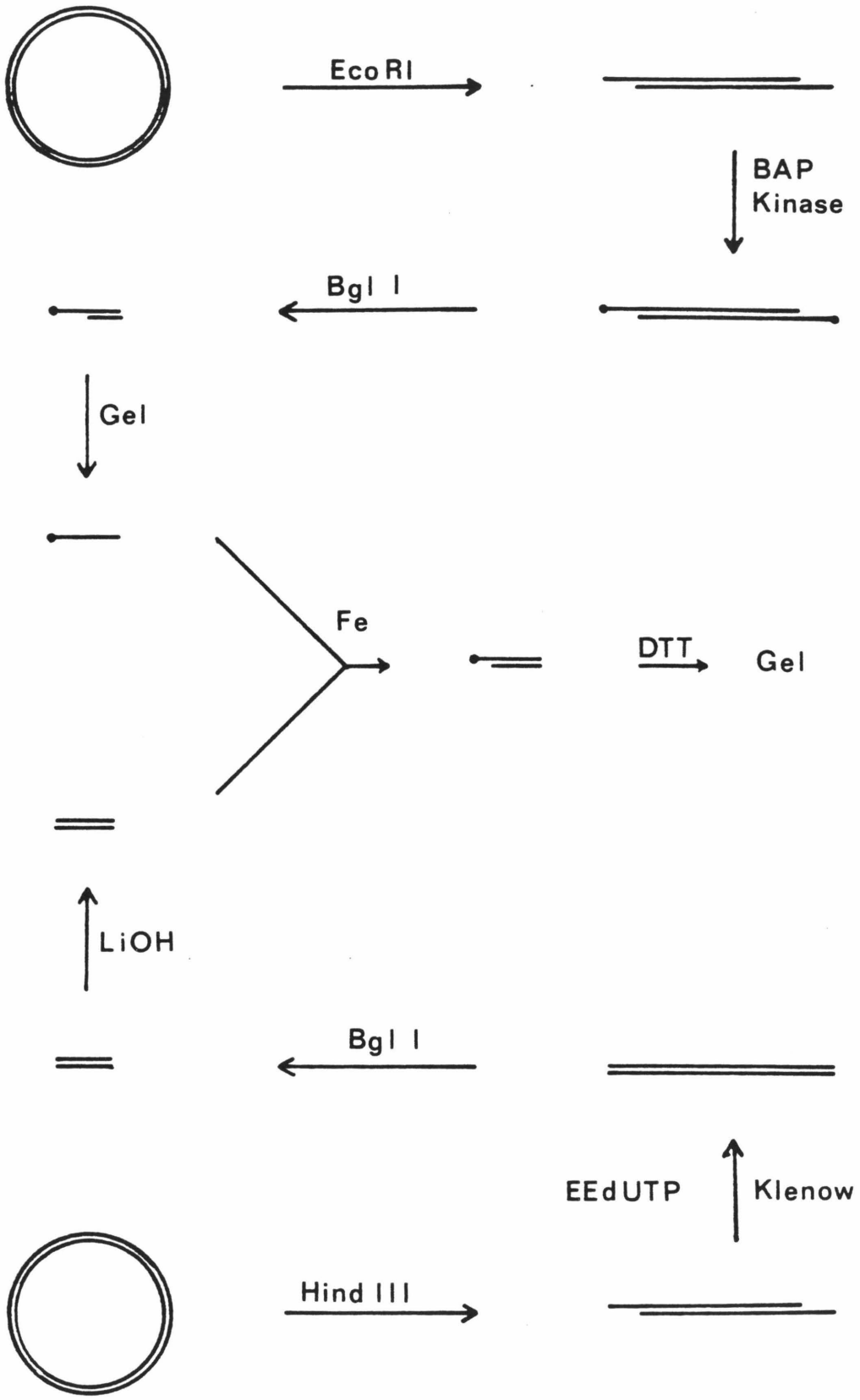

EcoRI
BAP
Kinase
Bgl I
Gel
Fe
DTT
Gel
LiOH
Bgl I
EEdUTP
Klenow
Hind III

Figure 27

and analyzed by electrophoresis through a 20% acrylamide denaturing gel. Specific cleavage should produce a fragment approximately 30 nucleotides long.

(c) An Alternative Cutting Probe. A cutting probe to increase the sensitivity of the cutting assay was synthesized with T4 polymerase. Ten micrograms of M13 mp8 RF was restricted with Hind III. Ten units of T4 polymerase were then added to the DNA solution, and the polymerase was allowed to hydrolyze the 3' ends of M13 mp 8. After a 2 min incubation at 37°C, EEdUTP, dATP, dGTP and dCTP were added to the reaction, and the polymerase was allowed to refill the ends of the linearized plasmid. The 60 min incubation at 37°C was followed by a 10 min incubation a 70°C to denature the polymerase. The DNA was re-restricted with Bgl I, and the 149 bp cutting probe was isolated, deprotected, and assayed as described in part (b). In this case, specific activity would be characterized by fragmentation of the ^{32}P labeled DNA at adenines 30-70 nucleotides from the Eco RI end of fragment.

Recombinant DNA Methodology.[52] All techniques needed for the handling and manipulation of DNA are described in detail in "Molecular Cloning: A Laboratory Manual".

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PROPOSITIONS

ABSTRACTS

PROPOSITION I: A systematic investigation of the relationship of the composition of flanking DNA sequences to the activity of a site specific enzyme.

PROPOSITION II: Site specific mutation of a U1 snRNA gene and a corresponding mutation in the splice site for 12s adeno early message is used to test the template theory of messenger RNA splicing.

PROPOSITION III: The use of chiral thiophosphate substrates to stereochemically probe the mechanism of Eco RI phosphodiester hydrolysis.

PROPOSITION IV: Methodology for the synthesis of nuclease resistant pp(A 2'p)$_2$A analogs, potential antitumor compounds.

PROPOSITION V: The synthesis of a photoactivated analog of aspartame capable of covalently binding to the receptors for sweet taste, and the possible use of this analog in the identification of the components of the sweet receptor.