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Existence of an Extended Series of Antitumor Compounds Which Bind to Deoxyribonucleic Acid by Nonintercalative Means†

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ABSTRACT: Viscometric titrations of bacteriophage PM2 closed circular DNA, in addition to spectrophotometric and fluorometric methods, were used to investigate the mode of DNA binding of a number of antitrypanosomal and antitumor compounds. Several classes of compounds were identified which failed to unwind PM2 DNA, which appeared to have a large DNA binding site of at least 4 base pairs and which often showed considerable selectivity of binding to poly[d(AT)] as opposed to poly[d(GC)]. The classes included the antiviral antibiotics distamycin and netropsin, bisamidines such as the trypanocidal drug berenil, phthalanilide bisamidines, aromatic bis(guanylhydrazones), and the bisquaternary ammonium heterocycles. It is proposed that the compounds all bind in the minor groove of the DNA double helix.

In recent years, Cain and co-workers (Cain et al., 1969, 1971) have synthesized a large number of compounds termed bisquaternary ammonium heterocycles [reviewed by Denny et al. (1979)]. Many of these compounds are highly active against the murine L1210 leukemia in mice, and in structure they resemble another group of antiprotozoal and antitumor compounds termed the phthalanilides (Bennett, 1965; Cain et al., 1969). On the basis of model building studies, Cain et al. (1969) proposed that these compounds bind to the minor groove of the DNA double helix, but experimental evidence was not advanced. Waring (1970) reported that the trypanocidal drug berenil did not unwind closed circular DNA, and subsequently several workers studying the antibiotics netropsin and distamycin concluded that they bound, without intercalation, in the minor groove of double-stranded DNA (Wartell et al., 1974; Zimmer, 1975). Since netropsin, like the bisquaternary ammonium heterocycles and the phthalanilides, contains terminal basic functions separating a largely coplanar aromatic skeleton, it seemed worthwhile to examine the DNA binding properties of these other classes. The viscometric analysis of closed circular DNA (Revet et al., 1971) has been

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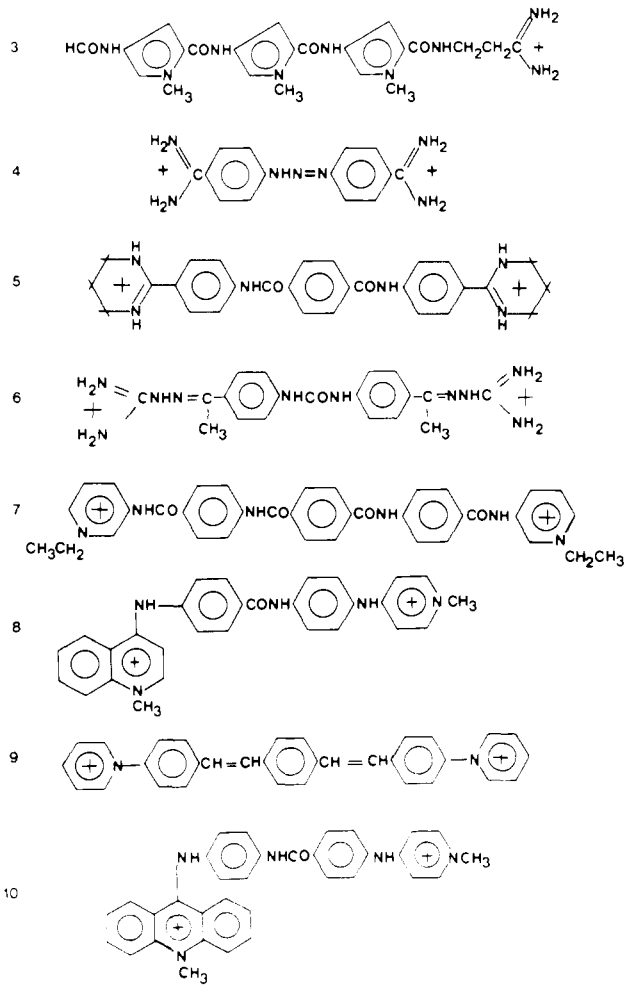
Chart I



combined with spectrophotometric and fluorometric studies to characterize this binding. Ethidium, 9-aminoacridine, and distamycin A have been used as reference compounds.

