Binding of the Nonprotein Chromophore of Neocarzinostatin to Deoxyribonucleic Acid†

Lawrence F. Povirk and Irving H. Goldberg*

ABSTRACT: The methanol-extracted, nonprotein chromophore of neocarzinostatin (NCS), which has DNA-degrading activity comparable to that of the native antibiotic, was found to have a strong affinity for DNA. Binding of chromophore was shown by (1) quenching by DNA of the 440-nm fluorescence and shifting of the emission peak to 420 nm, (2) protection by DNA against spontaneous loss of activity in aqueous solution, and (3) inhibition by DNA of the spontaneous generation of 490-nm fluorescence. Good quantitative correlation was found between these three methods in measuring chromophore binding. There was nearly a 1:1 correspondence between loss of chromophore activity and generation of 490-nm fluorescence, suggesting spontaneous degradation of active chromophore to a highly fluorescent product. Chromophore showed a preference for DNA high in adenine + thymine content in both fluorescence quenching and protection studies. NCS apoprotein, which is known to bind and protect active chromophore, quenched the 440-nm fluorescence, shifted the emission peak to 420 nm, and inhibited the generation of 490-nm fluorescence. Chromophore had a higher affinity for apoprotein than for DNA. Pretreatment of chromophore with 2-mercaptoethanol increased the 440-nm fluorescence sevenfold and eliminated the tendency to generate 490-nm fluorescence. The 440-nm fluorescence of this inactive material was also quenched by DNA and shifted to 420 nm, indicating an affinity for DNA comparable to that of untreated chromophore. However, its affinity for apoprotein was much lower than that of untreated chromophore. Both 2-mercaptoethanol-treated and untreated chromophore unwound supercoiled pMB9 DNA, suggesting intercalation by both molecules. Since no physical evidence for interaction of native neocarzinostatin with DNA has been found, it is likely that dissociation of the chromophore from the protein and association with DNA are important steps in degradation of DNA by neocarzinostatin.

While the base specificity of DNA cleavage by the protein antibiotic neocarzinostatin (NCS)[1] strongly suggests direct interaction of this drug with DNA (Poon et al., 1977; Hatayama et al., 1978; D'Andrea & Haseltine, 1978), no physical evidence for binding has yet been found (Maeda et al., 1975; L. S. Kappen and I. H. Goldberg, unpublished data). Recently, NCS was shown to contain a highly labile, nonprotein chromophore, which could be extracted from the lyophilized native drug with methanol and which was at least as active as native NCS in degrading DNA (Napier et al., 1979; Kappen et al., 1980). Furthermore, DNA degradation was much faster with isolated chromophore than with native or reconstituted NCS; in fact, the reaction was markedly inhibited by excess apoprotein. These results suggested that the protein might simply act as a carrier for the chromophore and that dissociation of chromophore from protein must precede any interaction with DNA. We therefore examined the interaction of isolated chromophore with DNA.

Experimental Procedure

Chromophore Preparation. A 2-mL ampule of clinical NCS (0.6 mg/mL in 0.015 M sodium acetate, pH 5.0), provided by Dr. W. T. Bradner of Bristol Laboratories, was lyophilized and then extracted at 0 °C for 10 min with 2 mL of methanol to remove chromophore, and the protein precipitate was pelleted by centrifugation. After a second methanol extraction, which was discarded, the precipitate (NCS apoprotein) was dissolved in 1 mL of distilled water and frozen. Chromophore was stored as the undiluted methanol extract at −70 °C. Exposure of chromophore solutions to fluorescent lighting was avoided.

2-Mercaptoethanol-treated chromophore was prepared by adding 2-mercaptoethanol to a final concentration of 10 mM to a solution of methanol-extracted chromophore at 8 °C. The 420-nm fluorescence of the chromophore gradually increased approximately sevenfold. After 20 min when the fluorescence had stabilized, the methanol and 2-mercaptoethanol were evacuated and the residue was redissolved in methanol. An equal volume of water was added, and the solution was lyophilized and redissolved in methanol or dimethylformamide.

Concentrations of apoprotein were determined from A_{277} (ϵ_{277} = 14 000 M^{-1}; Napier et al., 1979).

DNA. For minimization of light scattering, sonicated calf thymus DNA was used in most experiments. Four hundred milligrams of DNA, dissolved in 200 mL of 0.04 M sodium phosphate, pH 7.6, was sonicated in an ice bath with a Branson cell disruptor for 30 min. After four extractions with phenol, the DNA was dialyzed twice against the same phosphate buffer and then against 10 mM sodium acetate, pH 5. S_1 nuclease (80 IU, Calbiochem), NaCl (0.1 M), and $ZnCl_2$ (30 μM) were added, and the solution was incubated for 1 h at 37 °C to digest any single-stranded DNA. After four more phenol extractions, sodium acetate was added to 0.3 M and the DNA was precipitated with 2 volumes of ethanol, dissolved at a concentration of 10 mM nucleotide in 10 mM Tris, pH 8, and dialyzed against 0.1 M EDTA, pH 7, and then against the desired buffer. Where indicated, the DNA was denatured by heating it for 10 min in a boiling water bath and then transferring it to an ice bath.

Micrococcus luteus and *Clostridium perfringens* DNAs (Sigma) and synthetic DNAs (P-L Biochemicals) were used without further purification.

[^{3}H]Thymine–DNA was isolated from CHO cells. The cells were grown in roller bottles in Eagle's minimum essential medium (Gibco) plus 15% fetal calf serum. When the cells

† From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115. *Received April 22, 1980.* Supported in part by U.S. Public Health Service Research Grant GM 12573 from the National Institutes of Health. L.F.P. was supported by a National Science Foundation fellowship.

[1] Abbreviations used: NCS, neocarzinostatin; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

0006-2960/80/0419-4773$01.00/0

were about 25% confluent, the medium was replaced with medium containing 1 μCi/mL [*methyl*-^{3}H]thymidine (60 Ci/mmol). The cells were incubated for 4 days, harvested with trypsin, heated to 60 °C for 10 min, and lysed with 0.1% sodium dodecyl sulfate, and DNA was isolated by the technique of Marmur (1961) and stored frozen in 10 mM Tris, pH 8.

DNA Base Release Assays. Twenty microliters of 0.24 mM [^{3}H]thymine–DNA (10^5 cpm) was added to 20 μL of 30 mM 2-mercaptoethanol and 150 mM Tris, pH 8. Twenty microliters of chromophore in 20 mM Tris, pH 8, and 20% methanol were then added, and the mixture was incubated at 22 °C for 10 min. The extent of thymine release was then determined by paper chromatography as described previously (Povirk et al., 1978).

