Neocarzinostatin Chromophore Binds to Deoxyribonucleic Acid by Intercalation†

Lawrence F. Povirk, Nanibhushan Dattagupta, Benjamin C. Warf, and Irving H. Goldberg*

ABSTRACT: The nonprotein chromophore of neocarzinostatin was found to share many of the characteristics of classical intercalators in its interaction with DNA. Viscosity studies with PM2 DNA indicated that the DNA helix unwinding induced by the chromophore was 0.82 times that of ethidium or 21°. Electric dichroism of the chromophore–DNA complex showed that each bound chromophore molecule lengthened DNA by 3.3 Å and that absorbance transitions of the chromophore at 315–385 nm were oriented approximately parallel to DNA bases, as expected for an intercalated aromatic ring. Binding to DNA induced strong hypochromicity and a pronounced red shift in the absorbance spectrum of the chromophore. Spectrophotometric titrations suggested at least two types of chromophore binding sites on DNA; one type of site was saturated at $r_b = 0.125$ chromophore molecule/nucleotide, but binding to additional sites continued to at least $r_b = 0.3$. These physical–chemical studies were performed at pH 4–5 in order to keep the chromophore stable, but chromophore bound to an excess of DNA at pH 7 showed a stable absorbance spectrum identical with that seen at pH 4–5, suggesting that a similar type of binding occurs at neutral pH. Chromophore which had spontaneously degraded in pH 8 buffer did not bind to DNA at all, as judged by absorbance spectroscopy. The degree of protection afforded by DNA against spontaneous chromophore degradation implied a dissociation constant of approximately 5 μM for the DNA–chromophore complex at neutral pH and physiological ionic strength. Supercoiled DNA was nearly twice as effective as relaxed DNA in protecting chromophore from degradation, providing additional evidence for intercalation at neutral pH. Comparison of absorbance, fluorescence, and dichroism spectra suggests that the naphthalene ring system is the intercalating moiety.

Extraction of biologically active nonprotein chromophores from the protein antibiotics neocarzinostatin (NCS)[1] and auromomycin has greatly clarified the mechanism of action of these drugs (Napier et al., 1979; Kappen et al., 1980a,b; Ohtsuki & Ishida, 1980; Suzuki et al., 1980; Woynarowski & Beerman, 1980). The isolated NCS chromophore is even more active than the native chromophore–protein complex in degrading DNA, and a number of experiments with both isolated DNA and cultured cells support the view that the protein does not interact with DNA at all but serves mainly as a carrier to protect the labile chromophore from spontaneous degradation (Kappen et al., 1980a; Kappen & Goldberg, 1980; Povirk & Goldberg, 1980).

Recently we presented evidence for reversible binding of the biologically active NCS chromophore to DNA under conditions where it did not degrade the DNA, i.e., in the absence of the required sulfhydryl cofactors (Povirk & Goldberg, 1980). This binding unwound superhelical pMB9 DNA, suggesting intercalation by NCS chromophore. NCS chromophore contains 2-hydroxy-5-methoxy-7-methylnaphthoate and 2,6-dideoxy-2-(methylamino)galactose covalently linked to a highly unsaturated $C_{15}H_{10}O_4$ substituent of undetermined structure (Albers-Schönberg et al., 1980). The naphthoic acid derivative is a binuclear fused ring system potentially capable of intercalation. The $C_{15}H_{10}O_4$ substituent may also contain one or more aromatic rings. However, several compounds, including platinum complexes and covalently bound benzo[*a*]pyrenediol epoxide, have been reported to unwind DNA by inducing local denaturation rather than by intercalating (Cohen et al., 1979; Gamper et al., 1980). Therefore, we undertook a more detailed characterization of the DNA–chromophore complex.

Experimental Procedures

Preparation of Chromophore. Four ampules (8 mL) of clinical NCS were dialyzed overnight against distilled water, lyophilized, dissolved in 0.8 mL of 20 mM sodium citrate, pH 4, and again lyophilized. The white residue was resuspended in 0.8 mL of methanol and kept at 0 °C for 10 min, and the precipitate was pelleted by centrifugation. The colorless supernatant (0.68 mL) was decanted, and 0.17 mL of distilled water was added to improve its pipetting properties; the resulting solution contained NCS chromophore at a concentration of 0.20–0.26 mM and was stored at −70 °C.

The precipitate, when redissolved in 4 mM sodium acetate, pH 5, retained 30% of the initial NCS absorbance at 330–350 nm, indicating that approximately 70% of the chromophore had been removed. Usually the precipitate was immediately reextracted with 8 mL of acetic acid to remove remaining chromophore and dissolved in 8 mL of 0.1 M Tris, pH 8. This solution was subjected to XAD-7 chromatography as described by Napier et al. (1980) to obtain purified NCS apoprotein.

Although some chromophore extraction procedures result in preparations with high proportions of various inactive chromophore degradation products (Kappen et al., 1980a), there were several indications that the procedure described above produced nearly pure active chromophore. Analysis of the chromophore used in our studies by high-pressure liquid chromatography (kindly performed by Dr. M. A. Napier) revealed only one major (90%) and one minor (10%) peak, which eluted in the same positions as the major and minor active chromophore components obtained in an extraction of NCS with a 1:1 methanol–acetic acid mixture (Napier et al., 1981; Albers-Schönberg et al., 1980). No detectable amounts of the several inactive components found in methanol ex-

† From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 (L.F.P., B.C.W., and I.H.G.), and the Department of Chemistry, Yale University, New Haven, Connecticut 06511 (N.D.). *Received December 10, 1980.* This work was supported by U.S. Public Health Service Research Grants GM 12573 and CA 15583 from the National Institutes of Health. L.F.P. was supported by fellowships from the National Science Foundation and the National Cancer Institute.

[1] Abbreviation used: NCS, neocarzinostatin.

0006-2960/81/0420-4007$01.25/0

tractions of undialyzed, lyophilized clinical NCS (Kappen et al., 1980a) were seen. The presence of pH 4 citrate buffer apparently acted like acetic acid to stabilize the chromophore against degradation during extraction. The absorbance spectrum of our chromophore in methanol was indistinguishable from that of the purified major active component (Napier et al., 1981). Reconstitution of our chromophore and apoprotein preparations reproduced an absorption spectrum indistinguishable from that of native NCS and markedly different from the spectrum of free chromophore. Exposure of chromophore solutions to fluorescent lighting was avoided.

