Sherline, P., Bodwin, C. K., & Kipuis, O. M. (1974) *Anal. Biochem. 62*, 400–407.
Sternlicht, H., & Ringel, I. (1979) *J. Biol. Chem. 254*, 10540–10550.
Tanford, C. (1961) *J. Am. Chem. Soc. 83*, 1628–1636.
Taylor, E. W. (1965) *J. Cell Biol. 25*, 145–160.
Weingarten, M. D., Lokwood, A. H., Hwo, S., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858–1862.
Weisenberg, R., & Timasheff, S. N. (1970) *Biochemistry 9*, 4110–4115.
Wiche, G., Honig, L. S., & Cole, R. D. (1979) *J. Cell Biol. 80*, 553–563.
Wilson, L., & Meza, I. (1973) *J. Cell Biol. 58*, 709–719.
Wilson, L., Anderson, K., Grisham, L., & Chin, D. (1975) in *Microtubules and Microtubule Inhibitors* (Byers, M., & De Brabander, M., Eds.) pp 103–113, American Elsevier, New York.
Wyman, J. (1964) *Adv. Protein Chem. 19*, 224–285.
Zackoff, R. V., Weisenberg, R. C., & Deery, W. S. (1980) *J. Mol. Biol. 139*, 641–677.

Altered Physicochemical Properties of the Deoxyribonucleic Acid–Mitomycin C Complex. Evidence for a Conformational Change in Deoxyribonucleic Acid†

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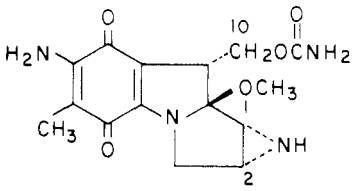
ABSTRACT: Binding of the antibiotic mitomycin C to sonicated calf thymus DNA results in increased viscosity and an unaltered sedimentation constant of DNA. Flow dichroism measurements of the mitomycin C–DNA complex indicate that the 310-nm absorbance vector of the chromophore of the bound drug is oriented at approximately 57.2° relative to the helix axis. A conclusion drawn from these results is that mitomycin C does not intercalate between base pairs, but rather, it is bound in one of the grooves. Binding of mitomycin C causes a number of changes which are DNA size dependent: (1) increased viscosity of sonicated, decreased viscosity of nonsonicated DNA; (2) unaltered sedimentation rate of sonicated, increased rate of nonsonicated DNA; (3) reduced electrophoretic mobility of nonsonicated DNA; (4) electron microscopic appearance of sonicated DNA–mitomycin complexes which is similar to that of control, while nonsonicated DNA complexes which display highly coiled, looped structures not seen in control nonsonicated DNA. These size-dependent effects are interpreted as indicative of conformational distortion of DNA at rare intervals, caused by a minor fraction of total bound mitomycin. The parallel use of sonicated and nonsonicated DNA as probes for certain effects of drug binding may be useful for detecting this type of phenomenon in general.

Mitomycin C (I) is a potent antibiotic and clinically useful antitumor agent (Remers, 1979). It inhibits cellular DNA synthesis selectively (Shiba et al., 1958) and interacts directly



I

with DNA by binding covalently to the individual strands ["monofunctional binding" (Szybalski & Iyer, 1964)], as well as inducing covalent cross-links between the complementary strands (Matsumoto & Lark, 1963; Iyer & Szybalski, 1963). The same types of interaction with DNA can be observed in vitro, but only if a reductive activating agent is added in situ, such as NADPH-dependent bacterial lysates (Iyer & Szybalski, 1964), certain chemical reducing agents (Iyer & Szybalski, 1964; Weissbach & Lisio, 1965), or NADPH-dependent rat liver microsomal preparations (M. Tomasz, unpublished results). These facts indicate that in vivo mitomycin C is converted by reductive metabolism into an active DNA-binding and cross-linking agent (Iyer & Szybalski, 1964). The cross-links were suggested to be the direct cause of the observed inhibition of DNA synthesis and death of bacteria (Iyer & Szybalski, 1963), although the monofunctional binding greatly predominates (10–100-fold) over the cross-links (Szybalski & Iyer, 1964; Weissbach & Lisio, 1965; D. J. Kaplan and M. Tomasz, unpublished results). Indeed, more recently the monofunctional attachment itself has also been implicated as biologically significant damage (Weiss et al., 1968; Kinoshita et al., 1971; Mercado & Tomasz, 1972; Small et al., 1976).

The active form of mitomycin is generated by reduction of the quinone system of I. The reactive, short-lived species has not been isolated or characterized (Iyer & Szybalski, 1964). Covalent interactions with DNA occur in this reduced state (Iyer & Szybalski, 1964; Weissbach & Lisio, 1965), but in the presence of air, the DNA-bound drug is reoxidized to a quinone as seen by the ultraviolet spectral properties of the isolated DNA–mitomycin complexes (Tomasz et al., 1974). The likely structure of the monofunctionally bound drug to DNA is shown in II, as inferred from the ultraviolet spectra, from mitomycin analogue binding studies (Lipman et al., 1978), and from model reactions of mitomycin (Hornemann et al., 1979; Hashimoto et al., 1980; Tomasz & Lipman, 1981). A second alkylating function, assumed because of the observed

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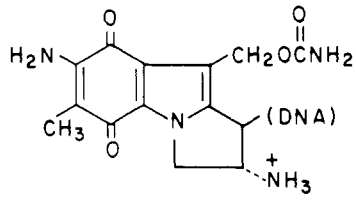

II

cross-links in DNA, is not obvious although postulated to be the C_{10} position (II) (Iyer & Szybalski, 1964; Moore, 1977). The binding to DNA and RNA is virtually guanine specific (Tomasz et al., 1974; Lipman et al., 1978). The number of cross-links increases with increasing G + C content of DNA (Iyer & Szybalski, 1964; Lown et al., 1976). The binding site(s) of guanine is (are) not known. In addition to its covalent affinity to DNA, activated mitomycin C also displays noncovalent, electrostatic binding to nucleic acids in vitro, observable at high drug concentratons and/or low ionic strength (Lipman et al., 1978).

Some effects of exposure to mitomycin on the macromolecular properties of DNA have been noted: reversible denaturation (Matsumoto & Lark, 1963; Iyer & Szybalski, 1963, 1964), increased T_m (Cohen & Crothers, 1970; Tomasz et al., 1974; D. J. Kaplan and M. Tomasz, unpublished results), and broadened melting profiles (Tomasz et al., 1974; D. J. Kaplan and M. Tomasz, unpublished results), all attributed to cross-links in DNA. Altered circular dichroism spectra were also reported (Mercado & Tomasz, 1977). We studied a variety of additional properties of mitomycin-modified DNA in order to test whether mitomycin is intercalated and, in a broader sense, whether its binding induces any characterizable conformational change of DNA.