Fluorescence Studies. All measurements were performed on a Perkin-Elmer 512 spectrofluorometer. For fluorescence quenching studies, small volumes of concentrated DNA (2–10 mM) were added to a solution of chromophore and the spectra were recorded. Corrections were made for dilution of chromophore and were never more than 10%. The absorbance at the excitation wavelength never exceeded 0.05. Kinetic measurements of fluorescence changes were made by adding chromophore in methanol to aqueous buffer solutions containing DNA or other components. Kinetic constants (τ) were usually determined from logarithmic plots of fluorescence vs. time, which could be described by a single exponential. For very slow reactions ($\tau > 2$ h) the rate $k = 1/\tau$ was calculated by dividing the initial observed rate of increase by the expected final fluorescence, as measured in samples which had been incubated overnight or at elevated temperature until their fluorescence had stabilized. In experiments with native NCS, the initial rate was determined and then 2-propanol was added to 3 M to allow the reaction to go rapidly to completion.

Excitation was always at 340 nm for 420- or 440-nm emission and at 380 nm for 490-nm emission.

Superhelix Titrations. 2-Mercaptoethanol-treated or untreated chromophores, both prepared from NCS which had been dialyzed against 20 mM sodium citrate, pH 4, were evacuated to dryness and dissolved in a 1:1 mixture of distilled water and dimethylformamide. By use of appropriate dilutions of these stock solutions, 5–20% sucrose gradients, containing 10% dimethylformamide, 20 mM sodium citrate, pH 4, and various concentrations of chromophore, were prepared at 4 °C. A 0.1-μg amount of pMB9 [^{3}H]DNA (1400 cpm) in 20 μL of the same buffer was layered atop each gradient, and they were centrifuged at 47 500 rpm for 5.5 h at 4 °C in a Beckman SW 50.1 rotor. Fractions of 0.2 mL were collected, and their radioactivity was counted after addition of 0.3 mL of distilled water and 4 mL of Scintiverse (Fisher Scientific Co.). pMB9 DNA was a generous gift of Dr. L. S. Kappen.

Results

Spontaneous Degradation of Chromophore and Protection by DNA. In methanol, the dominant fluorescence emission of the chromophore is at 420 nm (excitation at 340 nm), with a weaker fluorescence at 490 nm (excitation at 380 nm) (Napier et al., 1979). Upon dilution to an 80% aqueous solution, the fluorescence emission peak was immediately shifted from 420 to 440 nm and the intensity of fluorescence at 490 nm increased with continued incubation in aqueous buffer to approximately 10 times its initial value. The rate of this increase was strongly dependent on pH and temperature (Table I). Although the extent of increase of 490-nm fluorescence was reproducible, the kinetics of the increase were different for different chromophore preparations, even those extracted from the same lot of NCS. For some preparations the fluorescence increase was a simple exponential function of time (τ = 12 s at pH 8), but most preparations also showed varying proportions of a component with a lifetime τ = 30 s. The differences are presumably due to as yet undetermined details of the extraction procedure.

Table I: Effect of pH and Temperature on Chromophore Stability[a]

buffer	τ 8 °C	τ 25 °C
20 mM sodium citrate, pH 4	35 h	81 min
20 mM sodium acetate, pH 5	8.2 h	
20 mM Tris-HCl, pH 8	4.2 min	12 s

[a] 0.1 mL of a methanol-extracted chromophore was added to 0.4 mL of buffer. The 490-nm fluorescence was continuously recorded and time constants (τ) for the appearance of 490-nm fluorescence were determined. On the assumption that the appearance of 490-nm fluorescence corresponds to chromophore inactivation, τ also represents the mean lifetime of active chromophore in solution.

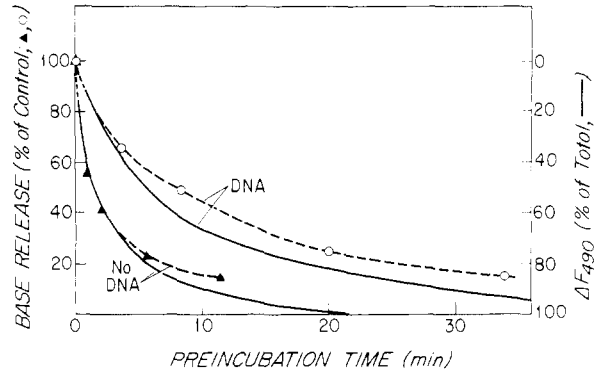

FIGURE 1: Protection by DNA against chromophore inactivation and generation of 490-nm fluorescence. 0.14 mL of methanol-extracted chromophore was added to 0.56 mL of 20 mM Tris, pH 8, containing either 0 or 0.14 mM calf thymus DNA at 8 °C. The fluorescence at 490 nm was continuously monitored, and at various times 20-μL aliquots were removed and assayed for release of thymine from [^{3}H]thymine–DNA. In the zero-time samples (i.e., aliquots of the preincubation solutions taken immediately after mixing) 0.45% of the total thymine was released when DNA was present in the preincubation and 0.62% when DNA was absent. The background release seen in the absence of chromophore (0.03%) was subtracted.

When chromophore was assayed for DNA base-releasing activity after incubation in aqueous buffer, there was a strong correlation between loss of activity and appearance of 490-nm fluorescence (Figure 1). Presence of DNA during the preincubation inhibited both of these processes. These results suggest that the chromophore spontaneously degrades in aqueous solution, forming a highly fluorescent inactive product, and that DNA binds the active chromophore, protecting it from this degradation. Since [^{3}H]DNA used to assay for base release was not present during the preincubation, chromophore bound to unlabeled DNA must have dissociated and reassociated with [^{3}H]DNA (present at a twofold higher concentration) shortly after mixing, in order to effect [^{3}H]thymine release. Therefore, binding of active chromophore to DNA is reversible. The chromophore preparation used in this experiment appeared to contain two active components which degrade at different rates, since neither the 490-nm fluorescence generation nor the loss of activity could be described by a single exponential. These two components appear to correspond to the two active fractions obtained in a separation of acetic acid extracted chromophore by high-pressure liquid chromatography (Albers-Schönberg et al., 1980; M. A. Napier, L. F. Povirk, and I. H. Goldberg, unpublished experiments).

