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Synthesis, DNA-binding and biological activity of a double intercalating analog of ethidium bromide

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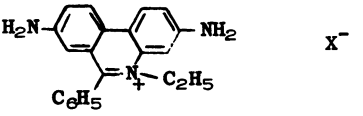
ABSTRACT

A bis-phenanthridinium salt has been synthesized and its DNA-binding studied. Evidence provided by UV and CD spectra, by thermal denaturation profiles and by equilibrium dialysis of the drug-DNA complex lead to the conclusion that both phenanthridine moieties intercalate in the helix. The double intercalator appears to be less potent than ethidium chloride as an inhibitor of nucleic acid synthesis in cultured L1210 cells, though it is more potent than a monomeric analog. The low potency may be due to a low cell influx rate.

INTRODUCTION

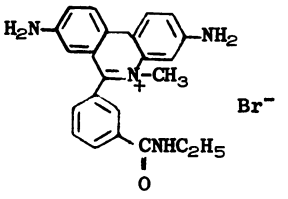
Ethidium cation, originally synthesized and still used as a trypanocidal drug, has achieved additional interest because it forms an intercalation complex with DNA[1] and inhibits DNA and RNA synthesis.[2,3] This inhibition is apparently due to blockage of template function by the intercalated drug.



Ethidium Halide

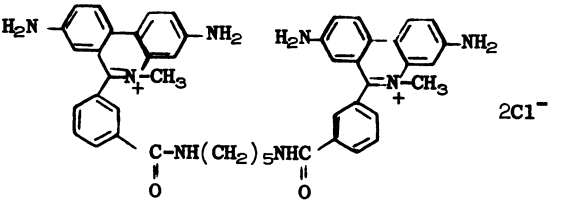
A drug with two phenanthridinium groups connected so that both would intercalate should have a much higher affinity for DNA, and would thus be expected to inhibit template function to an even greater extent. Double-intercalating molecules based on acridines,[4-11] ellipticines[12] and anthracyclines[13] have been reported. Two bisquinolyl derivatives[14] have also been prepared, but did not appear to doubly-intercalate. Le Pecq et al[15a] have synthesized a bis-phenanthridinium compound in which an alkylamino chain connects the ring nitrogens, and have concluded[15b] that it does not double-intercalate in DNA because of steric constraints.[16]

Examination of molecular models and available data on the biological activity of substituted phenanthridinium salts led us to the conclusion that, to give the most effective double intercalator, two ethidium cations should be connected through the meta positions of the 6-phenyl groups. Substitutents at this position cause minimal interference with intercalation[17] and as the two phenyl carbons effectively form part of the linking chain, the length of the flexible connecting chain can be minimized. In this paper we report the synthesis and DNA-binding characteristics of a bis[6-(3-substituted phenyl)]phenanthridinium salt (II) and its monomeric analog (I). Space filling CPK models indicate that the connecting link in II is long enough to allow double intercalation with at least two base pairs between the planar phenanthridinium moieties.



I

N-Ethyl-3-(3,8-diamino-5-methylphenanthridinium-6-yl) benzamide bromide (SR 2183)



II

N,N'-Bis[3-(3,8-diamino-5-methylphenanthridinium-6-yl) benzoyl]-1,5-diaminopentane dichloride (SR 2166)

MATERIALS AND METHODS

Spectra were determined on the following instruments: Fluorescence on Aminco Bowman Spectrophotofluorimeter (IP 21 photomultiplier tube and xenon lamp) with entrance and exit slit widths of 4.76 mm, photomultiplier slit width of 1.59 mm, excitation and emission wavelengths of 300 nm and 590 nm respectively; NMR, on a Varian XL-100-15 spectrometer equipped with Fourier transform; CD, on a Durrum-Jasco ORD/UV spectrophotometer equipped with a Sproul Scientific SS-20 CD modification; UV-visible spectra and thermal denaturation curves, on a GCA-McPherson digital double-beam, ratio-recording spectrophotometer (EU-707-K), equipped with EU-707 SA automatic sample positioner, Lauda K-2/R constant temperature bath, Lauda R20 digital temperature regulator, and Brinkmann P-120 programmer.

