FLUOROSPECTROMETRIC STUDIES ON NEOTHRAMYCIN AND ITS REACTION WITH DNA

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The characteristics of neothramycin and its reaction mechanism with DNA were studied by fluorescence spectroscopy.

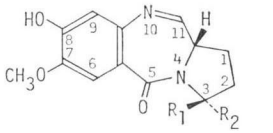
The fluorescence intensity of neothramycin is enhanced by the reaction with DNA. The reaction rate of the drug and DNA is rather slow; and is dependent upon both DNA and drug concentrations, and is stimulated by hydrogen ion. Analysis of the equilibrium reaction suggested that neothramycin possesses about one binding site per 2~3 base-pairs with homogeneous affinity and association constant of 4.7×10^3 M^{-1}; and no significant mutual interference of the binding sites seems to exist.

From the results of neothramycin's interaction with various polynucleotides, it was suggested that a guanine base and a double helix conformation of DNA are required for binding the antibiotic.

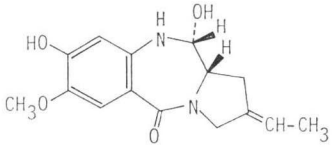
The current results and previous ones, concerning neothramycin-2'-deoxyguanosine adduct formation, suggested that the antibiotic reacts with 2-amino group of guanine base of DNA by dehydration in the manner of a bimolecular (Sn_2ca) equilibrium reaction.

Neothramycin[1] is a new antibiotic of the pyrrolo(1,4)benzodiazepine group, and shows potent antitumor effects on experimental neoplasms, particularly sarcoma 180 and Walker tumor 256. The tumor-inhibitory activity of neothramycin seems to be similar to those of other pyrrolo(1,4)benzodiazepine antibiotics, but the toxicity is lower than the others[2].

We have studied the mechanism of action of neothramycin, and observed that the antibiotic binds to double stranded DNA and shows a preferential block of RNA synthesis over DNA synthesis *in vivo* and *in vitro*[3,4]. Studies on the structure-activity relationship of neothramycin derivatives have suggested that neothramycin may covalently bind to DNA through C-11[4]. We have also investigated the interaction of the antibiotic with deoxynucleotides as a model of the drug-DNA binding, and found that



Neothramycin A R_1 = H, R_2 = OH
Neothramycin B R_1 = OH, R_2 = H



Tomaymycin

the antibiotic selectively reacts with 2'-deoxyguanosine in aqueous dimethyl sulfoxide. The major reaction product has been isolated, and the structure has been determined. It is a one-to-one adduct of neothramycin and 2'-deoxyguanosine, and a covalent aminal bond is formed between C-3 of the antibiotic and N-2 of 2'-deoxyguanosine by dehydration[5].

Since the fluorescence intensity of neothramycin or tomaymycin is greatly enhanced by double stranded DNA, we have examined the binding characteristics of neothramycin to DNA by fluorescence spectroscopy, and the results are presented in this publication. From the current results and previous studies on the reaction with 2′-deoxyguanosine, the reaction mechanism of neothramycin with DNA is proposed.

Materials and Methods

Chemicals

Neothramycin [A and B mixture (5.5: 4.5)], neothramycins A and B were prepared following the procedures described previously[1]. Tomaymycin was generously supplied by Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan. Calf thymus DNA, *E. coli* tRNA, poly[dG-dC], poly[dI-dC], poly[dA-dT] and poly[dG] were products of P-L Biochemicals, Milwaukee, Wis., U.S.A. The other reagents were of the highest grade available commercially.

DNA Solution

Calf thymus DNA was dissolved at a concentration of *ca.* 1 mg/ml in an aqueous medium, containing 50 mM NaCl and 10 mM disodium EDTA; and dialyzed overnight at 7°C against 50-fold volume of the same medium with subsequent dialysis against 50 mM NaCl solution. It was stored at 2°C. DNA concentrations were assayed as inorganic phosphate by the method of AMES[6]. Heat-denatured DNA was prepared by heating at 100°C for 5 minutes in a water bath, and then immediately chilled in an ice-bath.