Experimental Procedures

Materials. Bisquaternary ammonium heterocycles (compounds **7**–**10**, Chart I) were provided by Dr. B. F. Cain, Cancer Chemotherapy Laboratory, Auckland, and were pure as judged by thin-layer chromatography. The phthalanilide NSC 57153 was a gift from Dr. H. B. Wood to Dr. Cain, and DDUG[1] was a gift from Dr. E. Mihich. Berenil was provided by May and Baker Ltd. Distamycin was purchased from Boehringer Mannheim, Germany, and ethidium bromide, 9-aminoacridine hydrochloride, poly[d(AT)], poly[d(GC)], calf thymus DNA, and dextran sulfate (M_r 275000) were from Sigma Chemical Co. Phage T2 DNA was from Miles Laboratories. Phage PM2 DNA, prepared according to the method of Espejo et al. (1969), was kindly provided by Dr. A. R. Morgan, Department of Biochemistry, University of Alberta. It was stored frozen in 0.01 M SHE buffer (9.4 mM NaCl, 2 mM Na^+–Hepes, pH 7.0, and 20 μM EDTA).

Spectrophotometry and Fluorometry. Spectra were recorded in a Cary Model 15 spectrophotometer, using either 0.01 M SHE buffer or 0.2 M SHE buffer (199.4 mM NaCl, 2 mM Na^+–Hepes, pH 7.0, and 20 μM EDTA). Ethidium displacement assays were carried out in 0.01 M SHE buffer by using 1.26 μM ethidium and 1 μM DNA (in nucleotides) as previously described (Baguley & Falkenhaug, 1978; Cain et al., 1978). A Zeiss PMQ-2 spectrophotometer with a ZMF-4 fluorescence attachment was used for these assays (excitation at 546 nm and measurement at 595 nm). The C_{50} value is defined as the drug concentration which reduces the fluorescence of initially DNA-bound ethidium by 50%.

Viscometry. The method followed that of Waring (1976), which is based on that of Revet et al. (1971). The viscometer had a bore size of 0.4 mm and a flow time for distilled water of 92.6 s. It was filled with 1.3 mL of a dust-free solution of PM2 DNA (150–250 μM in phosphate) in 0.01 M SHE buffer. Agents were added from a microsyringe as concentrated (200–860 μM) solutions in water rather than buffer, since several drugs precipitated if dissolved directly in buffer. Flow times were measured 2–4 times for each addition, and the reduced viscosities were calculated by standard methods.

[1] Abbreviations used: DDUG, 4,4′-diacetyldiphenylureabis(guanylhydrazone); poly[d(AT)], poly(deoxyadenylic deoxythymidylic acid) (double stranded); poly[d(GC)], poly(deoxyguanylic deoxycytidylic acid) (double stranded); D/P, drug/phosphate ratio.

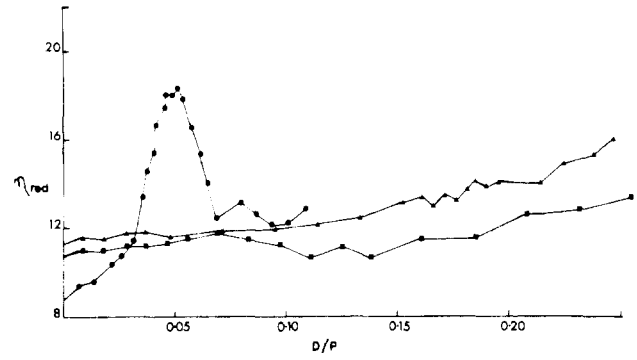


FIGURE 1: Effects of ethidium (●), distamycin A (▲), and compound **6** (■) on the viscosity of closed circular PM2 DNA. The reduced viscosity was calculated for each increment and expressed in terms of the drug/DNA phosphate (D/P) ratio.