Calf thymus DNA was purchased from Worthington Biochemical Corp. For use in spectral and thermal denaturation studies, it was dissolved in 0.01 M

phosphate buffer, pH 7.0 to give an approximately 4×10^{-3} M solution, which was filtered through a prewashed Celite pad on a fritted glass funnel; the exact concentration was determined by measuring absorbance assuming $\epsilon_{259} = 6800$. Aliquots were stored frozen at -20° and thawed just prior to use. For use in equilibrium dialysis experiments the DNA was dissolved in tris buffer (0.01 M tris pH 8.0, 0.001 M EDTA, 0.002 M or 0.012 M $MgCl_2$, 0.0015 M NH_4Cl), sonicated while cooling in an ice bath for a total of 6 mins at 100-150 watts with resting periods to prevent overheating and denaturation, filtered as described above, and finally dialyzed repeatedly against buffer until the dialysate absorbance at 260 nm was less than 0.002 absorbance units. The DNA solution showed $\epsilon_{260} = 6700$. Aliquots were stored as above.

Thermal Denaturation. An approximately 5.2×10^{-5} M drug solution was made by dissolving the necessary weight of drug in 0.5 ml of dimethyl sulfoxide (DMSO) and diluting to 5.0 ml either with 0.01 M Phosphate buffer, pH 7.0 or with the tris buffer described above, and used as described previously.[18] Teflon stoppers in the cells provided pressure seals at elevated temperatures.[6]

L1210 Nucleic Acid Synthesis Inhibition Assay was performed as described previously[19] except that the compounds were dissolved in a calculated amount of DMSO and then diluted with growth medium so that the final concentration of DMSO in the cell-drug mixture was 1%.

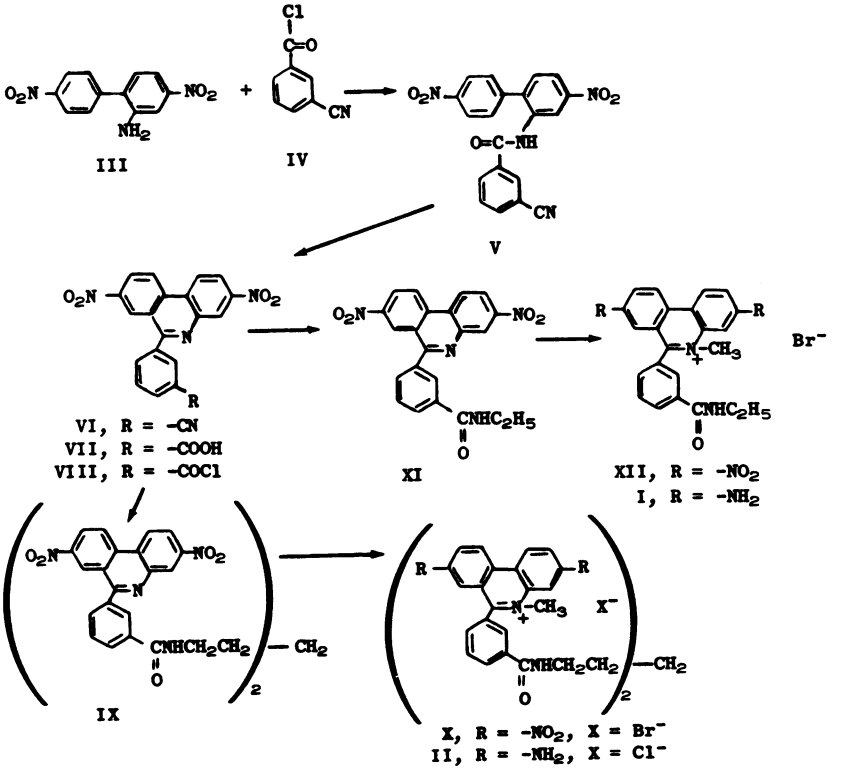
Equilibrium Dialysis experiments were carried out in two-chamber multicavity dialysis cells (National Scientific, 5 ml per chamber). Standard cellulose dialysis tubing (molecular weight cut off 12,000-14,000) was prepared by boiling in distilled water twice, then predialyzing in buffer overnight at 37° C. All glassware and quartz cuvettes were coated with a solution of dimethyldichlorosilane in toluene and the dialysis cells were periodically coated with Siliclad (Scientific Products) to prevent drug and DNA adsorption.

