## Mode of reversible binding of neocarzinostatin chromophore to DNA: base sequence dependency of binding

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ABSTRACT
　　The reversible binding of neocarzinostatin chromophore to polynucleotides was studied in order to understand the molecular basis of its base sequence-specificity in DNA damage production.  Studies of the spectroscopic and thermo-dynamic properties of chromophore-polynucleotide interactions reveal that the binding of the chromophore to poly(dA-dT) is qualitatively and quantitatively different from that to poly(dG-dC) (and poly(dI-dC)).  From these and other experiments using double-stranded mixtures of homopolynucleotides, it is proposed that the observed A-T specific intercalation might result from differential recognition of minor variations in the B-DNA type structure by the chromophore.

INTRODUCTION

　　　Neocarzinostatin, a DNA-damaging antitumor antibiotic, consists of an apo-protein and a tightly but noncovalently bound nonprotein chromophore (1,2). The chromophore (Figure 1) consists of four parts:  2-hydroxy-7-methoxy-5-methyl naphthoate and 2,6-dideoxy-2-(methylamino)galactose linked to a $C_{15}H_8O_4$ substituent consisting of an ethylene cyclic carbonate group and a highly strained ether epoxide attached to a novel bicyclo(7,3,0) dodecadiyne system (3,4). The chromophore, responsible for the antibiotic action (5,6), binds reversibly to DNA in the absence of sulfhydryl reagents (7,8).  Hydrodynamic studies (8) have shown that the chromophore unwinds DNA with an angle of 21°, suggesting an intercalative mode of binding.  Furthermore, electric dichroism measurements have shown that the DNA is lengthened 3.3 Å for each bound chromophore molecule and the chromophore lies essentially parallel to the DNA bases (8).  The naphthalene ring is the likely intercalating moiety, since the negative dichroism is restricted to a wavelength range (320 to 380 nm) corresponding to that characteristic of this part of the chromophore molecule (8).  Recent evidence suggests that intercalation occurs via the minor groove of B-DNA (9).
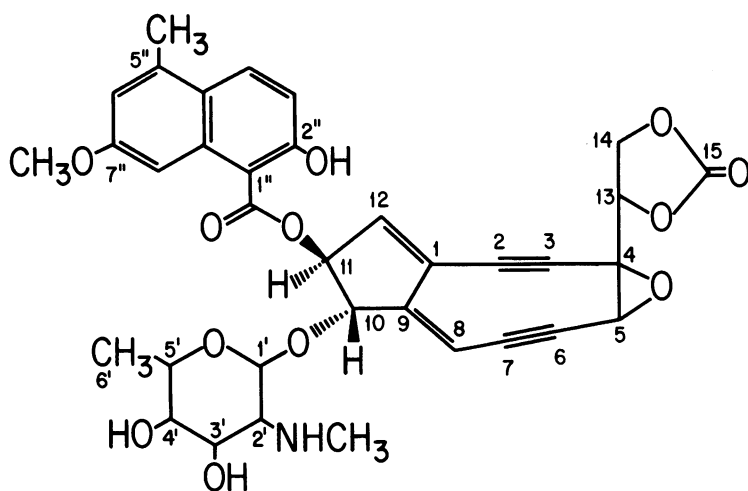
Figure 1. Structure of neocarzinostatin chromophore.

Binding to DNA plays an important role in determining the site of attack in the ensuing sulfhydryl activated processes of i) DNA strand breakage due to oxidative attack at the C-5' position of deoxyribose, ii) base release, and iii) covalent adduct formation with deoxyribose, all with a base preference T>A>>C>G (10-14). Both the reversible binding and the covalent interaction with DNA is base and structure specific (7,9-14). To understand the structural and chemical basis of the base specificity in the reversible binding of the chromophore to DNA, we undertook a detailed study of its binding to synthetic polynucleotides.

MATERIALS AND METHODS

The nonprotein chromophore of neocarzinostatin was obtained by methanol extraction of lyophilized (at 4°C) neocarzinostatin containing sodium citrate buffer, pH 4.0 (derived from clinical ampules obtained from Kayaku Antibiotics, Japan), as described earlier (8). Chromophore stock solutions were stored at -70°C and contained 0.25 mM chromophore and 20 mM sodium citrate, pH 4, in methanol. The purity of the chromophore was checked by high pressure liquid chromatography (15), and the concentration determined spectrophotometrically by adding an excess of apoprotein and measuring the absorbance at 340 nm ($\epsilon_{340}$ = 10,800 $M^{-1}cm^{-1}$) (8).