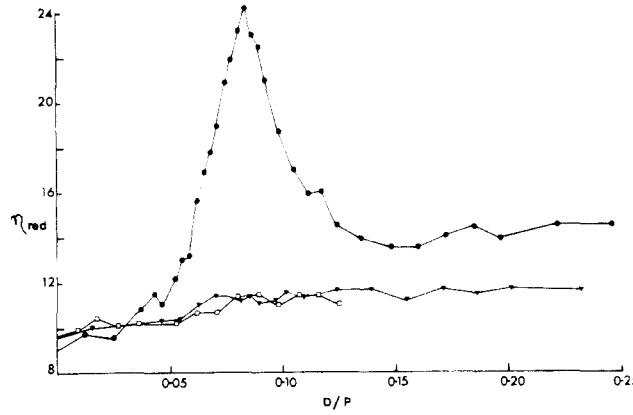


FIGURE 2: Effects of 9-aminoacridine (●), compound **5** (▼), and compound **7** (□) on the viscosity of closed circular PM2 DNA.

Results

Reference Intercalating Compounds. Ethidium (compound **1**, Chart I) is a trypanocidal drug with well characterized DNA binding properties (Sobell et al., 1977). When added to PM2 DNA, it caused the characteristic changes in viscosity (Figure 1) indicative of first unwinding of the negatively supercoiled form and secondly the formation of a positively supercoiled form. The binding ratio at the equivalence point, obtained with the DNA in a relaxed form with maximum viscosity, was 0.051 ethidium molecule/base pair, similar to that found by Waring (1976). Under these ionic conditions, ethidium has a binding constant for (relaxed) DNA of $\sim 3 \times 10^6$ M^{-1} (Baguley & Falkenhaug, 1978), consistent with

Table I: Compounds and DNA Binding Data

no.	name[a]	anion	C_{50} values[b] poly-[d(AT)]	T2 DNA	c.t. DNA	poly-[d(GC)]	unwinding angle[c] (deg)
1	ethidium	Br^-					26
2	9-amino-acridine	Cl^-	10	7.7	8.1	9.1	16
3	distamycin A	Cl^-	0.07	1.3	1.8	36	
4	berenil	Cl^-	0.90	1.5	2.6	5.7	
5	NSC 57153	Cl^-	2.2	2.5	4.0	11	
6	DDUG	Cl^-	0.43	0.66	0.69	1.3	
7	NSC 101327	Tos[d]	0.40	0.43	0.48	0.70	
8	SN 6999	Br^-	0.17	1.1	1.4	2.8	
9	SN 18071	Tos	0.20	0.59	0.76	2.1	
10	SN 13521	Cl^-	0.14	0.16	0.20	0.32	8

[a] Common name, NSC screening number, or laboratory screening number. [b] Micromolar drug concentration to decrease fluoresence of DNA-bound ethidium by 50% under standard conditions. c.t. = calf thymus; T2 = bacteriophage T2. [c] Unwinding angle based on the equivalence point if one was reached. [d] *p*-Toluenesulfonate salt.

Table II: Spectrophotometric Changes in the Presence of Calf Thymus DNA

compd	free drug-DNA complex D/P = 0.05 λ_{max} (nm)	$\epsilon_m \times 10^{-3}$	D/P = 0.5[c] λ_{max} (nm)	$\epsilon_m \times 10^{-3}$	dextran sulfate complex[d] λ_{max} (nm)	$\epsilon_m \times 10^{-3}$
5[a]	291	34	293	26	293	27
6[b]	310	50	317	43.5	316[a]	38
7[a]	304	60	314	43	306	40
8[a]	350	30	354	24	352	22.5
9[a]	370	70	379	48	360	34
10[a]	444	12	444	9.7	444	8.0

[a] Spectra recorded in 0.01 M ionic strength buffer, pH 7. [b] Data quoted from Dave et al. (1977) in 0.02 M buffer, pH 7. [c] In 0.2 M ionic strength buffer this form was weak or undetectable. [d] Maximal changes recorded in 0.01 M ionic strength buffer, pH 7, in the presence of 5–100 μg/mL dextran sulfate and 10 μM drug. Similar results were obtained in 0.2 M buffer, pH 7.

99.8% of the added ethidium being in the bound form.