A solution of DNA-drug complex (approximately 50:1 DNA(P)-drug ratio) was prepared volumetrically and serial dilutions made, creating samples for dialysis. Each of these samples was dialyzed against buffer in the cells described above. A DNA solution without drug was also made exactly in the same manner as the initial DNA-drug complex solution, then diluted volumetrically to about 1×10^{-4} M and used to determine spectrophotometrically the initial DNA concentration ($\epsilon_{260} = 6700$). The cells were longitudinally shaken in a thermostatted water bath at 37° C ± 0.5° for 48-72

hours until equilibrium was reached. Aliquots were taken from each side of each dialysis cell and the DNA concentration adjusted to exactly 1 x 10^{-4} M to take advantage of the large fluorescence enhancement of both I and II on binding to DNA. Final samples contained less than 2 x 10^{-6} M drug.

The samples were centrifuged at 20° for 2 mins at 800 g before the fluorescence was read in 1 cm quartz cuvettes. The room temperature was maintained at 20° C ± 0.5° during the reading time. Readings were corrected for background fluorescence from a dialyzed blank and concentrations were calculated using a fluorescence coefficient determined by a set of standards read along with the samples. Concentration ranges of the standards were similar to those in the samples. No deviation from linearity was observed. After correcting for dilutions, the fraction of bound drug (C_B/C_T) was calculated using the expression $C_B/C_T = (C_T-C_F)/C_T$ where C_T is total drug concentration, as determined from measurements of the drug-DNA complex side of the dialysis cell, and C_F is free drug concentration.

Synthesis. The syntheses of I and II outlined in the Scheme below, followed, in general, reported procedures.[20] The 2-amino-diphenyl (III)[21]



was condensed with IV to give amide V which was cyclized to phenanthridine VI. Hydrolysis of the nitrile to the corresponding carboxyl was accomplished by heating in 75% H_2SO_4; we had previously found in the isomeric p-substituted phenyl derivative, that alkaline hydrolysis gave an impure product. The acid VII was converted to VIII using the method of Bosshard et al.[22] The next two steps--condensation with 1,5-diaminopentane, yielding IX and quaternization to X--were successful only when glassware was well dried in the oven and solvents were freshly distilled. Final reduction to II proceeded well, but removal of the several impurities in the product was very difficult. The product was relatively insoluble in water, methanol or ethanol, but slightly more soluble in aqueous alcohols. Recrystallization effected almost no purification. Finally, repeated passing through Sephadex gave a pure product, removing the small amounts of "monomeric" materials easily, but requiring a number of treatments to remove the other contaminants. HPLC and TLC were used to monitor the process. It was difficult to remove solvents from the product. Even after drying at 130°/0.15 mm for 24 hr over P_2O_5 the NMR spectrum showed 4 H_2O and a trace of acetone (used in the last precipitation). Table I presents experimental details for the preparations of intermediates and final compounds.

RESULTS AND DISCUSSION

Spectra. The visible spectra of I and II are shown in Figure 1, the left ordinate applying to I and the right to II. The visible spectrum of II alone shows a shift to longer wave length and a significant hypochromicity compared to I or ethidium chloride. This is probably due to intramolecular stacking of the two chromophores. When II is bound to DNA the visible absorption maximum is at precisely the same wave length as in the complex of I with DNA. The shapes and amplitudes per chromophore of the DNA-drug complex spectra are very similar. The induced circular dichroism spectra of the DNA complexes of I and II are shown in Fig. 2. They are also nearly superposable, indicating that in the DNA complexes, the asymmetric environments of the two chromophores of II are very similar to that of I.