Polynucleotides (poly(dA-dT), alternating poly(deoxyadenylic-thymidylic acid) copolymer; poly(dG-dC), alternating poly(deoxyguanylic-deoxycytidylic

acid) copolymer; poly(dI-dC), alternating poly(deoxyinosinic-deoxycytidylic acid) copolymer; polydA·dT, double stranded mixture of homopolymers of deoxy-adenylic and thymidylic acids; polydG·dC, double-stranded mixture of homo-polymer of deoxyguanylic and deoxycytidylic acids) and dinucleoside monophos-phates, purchased from Pharmacia P.L. Fine Chemicals, were dissolved in 20 mM sodium acetate, pH 5.0 and extensively dialyzed against the same buffer. Poly(dA-dT) and poly(dI-dC) used for the NMR studies were synthesized enzymatically (16) and were purified by phenol extraction, ethanol precipita-tion and extensive dialysis against the required buffer. The CD spectral profiles of the polynucleotides at pH 5.0 in methanolic solutions, corres-ponding to the conditions used in the drug experiments, resembled those observed at neutral pH in the absence of methanol (16), suggesting there is probably no alteration of conformation due to low pH and methanol.

Spectrophotometric studies were carried out with a Perkin-Elmer Model 512A spectrophotometer. Spectrofluorometric measurements were done with a Perkin-Elmer spectrofluorometer. CD studies were done with an improved Cary-60 instrument with a higher sensitivity and data storage capacity (Aviv Associates, Lakewood, NJ). Ellipticity was expressed in terms of molar ellipticity, $[\theta]$.

$$[\theta] = (\theta_{obs} \cdot 100) / (1 \cdot c) ,$$

where $\theta_{obs}$ = observed ellipticity, $1$ = path length of the cell (1 cm) in decimeter and $c$ = concentration of the absorbing species in moles per liter (each of the reported spectra is an average of two runs).

Analysis of the Binding Data.

Spectrophotometric titrations of the chromophore with DNA were carried out at 324 nm where there is a maximum change (decrease) in absorbance. The association constant was determined from the following relation (17):

$$1/(e_f - e_{obs}) = 1/(e_f - e_b) + K_d/[(e_f - e_b) \cdot (c_{DNA} - c_{chrom}) \cdot r_b] \quad \text{(equation 1)}$$

where $e_f$ = extinction coefficient of the free chromophore,

$e_b$ = extinction coefficient of the bound chromophore,

$e_{obs}$ = extinction coefficient measured from the observed absorbance of the chromophore during the titration with DNA. $K_d$ is the intrinsic dissocia-tion constant for the complex, and c-terms denote the concentrations of the DNA and the chromophore, respectively. In some cases, the binding was quantita-tively expressed in terms of the apparent association constant ($K_{app} = r_b/K_d$), which includes both the intrinsic dissociation constant and the binding stoichiometry. $r_b$ denotes the binding stoichiometry and could be determined from the titration of the chromophore in the presence of an excess fixed

concentration (between 30 μM and 40 μM) of the nucleic acid, as described
earlier (9).    A plot of $1/(e_f-e_{obs})$ against $1/(c_{DNA}-c_{chrom})$ gives a straight
line; the ratio of the slope and the intercept of the line gives the $(K_d/r_b)$-
value.  The known value of $r_b$ gives the value of $K_d$.  The concentrations of the
polynucleotides were such that $c_{DNA}\gg c_{chrom}$, the condition at which the above
equation is valid.  For the construction of the Scatchard plot to evaluate the
binding constants, the concentration of the bound ligand $(c_b)$ was determined
from the relation: $c_b = \Delta A/(e_f-e_b)$, where $\Delta A$ = decrease in the absorbance of
the chromophore at 324 nm due to the addition of polynucleotide.

Analysis of the Kinetic Data.

    The reciprocal of the rate constant of the chemical degradation of the
chromophore in the absence and presence of the various polynucleotides was
determined from the semilog plot of the ratio of the observed fluorescence
change [at 490 nm where chromophore D (15), the highly fluorescent, aqueous
degradation product of chromophore A emits] at any time and the total
fluorescence change against that time (7).  The linearity of the plot over a
period of two to three half-lives of the reaction was taken as an index of the
first order nature of the degradation.

$^{31}P$ -NMR spectra.

    Proton noise decoupled $^{31}P$ NMR spectra were recorded at 107.9 MHz on a
Bruker FT-270MHz instrument using 90° pulses, at 4K data points and 1 sec pulse
delay.  All spectra were referenced to trimethylphosphate as internal standard.
The polynucleotides to be used for the spectral measurements were sonicated for
45 min under nitrogen and dialysed against the experimental buffer three to
four times.  Agarose gel electrophoresis (kindly performed by Dr. L.F. Povirk)
showed the length of the polymers to be 175-225 base pairs.


RESULTS

Binding Stoichiometry.