DNA. Sonicated, S_1 nuclease treated calf thymus DNA (Povirk & Goldberg, 1980) was used for spectral studies. For dichroism studies, this same DNA was fractionated on a Sepharose 4B column as described by Hogan et al. (1978). Bacteriophage PM2 DNA was prepared as described by Espejo et al. (1969). Supercoiled pMB9 and Col E1 plasmid DNAs were isolated as described by Blair et al. (1972). Relaxed, closed circular Col E1 was prepared by using the *Agrobacterium tumifaciens* relaxing enzyme as described previously (Povirk et al., 1980). Linear pMB9 DNA was prepared by sonicating supercoiled pMB9 for 20 s with the microtip of a Branson cell disruptor (Hyde & Hearst, 1978). This DNA had no detectable supercoiled molecules remaining, but sedimented as a monodisperse peak in neutral sucrose at approximately half the *s* value of nicked circular pMB9, and had 31% hyperchromicity. Thus, it consisted almost entirely of linear duplex DNA. All DNA concentrations are expressed in moles of nucleotide per liter.

Protein Determinations. For determination of extinction coefficients, 1-mL solutions of measured absorbance (A_{280} ~0.04–0.12) were prepared, and norleucine (10 nmol) was added as an internal standard. Duplicate samples were then hydrolyzed and subjected to amino acid analysis (kindly performed by W. E. McGovern, Biophysics Research Laboratory, Harvard Medical School, and the Division of Medical Biology, Brigham and Women's Hospital, Boston). The analysis was in all cases consistent with the known composition of NCS apoprotein (Meienhofer et al., 1972), and the concentration was determined from the alanine content, assuming 17 alanine residues per molecule. The maximum variation between duplicates was 6%.

DNA Unwinding. Reversible relaxation of PM2 DNA was monitored by viscometry (Waring, 1976). The flow time of a 2.5-mL solution of PM2 DNA (0.288 mM nucleotides) in 20 mM sodium citrate, pH 4, plus 10% methanol was measured in triplicate in a capillary viscometer. Serial additions of 10–40 μL of 0.6 mM ethidium bromide were made, and the point of full relaxation was determined from the point of maximum viscosity of the solution. DNA unwinding by chromophore was determined from its ability to decrease the amount of ethidium required to achieve full relaxation. The viscometer had a flow time of 90 s for water at 22 °C and 185 s for 9:1 water–methanol at 4 °C.

Electric Dichroism. Orientation of 310–385-nm absorption transition moments of the chromophore was determined by measuring the electric linear dichroism of chromophore bound to short (150 base pairs along) rodlike DNA (Hogan et al., 1979). A solution containing 150 μM DNA and 6 μM chromophore in 5 mM sodium acetate, pH 5, plus 1.6% methanol was prepared and kept at 4 °C, except during a brief (5 min) degassing. Very nearly quantitative binding of chromophore is expected under these conditions (Povirk & Goldberg, 1980), and this was confirmed by the observation that there was no detectable spontaneous degradation of chromophore for at least several hours, as monitored by either generation of 490-nm fluorescence (Povirk & Goldberg, 1980) or changes in the absorbance spectrum (Napier et al., 1981). This solution was placed in a modified temperature-jump apparatus at 4 °C and exposed to an electric field of 12–32 kV/cm, which oriented the DNA parallel to the field [see Hogan et al. (1978) for details]. The chromophore absorbance parallel to the field was measured before (A_0) and after ($A_\parallel$) orientation to give the reduced dichroism $\rho = {}^3/_2(A_\parallel - A_0)/A_0$. The angle α between the transition moment of the chromophore and the DNA helix axis is then given by $\rho = {}^3/_2(3\cos^2\alpha - 1)\Phi$, where Φ is a field-dependent orientation factor, $0 \leq \Phi \leq 1$ (O'Konski et al., 1959). ρ was determined at several field strengths, E, and extrapolated to $1/E = 0$ (i.e., to perfect DNA orientation).

For DNA alone at pH 5 this extrapolation gave $\rho = -1.15$ at 265 nm, very close to the values measured previously for DNA at neutral pH (Hogan et al., 1978). Because of the photolability of the chromophore, a fresh solution was used for each dichroism measurement, so that exposure to light (from a xenon lamp with monochromator) was reduced to several seconds. Longer exposures resulted in a loss of fine structure in both the absorbance and electric dichroism spectra of the chromophore–DNA complex.

Changes in DNA length induced by chromophore binding were measured under similar conditions by determining the rotational diffusion coefficient θ of the DNA in the presence and absence of chromophore. The DNA was oriented by rapid application of the electric field, and the orientation relaxation time, $\tau = 1/(6\theta)$, was determined by recording the absorbance of light polarized parallel to the field (Hogan et al., 1978). The equation of Broersma (1960) relating θ to length was used to calculate length changes for the complexes.

Chromophore Protection Assays. Recently we showed (Povirk & Goldberg, 1980) that binding of chromophore to DNA could be monitored by protection against spontaneous chromophore degradation. Chromophore (10 μL) in an 80% methanol–20% aqueous solution was added to 0.5 or 2.5 mL of DNA in sodium phosphate buffer which had been adjusted to an apparent pH of 7.0 with NaOH. Chromophore degradation rates were determined from kinetics of generation of 490-nm fluorescence (attributable to an inactive degradation product) with a fluorometer thermostated at 22 °C. These kinetics could be described by a single exponential whose rate constant ($R = 1/\tau$) is proportional to the fraction of unbound chromophore (Povirk & Goldberg, 1980).

DNA Strand Break Measurements. Reaction mixtures (100 μL) containing 30 μM DNA (a mixture of supercoiled ^{14}C-labeled Col E1 and enzymatically relaxed, closed circular ^{3}H-labeled Col E1), 10 mM 2-mercaptoethanol, 0.1 M NaCl, 50 mM Tris, pH 8, and 0–16 nM chromophore were prepared and incubated at 37 °C for 15 min. Chromophore (0–0.8 μM) was prepared from stock solutions by dilution in 20 mM sodium citrate at 4 °C, and 20 μL of these dilutions was added as the last component to incubation mixtures to yield the final concentrations indicated. The number of strand breaks per molecule was determined by measuring the loss of covalently closed relaxed or supercoiled molecules, using neutral sucrose gradients containing ethidium bromide (Povirk et al., 1979).