Fluorescence Spectroscopy

Corrected and uncorrected fluorescence spectra were recorded on a Hitachi spectrophotofluorometer model MPF-4, equipped with a data processor and a Hitachi 057 X-Y recorder. All measurements were carried out in a cuvette with 1-cm light path at an angle of 90° to excitation beam. The temperature of the sample compartment was controlled to ± 0.5°C by a Sharp TE-12 circulating bath.

The fluorescence quantum yield was calculated by comparing the absorbance at the excitation wavelength (316 nm) and the emission spectrum of an unknown sample with those of a standard of known quantum yield. Quinine, dissolved in 1 N H_2SO_4 (quantum yield 0.55) was used as a standard[7].

In kinetic experiments, the reaction was initiated by the addition of a small volume of antibiotic solution to a cuvette, containing DNA solution. The cuvette was kept in the temperature-controlled sample-compartment for at least 10 minutes prior to the drug addition.

Fluorescence Intensity of Neothramycin-DNA Complex

Since a fraction of neothramycin always remained unbound to DNA, using fluorometrically detectable concentrations of the antibiotic and soluble concentrations of DNA, the fluorescence intensity of neothramycin-DNA complex was calculated by the equation (7), described below.

Provided that all the binding sites on DNA are equivalent and noninterfering: *i.e.* the binding of one molecule of neothramycin to DNA does not affect the binding of a second molecule, the reaction of the antibiotic and DNA is expressed by

$$\mathrm{NTM} + \mathrm{DNA} \underset{}{\overset{\mathrm{Ka}}{\rightleftharpoons}} \mathrm{NTM \cdot DNA} \tag{1}$$

where NTM is neothramycin and Ka the association constant. Then

$$\mathrm{Ka} = \frac{[\mathrm{Ab}]}{[\mathrm{Af}]\,(\mathrm{n[P]} - [\mathrm{Ab}])} \tag{2}$$

where [Af] is a concentration of free neothramycin, [Ab] that of bound neothramycin or DNA, and (n[P]−[Ab]) that of free DNA, n the number of binding sites per DNA nucleotide.

With excess DNA, equation (2) is expressed by

$$\mathrm{Ka} = \frac{[\mathrm{Ab}]}{[\mathrm{Af}] \cdot \mathrm{n[P]}} \tag{3}$$

Assuming that the fluorescence increase parallels the amount of bound neothramycin, equations (4) and (5) are obtained.

$$[Ab]=\frac{\Delta F}{\Delta F_{max}}[At] \tag{4}$$

$$[Af]=[At]-[Ab]=\frac{\Delta F_{max}-\Delta F}{\Delta F_{max}}[At] \tag{5}$$

where [At] is a total concentration of free and bound neothramycin, ΔF the increase of neothramycin fluorescence by the presence of DNA, and ΔF_{max} the fluorescence intensity of neothramycin-DNA complex, from which that of free neothramycin was subtracted.

Substituting (4) and (5) into (3), equation (6) is obtained.

$$Ka=\frac{\Delta F}{(\Delta F_{max}-\Delta F)\cdot n[P]} \tag{6}$$

$$\therefore \quad \frac{1}{\Delta F}=\frac{1}{\Delta F_{max}\cdot Ka}\cdot\frac{1}{n[P]}+\frac{1}{\Delta F_{max}} \tag{7}$$

By plotting $1/\Delta F$ on the vertical axis and 1/[P] on the horizontal axis, $1/\Delta F_{max}$ value is obtained as an intercept on the vertical axis (*cf.* Fig. 4). [Ab] and [Af] can be calculated by equations (4) and (5), using ΔF and ΔF_{max} values.

Moreover, the equation (2) can be rearranged to give equation (8).

$$\frac{1}{[Af]}=Ka\left(n\frac{[P]}{[Ab]}-1\right) \tag{8}$$

The plots of 1/[Af] against [P]/[Ab] should be linear within this model; and the intercepts on the 1/[Af] and [P]/[Ab] axes represent Ka and 1/n, respectively.

Results

Fluorescence Characteristics of Neothramycin

The corrected and uncorrected fluorescence emission spectra of neothramycin A in 50 mM TES-KOH buffer, pH 7.2, are illustrated in Fig. 1. The emission maximum was observed at 420 nm (excitation at 316 nm). The spectral pattern of neothramycin B was the same as that of neothramycin A.