Table II: Effect of DNA and Other Additions on Chromophore Stability[a]

addition	concn (mM)	τ (s)
none		12
poly(dA-dT)·poly(dA-dT)	0.12	90
Cl. perfringens DNA (69% A+T)	0.12	47
calf thymus DNA (61% A+T)	0.12	55
M. luteus DNA (28% A+T)	0.12	27
poly(dG-dC) poly(dG-dC)	0.12	23
poly(dA)·poly(dT)	0.12	20
calf thymus DNA	0.4	130
+200 mM NaCl	0.4	11
+10 mM Mg^{2+}	0.4	12
200 mM NaCl alone		9
10 mM Mg^{2+} alone		12
calf thymus DNA	1.2	370
denatured calf thymus DNA	1.2	73
apoprotein	0.01	6000
bovine serum albumin	0.15	62

[a] 0.1 mL of methanol-extracted chromophore was added to 0.4 mL of 20 mM Tris, pH 8, containing the indicated additions at 25 °C, and the lifetime τ of the chromophore was determined from the kinetics of generation of 490-nm fluorescence.

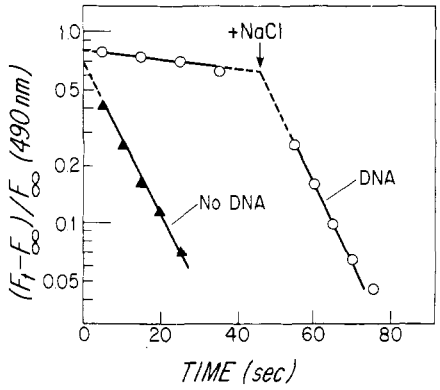

FIGURE 2: Effect of DNA and NaCl on generation of 490-nm fluorescence. 0.1 mL of methanol-extracted chromophore was added to 0.4 mL of 20 mM Tris, pH 8, containing 0 or 0.5 mM calf thymus DNA at 25 °C, and the 490-nm fluorescence was recorded. 20 μL of 5 M NaCl was added at the time indicated.

By use of a preparation whose degradation did follow a single exponential, protection by DNA was examined in more detail (Figure 2 and Table II). $MgCl_2$ or excess salt completely eliminated protection, even when added after DNA and chromophore, a result consistent with the hypothesis that protection was due to reversible DNA binding. DNAs with high proportions of adenine and thymine were more effective in chromophore protection, and native DNA was more effective than denatured DNA. The alternating polymer poly(dA-dT) was more effective than the homocopolymer poly(dA)·poly(dT). Rabbit tRNA was 20 times less effective than calf thymus DNA (data not shown). These are the same sequence preferences as were seen by Poon et al. (1977) in competition experiments, where degradation of a radioactive DNA was inhibited by addition of various unlabeled DNAs. However, they found poly(dA)·poly(dT) to be somewhat more effective than poly(dG-dC). Addition of DNA after generation of 490-nm fluorescence had gone to completion had no effect on its intensity. In 20 mM sodium citrate, pH 4, degradation was much slower than that at pH 8, but the degree of inhibition by DNA was about the same (data not shown).

Protection of Chromophore by Apoprotein. Kappen et al. (1980) have shown that apoprotein protects the chromophore from loss of activity in aqueous solution. Apoprotein also inhibited generation of 490-nm fluorescence much more effectively than either DNA or bovine serum albumin (Table II), suggesting rapid, specific, high-affinity binding of chromophore to apoprotein.

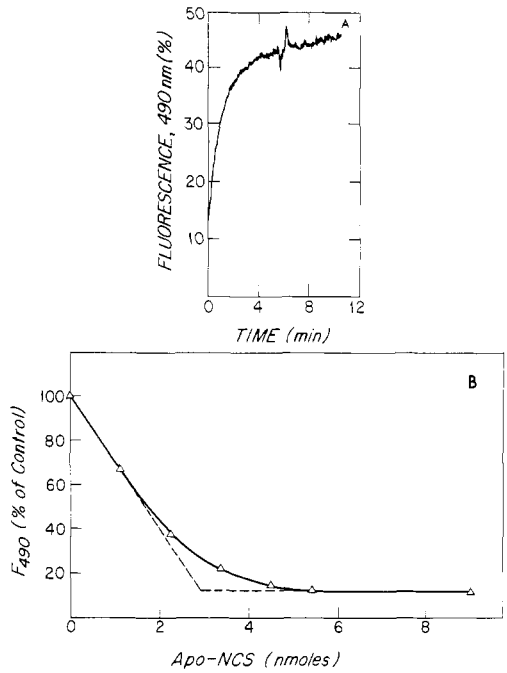

FIGURE 3: Effect of apoprotein on generation of 490-nm fluorescence. 0.1 mL of methanol-extracted chromophore was added to 0.4 mL of 20 mM Tris, pH 8, containing various amounts of apoprotein at 25 °C. (A) Plot of 490-nm fluorescence vs. time for a sample containing 2.1 nmol of apoprotein as a percentage of the final fluorescence of the sample containing no apoprotein. (B) Magnitude of 490-nm fluorescence 2 min after chromophore addition, as a function of the amount of apoprotein present.

When chromophore was added to solutions containing very small amounts of apoprotein, there was in the first few minutes an initial rapid increase in 490-nm fluorescence followed by a continuing increase at a rate at least 10 times slower (Figure 3). The rapid component was probably due to degradation of the excess free chromophore. Since protection data (Table II) indicate that chromophore–apoprotein binding is very tight, the degradation of the remaining, largely protein-bound chromophore would be expected to be much slower. Thus, the chromophore could be titrated with apoprotein by observing the magnitude of the rapidly generated 490-nm fluorescence (Figure 3B). On the assumption that apoprotein binds chromophore with a 1:1 stoichiometry (Kappen et al., 1980), this titration indicated a chromophore concentration of ~30 μM in the methanol extract, or half the concentration of NCS from which it was extracted. This result is in good agreement with data obtained from experiments in which NCS was reconstituted from separated chromophore and apoprotein and reconstitution was followed by isoelectric focusing (Kappen et al., 1980). The low yield of active chromophore was probably due partly to an initial chromophore deficiency in native NCS (Napier et al., 1980), partly to incomplete extraction, and partly to chromophore degradation during the extraction. Nevertheless, these data indicate that at least half of the NCS molecules contained a chromophore capable of generating a product with 490-nm fluorescence.

Addition of apoprotein to chromophore whose 490-nm fluorescence generation had already gone to completion had no effect on the fluorescence.