    Figure 2 shows the variation of $A_{340}-A_{350}$ against the concentration of
added chromophore at a fixed concentration of the polynucleotide.  The ratio of
the concentration of the chromophore corresponding to the breakpoint in the
line and the input concentration of the polynucleotide gives the binding stoi-
chiometry $(r_b)$ (9).  From Figure 2 it can be calculated that for poly(dG-dC)
the binding stoichiometry is 0.125 in contrast to the higher value of 0.25 for
poly(dA-dT).  Table I lists the binding stoichiometry values for the different
polynucleotide-chromophore interactions at different temperatures and percent-
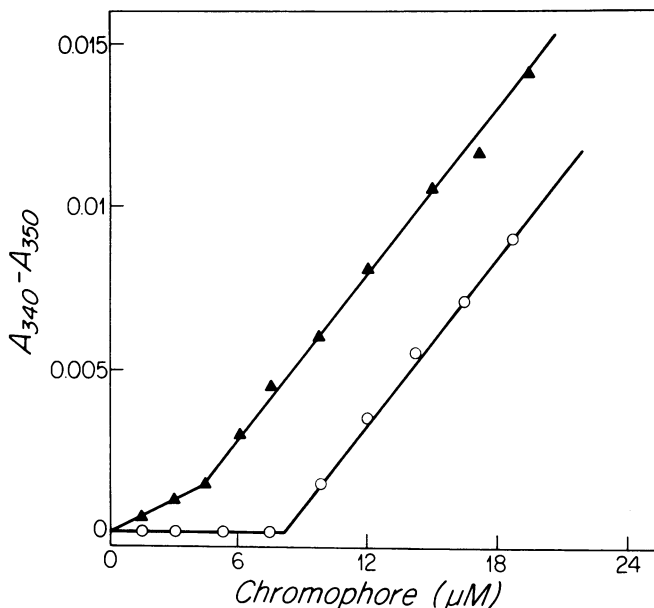ages of methanol.  It is clear from Table I that poly(dG-dC) and poly(dI-dC)

Figure 2. Determination of binding stoichiometry for chromophore-nucleic acid interaction. Plot of the differences in absorbances at 340 nm and 350 nm ($A_{340}$-$A_{350}$) against the concentration of chromophore added to poly(dA-dT) (O) and poly(dG-dC) (▲) at 14°C in 20 mM sodium acetate buffer, pH 5.0, containing 20% v/v methanol. The concentrations of poly(dA-dT) and poly(dG-dC) were 33 μM and 34.6 μM, respectively. $r_b$ was calculated from the plots as described in Materials and Methods.

can be grouped into one class with $r_b$ = 0.125, whereas for poly(dA-dT) $r_b$ = 0.25. Table I further suggests that neither the temperature nor the percentage of methanol exerts any significant influence on the stoichiometry. PolydA·polydT has a significantly lower binding stoichiometry than the corresponding alternating copolymer.

Binding Affinity as a Function of the Base Sequence.

The determination of binding stoichiometry ($r_b$) helps to determine the binding constant by means of equation 1, shown in the Materials and Methods. Table II lists the binding constants of the bound chromophore (measured from the plot of $1/(e_f-e_{obs})$ against $1/(c_{DNA}-c_{Chrom})$). The following features are apparent from Table II: i) the dissociation constant follows the order of base sequence G-C > I-C > A-T and exhibits the same trend when the percentage of methanol present in the system changes from 5 to 20%, ii) for a particular polynucleotide, the increase in the percentage of methanol leads to an increase in the dissociation constant, iii) the dissociation constants for poly(dG-dC)

TABLE I.   Binding Stoichiometry for Chromophore-Polynucleotide Interaction.[a]

| Polynucleotide | Methanol (%,v/v) | Temperature | Binding Stoichiometry $(r_b = \frac{[chromophore]}{[polynucleotide]})$ [b] |
|---|---|---|---|
| poly(dA-dT) | 5 | 5°C | 0.252 |
|  | 5 | 10°C | 0.254 |
|  | 5 | 14°C | 0.253 |
|  | 20 | 14°C | 0.244[c] |
| polydA·polydT | 5 | 14°C | 0.150 |
| poly(dG-dC) | 5 | 5°C | 0.120[c] |
|  | 5 | 14°C | 0.125 |
|  | 20 | 14°C | 0.130 |
| polydG·polydC | 5 | 14°C | 0.104 |
| poly(dI-dC) | 5 | 5°C | 0.123 |
|  | 5 | 14°C | 0.112[c] |
|  | 20 | 14°C | 0.125 |

[a] In all cases the buffer was 20 mM sodium acetate, pH 5.0.
[b] Terms in the square bracket denote the molar concentration.
[c] Average of duplicate determination.

and poly(dI-dC) are more affected than that for poly(dA-dT) by the increase in percentage of methanol, and iv) comparison between poly(dA-dT) and polydA·polydT with the corresponding set for G-C containing polymers clearly indicates that for polydA·polydT there is a marked increase in the dissociation constant compared to the alternating copolymer.  This is consistent with what has been reported for intercalators, such as daunomycin (18) and propidium (19), and may reflect the deviation from B-DNA structure in polydA·polydT (20,21).  To evaluate the influence of backbone conformation upon the affinity of chromophore for nucleic acid, the $K_{as}$ for the chromophore-polyA·polyU (A-DNA) interaction was estimated.  It is apparent from Table I that the binding is of an order of magnitude lower than for the other polymers reported here.