The relative fluorescence intensity of neothramycin at 420 nm under different pH values are shown in Fig. 2. Stronger fluorescence of the antibiotic was demonstrated at acidic pH values than at neutral pH, and the intensity was markedly decreased in alkaline solution; no significant shift of the emission λ_{max} was observed at various pH values.

Fig. 1. UV absorption and fluorescence emission (corrected and uncorrected) spectra of neothramycin A (5 μM) in 50 mM TES-KOH buffer, pH 7.2.

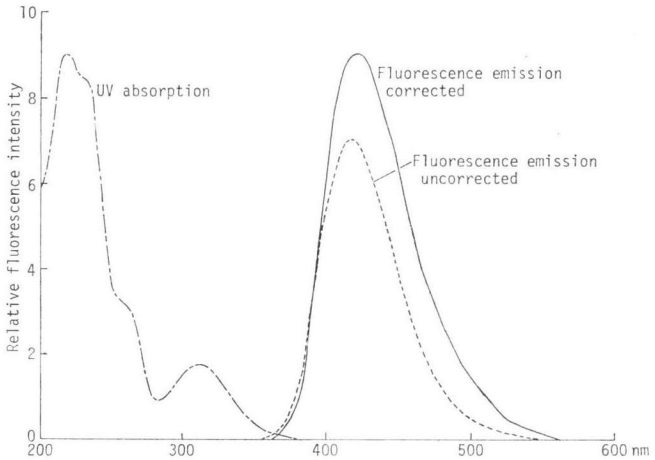


Fig. 2. The effect of pH on fluorescence intensity of neothramycin (5 μM) in 50 mM Tris-HCl buffer.

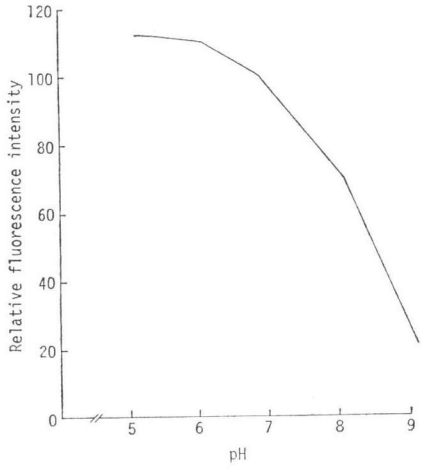


Table 1. Fluorescence quantum yields of neothramycins and tomaymycin.

Antibiotic	Quantum yield (ϕ)	Emission λ_{max} (nm)
Neothramycin [A & B (5.5 : 4.5)]	0.046	420
Neothramycin A	0.031	420
Neothramycin B	0.061	420
Tomaymycin	0.045	416

Table 2. Relative fluorescence intensity and λ_{max} of neothramycin A in various solvents.

Solvent	Emission λ_{max} (nm)	Relative fluorescence intensity
Dichloromethane	388	2.03
Ethyl acetate	390	1.53
Dioxane	390	3.88
Ethanol	411	5.91
Methanol	410	5.45
Water	420	1.00

Neothramycin 5 μM was used.

The fluorescence quantum yields were calculated at pH 7.0, using quinine as a standard: neothramycin (A: B=5.5: 4.5) 0.046, A 0.031, B 0.061, and tomaymycin 0.045 (Table 1). The relative fluorescence intensity and emission λ_{max} of neothramycin A in various solvents are presented in Table 2. The antibiotic exhibited stronger fluorescence in ethanol, methanol and dioxane than in dichloromethane, ethyl acetate, and water. The emission λ_{max} was 420 nm in water, *ca.* 410 nm in methanol and ethanol, and *ca.* 390 nm in the other solvents.

The relative fluorescence intensity of neothramycin paralleled to drug concentrations up to 100 μM in a neutral buffer. The fluorescence strength was decreased as the temperature was raised. The intensity of fluorescence at 30°C was approximately 84% of that at 20°C: the shift of the emission λ_{max} was not observed. The fluorescence intensity and emission λ_{max} were not changed at various ion (NaCl) strengths.