The fluorescence intensity per mole of ethidium ion is greatly enhanced on binding to DNA[23] and there is evidence[24] that this enhancement is due to the protection of the amine hydrogens from exchange with the solvent when intercalation occurs. Similar enhancement is observed

Table I. Preparation and Properties of Double Intercalator and Intermediates

Cpd. No.	Reactants	Solvent	Conditions	Purification	% Yield	m.p.[a] °C	TLC[b] R_f	Other
V	III[c] + IV[d]	C_6H_5Cl	Reflux 50 min	Recryst. HOAC	93	226.5-227.5	0.25 (A)	IR
VI	III, $POCl_3$	$C_6H_5NO_2$	Reflux, 2 hr	Triturate, EtOH	93	301-303	0.4 (A)	IR
VII	VI, 75% H_2SO_4	---	130°, 1.5 hr	Recryst. DMF-EtOH	65	335-343[e]	---	IR
VIII	VII, $SOCl_2$,DMF[f,g]	C_6H_5Cl	70-75°, 1.5 hr	Washed, pet. ether	96	amorphous 340-355d.	---	IR
IX	VIII, Et_3N,[f,g] $H_2N(CH_2)_5NH_2$	DMF[f]	Room temp. 16 hr.	Pptated from hot DMF with EtOH	68	amorphous 310-321d. (capillary)	0.3 (B)	IR, NMR Anal.[l]
X	IX, Me_2SO_4[g]	DMF[f]	170°, 1 hr DMF removed; Trit. aq HBr	---	81	---	0.6[o] (B)	IR
II	X, HBr,Fe	EtOH-H_2O	Reflux, 1 hr	Converted to Cl^-; Sephadex[i]	20	230-240[h]	0.1[p] (C)	NMR, HPLC[k] Anal.[m]
XI	VIII, $EtNH_2$	---	0°, 1 hr	Triturate, EtOH	86	amorphous 260-280d.	0.6 (B)	IR
XII	XI, Me_2SO_4[g]	DMF[f]	As for X	Converted to pseudo base, then back to Br^- salt	50	---	---	IR
I	XII, HBr, Fe	EtOH-H_2O	Reflux, 1 hr	Sephadex[j]	45	210-215[h]	0.3[p] (C)	IR, NMR HPLC[k], Anal.[n]

a. On Kofler-type hot stage, unless otherwise noted.
b. All spots detected as uv absorbing, unless otherwise noted. Solvent systems: A, $CHCl_3$; B, $CHCl_3$/MeOH (10/1); C, BuOH/HOAc/H_2O (4/1/1).
c. Ref. 21.
d. Prepared by action of $SOCl_2$ on the corresponding acid; m.p. 40-41° C.
e. Amorphous solid changed to fine needles at about 250°, which then melted 335-343° C.
f. Freshly distilled.
g. Glassware oven-dried overnight.
h. Exact temperature at which softening began was difficult to determine because the solid was very dark.
i. Sephadex LH-20 column, 36 cm long, 2.5 cm ID, flow rate 2 ml/min; solvent, MeOH/H_2O (10/1).
j. Sephadex LH-20 column, 80 cm long, 2.5 cm ID, flow rate 1 ml/min; solvent, MeOH. One passage of the material through this column separated faster moving and slower moving materials from I.
k. On HPLC column of μPorasil (Waters), 300 mm long, 4 mm ID, 2000 psi, flow rate 2 ml/min, solvent, MeOH/H_2O/HOAc (50/50/3), II had a retention time of 8.1 mins; I, 3.0 mins; ethidium chloride, 3.3 mins; propidium diiodide, 8.7 mins.
l. Anal. Calcd. for $C_{45}H_{32}N_8O_{10}$: % C, 64.0; H, 3.82; N, 13.3: Found, % C, 63.5; H, 3.78; N, 13.1.
m. Anal. Calcd. for $C_{47}H_{46}N_8Cl_2O_2.4H_2O$: % C, 62.9; H, 6.06; N, 12.5: Found: % C, 62.6, H, 5.88; N, 12.4.
n. Anal. Calcd. for $C_{23}H_{23}N_4OBr.1.4\ H_2O$: % C, 58.0; H, 5.46; N, 11.8; Br, 16.8: Found: % C, 57.7; H, 5.14; N, 11.6; Br, 17.4.
o. Yellow-orange fluorescence.
p. Bright red fluorescence.