Materials and Methods

Sources of DNA. Calf thymus DNA was used throughout this work unless indicated otherwise. *Calf thymus DNA* (Worthington, Freehold, NJ) was further purified by treatment with 40 units of RNase T_1 per mg of DNA per mL of 0.015 M NaCl–0.002 M sodium citrate, pH 7.4, for 1 h at 37 °C, followed by ethanol precipitation and repeated deproteinization by $CHCl_3$–isoamyl alcohol (Marmur, 1961). Its average molecular weight was 5.4×10^6 (see below). *Sonicated calf thymus DNA* was obtained by using a Branson Model W-185 sonicator for one preparation and Model W-140 for another, applying otherwise the same procedure (Müller & Crothers, 1968). The average molecular weights of the two preparations were 3.8×10^5 and 2.0×10^5, respectively, determined by the viscosity method (see below). [^{3}H]DNA from *Diplococcus pneumoniae* (biosynthetically labeled; $\sim 1.5 \times 10^4$ dpm/OD_{260}) was a gift of Dr. Alexander Tomasz, The Rockefeller University.

Mitomycin–DNA complexes with various binding ratios were prepared by using $Na_2S_2O_4$ as the reducing agent to activate mitomycin in the presence of DNA under anaerobic conditions (Iyer & Szybalski, 1964). A modified procedure (method B) and isolation method was used, as described previously (Tomasz et al., 1974). Binding ratios are defined as moles of antibiotic bound per mole of mononucleotide unit and were determined as in the same reference.

Ethidium–DNA complexes with varying binding ratios were prepared by mixing ethidium bromide and DNA solutions in 0.015 M NaCl–0.002 M sodium citrate, pH 7.4, in appropriate proportions (Aktipis & Kindelis, 1973).

Viscosity Measurements. A Zimm–Crothers (Zimm & Crothers, 1962) Model A rotary viscometer (Beckmann) was used. Constant temperature (25.0 °C) was provided by circulation of water through the jacket of the apparatus via a Lauda circulator bath. The buffer used was 0.015 M NaCl–0.002 M sodium citrate–9×10^{-4} M ethylenediaminetetraacetic acid (EDTA), pH 7.4. Intrinsic viscosity of each sample was calculated from a linearly analyzed plot of reduced viscosity vs. DNA concentration (c) by extrapolating it to $c = 0$ (Bloomfield et al., 1974a). The DNA concentration range used was 0.40–1.20 and 0.01–0.05 mg/mL for sonicated and unsonicated DNA, respectively.

Molecular weights of unsonicated DNA were calculated by the modified (Crothers & Zimm, 1965) Mandelkern–Flory equation (Mandelkern & Flory, 1952), those of sonicated DNA above 3×10^5 by the equation of Doty, McGill, and Rice (Doty et al., 1958), and those below 2×10^5 by the equation of Eigner and Doty (Eigner & Doty, 1965).

Ultracentrifugation was carried out by using a Beckman L3-50 preparative ultracentrifuge equipped with an SW 27 rotor, in a continuous isokinetic gradient of sucrose (10–35%) in 0.017 M potassium phosphate buffer, pH 7.4 (Clark & Lange, 1976). DNA (200 μg) in 1.0 mL of buffer was layered over the sucrose gradient containing the same buffer in 38.5-mL tubes. All runs were conducted at 24 500 rpm, 14 °C. Fractions from the tubes were collected after puncturing the bottom via a Buchler piercing unit and were analyzed for the absorbance at 260 nm. The distance of sample peaks from the top of the gradient was calculated from the experimentally determined value of 0.1 cm per 0.50 mL of liquid collected. ^{3}H-Labeled DNA-containing fractions were counted by scintillation counting (10 mL of Aquasol-2 for 1-mL fractions). The sedimentation rates of all samples were highly reproducible and remained constant with varying time of sedimentation. Sedimentation coefficients were calculated as outlined by Burgi and Hershey (Burgi & Hershey, 1963).

Flow dichroism was measured as described by Gabbay (Gabbay et al., 1976a,b). The reduced dichroism (ρ) is defined as

$$\rho = \frac{A_\perp - A_\parallel}{A}$$

where $A_\perp$ is the absorbance when the light is perpendicular to the direction of flow and $A_\parallel$ is that when the light is parallel. A is the absorbance of nonpolarized light. [Note that this definition of ρ is the negative of that used by a number of other authors, e.g., Callis & Davidson (1969).] The shear rates involved in these experiments were calculated according to the equation of Davison (1959).

Polyacrylamide gel (3.5%) electrophoresis was carried out as described (Maniatis et al., 1975). Unsonicated DNA samples were electrophoresed for 6–9 h at ~80 V while sonicated samples required only 2–3 h. Ethidium bromide solution was used to detect the position of DNA on the gel slabs. The gels were photographed under ultraviolet illumination as usual. The distances moved by the middle of the bands were measured with a metric ruler. The relative mobility is defined as the distance traversed by the sample divided by the distance traversed by control DNA.

Electron microscopy was performed in the laboratory of Dr. Alexander Tomasz, The Rockefeller University, New York, on a Sieman 101 electron microscope, using magnification of 50000×. The Kleinschmidt procedure, as modified by Davis and Hyman (Davis & Hyman, 1971), was used for the preparation and spreading of DNA samples. Grids were shadowed at an angle of 5° with platinum and palladium. DNA concentration in the solutions used was 0.5 μg/mL.

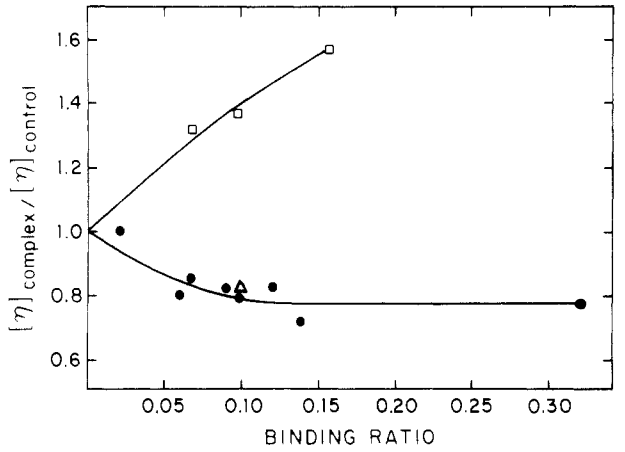

FIGURE 1: Ratio of intrinsic viscosity of complex to that of control DNA ($[\eta]_{complex}/[\eta]_{control}$) as a function of binding ratio. (□) Sonicated DNA–mitomycin complexes; (●) unsonicated DNA–mitomycin complexes; (△) the latter, prepared in the presence of catalase (50 μg of catalase/μmol of DNA was added to the activation reaction mixture; see Materials and Methods). The molecular weights of sonicated and unsonicated DNA used were 2×10^5 and 5.4×10^6, respectively.