The binding constant and the stoichiometry were also determined by Scatchard analysis (22).  The $K_d$ and $r_b$ values for some polynucleotide-

TABLE II.   Dissociation Constant for the Chromophore-Polynucleotide
Interaction.[a]

| Polynucleotide | Methanol (% v/v) | $K_{as}$ $(= r_b/K_d)$[b] $(10^5 M^{-1})$ | $K_d$[c] (μM) |
|---|---|---|---|
| poly(dA-dT) | 5 | 3.02 | 0.83 |
|  | 20 | 2.5 | 0.98 |
| polydA·polydT | 5 | 0.26 | 5.6 |
| poly(dG-dC) | 5 | 0.35 | 3.6 |
|  | 20 | 0.21 | 6.0 |
| polydG·polydC | 5 | 0.23 | 4.5 |
| poly(dI-dC) | 5 | 1.15 | 1.1 |
|  | 20 | 0.53 | 2.4 |
| polyA·polyU | 5 | 0.038 |  |

[a]All measurements were done at 14°C in 20 mM sodium acetate buffer, pH 5.0, containing the specified percentage of methanol.
[b]$K_{as}$ denotes the apparent binding constant and includes the binding stoichiometry.  It is the reciprocal of the slope and the intercept of line, such as shown in Figure 3.
[c]$r_b$-values used for the calculation of $K_d$ were taken from Table I.

chromophore interactions, determined from the Scatchard plot, are reported in Table III.  The agreement between the $K_d$ and $r_b$ values determined by the two methods suggests an internal consistency of the methods.

The spectrophotometric titrations to evaluate the affinity constants were carried out near pH 5.0 in order to avoid degradation of the free chromophore at neutral or alkaline pH.  However, comparison of the rate constants of the degradation of the chromophore (carried out at neutral pH) in the presence of various nucleic acids and under identical conditions of pH, ionic strength, temperature, and percentage of methanol and nucleotide/drug values also reveals the relative DNA binding affinities of the chromophore.  Based on the following scheme, which is well-supported by earlier reported experiments (7),

Chrom + DNA $\rightleftharpoons$ (Chrom-DNA)$_{complex}$
      $\downarrow$
(Chrom)$_{degraded}$
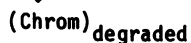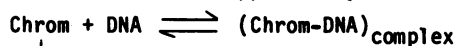
a relation is suggested between the dissociation constant and the rate of

TABLE III.  Determination of Binding Parameters for Chromophore-Polynucleotide
Interactions by Scatchard Plot.[a]

| Polynucleotide | Temperature | Dissociation Constant $K_d$ in $\mu$M) | Binding Stoichiometry $(r_b)$ |
|---|---|---|---|
| poly(dA-dT) | 14°C | 0.74 (0.83)[b] | 0.29  [0.25][c] |
| poly(dG-dC) | 5°C | 1.64 (2.04) | 0.115 [0.125] |
| polydA·polydT | 14°C | 4.93 (5.6) | 0.13  [0.15] |

[a]All measurements were done in 20 mM sodium acetate buffer, pH = 5.0,
containing 5% v/v methanol.
[b]Calculated from equation 1.
[c]Determined from the spectrophotometric titration of the chromophore in the
presence of a fixed concentration of the nucleic acid (as shown in Table I).

degradation of the chromophore in the presence of polynucleotides:  $K_d$ <
$[DNA]_T/[(R_D/R_0)-1]$, where $R_0$ and $R_D$ denote the time constants for the
degradation kinetics in the absence and presence of nucleic acid, respectively.
Table IV summarizes the time constants and the $K_d$-values determined therefrom
for different polynucleotide-chromophore interactions.  The calculated $K_d$
values lie in the same range as those determined from the direct evaluation of
$K_d$ at pH 5.0.  There is also a consistency in the relative binding affinities
of the polynucleotides for the chromophore at both pHs of 5.0 and 7.0.  Table
IV also shows an enhanced stability of the chromophore in the presence of the
dinucleoside monophosphate, $dA_pT$, in comparison with $dT_pA$, presumably
reflecting a base sequence preference for the binding of the chromophore.
Effect of Temperature on the Binding Affinity.
      Having established the validity of the methods to evaluate the influence
of base composition on the affinity of chromophore-polynucleotide interaction,
we attempted to measure the thermodynamic parameters, such as the Gibbs free
energy ($\Delta$G), and enthalpy ($\Delta$H) changes associated with the binding in order to
account for two observed characteristics: i) A-T specific nature of the binding
and ii) change in the binding stoichiometry values from 0.25 for poly(dA-dT) to
0.125 for poly(dG-dC) and poly(dI-dC). $\Delta$H could be quantitatively evaluated
from the variation of the association constants with temperature by means of
the Van't Hoff equation:
      $[d \ln (K_{as})/dT] = \Delta H/RT^2$ (equation 2).
For this purpose, the affinity constants at three different temperatures were
determined using equation 1.  Since there is no significant change of $r_b$ with
temperatures from 5°C to 14°C (Table I), it is justifiable to plot $\ln K_{as}$ as a
function of 1/T to get the $\Delta$H-value.  The labile nature of the chromophore (7),