Quantitative Fluorometric Studies on the Drug-DNA Reaction

The fluorescence intensity of neothramycin was enhanced, and a little blue shift of the emission λ_{max} was observed by the presence of native calf thymus DNA; the degrees of fluorescence increase were dependent upon DNA concentrations (Fig. 3).

Using the equation (7) described in Materials and Methods, the relative fluorescence increase of neothramycin by the presence of DNA in a range of DNA concentrations (100~1,000 μM·P) were obtained (Fig. 4). The enhancement of intensity (ΔF) depended upon DNA concentrations, showing a double reciprocal linear relationship. The relative fluorescence intensity of neothramycin-DNA complex was calculated to be 4.3 fold to that of free neothramycin at the same concentration: *i.e.* ΔF_{max} was 3.3.

In the case of tomaymycin, the antibiotic completely bound to excess DNA, and the relative fluorescence strength of the drug-DNA complex was found to be 3.0 fold to that of free tomaymycin at the same concentration.

Fig. 3. Enhancement of neothramycin fluorescence by the presence of DNA.

The composition of solution was 10 μM neothramycin, 30 mM phosphate buffer, pH 7.0, 1.5 mM EDTA, 0.1 M NaCl, and calf thymus DNA as indicated in the graph. It was incubated at 25°C for 16 hours.

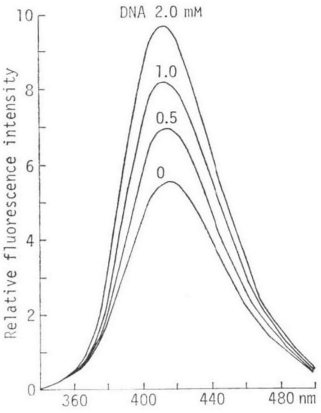


Fig. 4. The relationship between enhancement of neothramycin fluorescence and DNA concentrations.

The reaction mixture consisted of 6.64 μM neothramycin, 30 mM phosphate buffer, pH 7.0, 1.5 mM EDTA, 0.1 M NaCl, and calf thymus DNA. It was incubated at 25°C for 48 hours. The reaction reached an equilibrium state within 48 hours.

ΔF: The increase of neothramycin fluorescence.

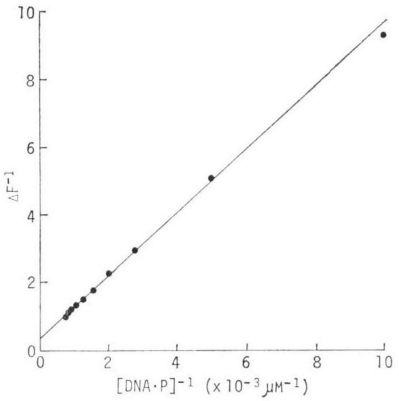


Fig. 5. Double reciprocal plots for neothramycin binding to DNA.

The solution was composed of 30 mM phosphate buffer, pH 7.0, 1.5 mM EDTA, and 0.1 M NaCl; incubated at 25°C for 48 hours.

[NTMf] represents the molar concentration of free neothramycin, [NTMb] that of bound neothramycin, and [DNA·P] that of DNA·P.

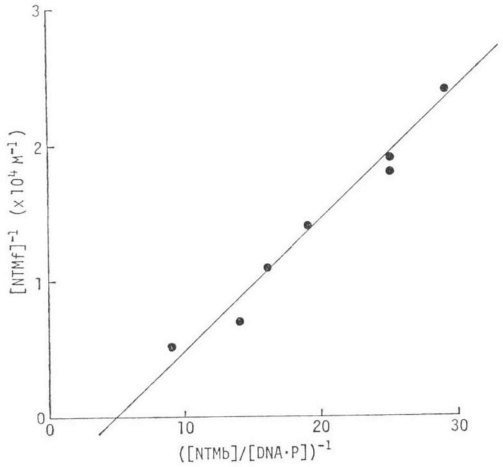
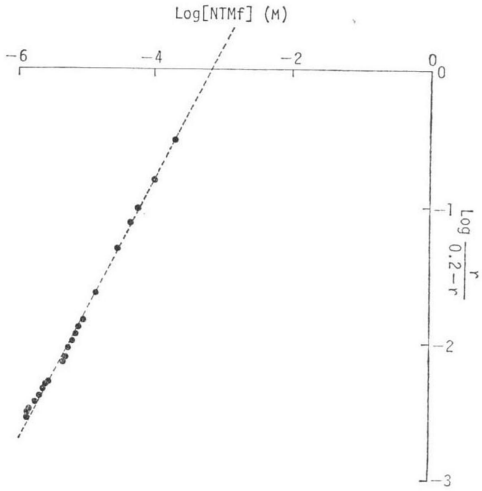