9-Aminoacridine (compound **2**) shows a variety of growth inhibitory and mutagenic properties, mainly in bacteria (Albert, 1966). It causes similar changes in the viscosity of PM2 DNA to those caused by ethidium (Figure 2) except that the equivalence point is 0.083 drug molecule/base pair. The difference is not due to reduced binding, since the binding constant to relaxed DNA at this ionic strength is 5×10^5 M^{-1} (Wilson, 1978) and 99% of the added drug should be bound. The difference in the equivalence point is presumably either the result of a lower unwinding angle [16°, as compared to 26° deduced by Wang (1974) for ethidium] or a consequence of some drug being bound in a nonintercalated form (Blake & Peacocke, 1968).

Distamycin A. This antibiotic (compound **3**, Chart I), together with netropsin, is best known for its antiviral properties and its selective binding to adenine–thymine-rich DNA (Zimmer, 1975). The ethidium displacement data in Table I show a pronounced selectivity for poly[d(AT)] over poly[d(GC)], with T2 phage DNA and calf thymus DNA giving intermediate values. Work by others (Zimmer, 1975) has shown that distamycin spans 4 base pairs in the DNA. Its lack of unwinding of PM2 DNA has been confirmed in this study (Figure 1).

Berenil. This trypanocidal drug (compound **4**) is one of a series of related bisamidines which are described in a review by Bennett (1965). Binding studies by Waring (1969) showed that at saturation 0.12 molecule of berenil bound to 1 nucleotide of double-stranded DNA, suggesting a site size of 4 base pairs. The drug does not unwind PM2 DNA (Waring, 1970). The ethidium displacement data (Table I) show that the drug binds more strongly to poly[d(AT)] than to poly[d(GC)], although the selectivity is not as great as with distamycin A.

Terephthalanilides. Compound **5** is representative of this class of compounds which show antitumor and trypanocidal activity (Bennett, 1965). The amide-linked aromatic skeleton, containing terminal positively charged groups, is thought to have a curved coplanar structure. Previous DNA binding studies (Sivak et al., 1963) indicated saturation at a ratio of 1 drug molecule/base pair. In the present work a biphasic change in the absorption spectrum of **5** was observed (Table II) with one change maximal at 1 molecule drug/base pair and a second change maximal at 1 molecule/10 or more base pairs. At an ionic strength of 0.2 M only the second of these transitions was evident. In the presence of dextran sulfate instead of DNA at either 0.01 or 0.2 M ionic strength, a spectral transition similar to the first of the transitions with DNA was observed. The change was maximal at a ratio of ~1 drug molecule/2 sulfate groups. The ethidium displacement data (Table I) indicate strong DNA binding, with a preference for poly[d(AT)]. No evidence of intercalation with PM2 DNA was found (Figure 2). The slight increase in reduced viscosity is consistent with stiffening or slight distortion of the helix, as has been observed with distamycin and netropsin (Zimmer, 1975).

Bis(guanylhydrazones). DDUG (compound **6**) is a highly active antitumor drug (Marxer, 1967; Mihich & Gelzer, 1968). DNA binding studies by Dave et al. (1977) have shown that **6**, like **5**, shows a biphasic shift in the absorption spectrum in the presence of DNA, the first form predominant at 1 drug molecule/DNA nucleotide and the second at 1 molecule/15 DNA nucleotides. We have confirmed these results and have shown in addition that the first form is almost absent at an ionic strength of 0.2 M and that the second form is absent in the presence of dextran sulfate. The ethidium displacement data show tight DNA binding but, in comparison with **5**, a reduced preference for poly[d(AT)] over poly[d(GC)]. DDUG does not unwind PM2 DNA (Figure 1).

Bisquaternary Ammonium Heterocycles. Compound **7** is a derivative of terephthalanilide, which is highly active against L1210 murine leukemia (Atwell & Cain, 1968) and is also a selective inhibitor of bacteriophage production following either infection or induction (Counsilman et al., 1974). Spectrophotometric studies (Table II) indicate two modes of binding to DNA at low ionic strength as found with compounds **5** and **6**. At 0.2 M ionic strength, conversion to only one form is observed, at a minimum ratio of 10 base pairs/drug molecule. Binding to dextran sulfate shows only one spectral form. Eithidium displacement indicates tight binding to DNA but only a slight preference for poly[d(AT)]. Binding to PM2 DNA is accompanied by a small increase in reduced viscosity (Figure 2).