Table I: Sedimentation Coefficients (S) of Sonicated DNA-Mitomycin C Complexes in Neutral Sucrose Density Gradients

	distance sedimented (cm)[a]	S
control DNA[b]	5.90 ± 0.05	7.6
complex, binding ratio 0.05	6.00 ± 0.05	7.7
complex, binding ratio 0.11	6.10 ± 0.05	7.9

[a] Time of run: 93.7 h. [b] Sonicated calf thymus DNA, molecular weight 3.8×10^5.

Results

(I) Experiments Probing Intercalation of Mitomycin C into DNA. (A) Viscosity of Sonicated DNA–Mitomycin Complexes. The intrinsic viscosity increases with increasing binding ratio (Figure 1), up to 1.6-fold of the DNA control value at the highest binding ratio (0.15) employed.

(B) Sedimentation velocity of sonicated DNA–mitomycin complexes (Table I) shows only a slight increase over that of control DNA. This increase corresponds exactly to the calculated value when the molecular weight is corrected for the mass of drug bound to DNA.

(C) Flow Dichroism of DNA–Mitomycin Complexes (Figure 2). Flow dichroism of control nonsonicated DNA was measured as a function of the shear rate at 260 nm. The flow dichroism of DNA–mitomycin complexes was measured both at 260 and at 310 nm. The latter wavelength is one of the absorption maxima of the DNA-bound drug (II; Tomasz et al., 1974). It is reasonable to assume that any dichroism at 310 nm is due to the drug chromophore alone since DNA has no absorption at this wavelength, i.e., $\rho_{310} = \rho_{drug}$. The experimental dichroism at 260 nm, however, is, in principle, the sum of that of DNA and bound drug. In order to obtain the reduced dichroism of *DNA alone*, we subtracted the absorbance of the drug itself from observed 260-nm absorbance values, utilizing the known ratio $A_{260}:A_{310} = 1.5$ for mitomycin attached to DNA (Tomasz et al., 1974). Therefore, the reduced flow dichroism of DNA at 260 nm [$\rho_{260(DNA)}$] is given as

$$\rho_{260(DNA)} = \frac{[A_{\perp(260)} - 1.5A_{\perp(310)}] - [A_{\parallel(260)} - 1.5A_{\parallel(310)}]}{A_{260} - 1.5A_{310}}$$

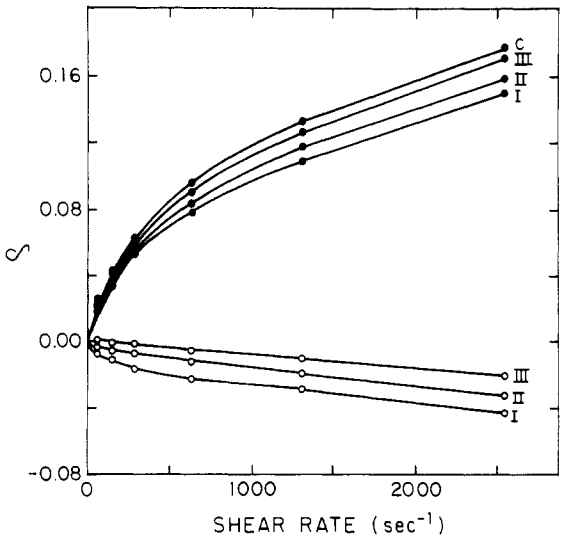

FIGURE 2: Reduced flow dichroism (ρ) vs. shear rate of a series of unsonicated DNA–mitomycin complexes. C, control DNA; I–III, complexes with binding ratios of 0.15, 0.09, and 0.06, respectively. (●) $\rho_{260(DNA)}$; (○) ρ_{310} ($=\rho_{drug}$).

Table II: Sedimentation Coefficients (S) of Unsonicated DNA-Mitomycin Complexes in Neutral Sucrose Density Gradients

	distance sedimented (cm)[a]	S
control DNA[b]	1.20 ± 0.05	15.0
control DNA	2.10 ± 0.05 (17 h)	15.0
complex, binding ratio 0.05	1.40 ± 0.05	17.5
complex, binding ratio 0.10	1.70 ± 0.05	21.2

[a] Time of run: 9.5 h except when indicated otherwise. [b] Calf thymus DNA, molecular weight 5.4×10^6.

These curves display the same large positive dichroism as shown by control DNA with a slight progressive decrease with increasing binding ratio. The ρ_{310} curves display low negative values. We calculated the angle (α) between the transition moment of mitomycin at 310 nm and the helical axis of DNA by using (Geacintov et al., 1978)

$$\frac{\rho_{310}}{\rho_{260(DNA)}} = \frac{3\cos^2\alpha - 1}{3\cos^2 90 - 1}$$

The values of α were calculated at the four highest shear rate points of each curve (Figure 2, curves I–III). While α is constant along the individual curves, it shows a slight apparent increase with the binding ratio (56.4°, 58.0°, and 61.1° from curves I, II, and III, respectively). Since curve III represents a complex having a very high binding ratio [0.15, the approximate limit of the extent of covalent binding (Lipman et al., 1978)] and indicates deformation of DNA as seen from the lowered $\rho_{260(DNA)}$ values, the average α from curves I and II is regarded as the better value. Thus, we conclude that the angle of the 310-nm transition vector of the mitomycin chromophore is approximately 57.2° relative to the DNA helix axis.

(II) Experiments Probing Mitomycin-Induced Conformational Changes of DNA. (A) Viscosity of Unsonicated DNA–Mitomycin C Complexes. In contrast to the viscosity increase displayed by sonicated DNA–mitomycin C complexes, unsonicated DNA complexes show a distinct *decrease* in viscosity (Figure 1), leveling off at a binding ratio of 0.10–0.15. The presence of catalase during complex formation does not abolish the decrease.