Results

Extinction Coefficient of the Chromophore. Although the chromophore has a distinct absorbance spectrum and its molecular weight (661) is known (Albers-Schönberg et al., 1980), the limited amount of material available made a direct weight determination impractical. Therefore, we chose to

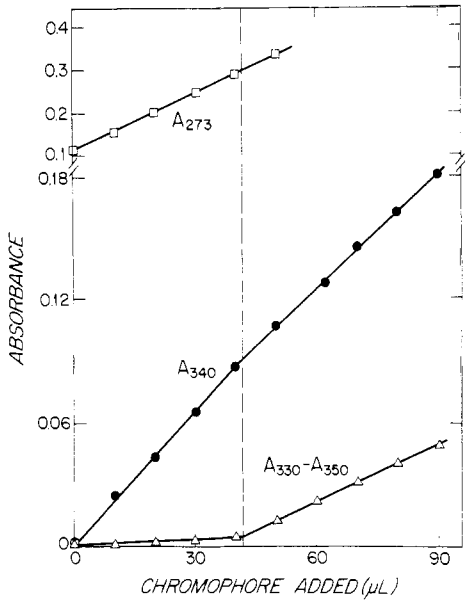

FIGURE 1: Titration of NCS apoprotein with isolated chromophore. Aliquots (10 μL) of chromophore were added to 1 mL of 20 mM sodium citrate, pH 4, containing 8.3 μM apoprotein (A_{278} = 0.12) which had been purified by XAD-7 chromatography. The ultraviolet–visible spectrum was recorded, and absorbance at the indicated wavelengths was plotted after correction (<10%) for dilution.

determine the extinction coefficient of the chromophore by titrating it with a known amount of apoprotein purified by XAD-7 chromatography. Amino acid analysis of an apoprotein sample with a measured A_{278} = 0.046 indicated a concentration of 3.2 μM; thus the extinction coefficient of apoprotein is ϵ_{278} = 0.046/3.2 μM = 14400 M^{-1}, in agreement with the value of 14000 M^{-1} previously determined for apoprotein obtained by simple methanol extraction (Napier et al., 1979). Chromophore bound to an excess of apoprotein has, like native NCS, an absorbance spectrum which is nearly flat between 330 and 350 nm, while the spectrum of free chromophore has a steep negative slope in this region (Figure 2A; (Napier et al., 1981); thus the difference $A_{330} - A_{350}$ is a sensitive indicator of chromophore binding to apoprotein. When an apoprotein solution with A_{278} = 0.12 was titrated with chromophore, a sharp inflection was seen in a plot of $A_{330} - A_{350}$ (Figure 1), indicating saturation of apoprotein with chromophore. At the point of saturation, A_{340} was 0.091. Thus, assuming a 1:1 chromophore–apoprotein complex was formed, the protein-bound chromophore has ϵ_{340} = (0.091/0.12)14400 M^{-1} = 10900 M^{-1}. By use of this extinction coefficient, the concentrations of all chromophore stock solutions were determined by adding an aliquot to an excess of apoprotein and measuring the A_{340}.

In the same titration, the A_{273} increased from 0.115 to 0.295 at saturation, indicating that 61% of the A_{273} of native holo-NCS is due to bound chromophore. The apoprotein-bound chromophore thus has ϵ_{273} = (14400 M^{-1})(0.295 − 0.115)/0.12 = 21600 M^{-1} and holo-NCS has ϵ_{273} = 35400 M^{-1}. This value is somewhat higher than the ϵ_{273} = 23000 M^{-1} previously determined for highly purified NCS (Napier et al., 1979), but that preparation was subsequently shown to be chromophore deficient (Napier et al., 1980).

In Figure 1, the ratio A_{340}/A_{273} was 0.091/0.295 = 0.31 at saturation. This ratio is the same as that of clinical NCS, indicating that clinical NCS is very nearly saturated with chromophore, in agreement with isoelectric focusing studies (Kappen et al., 1980a; Jung et al., 1980). NCS reconstituted as in Figure 1 to the point of saturation had an absorbance spectrum indistinguishable from that of clinical NCS, and amino acid analysis indicated an identical ϵ_{273} (34400 M^{-1}) for clinical and reconstituted NCS preparations.

Absorbance Spectrum of the DNA–Chromophore Complex. Under conditions (Povirk & Goldberg, 1980) where free chromophore was stable in aqueous solution (pH 4 and 4 °C), addition of excess DNA to dilute chromophore induced strong hypochromicity and a pronounced red shift in the absorbance spectrum (Figure 2A). The spectrum of DNA-bound chromophore was thus similar to that of apoprotein-bound chromophore but was more hypochromic (ϵ_{350} = 6800 M^{-1}) and had less fine structure at 250–270 nm, a shallower slope above 350 nm, and measurably greater absorbance at 375–390 nm. While for binding titrations by fluorescence spectroscopy (Povirk & Goldberg, 1980) it is difficult to eliminate the possibility that spectral changes may be due to a highly fluorescent minor contaminant, absorbance spectra (Figure 2) clearly show interaction between DNA and the major ultraviolet-absorbing compound in the preparation, which is almost certainly active chromophore.

In the presence of a sufficient excess of DNA, essentially the same spectrum of DNA-bound chromophore (Figure 2A) was always seen, at pH 4, 5, and 7, at NaCl concentrations as high as 0.4 M, or with methanol content as high as 20%. As with free chromophore, there appear to be many overlapping absorbance transitions, whose assignments are uncertain. However, the fluorescence excitation spectrum (emission at 420 nm) of DNA-bound chromophore (Figure 2C) had a broad maximum at 350 nm, closely corresponding to the broad absorbance band at 350 nm. Since the fluorescence probably arises from the naphthalene ring system (M. A. Napier and I. H. Goldberg, unpublished data), it is likely that this absorbance band is due largely to electronic transitions of the naphthalene rings. In the absorbance of DNA both the absorbance and the excitation bands were shifted to lower wavelength, although the emission peak is shifted to higher wavelength, from 420 to 440 nm (Povirk & Goldberg, 1980).

At pH 7, treatment of DNA-bound chromophore with 20 mM 2-mercaptoethanol converted the absorbance shoulder at 305 nm to an absorbance minimum, reducing the A_{305} by a factor of 2 (Figure 3). This result suggests that this absorbance band is attributable to the $C_{15}H_{10}O_4$ chromophore substituent, since this portion of the molecule is known to react with mercaptan (Albers-Schönberg et al., 1980). At longer times, a small increase in A_{350} was also seen. Like the isolated naphthoic acid derivative of NCS chromophore (M. A. Napier and I. H. Goldberg, unpublished data), 2-mercaptoethanol-treated chromophore is intensely fluorescent (Povirk & Goldberg, 1980). In the active chromophore the naphthalene fluorescence is strongly quenched, presumably through intramolecular physical interaction or competitive absorption by the $C_{15}H_{10}O_4$ substituent.

When the DNA–chromophore complex in pH 4 buffer was precipitated with ethanol, the supernatant had an absorbance spectrum resembling that of free chromophore, while the redissolved precipitate had no absorbance above 310 nm (data not shown). These results indicate that DNA–chromophore binding is fully reversible and that spectral changes are due to physical interaction with DNA rather than to chemical alteration of the chromophore.