Compound **8** is a highly active experimental antitumor agent (Cain et al., 1969) and like **7** it also inhibits the production of bacteriophage (Robertson, 1978). The ultraviolet absorption spectrum exhibits biphasic changes in the presence of DNA (Figure 4, Table II). The binding preference for poly[d(AT)] is very pronounced (Table I). The binding of **8** to poly[d(AT)]

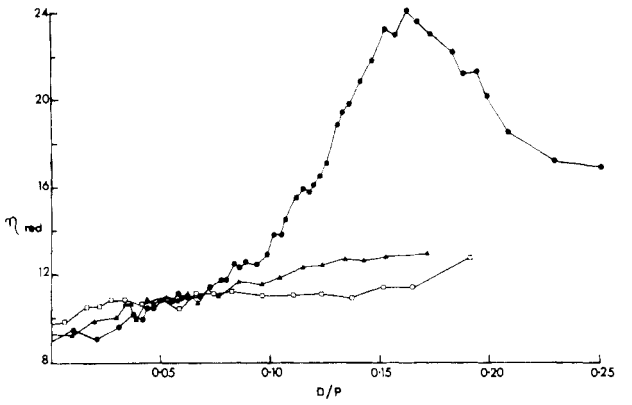


FIGURE 3: Effects of compound **8** (▲), compound **9** (□), and compound **10** (●) on the viscosity of closed circular PM2 DNA.

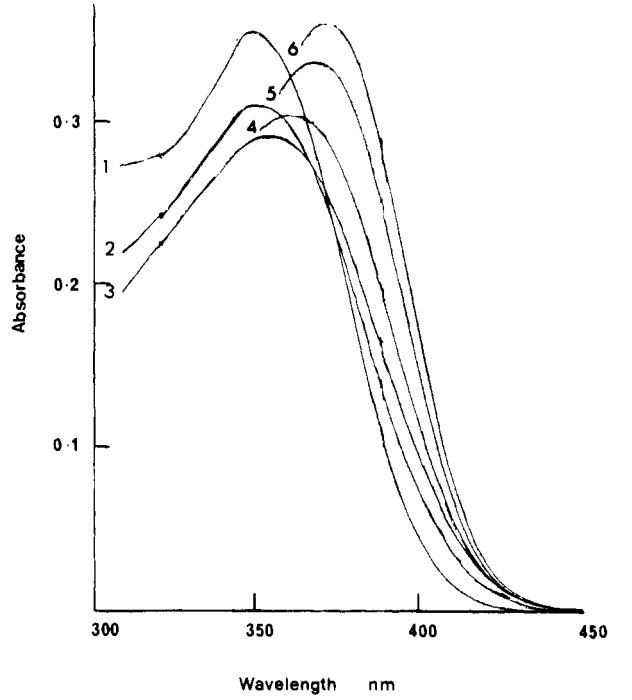


FIGURE 4: Absorption spectra of compound **8** in 0.01 M SHE buffer either alone (curve 1) or in the presence of calf thymus DNA at a drug/phosphate ratio of 1.0 (curve 2), 0.5 (curve 3), 0.25 (curve 4), 0.1 (curve 5), or 0.05 (curve 6).

in 0.2 M ionic strength buffer has been measured by a combination of spectrophotometric and equilibrium dialysis measurements (Figure 5). Analysis using the model of McGhee & von Hippel (1974) indicates a site size of 5 base pairs and a binding constant of $\sim 2 \times 10^6$ M^{-1}.

Compound **8** shows no evidence of intercalation (Figure 3). Two further compounds, the 6-amino derivative and the 6-chloro derivative (Atwell & Cain, 1973), have been tested in the PM2 DNA system with the same result (Braithwaite, 1978). The 6-amino derivative, as "quinolinium dibromide" (NSC 176319), has undergone extensive preclinical testing as an antitumor agent at the National Cancer Institute (Castles et al., 1976).