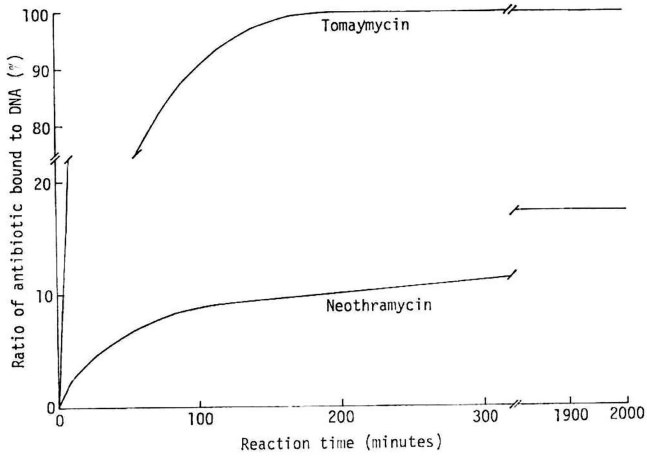
Fig. 6. Hill plots for the binding of neothramycin to DNA.

The experimental conditions were the same as shown in Fig. 5. r=[NTMb]/[DNA·P]



The amounts of neothramycin bound to DNA were calculated from the fluorescence intensity increase (ΔF) over a range of concentrations of neothramycin (1 ~ 100 μM) and DNA·P (100 ~ 1,700 μM) (Figs. 5 and 6). The double reciprocal binding plot of the data is illustrated in Fig. 5. There appeared to exist a linear relationship between $[\text{neothramycin}]_{\text{bound}}/[\text{DNA} \cdot \text{P}]$ and $[\text{neothramycin}]_{\text{free}}$. Native calf thymus DNA seemed to possess one binding site for the antibiotic per *ca.* 2 ~ 3 base-pairs with

Fig. 7. Reaction rates of neothramycin and tomaymycin with DNA.
The solution consisted of 1 mM calf thymus DNA, 1.5 mM EDTA, 0.1 M NaCl, 30 mM phosphate buffer, pH 7.0, and 1.0 μM neothramycin or 1.0 μM tomaymycin; incubated at 25°C.



homogeneous affinity: an association constant was *ca.* 4.7×10^8 M^{-1}. The Hill plot of the binding data (Fig. 6) indicated that no mutual interference of the binding sites appears to exist and neothramycin may not be intercalated between adjacent base-pairs of the double helix.

Reaction Characteristics of Neothramycin and DNA

As presented in Fig. 7, the reaction rate of neothramycin with DNA was slower than that of tomaymycin, suggesting that neothramycin shows the slowest reaction rate with DNA in the pyrrolo-(1,4)benzodiazepine group of antibiotics. The reaction rate of neothramycin with DNA was dependent upon both drug and DNA concentrations (Fig. 8).

The binding rate of neothramycin to DNA was highly dependent upon pH of the buffer used, and markedly promoted by hydrogen ion (H^+) in a range of pH 5~7 (Fig. 9). There appeared to exist a linear relationship between the initial reaction rate constants and pH of the buffer (Fig. 10). However, the reaction rate was not significantly changed in a concentration range of 10~100 mM NaCl in 5 mM TES-KOH buffer, pH 7.0 containing 1.5 mM EDTA.

The interactions of neothramycin with nucleic acid and synthetic deoxynucleotides were studied by fluorospectrometry, and the results are summarized in Table 3. The antibiotic bound to native DNA, but not significantly to heat-denatured DNA or tRNA (*E. coli*), suggesting that the double helix structure as in DNA is essential for the binding. The interaction with poly[dG-dC] was more intense than those with poly[dI-dC] and poly[dA-dT], suggesting that the deoxyguanosine moiety of DNA participates in the drug-DNA binding. However, poly[dG] showed less affinity for the antibiotic than poly[dG-dC], and no significant binding was observed with dATP, dCTP, dGTP or TTP. The results again suggested the importance of the structural conformation of nucleotide.