for I. The fluorescence intensity per mole of II alone is unusually small, only 9% of that of I. However, when II is bound to DNA, the fluorescence is enhanced far more than that of I, until at a DNA concentration of 10^{-4} M^{-1} and a DNA:drug ratio of 50:1 the fluorescence

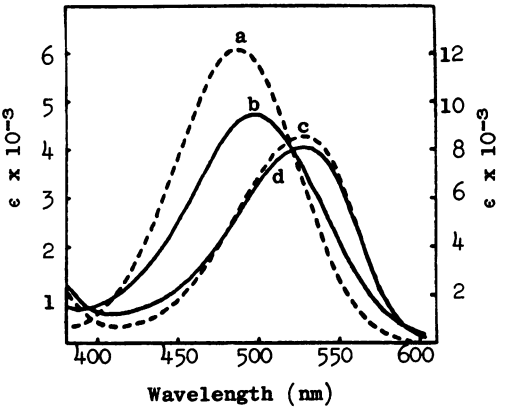


Fig. 1. Visible absorption spectra. --- I, left ordinate. —— II, right ordinate. (a,b) drug alone; (c) DNA(P)- I (10:1) complex; (d) DNA(P)-II (20:1) complex.

intensity per mole of II is approximately double that of I. This suggests that the interaction of the two chromophores in unbound II quenches the fluorescence, but that when II is bound to DNA, this mutual quenching is removed and the usual enhancement due to intercalation applies to both chromophores.

Binding Affinity. A first approximation to the effect of double intercalation on the DNA-binding constant is to assume that the standard free energies of binding of the two chromophores are simply additive. If this assumption were true, the intrinsic binding constant of the double intercalator would be the square of that for the single intercalator. However, consideration of several obvious factors suggests that non-additivity might be important. For example, the conformation of the linking group which permits double intercalation may not be the minimum energy conformation, or perhaps only one of several low energy conformations permits double intercalation. Another possibility would be that neither chromophore could attain the quality of fit possible in the absence of the other. Thus, although one expects a very high binding constant for a double intercalator, the square of the corresponding single intercalator binding constant can only be taken as a rough guide.

When the ratio of total drug concentration (C_T) to the DNA phosphate concentration (C_P) is small, the fraction of drug bound (C_B) should obey the equation

$$\frac{C_B}{C_T} = \frac{\sigma C_P}{1 + \sigma C_P}$$

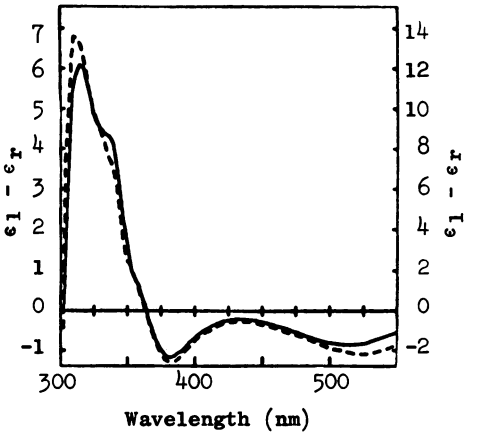


Fig. 2. Induced circular dichroism spectra. Legend as in Fig. 1.

where σ is the product of the intrinsic binding constant of an isolated binding site and the number of nucleotide phosphates per binding site. This product has been designated "affinity" by Müller and Crothers.[25] We find that fitting this equation to dialysis data from a serial dilution experiment gives a much more reliable estimate of σ than extrapolation of a Scatchard plot to r = 0, since the latter is usually curved.