Native NCS, even in a totally aqueous environment, also generated 490-nm fluorescence, at a rate 4000 times slower than that of isolated chromophore (Table III). Because chromophore was not very soluble in water, it was added as a methanol solution, resulting in a 4% methanol concentration in the reaction mixture; since the observed degradation rate

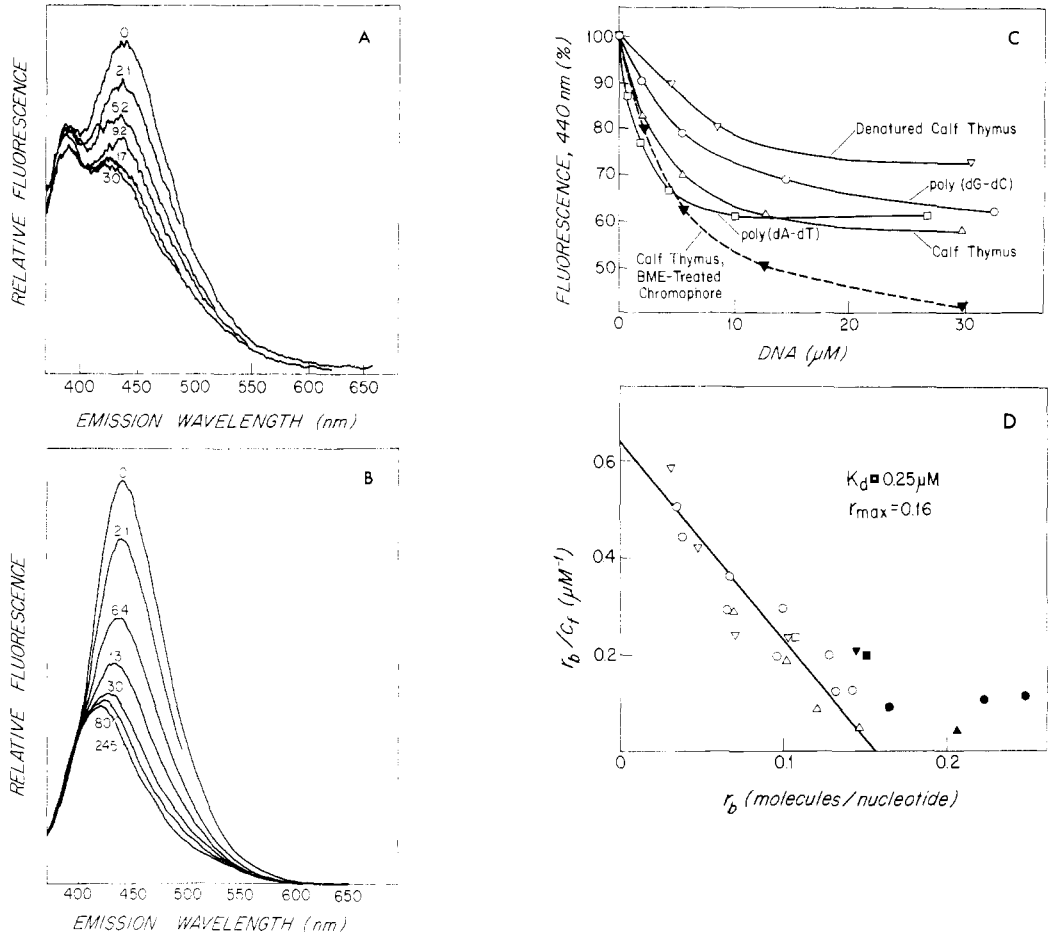

FIGURE 4: Quenching of chromophore fluorescence by DNA. (A) Various amounts of concentrated calf thymus DNA were added to a solution containing 2 mL of 20 mM sodium citrate buffer, pH 4, 0.45 mL of methanol, and 50 μL of methanol-extracted chromophore. The chromophore used in this experiment was prepared from NCS which had also been dialyzed against 20 mM sodium citrate, pH 4. (B) Calf thymus DNA was added to a solution containing 2 mL of buffer, 0.475 mL of methanol, and 25 μL of 2-mercaptoethanol-treated chromophore in dimethylformamide. (C) Fluorescence at 440 nm was measured after addition of calf thymus or synthetic polymer DNAs to the chromophore solution described in (A). The temperature was 8 °C, and excitation was at 340 nm. DNA concentrations in micromolarity are indicated for each spectrum. (D) Scatchard analysis of fluorescence quenching data for untreated chromophore and calf thymus DNA. Experimental conditions were the same as in (A) except that the total concentrations of chromophore were 1.2 (▽), 1.5 (□), 3 (○) and 6 (△) μM, assuming a concentration of 30 μM in the undiluted extract. r_b is the number of bound chromophore molecules per nucleotide and c_f is the concentration of free chromophore. Closed symbols indicate data taken at chromophore to DNA nucleotide ratios greater than 0.5.

was somewhat faster than that seen in 20% methanol, the rate in pure water is likely to be, if anything, even faster than that reported in Table III. High salt concentrations increased the rate of fluorescence generation fourfold for NCS but less than twofold for chromophore. A 3 M (24%) 2-propanol solution stabilized the chromophore against fluorescence generation but destabilized NCS. When both high salt and 2-propanol were present, the rates of fluorescence generation for NCS and chromophore were nearly identical, as though all chromophore–protein interactions had been eliminated. These results support the proposal (Kappen & Goldberg, 1979; Kappen et al., 1980) that 2-propanol accelerates NCS-induced DNA degradation by partially unfolding the protein, thus facilitating chromophore release. The finding that addition of excess apoprotein decreased the rate of generation of 490-nm fluorescence by NCS suggests that this generation was due to degradation of free chromophore in equilibrium with apoprotein-bound chromophore, rather than degradation of the native complex as such.

Changes in Chromophore Fluorescence Induced by DNA and Apoprotein. Under conditions where the aqueous solution of chromophore was fairly stable (pH 4 and 8 °C, Table I), addition of DNA quenched the 440-nm fluorescence and shifted the emission peak to 420 nm (Figure 4). The spectrum

Table III: Solvent Effects on Stability of Chromophore and NCS[a]

solvent	τ NCS	τ chromophore
20 mM Tris, pH 8	10.7 h	8 s
+200 mM NaCl	2.5 h	
+600 mM NaCl	2.6 h	~5 s
+3 M 2-propanol	122 s	51 s
+600 mM NaCl + 3 M 2-propanol	60 s	45 s
+2 μM apoprotein	80 h	

[a] 20 μL of native NCS (~60 μM) or 20 μL of methanol-extracted chromophore was added to 0.48 mL of the indicated solvents, and the lifetime τ was determined from kinetics of generation of 490-nm fluorescence.

of DNA-bound chromophore was thus similar to that of chromophore alone in organic solvents, suggesting that chromophore occupies a largely hydrophobic environment in its complex with DNA. Like other binding assays, fluorescence titrations indicated a preference by chromophore for native DNA and DNA high in adenine + thymine content.