Discussion

The current and previous experiments[4,5] have revealed that neothramycin binds to double stranded DNA but not significantly to single stranded DNA. The same tendency has been shown by intercalating

Fig. 8. Dependency of neothramycin-DNA binding on drug and DNA concentrations.

The composition of solution was 30 mM phosphate buffer, pH 7.0, 1.5 mM EDTA and 0.1 M NaCl. An adequate concentration of calf thymus DNA and 1.0 μM neothramycin were used in the experiment (A); and an adequate concentration of neothramycin and 1.0 mM calf thymus DNA were employed in the experiment (B). The reactions were performed at 25°C.

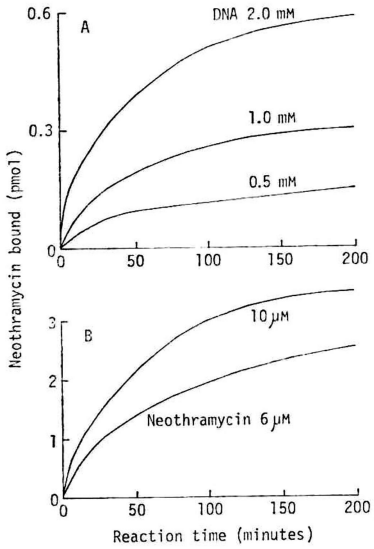


Fig. 9. pH-Dependency of reaction rate of neothramycin with DNA.

The reaction solutions were composed of 1.5 mM EDTA, 0.1 M NaCl, 10 μM neothramycin, 1.0 mM calf thymus DNA, and 30 mM citrate-NaOH buffer; incubated at 25°C.

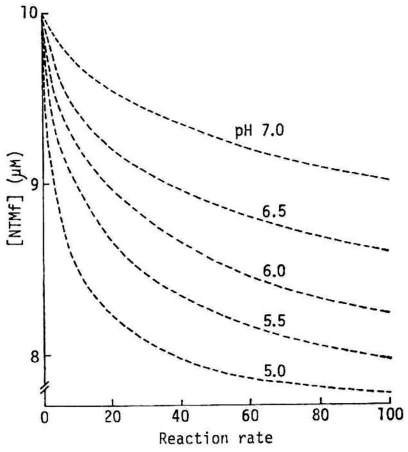


Fig. 10. Initial reaction rate constants of neothramycin with DNA at various pH's.

The initial rate values were obtained from the results of Fig. 9.

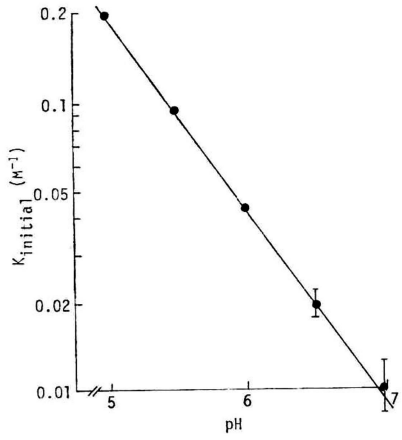


Table 3. Fluorescence intensity of neothramycin in the presence of various nucleotides.

Nucleotide	(F−Ff)/Ff (%)
Calf thymus DNA native	28.0
heat-denatured	0.0
E. coli tRNA	0.7
poly[dG-dC]	9.6
poly[dI-dC]	0.0
poly[dA-dT]	1.5
poly[dG]	2.6
dATP	0.0
dGTP	0.0
TTP	0.0

The reaction mixture contained: 10 μM neothramycin, 1.5 mM EDTA, 0.1 M NaCl, 30 mM citrate-NaOH buffer, pH 6.0, and 0.8 mM nucleotide. It was incubated at 25°C for 2 hours. The increase of neothramycin fluorescence by various nucleotides (F−Ff) was compared with neothramycin fluorescence without nucleotides (Ff).

agents, such as actinomycin D[10], acridine[11] and daunorubicin[12]. However, the interaction of neothramycin with DNA differs from intercalation in the following aspects: (1) The antibiotic does not significantly alter the melting profile (Tm) of DNA[4]. (2) Heating the drug-DNA complex does not easily release neothramycin[4]. (3) The reaction rate with DNA is extremely slow. Therefore, the

intercalation model for the neothramycin binding to DNA remains open to discussion. GLAUBIGER *et al.*[9] have opposed the idea of intercalation with anthramycin. The covalent bond formation between the antibiotic and DNA has been suggested[4,5].