The affinity can be shown to be equal to a weighted mean intrinsic binding constant, $< K >$, times the number, n, of binding sites per nucleotide phosphate.

$$\sigma = \Sigma_i n_i K_i = \left[\Sigma_i \left(\frac{n_i}{n}\right) K_i\right] n = < K > n$$

One might conclude that the number of binding sites for the double intercalator is less than that for monointercalators. However, the distinction must be made between the number of potential binding sites available to the first drug molecule and the number of binding sites blocked by a bound molecule. The former number is independent of the size of the ligand except for end effects, which are negligible for highly polymerized DNA. Thus the affinity can be used for direct comparison of the weighted mean binding constants for both double and single intercalators.

The limitation in this, or any method, of determining binding constants is the ability of the analytical method to measure the free drug concentration. Since the maximum free drug concentration is inversely related to the binding affinity, we have used magnesium ion to lower the affinity of these cationic drugs.

Table II. DNA-Binding Affinity (M^{-1})

	σ (2 mM $MgCl_2$)	σ (12 mM $MgCl_2$)
Ethidium	9.0 (3)[a] x 10^4	1.2 (4) x 10^4
I	7.1 (8) x 10^4	4.0 (3) x 10^4
II	> 5 x 10^6	> 5 x 10^6

a. Variation of least significant figure (standard deviation).

Table II shows the affinities determined for ethidium chloride and I at two concentrations of magnesium ion. Based on these results, if II double intercalates, one would expect the DNA binding affinity to be in the neighborhood of 10^8 M^{-1} in the presence of 12 mM magnesium chloride. An attempt to apply the dialysis dilution method to II gave only a lower limit to the affinity. At a DNA(P) concentration of 2 x 10^{-6} M and total drug concentration of 4 x 10^{-8} M we were unable to detect any fluorescence above background on the free drug side of the membrane. Fluorescence should have been detected if the free drug concentration were as much as 3 x 10^{-9} M. This would correspond to a binding affinity of 5 x 10^6 M^{-1}. Thus we conservatively conclude that a lower limit to the affinity is 5 x 10^6 M^{-1} under these conditions.

Thermal Denaturation. Another illustration of the large affinity of II for helical DNA is its effect on the helix-coil transition of DNA in 0.01 M phosphate buffer, shown in Fig. 3. The curve for II is clearly bimodal. Although it is not apparent in Fig. 3, careful analysis of the original data indicates a bimodal thermal denaturation curve for I, but with a much lower second transition temperature. A qualitative interpretation of this bimodal transition behavior[26-28] is that the first transition represents the unwinding of regions of DNA with little or no bound drug, with a consequential increase in the degree of saturation of the remaining helical segments. When the helical DNA is saturated, the unwinding process pauses until a temperature is reached where the helix stability is reduced to a value which permits dissociation of drug from helical regions, even in the presence of a large excess of drug. The remaining helical sections then unwind. Thus the temperature of the second transition should be related to the affinity of drug binding to helical DNA.

Such curves have been calculated using a nearest neighbor Ising model[27] for DNA; the theoretical curves obtained for binding constants of the order of 10^8 and higher have the appearance of curve D of Figures 3 and 4, but since this model assumes a homogeneous polymer, the transitions are much

sharper. It should be emphasized that ligand-induced shifts of helix-coil transitions to higher temperature indicate only that the affinity of the ligand for helix is greater than that for coil.[26,27,29]

Part of the effect on the helix-coil transition must be due to the added positive charge in II. In 0.01 M phosphate buffer the doubly charged drug propidium diiodide also has a much more profound effect on the helix-coil transition than does I; however, the effect is considerably less than for II. When the transition is observed in the presence of 2 mM magnesium ion where the importance of electrostatic interactions is reduced (Fig. 4), the difference between II and propidium diiodide is even more pronounced. Thus a significant proportion of the additional stabilization must be attributable to the second phenanthridinium group.