TABLE IV.   Kinetics of Degradation of Chromophore in the Absence and Presence of Polynucleotides.[a]

| Polynucleotide | P/D | Time Constant (sec) | $R_D/R_0$ | $K_d$ (μM) |
|---|---|---|---|---|
| none | --- | 19 | | |
| | --- | (7.0) | | |
| | --- | 96[b] | | |
| poly(dA-dT) | 9.5 | 690 | 36.3 | 0.67 |
| | 9.5 | (183) | (26.1) | (0.94) |
| polydA·polydT | 9.5 | 105 | 5.5 | 5.25 |
| poly(dG-dC) | 9.5 | 125 | 6.5 | 4.3 |
| polydG·polydC | 9.5 | 97 | 5.1 | 5.8 |
| poly(dI-dC) | 9.5 | 257 | 13.5 | 1.9 |
| | 9.5 | (123) | (17.5) | (1.4) |
| dApT | 136 | 382[b] | 4.0 | 1.6[c] |
| dTpA | 136 | 138[b] | 1.4 | 4.3[c] |

[a]P/D denotes the ratio of the molar concentrations of nucleic acid and chromophore.  A small volume of the stock solution of the chromophore was added to the fluorescence cuvette containing the required concentration of the DNA in 10 mM phosphate, pH 7.1 containing 5% v/v methanol at 14°C.  The concentration of the chromophore was constant in all cases (2.5 μM).  There was usually a time lag of 5-10 sec between the addition of the chromophore and the first point of record of the fluorescence at 490 nm.  For t < 50 sec, there is an uncertainty of about 15% in the values, while for t > 60 sec the uncertainty is 10%.  Numbers in the parentheses denote the values determined at 20°C and are the averages of duplicate determinations.  $K_d$ was calculated from the relation: $K_d < [DNA]_T/[R_D/R_0)-1]$.
[b]The buffer was 0.2 M Tris-HCl, pH 8.0 containing 20% v/v methanol at 8°C.
[c]$K_d$ is expressed in mM.

however, prevents the measurement of $K_{as}$ at T>14°C.  One illustrative example for poly(dA-dT) is shown in Figure 3.  The ΔH-values thus calculated from the slope of the straight line, such as shown for poly(dA-dT) in the inset of Figure 3, are summarized in Table V.  The other thermodynamic parameter,ΔF, listed in the Table was calculated from the relation:

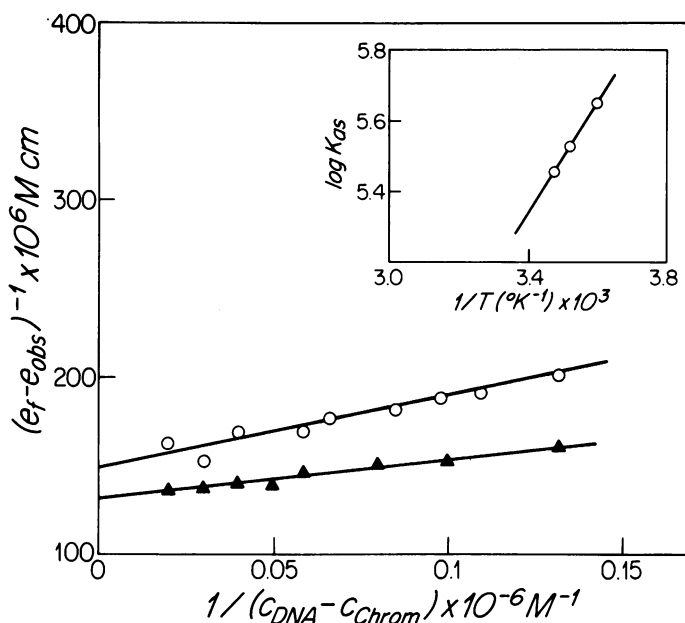$$\Delta G = -RT\ln K_{app} \quad \text{at } T = 14°C \qquad \text{(equation 3)}$$

Figure 3. Plot of $1/(e_f-e_{obs})$ against $1/(c_{DNA}-c_{Chrom})$ for the chromophore-poly(dA-dT) interaction at the temperatures 5°C (▲), and 14°C (0) (pH = 5.0, 20 mM sodium acetate buffer containing 5% v/v methanol). The inset shows the Van't Hoff's plot of the variation of log $K_{as}$ (= $r_b/K_d$) with 1/T (the recipro-cal of temperature) for the chromophore binding to poly(dA-dT) under the condi-tions mentioned above.