More intensive interaction of neothramycin with poly[dG-dC] than poly[dI-dC] and poly[dA-dT] suggests that 2-amino group of guanine base of DNA participates in the binding to DNA. The same base specificity has been found in the reaction with 2′-deoxynucleosides[5].

Most substances (proflavine, quinacrine *etc.*) show decrease of fluorescence intensity by binding to DNA, but some (ethidium bromide, acridine orange, *etc.*) show an increase[13~16]. Since the fluorescence is derived from the benzene ring of neothramycin, the benzene ring moiety may bind to DNA presumably by hydrogen bonding. However, the mechanism of fluorescence increase of neothramycin by DNA remains to be determined.

The fluorescence intensity and emission λ_{max} of the neothramycin-DNA complex are similar to those of the drug in ethanol and methanol, but differ from those in dichloromethane or ethyl acetate, suggesting that the antibiotic exists in the neighborhood of the phosphate-sugar backbone of DNA.

The reaction rate of pyrrolo(1,4)benzodiazepine antibiotics with DNA is slower than those of mitomycin C, actinomycin D and adriamycin[8]. Of the pyrrolo(1,4)benzodiazepine group, neothramycin exhibits the slowest reaction rate with DNA. Anthramycin, sibiromycin and tomaymycin possess unsaturated side chains at C-2, which may participate in the binding to DNA[8]. The absence of a C-2 side chain in the neothramycin molecule may be related to the slow reaction rate with DNA. The lower toxicity of neothramycin than the other pyrrolo(1,4)benzodiazepines might be due to the slower binding rate to DNA.

The reaction of neothramycin to DNA is catalyzed by hydrogen ion. Since the acid-base balance tends to be more acidic direction in cancer cells than in normal cells[17,18], neothramycin may bind to DNA more intensively in the tumor cells than in normal cells.

Neothramycin possesses carbinolamines at 3,4- and 10,11-positions: both may be chemically reactive in a similar manner. We have observed that the antibiotic may bind to DNA mainly through C-11 and to 2′-deoxyguanosine through C-3[4,5]. Both C-11 and C-3 may possess a potential reactivity with DNA. However, the steric hindrance of, and/or hydrogen bond formation with the double helix of DNA may lead to the complex formation through the C-11 more easily than through the C-3.

The reaction of neothramycin with DNA is similar to that with 2′-deoxyguanosine in the following characteristics: (1) slow reaction rate, (2) specificity for guanine base, (3) catalysis by hydrogen ion, (4) dependency on both drug and nucleotide concentrations, and (5) reversible reaction. Since the mechanism, therefore, seems to be similar in both reactions, the formation of neothramycin-DNA complex may involve the nucleophilic attack originating from 2-amino group of DNA guanine at C-11 of the antibiotic and dehydration and may be a bimolecular substitution reaction, catalyzed by hydrogen ion. Since most 2-amino groups of guanine are exposed to the narrow groove of DNA, neothramycin may be bound within the minor groove of the double helix. This is in accord with the reports by GLAUBIGER *et al.*[9] and by HURLEY *et al.*[19,20].

HURLEY[8] has assumed that the interaction of pyrrolo(1,4)benzodiazepine antibiotics with DNA occurs in at least two steps: the first being a non-covalent type of interaction and the second covalent attachment:

$$A + DNA \overset{K}{\rightleftharpoons} A \cdot DNA \overset{k}{\longrightarrow} A\text{-}DNA$$

where A·DNA is the reversible complex, A-DNA is the covalent complex, K an equilibrium constant and k the first-order rate constant. The current results suggest that the covalent binding of neothramycin and DNA is a bimolecular reaction. The complex with a covalent bond may be stabilized by hydrogen bonds between the drug and DNA.

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