Previous fluorescence studies (Povirk & Goldberg, 1980) suggested at least two types of chromophore binding sites on DNA with different affinities, and this result was confirmed by absorbance titrations (Figure 2B). Addition of small amounts of DNA (up to 1:1 ratio of chromophore to nucleo-

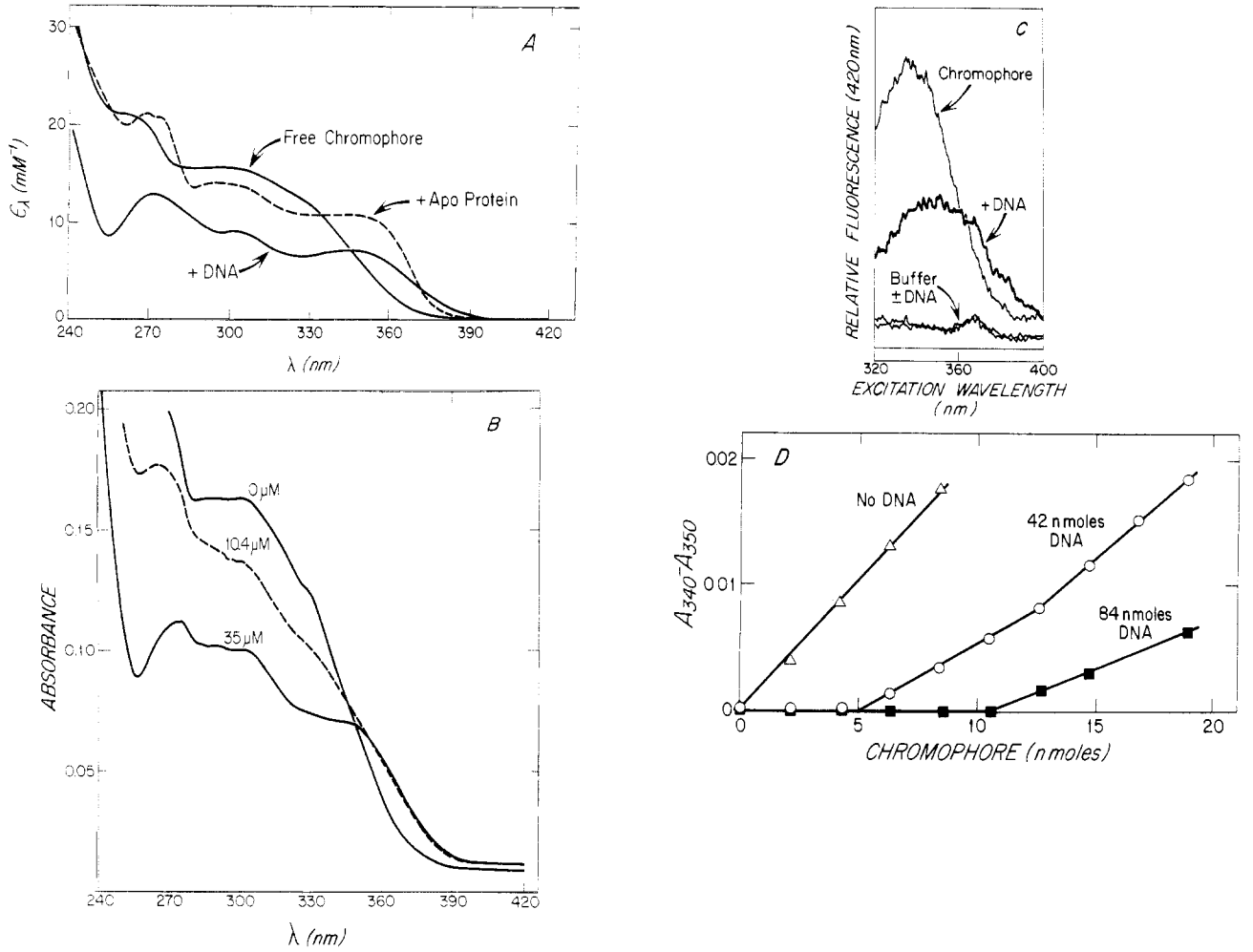

FIGURE 2: Effect of DNA on the absorbance spectrum of the chromophore. (A) Comparison of absorbance spectra of free, DNA-bound, and apoprotein-bound chromophore in 1 mL of 20 mM sodium citrate, pH 4, plus 2% methanol. Spectra of 3.6 μM chromophore before and after addition of 10 μL of 10 mM DNA (final concentration 100 μM) are direct tracings from the recording spectrophotometer (uncorrected for 1% dilution). The spectrum of 4.8 μM apoprotein-bound chromophore (apoprotein concentration = 6.9 μM) has been corrected for the concentration difference, so that all three spectra are plotted as the molar extinction coefficient ϵ_λ. For bound spectra, an equal concentration of DNA or apoprotein was added to the reference cuvette. (B) Effect of DNA on the chromophore spectrum at different chromophore/nucleotide ratios. Small volumes of concentration DNA were added to a solution of 10 μM chromophore (as well as to the reference cuvette) in pH 4 buffer plus 20% methanol to give the final DNA concentrations indicated. Spectra are uncorrected for dilution (<4%). (C) Fluorescence excitation spectra (emission at 420 nm) of free and DNA-bound chromophore in pH 4 buffer plus 10% methanol. The chromophore concentration was 12 μM and the DNA concentration 250 μM. (D) Titration of chromophore binding sites on DNA. Small volumes of concentrated chromophore were added to a 1-mL solution of pH 4 buffer containing the indicated concentrations of DNA. Plotted absorbances are corrected for dilution (<10%). All spectra were taken with cuvettes thermostated at 4 °C.

tide) resulted in a decrease in absorbance below 340 nm and an increase in absorbance above 350 nm. Further DNA additions caused a continued decrease below 340 nm but no change above 350 nm. Thus intermediate spectra were not simple linear combinations of bound and free spectra, implying existence of a secondary bound component with a distinct spectrum.

In order to determine the point of saturation of the highest affinity sites, small volumes of chromophore were added to solutions containing fixed amounts of DNA. In each case (Figure 2D) the $A_{340} - A_{350}$ was zero up to $r_{add} = 0.125$, where a sharp inflection occurred and the $A_{340} - A_{350}$ began to increase, but at a rate equivalent to only half that of free chromophore. Thus, the most preferred sites of DNA appeared to be saturated at $r_b = 0.125$ (reasonably close to the $r_{max} = 0.16$ estimated from fluorescence titration), but further additions of chromophore resulted in some other type of binding at higher r_b values.

In 20 mM citrate buffer, binding constants were too high to measure by absorbance titration; i.e., nearly vertical Scatchard plots were produced. A titration in citrate buffer plus 400 mM NaCl yielded $K_D = 1$ μM, $r_{max} = 0.30$, but again, no single isosbestic point was seen, and the shape of the Scatchard plot was strongly dependent on the wavelength chosen for computation of bound and free fractions. Thus, the significance of these numbers is doubtful.