A maximum estimate of the dissociation constant (K_d) may be obtained from the midpoint of the fluorescence quenching

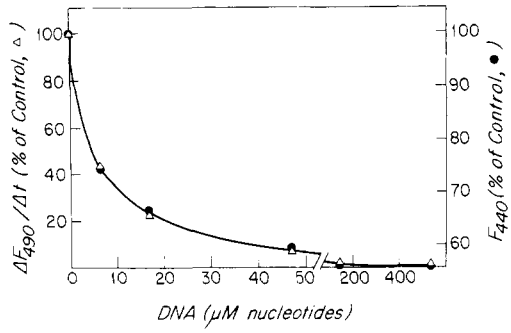

FIGURE 5: Protection and fluorescence quenching of chromophore by DNA. Small volumes of concentrated calf thymus DNA were added to a solution containing 0.4 mL of 20 mM sodium acetate, pH 5, 50 μL of methanol, and 50 μL of methanol-extracted chromophore at 8 °C. After each DNA addition, the 440-nm fluorescence or the rate of generation of 490-nm fluorescence was measured.

curves. Since the concentrations of bound and free chromophore should be equal at that point, K_d should be equal to the free DNA concentration and less than the total DNA concentration. Thus, the data indicate $K_d \leq 4$ μM for the untreated chromophore. This estimate assumes one binding site per nucleotide; if, as is likely, there are fewer binding sites, K_d must be considerably lower than this estimate.

For untreated chromophore, Scatchard analysis of fluorescence quenching data (Figure 4D) indicated a high-affinity component (K_d = 0.25 μM), which saturated at one chromophore per six nucleotides, a value consistent with an intercalative binding geometry (see below). Whenever the ratio of total added chromophore per DNA nucleotide exceeded 0.5, points consistently fell to the right of an otherwise linear Scatchard plot, suggesting an additional lower affinity, higher capacity binding component, probably electrostatic binding of chromophore to the outside of the DNA helix.

At pH 5, 490-nm fluorescence generation was fast enough to measure its inhibition by DNA, yet slow enough to allow measurement of DNA binding by 440-nm fluorescence quenching. Excellent correlation was found between these two measurements of DNA binding; 440-nm fluorescence was quenched to the same degree that 490-nm fluorescence generation was inhibited (Figure 5). These data suggest that the same molecule is responsible for both effects, or at least that the molecules producing the two effects have the same affinity for DNA.

Chromophore pretreated with 10 mM 2-mercaptoethanol in methanol at 0 °C has been shown to be rapidly and completely inactivated (Kappen & Goldberg, 1980). We found that this pretreated chromophore had about sevenfold higher 440-nm fluorescence in aqueous solution than untreated chromophore and showed no tendency to generate 490-nm fluorescence. However, except for a slightly greater degree of quenching, the effect of DNA on the fluorescence of 2-mercaptoethanol-treated chromophore, including a shift in the emission maximum from 440 to 420 nm, was very similar to that of untreated chromophore (Figure 4B). For both chromophores, ~4–5 μM DNA induced half-maximal quenching, indicating that the inactivation by 2-mercaptoethanol did not markedly affect the affinity of chromophore for DNA. The same spectrum of DNA-bound 2-mercaptoethanol-treated chromophore was obtained whether 2-mercaptoethanol treatment occurred in methanol or in an 80% aqueous solution or whether 2-mercaptoethanol was added before or after DNA.

2-Mercaptoethanol-treated chromophore differed markedly from untreated chromophore in its interaction with apoprotein. For untreated chromophore, addition of very low concentrations of apoprotein quenched the 440-nm fluorescence and shifted the emission peak to 420 nm (spectra not shown). As shown in Figure 6A, the quenching of 440-nm fluorescence reached its maximum extent when 1.5 nmol of apoprotein was added to a solution containing 50 μL of methanol-extracted chromophore. The sharp inflection in the curve indicates that binding of chromophore to protein was very tight and that the concentration of chromophore capable of binding to apoprotein was 1.5 nmol/50 μL = 30 μM in the methanol extract. This is the same value obtained by titration of 490-nm fluorescence generation and again suggests that the two effects are due to the same chromophore.

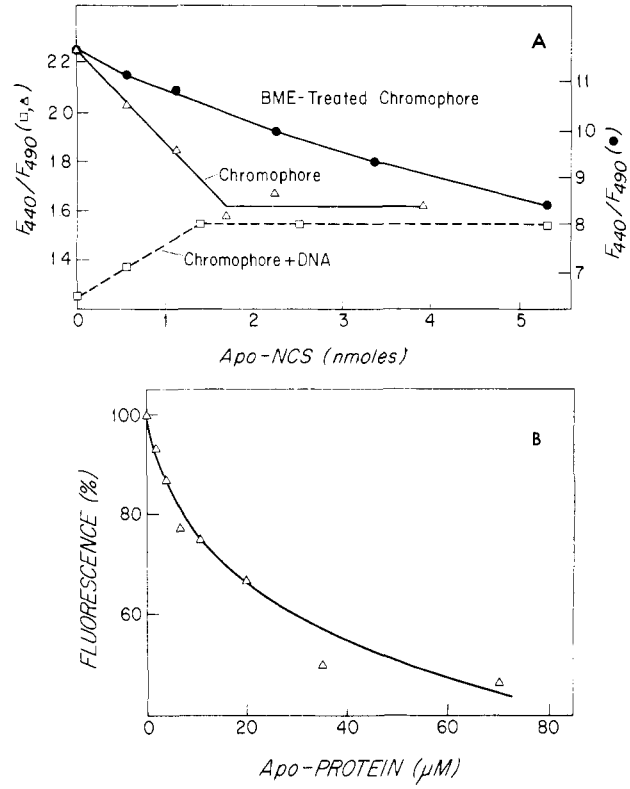

FIGURE 6: Effect of apoprotein on chromophore fluorescence. (A) Small volumes (10–40 μL) of 53 μM apoprotein in distilled water were added to a solution containing 0.4 mL of 20 mM citrate, pH 4, and 0 or 0.16 mM DNA, plus either 50 μL of methanol-extracted chromophore and 50 μL of methanol or 25 μL of 2-mercaptoethanol (BME)-treated chromophore in dimethylformamide and 75 μL of methanol. After each addition of apoprotein the fluorescence at 440 and 490 nm was measured. (B) Solutions containing 0.4 mL of 0 or 87 μM apoprotein in citrate buffer plus 50 μL of 2-mercaptoethanol-treated chromophore in methanol and 50 μL of methanol were mixed to yield a constant concentration of chromophore (~3 μM) and the desired final concentrations of apoprotein. The 440-nm fluorescence was measured at each concentration, with reference to a blank containing protein but no chromophore.