It is apparent from Table V that i) the binding to poly(dA-dT) is exo-thermic while that for poly(dI-dC) and poly(dG-dC) are endothermic and as a sequel to this, ii) the interaction with the latter is entropy driven. The magnitude of $\Delta H$ for the exothermic binding observed in the case of poly(dA-dT) is comparable to those reported for some other intercalators such as ethidium bromide (23). The opposite sign of the $\Delta H$-values for the binding of chromo-

TABLE V. Thermodynamic Parameters for Chromophore-Polynucleotide Associations.[a]

| Polynucleotide | $\Delta G$(Kcal/mol) | $\Delta H$(Kcal/mol) | $\Delta S^b$(eu) |
|---|---|---|---|
| poly(dA-dT) | -7.2 | -7.5 | - 1.0 |
| poly(dG-dC) | -5.9 | 9.8 | +54.7 |
| poly(dI-dC) | -6.6 | 6.4 | +45.3 |

[a] At 14°C in 20 mM sodium acetate, pH 5.0, containing 5% v/v methanol.
[b] Calculated from the relation $\Delta G = \Delta H - T\Delta S$.

phore to polynucleotides depending on their base composition was also indicated from the relative extent of stabilization of the chromophore by polynucleotides at different temperatures, e.g., it is clear from Table IV that the degree of stabilization of the chromophore ($R_D/R_0$ is a quantitative measure) by poly(dA-dT) decreases with the increase in temperature, whereas the reverse is found for the chromophore-poly(dI-dC) interactions.

The linear dependence of the absorbance of the chromophore at various wavelengths on its concentration argues against any aggregation of the free chromophore. In the absence of any such aggregation, it is justifiable to conclude that the observed difference in ΔH-values for the association of the chromophore with different polynucleotide reflects either the presence of additional noncovalent interactions in the case of binding to poly(dA-dT) compared to poly(dG-dC) and poly(dI-dC) or some gross conformational change leading to a high entropy change in poly(dG-dC) or poly(dI-dC), which upsets the positive enthalpy of binding. We, therefore, followed the spectral properties of the chromophore and the polynucleotides to obtain evidence for any possible distinctive features of binding or conformational change of the polynucleotides upon the binding of the chromophore.

CD Spectroscopy of Chromophore-Polynucleotide Complex.

Figure 4 shows the spectra of the chormophore alone and in the presence of poly(dA-dT) and poly(dG-dC). In the presence of the polynucleotides there is an increase in molar ellipticity in the positive band region of 300-340 nm. Induced Cotton effects similar to such an increase have been reported for drugs such as proflavin or ethidium bromide (24-26). The shape and strength of the CD band of the chromophore in the presence of polynucleotides depends on the nature of their base composition, e.g., i) there is a bathochromic shift of the crossover point by about 10 nm in the presence of poly(dA-dT), while such shifting is absent in the presence of poly(dG-dC), and ii) the peaks of the spectra are different, for poly(dG-dC) it is 305 nm whereas in case of poly(dA-dT) it is 312 nm. The spectra of the complexes shown in the figure correspond to the condition where the ligand is completely bound, as evident from the lack of further change upon addition of more polymer. The variation of the molar ellipticity (corresponding to the peak of the spectrum) as a function of concentration of added polynucleotide is indicative of a non-cooperative type of binding. The difference in the CD spectra of the chromophore in the presence of the two polynucleotides can be accounted for by two different types of binding, both inducing Cotton effects (24-26). Poly-(dI-dC) induces similar kinds of CD spectral changes as poly(dG-dC).
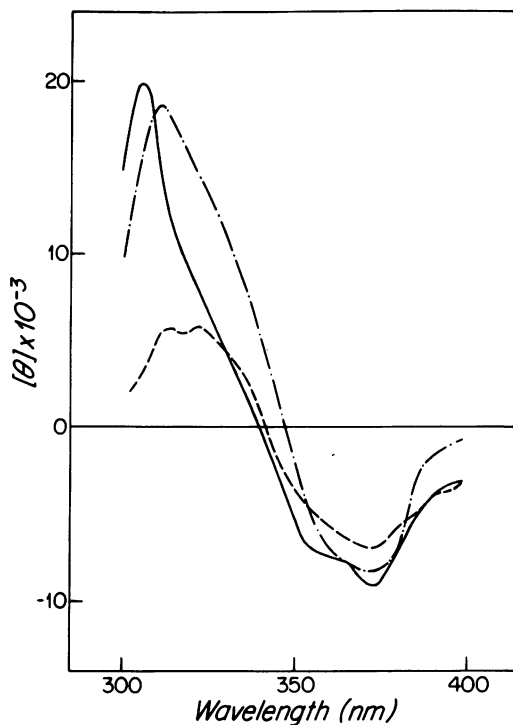
Figure 4. CD spectra of the chromophore (23 µM): a) alone (---); in the presence of b) poly(dA-dT) (-·-) (264 µM) and c) poly(dG-dC) (——) (350 µM). All spectra were recorded at 9°C in 20 mM sodium acetate buffer, pH 5.2, containing 10% v/v methanol. CD spectra of the polynucleotides in this region were subtracted to generate the above spectra.