Dichroism of the DNA–Chromophore Complex. Chromophore bound to short rodlike DNA oriented in an electric field showed negative electric dichroism from 315 to 385 nm; that is, application of the electric field resulted in a decrease in absorbance of light polarized parallel to the field [see Hogan et al. (1979)]. The apparent angles (α) between chromophore absorption transition moments at 315–385 nm and the DNA helix axis were between 64 and 76° (Figure 4A), similar to values seen with other known intercalators (Hogan et al., 1979). Since transition moments of aromatic rings are usually in the plane of the ring, these results are consistent with an intercalated ring system oriented roughly parallel to DNA bases. For DNA bases, $\alpha_{265} = 75°$ (Hogan et al., 1978). Since the precise orientation and number of transition moments for the chromophore is unknown, it is difficult to draw any definite geometric model for the complex.

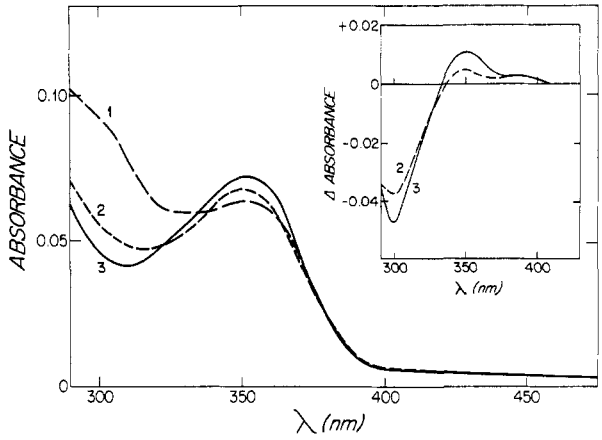

FIGURE 3: Effect of 2-mercaptoethanol on the spectrum of DNA-bound chromophore. Chromophore (final concentration 10 μM) was added to a 1-mL solution containing 230 μM DNA in 20 mM sodium phosphate, pH 7, at 4 °C, and the absorbance spectrum (1) was recorded; 20 μL of 1 M 2-mercaptoethanol was then added, and the spectrum was recorded after 5 min and after 20 min (spectra 2 and 3, respectively). Inset: Computed difference spectra (spectra 2 and 3 minus spectrum 1) for changes seen upon 2-mercaptoethanol addition.

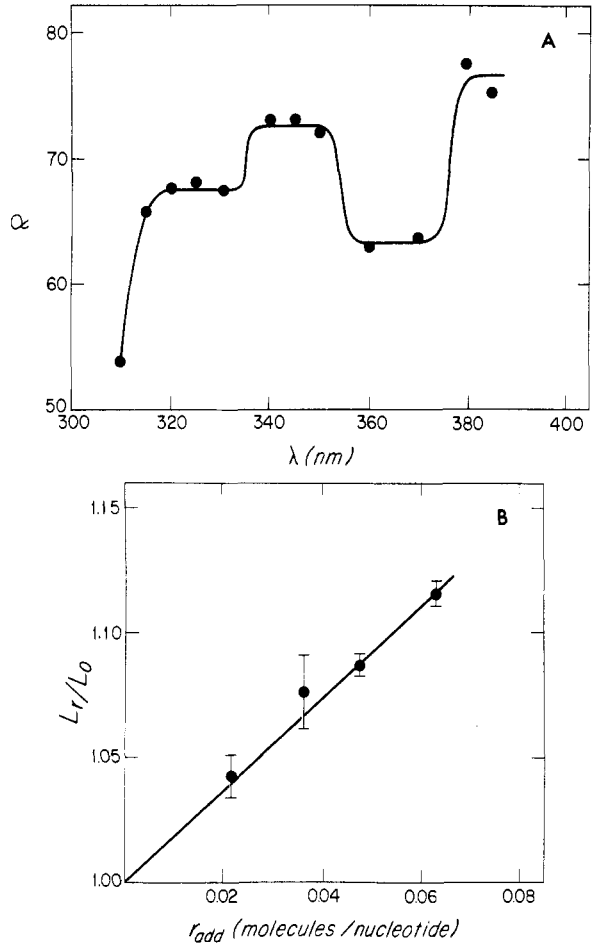

FIGURE 4: Electric dichroism of the DNA chromophore complex. (A) Dichroism spectrum of the complex (r_{add} = 0.04) at pH 5, plotted as the apparent angle α between the chromophore transition moments and the DNA helix axis, extrapolated to perfect DNA orientation (Hogan et al., 1978). (B) Changes in DNA length induced by chromophore binding. Chromophore (or an equivalent amount of methanol) was added to 75 μM DNA in 5 mM sodium acetate, pH 5, to give the r_{add} values indicated. DNA length was then determined from its orientation time in an electric field. Note that r_{add} is expressed in molecules per nucleotide rather than per base pair.

Bound chromophore had absorbance but no detectable dichroism at 310 nm, giving an apparent $\alpha \sim 54°$. Zero dichroism could also result from a randomly oriented transition or from two transitions with compensating parallel and perpendicular orientations.

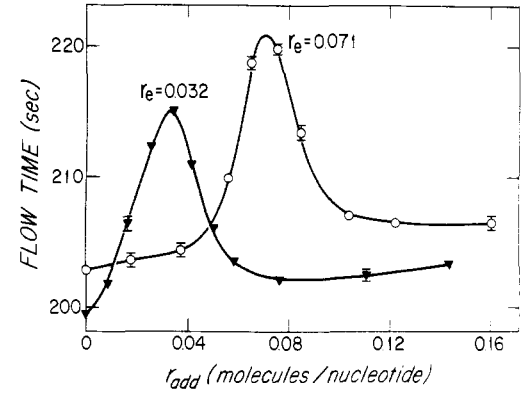

FIGURE 5: Unwinding of PM2 DNA by chromophore and ethidium bromide. Small volumes of concentrated ethidium were added to solutions containing PM2 DNA and 0 (○) or 0.048 (▼) chromophore molecule per nucleotide, and relaxation of the DNA was followed by the change in viscosity of the solution.

DNA Lengthening. The length of short rodlike DNA molecules was determined from their orientation relaxation time as measured by the rise of dichroism at 265 nm upon application of an electric field (Hogan et al., 1978). DNA length increased linearly with the amount of added chromophore (Figure 4B). At r_{add} = 0.05, or one chromophore per 10 base pairs, DNA length was increased by 9.5%. Thus each chromophore molecule lengthened DNA by very nearly the equivalent of one base pair or (3.4 Å)(0.095/0.10) = 3.3 Å, as expected for insertion of an aromatic ring between base pairs. Similar lengthening is seen with other intercalators (Hogan et al., 1979).