We conclude from the above results that both phenanthridinium groups of II intercalate in DNA and that the resulting enhancement of the binding affinity approaches the value expected for additivity of the standard free energy of binding of a monomer.

Inhibition of Nucleic Acid Synthesis. In spite of the high affinity of II for DNA, it is disappointingly ineffective as an inhibitor of nucleic acid synthesis in L1210 cells. The median effective dose for inhibition of DNA and RNA synthesis in L1210 cell culture by the compounds is shown in Table III.

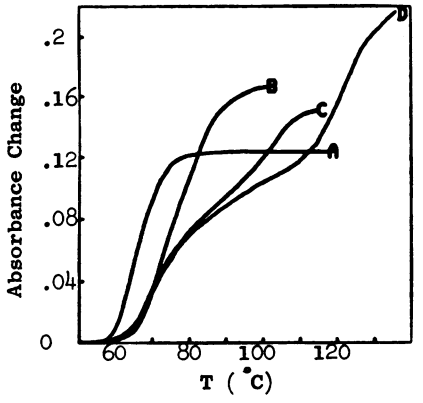


Fig. 3. In 0.01 M phosphate buffer

T_m: A, 67°; B, 76°; C, 81°; D, 98°.

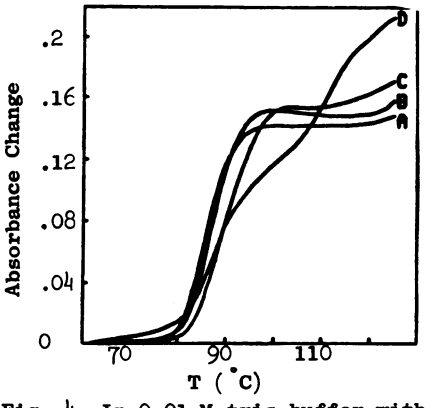


Fig. 4. In 0.01 M tris buffer with 2 mM $MgCl_2$

T_m: A, 86°; B, 87°; C, 90°; D, 97°.

Figs. 3 and 4. Effect of drugs on the helix-coil transition temperature. A, DNA alone; B, DNA(P)-I (10:1); C, DNA-propidium diiodide (20:1); D, DNA-II (20:1).

Table III. Median Effective Dose (μM) for Nucleic Acid Synthesis Inhibition in L1210 Cells

	DNA	RNA
Ethidium	31 (25-38)[a]	11 (10-12)[a]
I	200 (100-400)	50 (40-63)
II	100 (80-126)	17 (15-20)
Propidium	> 1000	> 1000

a. ± one standard deviation in log ED_{50}.

The ED_{50} values were derived from a least squares fit of a log-normal cumulative probability curve to the results of several experiments. This method assumes that the response of the cell population to the drug is log-normally distributed about the log ED_{50}. The standard deviation of the distribution is an additional parameter for evaluation of the drugs. Substitution of the N-ethyl amide moiety in the 3-position of the phenyl group (compare I with ethidium chloride) reduces the ability to inhibit nucleic acid synthesis in general, while maintaining the differential between RNA and DNA synthesis inhibition. Most of the efficacy for RNA synthesis inhibition is regained in going from I to II, but the width of the population distribution is significantly greater. Since the DNA binding affinity of I is comparable to that of ethidium and that of II is much greater, perhaps the reduced inhibitory effect of these drugs is due to lower cellular or nuclear uptake rates. The extremely low activity of propidium diiodide in the nucleic acid synthesis inhibition assay is consistent with the hypothesis that the double positive charge of II slows its transport across cell membranes. The increased standard deviation of the log-normal population distribution would also be consistent with this hypothesis, since the diversity of the cells with respect to membrane permeability would be superposed on the diversity with respect to primary enzyme inhibition.

An isolated enzyme assay would avoid this problem, but the only isolated RNA polymerase available is bacterial in origin and we prefer to focus our attention on RNA synthesis in mammalian cells. Thus we are developing assays which should determine the importance of drug transport rates on RNA and DNA synthesis inhibition in cell culture.

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