When apoprotein was added to a solution containing chromophore and DNA, the fluorescence at 440 nm was increased from the lower value seen with DNA alone to the value seen with NCS alone. This result suggests that apoprotein binds the same chromophore as DNA but much more tightly.

Low concentrations of apoprotein had less effect on 2-mercaptoethanol-treated chromophore (Figure 6A). Although considerable quenching was seen at higher concentrations of aproprotein (Figure 6B), the emission peak remained at 440 nm (spectra not shown). These results suggest that 2-mercaptoethanol-treated chromophore binds to apoprotein with a much lower affinity than untreated chromophore and in a qualitatively different manner. In fact, the interaction may be largely nonspecific, since the concentrations of apoprotein

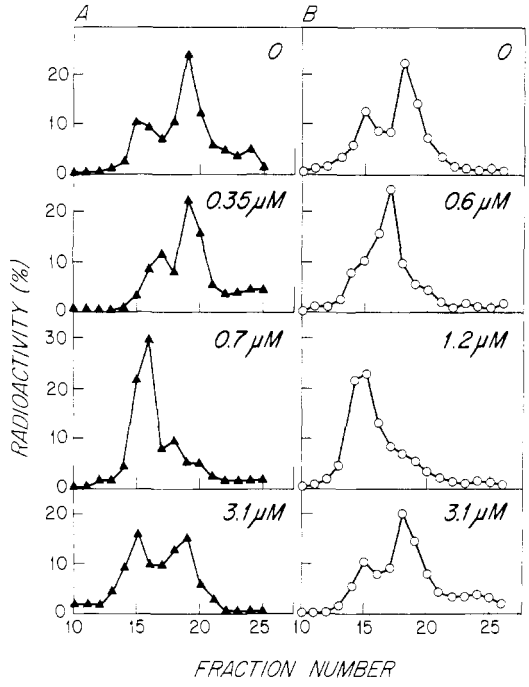

FIGURE 7: Unwinding of closed circular DNA by chromophore. pMB9 DNA was sedimented in gradients containing the indicated concentrations of untreated (A) or 2-mercaptoethanol-treated (B) chromophore. Sedimentation is to the right.

required to quench the fluorescence of 2-mercaptoethanol-treated chromophore were not much lower than the concentration of bovine serum albumin required to protect active chromophore (Table II).

Unwinding of DNA by Chromophore. When pMB9 DNA (70% supercoiled closed circles and 30% nicked circles) was sedimented in gradients containing an appropriate concentration of either 2-mercaptoethanol-treated or untreated chromophore, the sedimentation rate of closed circles was decreased and they cosedimented with nicked circles (Figure 7). At higher chromophore concentrations, the closed circles reverted to their orginal sedimentation rate. This pattern, indicating a relaxation and reversal of superhelical turns, is characteristic of ligands which unwind helical DNA (Waring, 1970). While the highest concentration of 2-mercaptoethanol-treated chromophore produced a sedimentation profile nearly identical with the control profile, the profile seen with the same concentration of untreated chromophore indicated a transfer of material from the supercoiled to the relaxed circular peak. This effect was probably due to nicking of DNA by chromophore at a very slow rate during sedimentation. Unwinding of DNA suggests that both untreated and 2-mercaptoethanol-treated chromophores intercalate, although additional data will be required before other modes of binding can be definitely ruled out. Dichroism and viscosity studies of the complex of untreated chromophore with DNA (L. F. Povirk, N. Dattagupta, and I. H. Goldberg, unpublished experiments) indicate negative electric dichroism, DNA lengthening, and a DNA helix unwinding angle all quantitatively similar to those obtained with other known intercalators.

Discussion

The methanol-extracted nonprotein chromophore of NCS has been shown to be a mixture of active and inactive components (Kappen et al., 1980). The most convincing evidence for binding of the active component to DNA is the protection of chromophore activity by DNA (Figure 1). There is circumstantial evidence that the effects induced by DNA on chromophore fluorescence are also due primarily to binding of the active chromophore. The close correspondence between the kinetics of generation of 490-nm fluorescence and those of activity loss both in the presence and in the absence of DNA (Figure 1) suggests that fluorescence generation is an indication of spontaneous degradation of active chromophore. The close correspondence of 440-nm fluorescence quenching by DNA with the inhibition of generation of 490-nm fluoresence (Figure 5) suggests that quenching is also a result of interaction of DNA with active chromophore. Furthermore, both of these fluorescence effects could be titrated with apoprotein (Figures 3 and 6) and both titrations indicated a chromophore concentration in the methanol extract of 30 μM, about one-half the concentration of the NCS from which it was extracted. This result agrees with the finding of Kappen et al. (1980) that the active component of chromophore appears to be the major ultraviolet-absorbing peak in a separation of chromophore by high-pressure liquid chromatography; therefore, it is probably present in a majority of native NCS molecules. Finally, titrations of identical chromophore preparations by absorbance spectroscopy show that DNA (at concentrations similar to those required to quench chromophore fluorescence) induces a bathochromic shift and hypochromicity of 45% in the near-ultraviolet spectrum of the chromophore (B. C. Warf, L. F. Povirk, and I. H. Goldberg, unpublished experiments). These results strongly suggest DNA binding by the active chromophore, which is the major ultraviolet-absorbing component of the preparation (Kappen et al., 1980). None of these results rigorously eliminates the possibility that one or both of the fluorescence effects result from an inactive chromophore component, but if this is the case, then this component must have nearly the same affinity for both DNA and apoprotein as the active component. However, reconstitution studies (Kappen et al., 1980) indicate that only active chromophore can recombine with apoprotein to change its isoelectric point to that of native NCS. Chromophore preparations inactivated by heat or 366-nm light have no effect.