Helix Unwinding Angle of the Chromophore. Although we have previously shown that NCS chromophore removes and reverses superhelical turns in pMB9 DNA (Povirk & Goldberg, 1980), we did not determine the unwinding angle because the complexity of DNA–chromophore binding makes accurate determination of the binding isotherm very difficult (see above). The PM2 viscosity assay for DNA unwinding (Waring, 1976) has the advantage that very high DNA concentrations (0.288 mM) can be used, so that binding of serially added ligand is virtually quantitative and an accurate determination of the binding constant is not required. However, the NCS chromophore is soluble at high concentrations only in organic solvents such as methanol, which itself causes substantial increases in the viscosity of aqueous solutions. Therefore, we chose to measure the unwinding angle by partially relaxing PM2 DNA by addition of a fixed amount of chromophore and completing the titration with ethidium bromide (Figure 5). In the solvent system used, which included 10% methanol, full relaxation by ethidium alone occurred at r_e = 0.071. [With no methanol, at neutral pH and room temperature we reproduced the value r_e = 0.055 reported by Mong et al. (1980).] When chromophore was added to give r = 0.048 chromophore molecule/nucleotide, the amount of ethidium required to achieve full relaxation was reduced to r_e = 0.032. Thus each chromophore unwound the DNA by the equivalent of (0.071 − 0.032)/0.048 = 0.82 ethidium molecule, and the chromophore unwinding angle is (0.82)(26°) = 21°, well within the range measured for other intercalators (Waring, 1970).

Although it is possible to postulate more complex tertiary interactions between DNA, chromophore, and ethidium to explain the results in Figure 5, the finding that chromophore

Table I: Effect of Salt on Protection of Chromophore by DNA[a]

buffer concn[b] (mM)	τ[c] (s) no DNA	τ[c] (s) DNA[d]	K_D[e] (μM)
20	16	225	0.7
120	19	55	4.5
400	20	33	13

[a] Chromophore (10 μL of 0.2 mM) was added to 2.49 mL of buffer and its degradation monitored by generation of 490-nm fluorescence (Povirk & Goldberg, 1980). [b] Phosphate concentration of sodium phosphate buffers, pH 7. [Na^+] is ~1.5 times larger. [c] Lifetime of the active chromophore in solution. [d] 69 μM calf thymus DNA. [e] Apparent dissociation constant for the DNA-chromophore complex, assuming one binding site per eight nucleotides (see text).

is known to be capable of unwinding DNA (Povirk & Goldberg, 1980) and the observation that chromophore shifted the ethidium titration curve to lower r_{add} values with little change in its shape both suggest that partial PM2 DNA unwinding by chromophore is the most reasonable interpretation of the data. It should be noted that the data cannot be explained by displacement of ethidium from DNA binding sites by chromophore, since such an effect would prevent unwinding entirely, or at least would increase rather than decrease the concentration of ethidium required (Braithwaite & Baguley, 1980).

Effects of Salt and Superhelicity on DNA–Chromophore Binding. In order to keep chromophore stable, physical studies described were pefrormed at 4 °C and pH 4–5. At neutral pH and room temperature, chromophore spontaneously degrades rapidly, but since the DNA-bound chromophore is stable, binding can be assayed by the decrease in the rate of generation of a 490-nm fluorescent chromophore degradation product, using the equation (Povirk & Goldberg, 1980)

$$K_D = \frac{[DNA]_f^*}{(R_0/R_D) - 1}$$

where K_D is the dissociation constant, R_D and R_0 are the chromophore degradation rates in the presence and absence of DNA, and $[DNA]_f^*$ is the concentration of free binding sites on DNA. Since DNA is in vast excess and there is 0.125 high affinity chromophore binding site per nucleotide (Figure 2C), $[DNA]_f^*$ is 0.125 times the total DNA nucleotide concentration. Even if this site size is inaccurate, it would change only the absolute and not the relative values of K_D obtained by this method. Degradation rates were determined from kinetics of generation of 490-nm fluorescence, which could be described by a single exponential with time constant $\tau = 1/R$.

In buffer containing 20% methanol we previously found that 200 mM NaCl completely eliminated chromophore protection by DNA (Povirk & Goldberg, 1980). However, at a much lower methanol concentration (0.4%), fairly tight binding (K_D = 4.5 μM) was still seen near physiological ionic strength ([Na^+] = 180 mM) and neutral pH (Table I). The K_D was roughly proportional to the salt concentration, suggesting that electrostatic forces play an important role in chromophore–DNA binding. The amino group of the (methylamino)-galactose substituent of the chromophore (Albers-Schönberg et al., 1980) is expected to be positively charged at neutral pH and below.

Although the spectrum of DNA-bound chromophore was the same at pH 7 and 4, we sought additional evidence for intercalation at pH 7. At very low r_b values, intercalators show an enhanced affinity for supercoiled DNA (Hinton & Bode, 1975). This effect is thought to be due to the decrease in

Table II: Effect of Superhelicity on Protection of Chromophore by DNA[a]

pMB9 DNA	τ[b] (s)	K_D (μM)
none	25 ± 1	
supercoiled	256 ± 3	1.95
sonicated	156 ± 6	3.43

[a] Chromophore (10 μL of 40 μM) was added to 0.49 mL of 70 mM sodium phosphate, pH 7, containing DNA (144 μM) as indicated. [b] Average of triplicate determinations ± standard deviation. See Table I for explanation of τ and K_D.

Table III: Effect of Superhelicity on DNA Breakage by NCS Chromophore[a]

chromophore (nM)	breaks/molecule[b] supercoiled	breaks/molecule[b] relaxed	ratio[c]
0	(0.107)[b]	(0.089)[b]	
2	0.103	0.071	1.52
4	0.179	0.118	1.52
8	0.552	0.368	1.46
16	1.276	0.875	1.50
			av 1.50 ± 0.03

[a] A mixture of supercoiled ^{14}C-labeled Col E1 DNA and relaxed ^3H-labeled Col E1 DNA was treated with chromophore in the presence of 2-mercaptoethanol, and the number of breaks in each was determined from loss of covalently closed molecules. [b] Breakage seen at zero chromophore concentration (in parentheses) has been subtracted. [c] Breaks in supercoiled DNA/breaks in relaxed DNA.

torsional free energy of supercoiling that occurs when an intercalating molecule partially unwinds a supercoiled DNA molecule; therefore, its magnitide should be similar for molecules with similar unwinding angles, regardless of other details of the binding mechanism. Hinton & Bode (1975) found that ethidium bromide at the lowest measurable r_b value (~0.025) had a 2.3-fold higher affinity for supercoiled than for relaxed DNA, in a buffer containing 100 mM NaCl. In a buffer of similar ionic strength and at $r_b \sim 0.01$, protection experiments indicated that supercoiling increased the apparent affinity of DNA for chromophore by a factor of 1.75 (Table II). Since the supercoiled DNA used in the experiment had 20% contamination with nicked circles, the actual enhancement in affinity must have been slightly larger, approximately (1.75 − 0.2)/0.8 = 1.95. Considering the slightly smaller unwinding angle of NCS chromophore, these data seem quantitatively consistent with earlier data for ethidium and strongly suggest that chromophore intercalation occurs at neutral as well as acid pH and at reasonably high ionic strength.