(B) Sedimentation Coefficient of Unsonicated DNA–Mitomycin C Complexes. In contrast to the sonicated DNA case

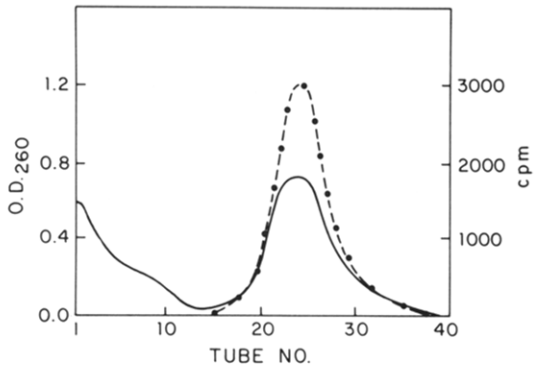

FIGURE 3: Sucrose density gradient sedimentation of DNA–mitomycin C complex prepared from "mixed DNA" (see text). Conditions: 24 500 rpm, 14 °C, 49 h. (—) OD_{260}; (●--●) cpm.

(Table I), the sedimentation coefficient shows a marked increase with increasing binding ratio (Table II) which is beyond the slight increase expected due to increased mass by the bound drug. Specifically, the calculated increases of distance are negligible (less than 4% for both the 0.05 and 0.10 binding ratios), while the corresponding actual increases are 17 and 42%, respectively (Table II).

(*C*) *Lack of Aggregation of DNA–Mitomycin C Complexes.* ^{3}H-Labeled sonicated pneumococcal DNA was mixed with unsonicated calf thymus DNA in approximately 1:1 OD unit proportion. This "mixed DNA" was used to prepare a DNA–mitomycin complex, binding ratio 0.08. This was submitted to sedimentation in a neutral sucrose density gradient. Since the radioactive counts are located only at the slow-sedimenting, sonicated DNA position (Figure 3), it is apparent that the shorter, radioactive DNA–mitomycin complex does not aggregate with the nonradioactive DNA–mitomycin complex.

(*D*) *Electrophoretic Mobility of DNA–Mitomycin C Complexes.* (a) Sonicated DNA: On 3.5% polyacrylamide gels, the complexes run slower than control DNA (Figure 4a). This effect is expressed quantitatively as a plot of mobility relative to control (μ) vs. binding ratio (Figure 5). (b) Unsonicated DNA: A much greater reduction of relative mobility is seen with this class of complexes (Figures 4b and 5). Values obtained with *Escherichia coli* DNA fall on the same curve as that obtained with calf thymus DNA of the same average size (Figure 5). In contrast to these mitomycin complexes, ethidium complexes of calf thymus DNA show only a slight decrease of electrophoretic mobility (Figure 5).

(*E*) *Electron Microscopy of DNA–Mitomycin Complexes.* Panels a and b of Figure 6 show electromicrographs of *unsonicated* control DNA and *unsonicated* DNA–mitomycin complexes, binding ratio 0.1, respectively. It is apparent that the DNA–drug complex shows highly coiled, looped structures, in contrast to the generally smooth contour of control DNA. No such difference in appearance is visible in the case of the *sonicated* DNA–mitomycin complex, binding ratio 0.1, vs. its control (Figure 6c,d). These results were found to be qualitatively invariant with different preparations.

Discussion

Experiments Probing Intercalation of Mitomycin into DNA. Intercalation of drug molecule between base pairs of DNA causes a set of changes of DNA properties. Most characteristic among these changes is an increase of viscosity and a decrease of the sedimentation coefficient, interpreted as due to increased length of the intercalated drug–DNA complex compared to

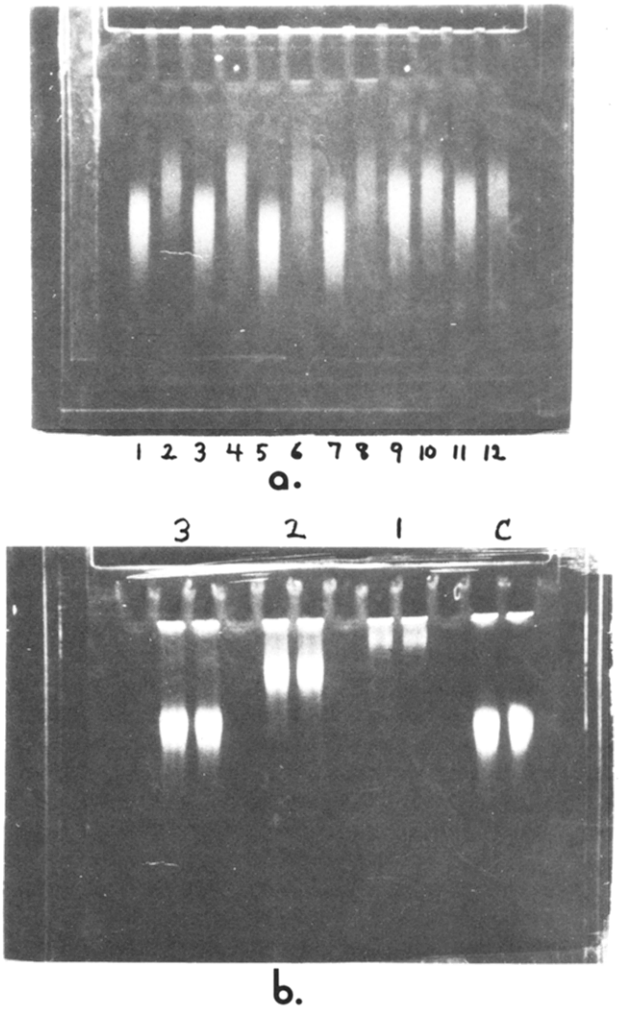

FIGURE 4: (a) Gel electrophoresis of sonicated DNA–mitomycin C complexes. *All* odd numbered slots contain control DNA; all other slots contain complexes: slots 2 and 4, binding ratio 0.09; slots 6 and 8, binding ratio 0.21; slots 10 and 12, binding ratio 0.01. (b) Gel electrophoresis of unsonicated DNA–mitomycin complexes. Slots C contain control DNA. Slots 1, binding ratio 0.16; slots 2, binding ratio 0.05; slots 3, binding ratio 0.01.