To determine whether binding of chromophore leads to any gross alteration in the polynucleotide structure, CD difference spectra of the polynucleotides in the presence of the chromophore were computed (data not shown). No significant change in the band shape of the CD spectra of the polynucleotides due to binding could be detected, suggesting the absence of any such gross structural alteration, such as B → Z transition, for poly(dG-dC) or poly(dA-dT).

$^{31}$P-NMR Spectra of the Chromophore-Polynucleotide Complex.

The difference in the CD spectral profile of the chromophore in the presence of poly(dA-dT) and poly(dG-dC) prompted us to look at other spectral properties of the polynucleotide that are sensitive to the binding. Earlier reports have shown that associated with the binding of a drug to DNA, there is a change in the phosphodiester backbone; this change manifests itself in the
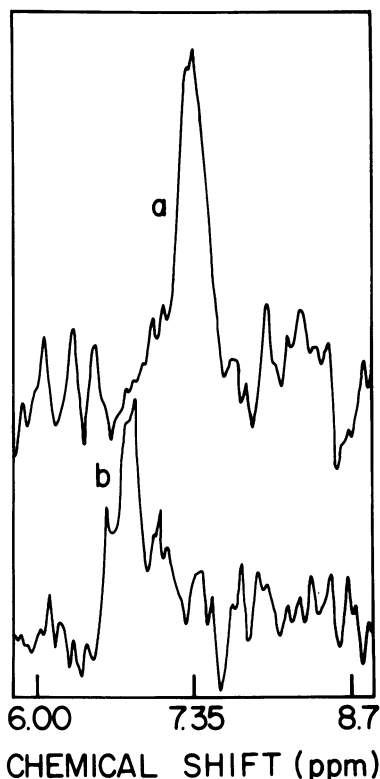
Figure 5. ^{31}P-NMR spectra (2,500 scans) of poly(dA-dT) in the absence (a) and presence (b) of the chromophore. Poly(dA-dT) concentration was 8.5 mM, and P/D was 18. Spectra were recorded at 15°C in 20 mM citrate buffer, pH 4.8 containing 10% v/v methanol. Pulse delay between scans was 1.0 sec.

CHEMICAL SHIFT (ppm)

[31]P chemical shift of the DNA (27,28). Figure 5 shows the ^{31}P-NMR spectra of the poly(dA-dT) in the absence and presence of the chromophore. There is a downfield shift of the peak in the latter case, comparable to that observed for the binding of an intercalator (27). In the presence of methanol it was difficult to get two well-resolved peaks corresponding to the dinucleotide repeat unit, ApT and TpA, as has been reported in the case of poly(dA-dT) alone (27). Also, it is clear from figure 5 that the presence of the chromophore has further broadened the peak, thereby making it difficult to comment on the site of binding, i.e., ApT or TpA. Table VI summarizes the changes in the ^{31}P chemical shift for both poly(dA-dT) and poly(dI-dC). Two features are apparent from the table: i) the shift for poly(dI-dC) is in the upfield direction as opposed to the downfield shift for poly(dA-dT); ii) there is a concentration dependence of the observed shifts, suggesting that they result from polymer-ligand binding and are not due to artifacts.

TABLE VI.   Changes in Chemical Shift of Phosphorus of Polynucleotides in the Presence of Chromophore.[a]

| Polynucleotide | P/D | Change in Chemical Shift (ppm) (upfield from trimethylphosphate) |
|---|---|---|
| poly(dA-dT) | 25 | 0.24 (downfield) |
|  | 18 | 0.62 (downfield) |
| poly(dI-dC) | 28 | 0.15 (upfield) |
|  | 18 | 0.45 (upfield) |

[a]All spectra were recorded at 15°C in 20 mM citrate buffer, pH 4.8, containing 10% v/v methanol.


DISCUSSION

The major conclusion from the above results is that the binding of neocarzinostatin chromophore to polynucleotide, both in nature and magnitude, depends on the base sequence.  All the results are consistent with previous reports indicating an A-T binding specificity of the chromophore (7, 10-14).  The observed sequence specificity in terms of DNA damage is most readily explained by differences in the modes of binding of the chromophore to the polynucleotides.