As a positive test of binding of compound **8** under the conditions employed, it was added to a complex of PM2 DNA with ethidium (Figure 6). Addition reversed the changes in the viscosity of DNA caused by ethidium, producing a new equivalence point (relaxed DNA) when 0.14 mol of drug was added per mol of DNA phosphate. On the assumption that the ethidium binding ratio is 0.051 at this point and that the site size of **8** is 5 base pairs, the equation of McGhee von

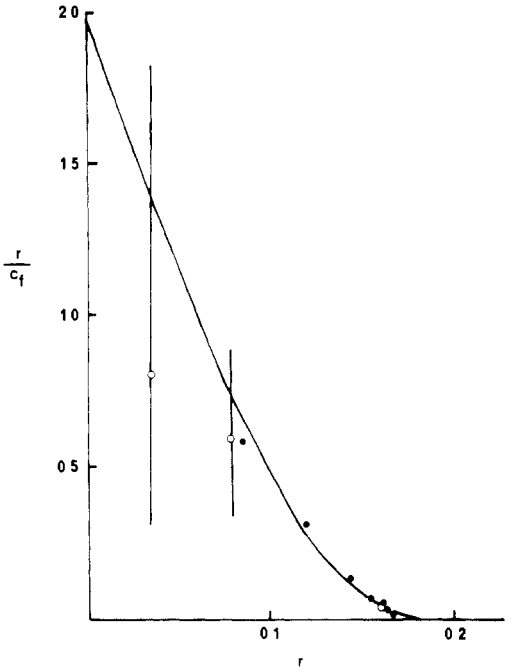


FIGURE 5: Scatchard plot for the interaction of compound **8** with poly[d(AT)] in 0.2 M SHE buffer. The isosbestic point for spectrophotometric measurements (closed circles) was 365 nm, and the absorbance was also measured at 385 nm to distinguish free and bound drug. Three equilibrium dialysis measurements (open circles) were also made utilizing two-chambered Teflon cells and a Visking dialysis membrane. Because of the low absorbance of drug in the DNA-free side, there were large errors (indicated by the vertical lines) in the estimates of the free drug concentration (c_f). r refers to the binding ratio in drug molecules per DNA base pair.

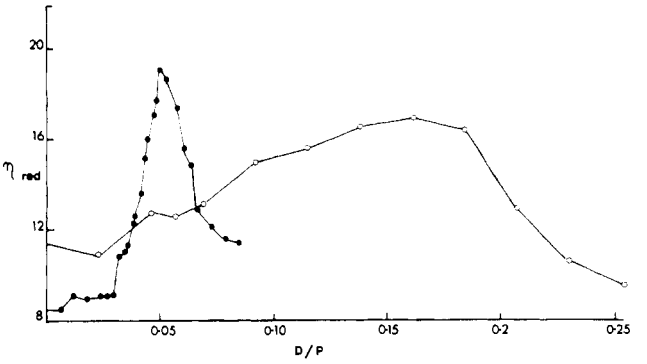


FIGURE 6: Effects of addition of compound **8** (○) on the viscosity of a complex of ethidium with closed circular PM2 DNA. The DNA solution (initially 288 μM) was first titrated with 229 μM ethidium (●) to a drug/phosphate ratio of 0.085. The resultant positively supercoiled DNA (267 μM) was titrated with 860 μM compound **8** (○).

Hippel (1974) for competing ligands may be utilized to calculate the binding constant for relaxed PM2 DNA at 0.01 M ionic strength. This value (8×10^6 M^{-1}) is very approximate since there is considerable site heterogeneity in PM2 DNA.

Compound **9** is a further example of a bisquaternary ammonium heterocycle which is an active experimental antitumor agent (Denny et al., 1979). The ultraviolet spectrum shows biphasic changes in the presence of DNA, and the ethidium displacement data show a selectivity for poly[d(AT)] which is similar to that shown by compound **8**. The main interest of this compound is that it is incapable of forming hydrogen bonds. It does not unwind PM2 DNA (Figure 3).