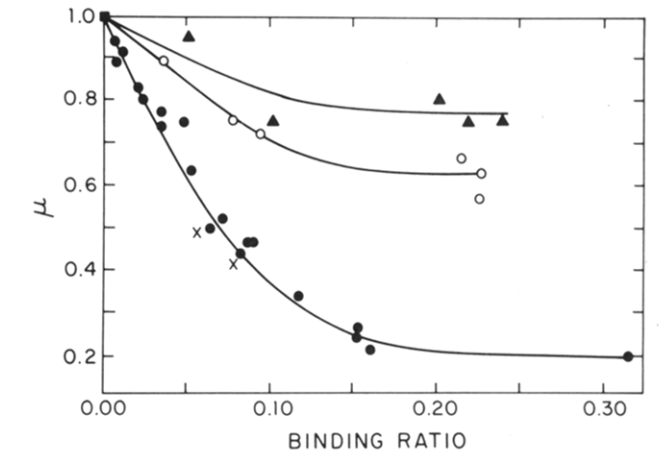

FIGURE 5: Electrophoretic mobility of DNA–mitomycin and DNA–ethidium complexes relative to control DNA (μ) as a function of drug binding ratio. (●) Unsonicated calf thymus DNA (M_r 3.6 × 10^6)–mitomycin complexes; (×) *E. coli* DNA (M_r 3.6 × 10^6)–mitomycin complexes; (▲) unsonicated calf thymus DNA–ethidium complexes; (○) sonicated calf thymus DNA–mitomycin complexes.

that of the parent DNA (Gale et al., 1972). In the case of mitomycin, using sonicated DNA, we observed increased viscosity but no decrease of the sedimentation coefficient.[1]

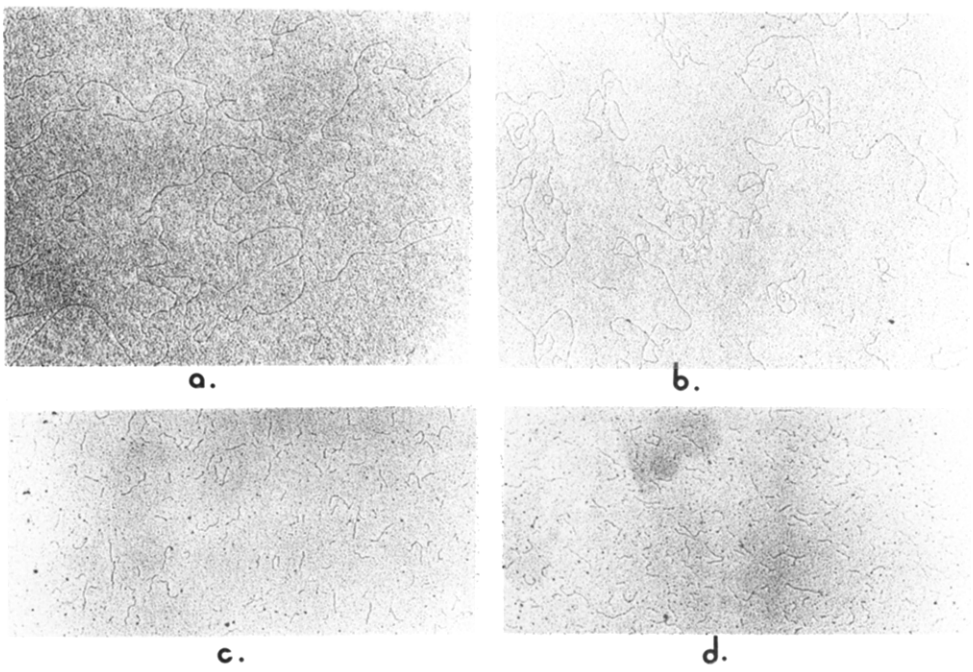

FIGURE 6: Electron micrographs. (a) Control unsonicated calf thymus DNA. (b) Unsonicated calf thymus DNA–mitomycin complex, binding ratio 0.1. (c) Control sonicated calf thymus DNA. (d) Sonicated calf thymus DNA–mitomycin complex, binding ratio 0.1.

Since viscosity increase in itself may be caused by nonintercalating drugs [e.g., see Zunino et al. (1977) and Dattagupta et al. (1978)], these experiments did not confirm intercalation by mitomycin. An independent, elegant test, namely, the unwinding of superhelical DNA by intercalators (Waring, 1970), is not applicable for this drug since it was reported (Lown & Weir, 1978) that during the reductive activation process, necessary to effect binding, superhelical DNA is relaxed, due presumably to a low level of nicking, caused by superoxide and/or H_2O_2 generated in situ.

As an alternative further test for intercalation, we measured the flow dichroism of mitomycin–DNA complexes. In this method, DNA is oriented by flow in a shear gradient so that its helical axis is partially lined up in the direction of the flow. Absorption of parallel and perpendicularly polarized light by the flowing DNA solution is measured separately. Since the electronic transition moments of the bases are perpendicular to the helical axis of DNA, greater absorbance is observed by using perpendicularly polarized light than parallel polarized light (Bloomfield et al., 1974b). In the case of intercalation, the electronic transition moment of the intercalating drug is parallel to that of the bases, and, consequently, the magnitude and sign of the flow dichroism of the DNA bases, observed at 260 nm, are similar to those of the drug chromophore, observed at a wavelength unique to the latter. Thus, this type of measurement can serve as a probe for intercalation by new drugs (Bloomfield et al., 1974c). We used unsonicated rather than sonicated DNA in our experiments since the fractional orientation of DNA increases with increasing molecular weight (Bloomfield et al., 1974b). Our measured dichroism of control DNA at 260 nm (Figure 2) reproduces well the published curves (Callis & Davidson, 1969). The slight decrease of dichroism of DNA [$\rho_{260(DNA)}$] in the DNA–mitomycin complexes with increasing binding ratio may indicate progressive deformation of DNA due to drug binding, causing it to be less orientable. This phenomenon will be discussed in the following section. The dichroism of the bound mitomycin in the complexes (ρ_{310}) is lower and has a sign opposite to that of DNA, indicating that the transition moment of the 310-nm absorption band of the drug is not oriented parallel to the bases. This transition is a π–π^* type, as indicated by the value of the extinction coefficient ($\sim$11 000; Tomasz et al., 1974). Such a value is too high for n–π^* transitions (Cantor & Schimmel, 1980). Furthermore, the unbound hydrolysis derivative of mitomycin C, a known compound having the same spectra as the DNA-bound drug (II) (Tomasz et al., 1974), shows a 5-nm red shift upon change from nonpolar (methanol) to polar (aqueous) solvent, another diagnostic test for π–π^* (Cantor & Schimmel, 1980). Consequently, the 310-nm transition moment lies in the plane of the conjugated ring system of DNA-bound mitomycin (II). We can then conclude from our data that the largely planar drug molecule is not oriented parallel to the bases and therefore is not intercalated. The angle of orientation is approximately 57.2° (see Results), suggesting that the drug is bound in one of the grooves of DNA. The observed increased viscosity is not inconsistent with this, since certain nonintercalating drugs have been shown to cause such an effect, presumably due to stiffening and/or lengthening of DNA (Zunino et al., 1977; Dattagupta et al., 1978; Reinert, 1972; Zimmer, 1975).