Hydrodynamic and electric dichroic measurements (8) have indicated an intercalative mode of binding for the chromophore to natural DNA.  Comparison of the binding constants ($K_{as}$), free energies ($\Delta G$) and enthalpies ($\Delta H$) for the polynucleotide-chromophore interactions (Tables IV and V) suggest an additional stabilization force (greater in magnitude than that corresponding to an H-bond) in the case of poly(dA-dT)-chromophore binding relative to chromophore-poly-(dI-dC) (or poly(dG-dC)) binding.  These experimental observations are consistant with an intercalative mode of binding in the case of poly(dA-dT), since it has been shown both experimentally and theoretically that intercalation imparts further stability to the drug-DNA complex (23).  The opposite nature of the ^{31}P-chemical shifts for poly(dA-dT) and poly(dI-dC) in the complexes with the chromophore also suggests different modes of binding. The observed downfield shift of the ^{31}P-NMR peak of poly(dA-dT) in the presence of chromophore is akin to a similar shift reported for other intercalator-polynucleotide (or natural DNA) interactions (27,28).  On the other hand, the upfield shift observed in the case of poly(dI-dC) is reminiscent of a similar shift in the nucleic acid observed in the presence of external binding agents, such as tetralysine (28) or netropsin (29).  The difference in the mode of

binding may also manifest itself in the change in $K_d$-value with the percentage of methanol. For poly(dG-dC) and poly(dI-dC), the magnitude of change is relatively larger than that for poly(dA-dT), as might be expected for a more externally binding or partially intercalated agent.

The observed increase in the CD band in the 300-350 nm region of the of the chromophore in the presence of polynucleotides can be ascribed to either externally bound or intercalated chromophore, because in both cases the naphthalene moiety can stack upon each other along the helix (externally bound) or with the bases (intercalated) to give rise to such effects (24-26). However, the observed difference in magnitude and nature of the Cotton effects is definitely indicative of the difference in the environments of stacking and, therefore, lends support to the proposition of different binding modes for poly(dA-dT) and poly(dG-dC). It may be suggested, therefore, that the origin of sequence specificity in the reversible binding of chromophore to poly(dA-dT) can probably be attributed to the additional stabilization arising out of A-T specific intercalative binding of the chromophore.

It is clear that the chromophore has a preferred affinity for the B-DNA type of structure as evident from the facts that: i) it binds poorly to polyA·polyU; ii) it does not induce B→Z (or any other major conformational) transition; in fact, it induces a Z→B transition in poly(dG-5-methyldC) (D. Dasgupta, manuscript in preparation); iii) the binding to polydA·dT, which probably has an aberrant B-DNA structure (20,30,31), is poorer than to poly(dA-dT). Since the alternating copolymers studied here appear to have B-DNA type structures by 2D-NMR (32), it is unlikely that the sequence-specific intercalation results from gross changes in the B-DNA type structure in the polymers. However, X-ray fiber diffraction and nuclease digestion studies of the alternating copolymers suggest that there are base sequence dependent minor variations from the classical B-DNA structure (21,33). Such structural micro-heterogeneity could be recognized by the chromophore and lead to the observed differences in the mode of binding. It should be pointed out, however, that the preferential stacking of the naphthalene ring of the chromophore with the A-T base pair compared to G-C or I-C base pairs might also play a role in the selective intercalation into poly(dA-dT).

Extrapolating the above discussion to natural DNA, one might suggest that base sequence-dependent minor variations in structure along the DNA helix (34,35) could account for the different drug binding properties. Such alterations in the microstructure of DNA appear to be responsible for the attack site specificities of nucleases and intercalating drugs (36-38). In this regard, it

is of particular interest that neocarzinostatin chromophore shares with micro-
coccal nuclease a strong preference for thymine and adenine in DNA, especially
in alternating sequence (39,40), and there is a 3'-phosphate at the strand
break.  The base sequence-dependent alteration in the binding affinity, as re-
corded by the polynucleotide-chromophore binding studies, is clearly reflected
in the observation that not all the T-residues in DNA restriction fragments are
attacked with equal efficiency by the chromophore (12,13).  In this regard, the
studies with dinucleoside monophosphate reported here are in agreement with DNA
strand breakage analysis showing that adenine is more frequently found on the
5' side of the thymine attacked by neocarzinostatin chromophore (13), although
no absolute base sequence specificity could be detected.

   Finally, it might be pointed out that the differences in binding constants
(Tables II and IV) and enthalpy of binding (Table V) of chromophore to
poly(dG-dC) and poly(dI-dC) imply that the 2-$NH_2$ group of guanine in the minor
groove of DNA interferes with chromophore binding and may account, in part, for
the difference in binding between G·C and A·T containing polymers.

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