Acridine Bisquaternary Salts. Compound **10** (Chart I) is analogous to **8** except that the extra ring of the acridine nucleus forces the molecule to adopt a non-coplanar configuration. It

is inactive as an antitumor agent, but several closely related derivatives are active (Cain et al., 1971). Spectral studies (Table II) indicate a biphasic change when **10** binds to DNA and a large apparent site size. The ethidium displacement data indicate only a slight binding preference for poly[d(AT)] (Table I). Addition of **10** to PM2 DNA causes unwinding (Figure 3) but only at high drug/DNA ratios. It is possible that the drug binds to DNA by two different modes, with an intercalative mode at a high binding ratio. Alternatively, compound **10** may bind to a highly distorted site of the DNA double helix, as has been suggested for the steroidal diamines (Dattagupta et al., 1978).

Discussion

The DNA binding and DNA unwinding studies outlined in this study suggest the existence of an extended series of DNA binding compounds with the following properties.

(1) They are all aromatic, largely coplanar molecules with at least one basic charge. They may have an overall curved linear shape (Cain et al., 1969).

(2) They bind to closed circular DNA with little or no unwinding and hence differ from intercalating agents.

(3) Their binding to DNA at 0.01 M ionic strength is accompanied by changes in the ultraviolet absorption spectrum, displacement of ethidium, and, in several cases studied, elevation of the DNA denaturation temperature. Binding to DNA in 0.2 M ionic strength buffer is also detectable by changes in the absorption spectrum and by ethidium displacement (data not shown).

(4) As measured by ethidium assays, they bind selectively to poly[d(AT)] as compared to the other DNA types tested. Binding of some drugs to DNA can cause quenching of the fluorescence of bound ethidium, as well as displacement of ethidium (Baguley & Falkenhaug, 1978). In the case of this series, quenching of fluorescence is minor as compared to displacement (results not shown), and the dA·dT selectivity is thus not a result of differential quenching effects. The selectivity in some cases is highly pronounced and is also observed at higher ionic strength. Although specific hydrogen bond formation may contribute to this selectivity (Zimmer, 1975), the results with compound **9** indicate that it is not essential.

(5) Spectrophotometric data indicate a large DNA site size which varies approximately with the linear dimensions of the molecule: e.g., 4 base pairs for distamycin A and berenil and 5 base pairs for compound **8**.

The biphasic spectral changes shown when these compounds bind to DNA in 0.01 M buffer indicate two modes of binding. The form at high drug binding ratios (maximal between 1 and 2 drug molecules/DNA base pair) is presumably a result of cooperative stacking of cationic drug molecules on a polyanionic surface. Spectral changes in these compounds have also been observed in the presence of the polyanion dextran sulfate (Table II). The spectral form observed at high binding ratios to DNA is, in general, intermediate between that seen with dextran sulfate and that seen with DNA at low binding ratios. It is suppressed when the experiments are carried out in 0.2 M ionic strength buffer.

The second form of DNA binding, observed at low binding ratios, is consistent with the notion that these molecules bind to the minor groove of the DNA double helix. The ethidium displacement data for phage T2 DNA are intermediate between those for poly[d(AT)] and calf thymus DNA (phage T2 DNA has a higher adenine–thymine content than calf thymus DNA), indicating that the presence of partially glucosylated (hydroxymethyl)cytosine groups (Sinsheimer, 1960) in the major groove of the phage T2 DNA does not influence drug binding. Furthermore, the depth of the minor groove is presumably greater adjacent to adenine–thymine base pairs, since the sterically demanding 2-amino group of guanine is absent. Thus, steric effects would provide a basis for dA·dT selectivity in the minor groove but not in the major groove.

There is now an abundance of analogues of the compounds discussed here which presumably bind to DNA by similar means. Recent studies on a series of bisquaternary ammonium heterocycles (Denny et al., 1979) and also on a series of aromatic bis(guanylhydrazones) (Denny & Cain, 1979) have indicated a high degree of correlation between DNA binding (as measured by ethidium displacement) and biological activity, with lipophilic–hydrophilic balance constituting a second important term. Thus, minor groove DNA binding appears to be biologically significant and will undoubtably form the basis for design of further antiprotozoal and antitumor agents.