Preferential Cleavage of Supercoiled DNA. If intercalative binding is involved in DNA cleavage by chromophore, enhanced affinity for supercoiled DNA should result in enhanced breakage of supercoiled DNA when a mixture of supercoiled and relaxed molecules is treated with chromophore (Povirk et al., 1979). In such experiments (Table III), supercoiled DNA was broken at a rate 1.5 times greater than that of relaxed DNA. While this result qualitatively supports involvement of intercalative binding in the cleavage reaction, the enhancement is somewhat lower than would be expected from either ethidium binding data (Hinton & Bode, 1975) or chromophore protection data (Table II) and is the same as the enhancement seen with bleomycin, which shows a much smaller, 12°, unwinding angle (Povirk et al., 1979). Possible reasons for this apparent discrepancy are discussed below.

Spontaneously Degraded Chromophore. When chromophore was added to pH 8 buffer at room temperature and the

spontaneous degradation reaction (Povirk & Goldberg, 1980) allowed to go to completion, the resulting product(s) showed an altered absorbance spectrum with a peak at 380 nm (not shown). Subsequently added DNA, even at a concentration of 200 μM, had no effect on this spectrum, suggesting that spontaneously degraded chromophore had lost its ability to bind to DNA. DNA also has no effect on the fluorescence of this material (Povirk & Goldberg, 1980).

Discussion

The extent of DNA unwinding and lengthening induced by chromophore binding and the dichroism of the chromophore–DNA complex leave little doubt that part of the NCS chromophore molecule intercalates. Less certain, however, is the identity of the intercalating moiety and the involvement of intercalative binding in the DNA cleavage reaction.

NCS chromophore (Albers-Schönberg et al., 1980) has two substituents potentially capable of intercalation: a naphthoic acid derivative linked as the carboxyl ester to the rest of the molecule and a $C_{15}H_{10}O_4$ substituent whose structure is unknown but whose formula indicates a great deal of unsaturation with possible aromatic components. There is circumstantial evidence that the naphthalene ring system is the intercalating moiety. Like other substituted naphthoic acids, the derivative found in NCS chromophore, which can be split off and separated from the rest of the molecule, has intense fluorescence at 440 nm in its uncharged form (M. A. Napier and I. H. Goldberg, unpublished data). Therefore it is likely that the 440-nm fluorescence of NCS chromophore (which is shifted to 420 nm upon addition of DNA) is attributable to the naphthalene ring system and that the absorbance band of DNA-bound chromophore at 350 nm which is closely coincident with the excitation band for 420-nm fluorescence is also due largely to absorbance by this ring system. Thus the negative dichroism (Figure 4A) above 340 nm suggests that the naphthalene ring system is oriented parallel to DNA bases, making it a likely candidate for the intercalating moiety. Evidence for intercalation by (trans) naphthylvinylpyridines (White et al., 1971) and by naphthylimide (Gabbay et al., 1973) has been reported previously. Although these are both three-ring planar structures, the naphthylvinylpyridines have, like NCS chromophore, a nonaromatic linkage between the naphthalene rings and the rest of the molecule.

Conversely, no dichroism was seen at 310 nm, where the $C_{15}H_{10}O_4$ substituent has strong absorbance (M. A. Napier and I. H. Goldberg, unpublished data) . This substituent is known to react with mercaptan (Albers-Schönberg et al., 1980), and treatment of either free (M. A. Napier and I. H. Goldberg, unpublished data) or DNA-bound chromophore (see above) with 2-mercaptoethanol results in loss of an absorbance band near 305 nm. Loss of absorbance may reflect elimination of a double bond and partial destruction of aromaticity, but whatever the structural change in the $C_{15}H_{10}O_4$ substituent is, it has very little effect on the ability of the chromophore to bind to and unwind DNA (Povirk & Goldberg, 1980). Thus the $C_{15}H_{10}O_4$ substituent seems a less likely candidate for intercalation. However, definitive resolution of this question must await more complete structure determinations for both active and 2-mercaptoethanol-treated chromophore. Atomic absorption spectroscopy (Napier et al., 1981) indicates that extracted NCS chromophore does not contain stoichiometric amounts of transition metals. Although it is difficult to rigorously eliminate the possibility that trace metal contaminants of buffer solutions may be involved in DNA–chromophore binding, there is no evidence that this is the case. Binding occurs in buffers containing either citrate (Povirk & Goldberg, 1980) or EDTA (L. F. Povirk and I. H. Goldberg, unpublished results), using DNA which has also been dialyzed against EDTA. Furthermore, a number of chelators have been tested and found to have no effect on DNA cleavage by NCS (Kappen & Goldberg, 1978).

Like other intercalators (Hinton & Bode, 1975; Hyde & Hearst, 1978), NCS chromophore shows enhanced affinity for superhelical DNA. Assuming that enhanced affinity is due to reduction of torsional stress in DNA upon intercalation (Bauer & Vinograd, 1968), the ratio r_b'/r_b of binding to supercoiled and relaxed forms at a given concentration of free ligand should be given by a partition function (Povirk et al., 1979)

$$r_b'/r_b = e - A\phi/kT \quad (1)$$

where ϕ is the unwinding angle and A is the torsional free energy change per degree of unwinding. For ethidium in 0.1 M NaCl at the lowest measurable r_b values, $r_b'/r_b = 2.3$ (Hinton & Bode, 1975), and $\phi = 26°$ (Wang, 1974b). For NCS chromophore, $\phi = 21°$; therefore r_b'/r_b should be $\sim$1.95, exactly the value estimated from protection studies.

However, enhanced cleavage of supercoiled DNA by NCS chromophore is clearly less, a factor of only 1.5 (Table III). Several other intercalators have also shown enhanced reaction rates with supercoiled DNA, but the degree of enhancement is not consistent with the simple model of enhanced binding described in eq 1. Two intercalating psoralen derivatives each show 2-fold greater affinity for supercoiled DNA, but at the same ionic strength covalent photobinding is enhanced by a factor of 2 for (aminomethyl)trimethylpsoralen but by only a factor of 1.3 for trimethylpsoralen (Hyde & Hearst, 1978). NCS chromophore (Table III) and bleomycin (Povirk et al., 1979) each show 1.5-fold enhanced cleavage of supercoiled DNA, even though the unwinding angle of NCS chromophore is nearly twice as large. Camptothecin, also suspected to intercalate, shows a 10-fold greater rate of photolytic cleavage with supercoiled DNA than with relaxed DNA (Lown & Chen, 1980). Finally, phleomycin and Fe^{2+} enhanced cleavage of supercoiled DNA by factors of 1.35 $\pm$ 0.07 and 2.5 $\pm$ 0.4, respectively (L. F. Povirk and I. H. Goldberg, unpublished experiments), but phleomycin shows no physical evidence for intercalation (Povirk et al., 1981), and although the mechanism of DNA cleavage by Fe^{2+} is uncertain, it is not likely to involve intercalation.