An additional supporting fact for the nonintercalative nature of mitomycin binding is an earlier observation that the ultraviolet spectrum of the DNA-bound mitomycin derivative is very similar to that of free drug derivatives having the same chromophore (Tomasz et al., 1974), in contrast to known intercalators which show a bathochromic and hypsochromic shift of their spectra upon binding (Gale et al., 1972; Müller & Crothers, 1968). Considering all these facts, old and new, it is evident that the covalently bound form of mitomycin C is not intercalated between the base pairs of DNA.

It should be added that this conclusion is based on measurements which reflect average properties of a population of molecules. Since the predominant mode of binding of mito-

[1] Recent work (Fulmer et al., 1981) showed that at low ionic strength (10^{-2} M or below) short DNA rods show marked deviation from the expected hydrodynamic behavior. Since our measurements were all conducted at ionic strength above the critical value of 10^{-2} M (see Materials and Methods), this phenomenon is not likely to affect the interpretation of our results.

Table III: Qualitative Summary of the Size-Dependent Effects of Mitomycin C on DNA Properties

property	sonicated DNA	unsonicated DNA
viscosity	increase	decrease
sedimentation coefficient	no change[a]	increase[a]
electrophoretic mobility	slight decrease	large decrease
electron microscopic appearance	no change	looped, entangled regions

[a] After correction for increased mass due to bound drug.

mycin is monofunctional [e.g., at a binding ratio of 0.06–0.08, the cross-links correspond only to about 3% of the overall binding (D. J. Kaplan and M. Tomasz, unpublished results); somewhat higher estimates were given in Szybalski & Iyer (1964) and Weissbach & Lisio (1965)], the results characterize monofunctionally bound mitomycin.[2]

Mitomycin-Induced Conformational Changes of DNA. The observed contrasting behaviors of low molecular weight vs. high molecular weight DNA–mitomycin complexes are summarized in Table III. The differential effect of mitomycin on the hydrodynamic behavior of low and high molecular weight DNA parallels closely the effects of actinomycin D, described by Müller & Crothers (1968). Using the analogous system, i.e., low and high molecular weight calf thymus DNA–actinomycin complexes, they observed increased viscosity and slightly decreased sedimentation rate in the former case and decreased viscosity and increased sedimentation rate in the latter (cf. our Table III). The authors offer the following explanation.

The key to this peculiar effect is to be found in the limited flexibility of the DNA molecule. Low molecular weight (approximately 10^5) DNA approaches the character of a rigid rod while higher molecular weight DNA behaves as a more flexible, coiled chain. Actinomycin intercalates DNA, increasing its length, hence the increased viscosity and decreased sedimentation coefficient of low molecular weight DNA. In the case of high molecular weight DNA (approximately 10^6 and higher), these changes are superimposed by an *opposing* effect: the coiling polymer chain is fixed into loops by intramolecular interactions, involving the peptide "arms" of actinomycin, thereby decreasing the radius of gyration. This results in the observed decreased viscosity and increased sedimentation rate.

The key concept of this hypothesis, that is, the differential flexibility of short vs. long DNA, can be applied analogously to explain the present observations with mitomycin C. Since mitomycin C, however, is a very different drug from actinomycin, the details of our explanation are also different as follows:

The increased viscosity of sonicated DNA–mitomycin complexes is due to stiffening and/or lengthening of DNA by groove-bound mitomycin, as discussed in the previous section. With high molecular weight DNA, this effect is superimposed by an opposing one, resulting in overall decrease of viscosity. Viscosity decrease at high molecular weights may be caused by two possible factors according to Müller & Crothers (1968): the coiling polymer chain is contracted by intramolecular interactions (as in the case of actinomycin) or the flexibility is increased somewhat by the binding of the drug. In the present case, the former is quite unlikely, since, in contrast to actinomycin D, mitomycin is a very small molecule. Therefore, it is hard to envisage any significant multiple secondary interactions of bound mitomycin with another section of the same DNA molecule. Such interactions would be necessary to overcome the simultaneous repulsion between the two sections of DNA as they have to come very close for intramolecular interaction. We propose, rather, the second possibility, i.e., that binding of mitomycin C confers increased flexibility on high molecular weight DNA. This must be accomplished by a small, unique fraction of the total DNA-bound mitomycin rather than by all in a uniform manner. (Remember that the uniform effect of bound mitomycin is to *increase* the viscosity, as seen with sonicated DNA. The opposing one, since evident only at high molecular weight, should be rare, and different.) The fact that the viscosity decrease levels off with increasing binding ratio (Figure 1) supports this argument.[3]

This model implies that there are certain segments within the drug–DNA complex which are conformationally different (more flexible) than the rest, and these are the source of the observed greater overall flexibility. It is easy to see, qualitatively, why this phenomenon is not detected with low molecular weight (2×10^5) DNA: If the number of such segments per DNA mass is fixed and lower than one per 2×10^5 daltons ($\sim$200 bp), then the fraction of molecules containing such a segment is lower than that in the high molecular weight (5×10^6) preparation. For example, if the number of segments is one per 2000 base pairs, then, by simplest approximation, the fraction is only 10% compared to 100% in the high molecular weight sample.

We probed another possible explanation for the viscosity decrease, namely, the occurrence of rare single-strand breaks, located close to one another, in opposite strands, and therefore manifested as double-stand breaks. Since catalase reportedly inhibits the rare single-strand breaks occurring during preparation of mitomycin–DNA complexes (Lown et al., 1976), we tested its effect on the observed viscosity decrease. No effect was found, ruling out this alternative explanation.

The increased sedimentation rate of nonsonicated DNA–mitomycin C complexes (Tables II and III) is consistent with the hypothesis of increased flexibility of high molecular weight DNA induced by mitomycin, since it is known that more coiled DNA provides less frictional resistance and sediments faster (Bloomfield et al., 1974d). Again, as expected, no such effect was detectable in the low molecular weight DNA complexes. A test for aggregation between molecules as an alternative cause for the increased sedimentation coefficient was negative (see Results).

The increased flexibility phenomenon also provides an explanation as to why the flow dichroism of nonsonicated DNA decreases slightly with increasing binding ratio, as noted in the preceding part: more flexible DNA is less orientable by the flow.

In addition to the effects on hydrodynamic behavior, mitomycin induced another type of size-dependent change in DNA: reduction of electrophoretic mobility on polyacrylamide gels. The slight reduction of mobility of the sonicated DNA–mitomycin complexes (Figures 4a and 5) may be explained by partial charge neutralization since DNA-bound mitomycin is a cation (II) (Lipman et al., 1978). The similar

[2] The reported (Lipman et al., 1978) noncovalent, electrostatic attachment of activated mitomycin to DNA can also be discounted as cause of the present observations since it is not appreciable at the low binding ratios used in the present work.