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Correlation of Functional Elastin Messenger Ribonucleic Acid Levels and Rate of Elastin Synthesis in the Developing Chick Aorta†

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ABSTRACT: Thoracic aortas from 8- to 16-day chick embryos were incubated in vitro for 30 min with [^{3}H]valine, and the newly synthesized, labeled proteins were subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate and analysis with affinity-purified, elastin-specific antibody. The results demonstrate that at day 8 ~21% of the incorporated [^{3}H]valine was found in tropoelastin (70 000 daltons). In the following 8 days of development, there was a significant increase in the relative incorporation into tropoelastin so that at day 16, 43% was now found in tropoelastin. mRNA was isolated from aortas of various age embryos by digestion of the tissue with proteinase K in sodium dodecyl sulfate followed by chromatography on oligo(dT)-cellulose in order to investigate possible control mechanisms. The mRNA was translated in a reticulocyte lysate system, and the incorporation of [^{3}H]valine into products which were precipitable by either trichloroacetic acid or the elastin-specific antibody was determined. When 8-day aorta mRNA was translated, 18% of the trichloroacetic acid precipitable [^{3}H]valine was also immunoprecipitable. This value increased to 36% at day 10 and 45% at day 16. The injection of 150 μg of hydrocortisone 21-phosphate into 8-day eggs produced a significant increase in both the relative rate of tropoelastin synthesized by the isolated aortas and the amount of immunoprecipitable protein synthesized in the reticulocyte lysate system in response to mRNA isolated from the aortas of the treated embryos. The close agreement between the values determined from aortas incubated in vitro and from isolated mRNA translated in a cell-free heterologous system indicates that the observed changes in tropoelastin synthesis during development and after hydrocortisone administration are governed by the elastin mRNA content of the aortas.

Elastin is a vital component of the major blood vessels and lungs of vertebrates. A single 70 000-dalton polypeptide, designated tropoelastin, appears to be a soluble intermediate in the synthesis of the protein (Sandberg et al., 1969; Murphy et al., 1972; Rucker et al., 1973; Smith & Carnes, 1973; Foster et al., 1975; Narayanan & Page, 1976; Rosenbloom & Cywinski, 1976; Uitto et al., 1976), although the existence of a larger precursor has been reported (Foster et al., 1976, 1978). After secretion from the cell, the soluble elastin chains are extensively cross-linked by the enzymatic oxidation of lysine residues, resulting in highly insoluble elastin fibers (Partridge, 1962; Franzblau et al., 1965; Miller et al., 1965; Partridge et al., 1966; Pinnell & Martin, 1968). These mature fibers are responsible for the rubberlike resilience of the aorta and other arteries.

Microscopic observations and biochemical analysis of the embryonic chick aorta indicate that elastin biosynthesis follows a characteristic developmental pattern during embryogenesis. During the first 6 days of development, the aorta appears to be composed of a homogeneous population of rounded cells, and elastin fibers cannot be detected definitively by specific histologic staining techniques. After day 7 or 8 of development, however, the cell population appears more heterogeneous, smooth muscle cells become evident, and elastin fibers become readily discernible in the extracellular matrix (Karrer & Cox, 1961; Kadar & Veress, 1974). From day 8 to 16 of development, the rate of tropoelastin synthesis in the aorta increases dramatically and intercellular elastin fibers increase in both number and size. This characteristic pattern can be altered by administration of hydrocortisone in ovo at day 8 of development so that the relative rate of tropoelastin synthesis in the 9-day embryo becomes similar to that of a 13-16-day embryo (Eichner et al., 1978).

The biochemical mechanisms involved in this rise in tropoelastin synthesis during embryogenesis or following hydrocortisone administration have not been examined to date. Recent evidence from several developmental and hormonally induced systems has demonstrated changes in the synthesis of specific proteins which closely parallel changes in the abundance of their respective mRNAs (Chan et al., 1973; Alton & Lodish, 1977; Soh & Sarkar, 1978). Proteins synthesized by freshly isolated aortas of various ages incubated in vitro were compared with the products synthesized in a rabbit reticulocyte lysate system in response to mRNA isolated from the same age aortas in order to investigate whether the

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