NCS has been shown to break DNA primarily at thymine residues and to a lesser extent at adenine residues (Poon et al., 1977; Hatayama et al., 1978; D'Andrea & Haseltine, 1978). This specificity is probably at least partly due to the higher affinity of chromophore for DNA rich in adenine + thymine (Table II and Figure 4C). The unwinding of closed circular DNA suggests that chromophore intercalates (Figure 7), and like other intercalators (Krugh, 1972; Krugh & Reinhardt, 1975) it appears to prefer an alternating purine–pyrimidine sequence (Table II). However, it is unlikely that the rather complex base sequence specificity of DNA cleavage by NCS (Hatayama et al., 1978; D'Andrea & Haseltine, 1978) can be explained on the basis of intercalation alone.

While anthracycline intercalators have been reported to cleave DNA in the presence of reducing agents (Lown et al., 1976; Someya & Tanaka, 1979; Mong et al., 1980), none are nearly as efficient as NCS chromophore. Greater DNA cleavage is produced by 5 nM chromophore [based on the concentration of extracted NCS (Kappen et al., 1980)] than is produced by 100 μM adriamycin, carminomycin, marcellomycin, or aclacinomycin under similar conditions (Mong et al., 1980). On the other hand, the concentrations required for DNA unwinding by chromophore and by the anthracyclines are similar, on the order of 1–10 μM (Figure 7; Mong et al., 1980). While for the anthracyclines it is uncertain whether DNA cleavage or simple intercalative binding is more significant biologically, for NCS chromophore DNA cleavage is almost certainly the more significant effect. NCS is

functionally similar to the glycopeptide antibiotic bleomycin in that both drugs induce DNA strand breaks which are accompanied by release of free bases, primarily thymine (Müller et al., 1972; Takeshita et al., 1978; Ishida & Takahashi, 1976; Poon et al., 1977; Hatayama et al., 1978; D'Andrea & Haseltine, 1978). Both drugs are activated by sulfhydryls and oxygen (Onishi et al., 1975; Beerman & Goldberg, 1974; Kappen & Goldberg, 1978; Burger et al., 1978) and inhibited by α-tocopherol [Kappen & Goldberg (1978) and unpublished data], but both bleomycin and NCS chromophore bind reversibly to DNA in the absence of reducing agents and both appear to contain intercalating moieties (Figure 7; Povirk et al., 1979). DNA degradation by bleomycin involves oxidation of iron chelated in an iron–bleomycin complex (Sausville et al., 1978), but there is no evidence for involvement of transition metals in the action of NCS and a variety of chelators have no effect (Kappen & Goldberg, 1978).

Although fluorescence quenching data yielded a reasonable Scatchard plot (Figure 4D) parameters obtained from this analysis (K_d = 0.25 μM; r_{max} = 0.16) must be regarded as approximate, since two types of binding appear to be present, and these may have different degrees of quenching. As noted above, the more conservative procedure of inferring a maximum binding constant from the midpoint of the fluorescence quenching curve yields $K_d \leq 4$ μM. If we assume that chromophore bound to DNA is completely stable to degradation, protection data may also be used to calculate K_d. The rate of degradation in the presence of DNA should be proportional to the fraction of chromophore which is free, i.e.

$$\frac{R_D}{R_O} = \frac{C_f}{C_b + C_f}$$

where R_D and R_O are the degradation rates in the presence and absence of DNA and C_f and C_b are the concentrations of free and bound chromophore. Thus

$$K_d = \frac{C_f[DNA]_f}{C_b} = \frac{[DNA]_f}{(R_O/R_D) - 1}$$

Strictly speaking, $[DNA]_f$ should be expressed as the concentration of unoccupied binding sites. Since the size and number of binding sites on DNA are uncertain, $[DNA]_f$ cannot be calculated, even if the concentration of bound chromophore is known. It must be, however, less than the total DNA concentration, $[DNA]_T$. Thus

$$K_d \leq \frac{[DNA]_T}{(R_O/R_D) - 1}$$

We take the most liberal estimate of one potential binding site per nucleotide, as $[DNA]_T$ is expressed in moles of nucleotides per liter. If several components of chromophore are present, with different affinities for DNA, R_D will be a reflection of the most loosely bound component, and again K_d will be overestimated.

For protection of chromophore (Table II), we found R_O/R_D = 55 s/12 s = 4.5 in the presence of 0.12 mM DNA; therefore, we estimate $K_d \leq 0.12$ mM/3.5 = 33 μM in 20 mM Tris, pH 8, plus 20% methanol at 25 °C. In the same buffer, a similar computation for apoprotein yields $K_d \leq 0.01$ mM/499 = 20 nM for the chromophore–apoprotein complex. With no methanol present (Table III), we estimate $K_d \leq 4$ μM/(80 h/8 s) = 0.1 nM for the same complex. The estimate $K_d \leq 33$ μM computed for the DNA–chromophore complex is somewhat higher than the $K_d \leq 4$ μM estimated from fluorescence quenching (Figure 4). This difference is probably due to the higher temperature and higher concentration of the salt-containing chromophore extract used in the protection studies. The binding constant may also be pH dependent. When quenching and protection were measured under the same conditions, they indicated the same extent of chromophore binding at several DNA concentrations (Figure 5).

In spite of the uncertainties in binding calculations, it is clear that the chromophore binds much more tightly to apoprotein than to DNA. It may seem surprising that chromophore introduced as native NCS degrades DNA at all. However, the reaction of isolated chromophore with DNA is extremely fast, being complete in less than 1 min at 37 °C (Kappen et al., 1980). Therefore, even with apoprotein present, the relatively small proportion of active chromophore bound to DNA at any one time may be sufficient to permit a moderate rate of DNA degradation. On the other hand, the precise sequence of events leading to DNA degradation is unknown. These presumably include dissociation of chromophore from apoprotein, association with DNA, reduction of chromophore by sulfhydryls, and oxidation of reduced chromophore to some highly reactive species. It is possible that reduction precedes and facilitates release of chromophore from apoprotein; the reduced chromophore intermediate may be similar in structure to the inactive 2-mercaptoethanol-treated chromophore, which has greatly reduced affinity for apoprotein but the same affinity for DNA as active chromophore (Figures 4 and 6). Elucidation of the correct reaction sequence will require more detailed kinetic data.