[3] Theoretically, a minimum should be apparent, similar to the results of Müller & Crothers (1968). Our data are not extensive enough to ascertain the presence of a minimum, but do not exclude it.

behavior of ethidium–DNA complexes has been rationalized this way (Lippard et al., 1976), although direct comparison of ethidium and mitomycin C indicates a somewhat greater effect by the latter (Figure 5). The role of charge in reducing the migration seems unimportant, according to a study by Loucks et al. (1979). They report that actinomycin D and a variety of other antibiotics all reduce the migration of restriction fragments on polyacrylamide gels. They conclude that the distortion of the DNA helix by the drugs is probably the main factor, but the mechanism underlying this phenomenon is unclear. The present observation, namely, that mitomycin induces a much greater reduction of mobility of high molecular weight DNA than that of the sonicated DNA, may be explained again by the increased flexibility of the former as concluded from the hydrodynamic data. It is known that tertiary isomers of DNA such as linear, open-circular, and superhelical circular forms are separated on electrophoretic gels: the linear form moves fastest, "end-on" through the gel, with minimal frictional resistance, while the others, with lower radius of gyration, slow down (Dingman et al., 1972; Johnson & Grossman, 1977; Aaij & Borst, 1972). The high molecular weight DNA–mitomycin complexes, having increased coiling and bending flexibility, may slow down for the same reason. Due to the complexities of gel electrophoresis (Loucks et al., 1979), however, this interpretation, while consistent with the hydrodynamic results, is tentative.

The model of mitomycin-induced, size-dependent flexibility of DNA receives additional support from the electron micrographs (Figure 6a–d, Table III). The contrast between the highly coiled, looped, entangled structures of the nonsonicated complex and the unchanged, controllike appearance of the sonicated complex demonstrates directly what was deduced from the hydrodynamic behavior of the system: increased flexibility at rare, limited segments can only manifest itself in high molecular weight DNA, i.e., in molecules having long-range physical continuity.

The nature of the perturbations which give rise to the rare, flexible segments in the DNA–mitomycin complex is subject to speculation. A possible cause of such "rare defects" may be the cross-links. Very little information exists about their chemical nature, except that their formation is facilitated by increasing G + C content of DNA (Iyer & Szybalski, 1963; Lown et al., 1976) and they are heat and alkali stable (Iyer & Szybalski, 1963). One can only speculate that one or perhaps more cross-links, formed in cooperative fashion, cause distortions of DNA, giving rise to the observed properties.

Another possible clue to the origin of the distortions may be the previously observed circular dichroism (CD) of the complexes of mitomycin with poly[d(G-C)]·poly[d(G-C)] in our laboratory (Mercado & Tomasz, 1977). These studies indicated that mitomycin induces a change of CD of this polynucleotide which is similar to that induced by high salt or ethanol concentration. The CD change by these latter agents was interpreted as due to a cooperative conformational change of poly[d(G-C)]·poly[d(G-C)] (Pohl & Jovin, 1972; Pohl, 1976) from the B to a new form. There is increasing evidence (Patel et al., 1979; Simpson & Shindo, 1980) that this new form in solution is the same as Z-DNA, first discovered as the crystal structure of d(CpGpCpGpCpG) by Rich and his collaborators (Wang et al., 1979). The Z conformation differs strikingly from the typical right-handed DNA B structure in that the helix is left handed. In solution, it has only been observed for alternating G-C sequences so far. The mitomycin-induced CD change of poly[d(G-C)]·poly[d(G-C)] is highly suggestive that binding of the drug has a similar effect on the polymer as high salt or ethanol concentration, i.e., causes it to change from B to Z conformation. The same type of CD change, but much smaller in magnitude, was also observed in calf thymus, *E. coli*, and T-2 phage DNA–mitomycin complexes (Mercado & Tomasz, 1977) which suggests that the same conformational change occurs in DNA to a limited extent, specifically at alternating G-C sequences of sufficient length. It was shown recently (Klysik et al., 1981) that the Z structure of inserted oligomers of d(C-G) *can* exist in close proximity to the B structure within DNA restriction fragments and within a recombinant plasmid when high salt concentration prevails. It was also indicated that B/Z junction regions, estimated to be ~11 base pairs long, have intermediate, unknown conformation (Klysik et al., 1981). It is possible that similar junctions exist in mitomycin–DNA complexes, and they represent points of increased flexibility within DNA since Rich and collaborators (Wang et al., 1979) predicted unstacked, kinked structure for B/Z junctions.

While these hypotheses require further critical testing (for example, the probing of the presence of Z-DNA in mitomycin–DNA complexes by means other than CD, which alone is not definitive), the present work demonstrates that mitomycin induces a conformational distortion in rare segments of DNA. This effect is distinct from the "macroscopic", overall effect of the drug on DNA properties. The key to its detection is the differential hydrodynamic behavior of sonicated and nonsonicated calf thymus DNA when complexed with mitomycin. The interaction of actinomycin D with the same two DNAs provides a striking precedent of this phenomenon. Specific effects of antibiotic binding on specific segments of DNA may be more general (Wells et al., 1980). Using the pair of sonicated and nonsonicated calf thymus DNA as probes for size-dependent hydrodynamic behavior as in the case of actinomycin D and now of mitomycin C could serve as a sensitive test for detection of such phenomenon.

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References

Aaij, C., & Borst, P. (1972) *Biochim. Biophys. Acta 269*, 192–200.

Aktipis, S., & Kindelis, A. (1973) *Biochemistry 12*, 1213–1221.

Bloomfield, V. A., Crothers, D. M., & Tinoco, I., Jr. (1974a) *Physical Chemistry of Nucleic Acids*, pp 225–234, Harper and Row, New York.

Bloomfield, V. A., Crothers, D. M., & Tinoco, I., Jr. (1974b) *Physical Chemistry of Nucleic Acids*, pp 248–252, Harper and Row, New York.

Bloomfield, V. A., Crothers, D. M., & Tinoco, I., Jr. (1974c) *Physical Chemistry of Nucleic Acids*, pp 437–439, Harper and Row, New York.

Bloomfield, V. A., Crothers, D. M., & Tinoco, I., Jr. (1974d) *Physical Chemistry of Nucleic Acids*, p 215, Harper and Row, New York.

Burgi, E., & Hershey, A. D. (1963) *Biophys. J. 3*, 309–321.

Callis, P., & Davidson, N. (1969) *Biopolymers 7*, 335–352.

Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry, Part II*, pp 386–388, W. H. Freeman, San Francisco.

Clark, R. W., & Lange, C. S. (1976) *Biochim. Biophys. Acta 454*, 567–577.
Cohen, R. J., & Crothers, D. M. (1970) *Biochemistry 9*, 2533–2539.
Crothers, D. M., & Zimm, B. H. (1965) *J. Mol. Biol. 12*, 525–536.
Dattagupta, N., Hogan, M., & Crothers, D. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4286–4290.
Davis, R. W., & Hyman, R. W. (1971) *J. Mol. Biol. 62*, 287–301.
Davison, P. F. (1959) *Proc. Natl. Acad. Sci. U.S.A. 45*, 1560–1568.
Dingman, W. C., Fisher, M. P., & Kakefuda, J. (1972) *Biochemistry 11*, 1242–1250.
Doty, P., McGill, B. B., & Rice, S. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 432–438.
Eigner, J., & Doty, P. (1965) *J. Mol. Biol. 12*, 549–580.
Fulmer, A. W., Benbasat, J. A., & Bloomfield, V. A. (1981) *Biopolymers 20*, 1147–1159.
Gabbay, E. J., Adawadkar, P. D., & Wilson, W. D. (1976a) *Biochemistry 15*, 146–151.
Gabbay, E. J., Grier, D., Fingerle, E. D., Reimer, R., Levy, R., Pearce, S., & Wilson, W. D. (1976b) *Biochemistry 15*, 2062–2070.
Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H., & Waring, M. J. (1972) *The Molecular Basis of Antibiotic Action*, pp 193–198, Wiley, New York.
Geacintov, N. E., Gagliano, A., Ivanovic, V., & Weinstein, I. B. (1978) *Biochemistry 17*, 5256–5262.
Hashimoto, Y., Shudo, K., & Okamoto, T. (1980) *Chem. Pharm. Bull. 28*, 1961–1962.
Hornemann, U., Keller, P. J., & Kozlowski, J. F. (1979) *J. Am. Chem. Soc. 101*, 7121–7124.
Iyer, V. N., & Szybalski, W. (1963) *Exp. Cell Res. 32*, 192–196.
Iyer, V. N., & Szybalski, W. (1964) *Science (Washington, D.C.) 145*, 55–58.
Johnson, P. H., & Grossman, L. I. (1977) *Biochemistry 16*, 4217–4225.
Kinoshita, S., Uzu, K., Nakano, D., & Takahashi, T. (1971) *J. Med. Chem. 14*, 109–112.
Klysik, J., Stirdivant, S. M., Larson, J. E., Hart, P. A., & Wells, R. D. (1981) *Nature (London) 290*, 672–677.
Lipman, R., Weaver, J., & Tomasz, M. (1978) *Biochim. Biophys. Acta 521*, 779–791.
Lippard, S. J., Bond, P. J., Wu, K. C., & Bauer, W. R. (1976) *Science (Washington, D.C.) 194*, 726–728.
Loucks, E., Charonas, G., Blakesley, R. W., Wells, R. D., & van de Sande, J. H. (1979) *Nucleic Acids Res. 6*, 1869–1879.
Lown, J. W., Begleiter, A., Johnson, D., & Morgan, A. R. (1976) *Can. J. Biochem. 54*, 110–119.
Lown, W. J., & Weir, G. (1978) *Can. J. Biochem. 56*, 296–304.
Mandelkern, L., & Flory, P. J. (1952) *J. Chem. Phys. 42*, 212–221.
Maniatis, T., Jeffrey, A., & van de Sande, H. (1975) *Biochemistry 14*, 3787–3794.
Marmur, J. (1961) *J. Mol. Biol. 3*, 208–218.
Matsumoto, I., & Lark, K. G. (1963) *Exp. Cell Res. 32*, 192–196.
Mercado, C. M., & Tomasz, M. (1972) *Antimicrob. Agents Chemother. 1*, 73–77.
Mercado, C. M., & Tomasz, M. (1977) *Biochemistry 16*, 2040–2046.
Moore, H. W. (1977) *Science (Washington, D.C.) 197*, 527–532.
Müller, W., & Crothers, D. M. (1968) *J. Mol. Biol. 35*, 251–290.
Patel, D. J., Canuel, L. S., & Pohl, F. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2508–2511.
Pohl, F. M. (1976) *Nature (London) 20*, 265–366.
Pohl, F. M., & Jovin, T. M. (1972) *J. Mol. Biol. 67*, 375–396.
Reinert, K. E. (1972) *J. Mol. Biol. 72*, 593–607.
Remers, W. A. (1979) *The Chemistry of Antitumor Compounds*, Vol. 1, pp 221–276, Wiley, New York.
Shiba, S., Terawaki, A., Taguchi, T., & Kawamata, J. (1958) *Biken J. 1*, 179–181.
Simpson, R. T., & Shindo, H. (1980) *Nucleic Acids Res. 8*, 2903–2103.
Small, G., Setlow, J. I., Kooistra, J., & Shapanka, R. (1976) *J. Bacteriol. 125*, 643–654.
Szybalski, W., & Iyer, V. N. (1964) *Microb. Genet. Bull. No. 21*, 16–17.
Tomasz, M., & Lipman, R. (1981) *Biochemistry 20*, 5056–5061.
Tomasz, M., Mercado, C. M., Olson, J., & Chatterjie, N. (1974) *Biochemistry 13*, 4878–4887.
Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., Van Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature (London) 282*, 680–686.
Waring, M. J. (1970) *J. Mol. Biol. 54*, 247–279.
Weiss, M. J., Redin, G. S., Allen, G. K., Jr., Dornbush, A. C., Lindsay, H. S., Poletto, J. F., Remers, W. A., Roth, R. H., & Sloboda, A. E. (1968) *J. Med. Chem. 11*, 742–745.
Weissbach, A., & Lisio, A. (1965) *Biochemistry 4*, 196–200.
Wells, R. D., Goodman, T. C., Hillen, W., Horn, G. T., Klein, R. D., Larson, J. E., Müller, U. R., Neuendorf, S. K., Panayotatos, N., & Stirdivant, S. M. (1980) *Prog. Nucleic Acid Res. Mol. Biol. 24*, 167–259.
Zimm, B. H., & Crothers, D. M. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 905–911.
Zimmer, C. (1975) *Prog. Nucleic Acid Res. Mol. Biol. 15*, 285–318.
Zunino, F., Gambetta, R., Mario, A. D., Velich, A., Zaccaro, A., Quadrifoglio, F., & Crescenzi, V. (1977) *Biochim. Biophys. Acta 487*, 38–46.