To reconcile these disparate data, we consider a generalized model for DNA strand breakage by a bound intercalating molecule

$$\mathrm{I} + \mathrm{DNA} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \mathrm{C_{out}} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \mathrm{C_{in}} \xrightarrow{k_4} \text{intermediate} \xrightarrow{k_5} \text{strand break} \quad (2)$$

where I is the intercalator, C_{out} and C_{in} are the outside-bound and intercalated forms, respectively, of the DNA–intercalator complex, and k_4 is the rate constant for the chemical reaction between the intercalator and DNA which is eventually expressed as a strand break. The model is analogous to that presented by Wang (1974a) for DNA-nicking enzymes. The equilibrium binding constant is given by $1/K_D = K_{12}(1 + K_{23})$ (Li & Crothers, 1974), where $K_{12} = k_{12}/k_{21}$ and $K_{23} = k_{23}/k_{32}$. The partition equation (eq 1) predicts that supercoiling should change K_{23} (and therefore K_D) by a factor related simply to the unwinding angle but does not predict how the individual rate constants k_{23} and k_{32} will change. If $k_4 \gg k_{32}$, enhancement of strand breakage will reflect only the change in k_{23}, not the change in k_{32}. Thus enhancement of breakage may

be less than enhancement of binding. Conversely, supercoiling may increase k_5, that is, increase the rate of conversion of labile intermediates into strand breaks, for example, by increasing the "breathing" rate and/or the mechanical stress in the DNA molecule. Such phenomena may explain the anomalously large enhancement of breakage seen with camptothecin as well as the enhancement seen with nonintercalators. In light of these complications, enhanced breakage of supercoiled DNA must be regarded as suggestive but not conclusive evidence for involvement of intercalative binding in DNA breakage.

The findings that DNA lengthening by NCS chromophore is nearly the equivalent of one base pair and that unwinding is comparable to that of ethidium indicate that K_{23} is significantly greater than 1; that is, most of the DNA-bound chromophore is intercalated, and intercalation makes a significant contribution to the binding energy. It is also possible that intercalation plays a more specific role in positioning the redox-active $C_{15}H_{10}O_4$ substituent for attack on DNA sugars (Hatayama & Goldberg, 1980).

References

Albers-Schönberg, G., Dewey, P. G., Hensens, O. D., Leisch, J. M., Napier, M. A., & Goldberg, I. H. (1980) *Biochem. Biophys. Res. Commun. 95*, 1351.

Bauer, W., & Vinograd, J. (1968) *J. Mol. Biol. 33*, 141.

Blair, D. G., Sherall, D. J., Clewell, D. B., & Helinski, D. R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2518.

Braithwaite, A. W., & Baguley, B. C. (1980) *Biochemistry 19*, 1101.

Broersma, S. (1960) *J. Chem. Phys. 32*, 1626.

Cohen, G. L., Bauer, W. R., Barton, J. K., & Lippard, S. J. (1979) *Science (Washington, D.C.) 203*, 1014.

Espejo, R. T., Canelo, E. S., & Sinsheimer, R. L. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 1164.

Gabbay, E. J., DeStefano, R., & Baxter, C. S. (1973) *Biochem. Biophys. Res. Commun. 51*, 1083.

Gamper, H. B., Straub, K., Calvin, M., & Bartholomew, J. C. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2000.

Hatayama, T., & Goldberg, I. H. (1980) *Biochemistry 19*, 5890.

Hinton, D. M., & Bode, V. C. (1975) *J. Biol. Chem. 250*, 1061.

Hogan, M., Dattagupta, N., & Crothers, D. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 195.

Hogan, H., Dattagupta, N., & Crothers, D. M. (1979) *Biochemistry 18*, 280.

Hyde, J. E., & Hearst, J. E. (1978) *Biochemistry 17*, 1251.

Jung, A., Lewis, R. S., & Kohnlein, W. (1980) *Biochim. Biophys. Acta 608*, 147.

Kappen, L. S., & Goldberg, I. H. (1978) *Nucleic Acids Res. 5*, 2959.

Kappen, L. S., & Goldberg, I. H. (1980) *Biochemistry 19*, 4786.

Kappen, L. S., Napier, M. A., & Goldberg, I. H. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1970.

Kappen, L. S., Napier, M. A., Goldberg, I. H., & Samy, T. S. A. (1980b) *Biochemistry 19*, 4780.

Li, J. L., & Crothers, D. M. (1969) *J. Mol. Biol. 39*, 461.

Lown, J. W., & Chen, H.-H. (1980) *Biochem. Pharmacol. 29*, 905.

Meienhofer, J., Maeda, H., Glaser, C. B., & Kuormizu, K. (1972) *Science (Washington, D.C.) 178*, 875.

Mong, S., Huang, C. H., Prestayko, A. W., & Crooke, S. T. (1980) *Cancer Res. 40*, 3313.

Napier, M. A., Holmquist, B., Strydom, D. J., & Goldberg, I. H. (1979) *Biochem. Biophys. Res. Commun. 89*, 635.

Napier, M. A., Kappen, L. S., & Goldberg, I. H. (1980) *Biochemistry 19*, 1767.

Napier, M. A., Holmquist, B., Strydom, D. J., & Goldberg, I. H. (1981) *Biochemistry* (in press).

Ohtsuki, K., & Ishida, N. (1980) *J. Antibiot. 33*, 744.

O'Konski, C. T., Yoshiro, K., & Orttung, W. H. (1959) *J. Phys. Chem. 63*, 1558.

Povirk, L. F., & Goldberg, I. H. (1980) *Biochemistry 19*, 4773.

Povirk, L. F., Hogan, M., & Dattagupta, N. (1979) *Biochemistry 18*, 96.

Povirk, L. F., Hogan, M., Dattagupta, N., & Buechner, M. (1981) *Biochemistry 20*, 665.

Suzuki, H., Miura, K., Kumada, Y., Takeuchi, T., & Tanaka, N. (1980) *Biochem. Biophys. Res. Commun. 94*, 255.

Wang, J. C. (1974a) *J. Mol. Biol. 87*, 797.

Wang, J. C. (1974b) *J. Mol. Biol. 89*, 783.

Waring, M. (1970) *J. Mol. Biol. 54*, 247.

Waring, M. J. (1976) *Eur. J. Cancer 12*, 995.

White, H. L., White, J. R., & Cavallito, C. J. (1971) *Prog. Mol. Subcell. Biol. 2*, 262.

Woynarowski, J. M., & Beerman, T. A. (1980) *Biochem. Biophys. Res. Commun. 94*, 769.