Perhaps even more surprising is the extreme rapidity of spontaneous chromophore degradation, a matter of a few seconds at pH 8 in aqueous solution (Table III). This result explains why such high sulfhydryl concentrations (10 mM) are required for maximum chromophore activity (Kappen et al., 1980); chromophore activation by sulfhydryls must be very fast in order to compete with the spontaneous degradation reaction. The chemical nature of the degradation is unknown, but the data are consistent with a simple base-catalyzed hydrolysis. We have not rigorously eliminated the possibility that oxygen is required, but bubbling solutions with argon or oxygen had no effect on the degradation rate (data not shown).

In spite of its extreme lability, chromophore is as active as native NCS in inhibiting HeLa cell DNA synthesis (Kappen et al., 1980). The most likely explanation is that chromophore spends most of its time nonspecifically bound to proteins, membranes, or other hydrophobic environments, where it is relatively stable. A similar situation may exist when NCS is administered clinically. Although chromophore appears to have about 1000-fold higher affinity for apoprotein than for bovine serum albumin (Table II), the dilution of NCS during clinical administration might easily be sufficient to shift the equilibrium so that most chromophore is nonspecifically bound to serum proteins or other structures. Clearly, these binding equilibria must be taken into account in evaluating NCS pharmacokinetics.

Added in Proof

Chromophore used in DNA fluorescence quenching studies (Figure 4) was extracted from NCS which had been dialyzed against pH 4 citrate. Analysis by high-pressure liquid chromatography (M. A. Napier and I. H. Goldberg, unpublished experiments) indicates that such preparations contain only active chromophore, with no detectable inactive contaminants.

References

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

Beerman, T. A., & Goldberg, I. H. (1974) *Biochem. Biophys. Res. Commun. 59*, 1254.
Burger, R. M., Peisach, J., & Horwitz, S. B. (1978) *J. Biol. Chem. 253*, 4830.
Chien, M., Grollman, A. P., & Horwitz, S. B. (1977) *Biochemistry 16*, 3641.
D'Andrea, A. D., & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3608.
Hatayama, T., Goldberg, I. H., Takeshita, M., & Grollman, A. P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3603.
Ishida, R., & Takahashi, T. (1976) *Biochem. Biophys. Res. Commun. 68*, 256.
Kappen, L. S., & Goldberg, I. H. (1978) *Nucleic Acids Res. 5*, 2959.
Kappen, L. S., & Goldberg, I. H. (1979) *Biochemistry 18*, 5647.
Kappen, L. S., & Goldberg, I. H. (1980) *Biochemistry* (third paper of three in this issue).
Kappen, L. S., Napier, M. A., & Goldberg, I. H. (1980) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Krugh, T. R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1911.
Krugh, T. R., & Reinhardt, C. G. (1975) *J. Mol. Biol. 97*, 133.
Lown, J. W., Sim, S., Majumdar, K. C., & Chang, R. C. (1976) *Biochem. Biophys. Res. Commun. 76*, 705.
Maeda, H., Aikawa, S., & Yamashita, A. (1975) *Cancer Res. 35*, 554.
Marmur, J. (1961) *J. Mol. Biol. 3*, 208.
Mong, S., DuVernay, V. H., Strong, J. E., & Crooke, S. T. (1980) *Mol. Pharmacol. 17*, 100.
Müller, W. E. G., Yamazaki, Z., Breter, H.-J., & Zahn, R. K. (1972) *Eur. J. Biochem. 31*, 518.
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.
Onishi, T., Iwata, H., & Takagi, Y. (1975) *J. Biochem. (Tokyo) 77*, 745.
Poon, R., Beerman, T. A., & Goldberg, I. H. (1977) *Biochemistry 16*, 486.
Povirk, L. F., Kohnlein, W., & Hutchinson, F. (1978) *Biochim. Biophys. Acta 521*, 126.
Povirk, L. F., Hogan, M., & Dattagupta, N. (1979) *Biochemistry 18*, 96.
Sausville, E. A., Peisach, J., & Horwitz, S. B. (1978) *Biochemistry 17*, 2740.
Someya, A., & Tanaka, N. (1979) *J. Antibiot. 32*, 839.
Takeshita, M., Grollman, A. P., Ohtsubo, E., & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5983.
Waring, M. (1970) *J. Mol. Biol. 54*, 247.

Requirement for Reducing Agents in Deoxyribonucleic Acid Strand Scission by the Purified Chromophore of Auromomycin†

Lizzy S. Kappen, Mary A. Napier, Irving H. Goldberg,* and T. S. A. Samy

ABSTRACT: By methanol extraction and high-pressure liquid chromatography a nonprotein chromophore has been obtained from the antitumor protein antibiotic auromomycin (AUR) which possesses the cytotoxic and the in vivo and in vitro deoxyribonucleic acid (DNA) strand scission activities of the parent material. The rate of DNA strand breakage by the purified chromophore is markedly stimulated by reducing compounds (maximally at ~0.1 mM dithiothreitol), but DNA strand scission activity is lost upon pretreatment of the chromophore with these agents. Apoprotein specifically protects against such inactivation but blocks the activity of both the stimulated and unstimulated reactions, presumably by complexing the chromophore and making it less available to the target DNA. Dithiothreitol-dependent scission of DNA by chromophore is faster and more complete at 0 °C than at 37 °C. The reaction at 0 °C is almost entirely dependent on the presence of a reducing compound. Although 2-mercaptoethanol does not stimulate the reaction of either AUR or its chromophore at 37 °C, it has a significant stimulatory effect at 0 °C.

The antitumor antibiotic auromomycin (AUR)[1] is a polypeptide of molecular weight 12 500 produced by *Streptomyces macromomyceticus*, which also produces macromomycin (MCR) (Yamashita et al., 1979). AUR is identical with MCR in molecular weight, isoelectric point (p*I* of 5.4), and amino acid composition but differs from it by possessing UV–visible absorption above 300 nm (broad peak at ~355 nm). AUR can be converted into a material with the chemical,

† From the Department of Pharmacology, Harvard Medical School, and the Sidney Farber Cancer Institute, Boston, Massachusetts 02115. *Received May 30, 1980.* Supported by U.S. Public Health Service Research Grants GM 12573 and CA 22406 from the National Institutes of Health; M.A.N. is the recipient of an IPH Fellowship.

[1] Abbreviations used: AUR, auromomycin; NCS, neocarzinostatin; MCR, macromomycin; DTT, dithiothreitol; BME, 2-mercaptoethanol; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/80/0419-4780$01.00/0