Presta, L. G., & Rose, G. D. (1988) *Science 240*, 1632.
Schevitz, R., Otwinowski, Z., Joachimiak, A., Lawson, C. L., & Sigler, P. B. (1985) *Nature 317*, 782.
Shoemaker, K. R., Fairman, R., Kim, P. S., York, E., Stewart, J., & Baldwin, R. L. (1987) *Cold Spring Harbor Symp. Quant. Biol. 52*, 391.
Wade-Jardetzky, N., Bray, R. P., Conover, W. W., Jardetzky, O., Geisler, N., & Weber, K. (1979) *J. Mol. Biol. 128*, 259.
Weiss, M. A., Sauer, R. T., Patel, D. J., & Karplus, M. (1984) *Biochemistry 23*, 5090.
Wuthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, pp 17, 135, Wiley, New York.
Zhang, R.-G., Joachimiak, A., Lawson, C. L., Schevitz, R. W., Otwinowski, Z., & Sigler, P. B. (1987) *Nature 327*, 591.

Mechanism of Inhibition of DNA Gyrase by Quinolone Antibacterials: Specificity and Cooperativity of Drug Binding to DNA

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ABSTRACT: Although the functional target of quinolone antibacterials such as nalidixic acid and norfloxacin has been identified as the enzyme DNA gyrase, the direct binding site of the drug is the DNA molecule [Shen, L. L., & Pernet, A. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 307–311]. As described in this paper, binding specificity and cooperativity of quinolones to DNA were further investigated with the use of a variety of DNA species of different structures and different base compositions. Results show that the drug binding specificity is controlled and determined largely by the DNA structure. The drug binds weakly and demonstrates no base preference when DNA strands are paired. The drug binds with much greater affinity when the strands are separated, and consequently, binding preference emerges: it binds better to poly(G) and poly(dG) over their counterparts including poly(dI). The results suggest that the drug binds to unpaired bases via hydrogen bonding and not via ring stacking with DNA bases. The weak binding to relaxed double-stranded DNA and the stronger binding to single-stranded DNA are both nonspecific as they do not demonstrate binding saturation and cooperativity. The specific type of binding, initially demonstrated in our previous publication with the supercoiled DNA and more recently with complex formed between linear DNA and DNA gyrase [Shen, L. L., Kohlbrenner, W. E., Weigl, D., & Baranowski, J. (1989) *J. Biol. Chem.* (in press)], occurs near the drug's supercoiling inhibition concentration. As shown in this paper, binding saturation curves of this type are highly cooperative (with Hill constant greater than 4). This form of binding represents a specific mode of drug binding which determines the drug's biological potency.

Quinolones, a series of nalidixic acid analogues, have become a major class of synthetic antibacterial agents which are under extensive clinical development (Hooper & Wolfson, 1985; Hooper, 1986; Neu, 1987; Fernandes, 1988). These drugs are considered to have a bright future owing to their extremely potent antibacterial activity, rapid bactericidal effects, and low incidence of resistance development (Wolfson & Hooper, 1985). Nalidixic acid, the first member of the quinolone family, was synthesized 25 years ago (Lesher et al., 1962). Little progress was made in the development of this class of drug until the discovery of DNA gyrase (Gellert et al., 1976). Subsequent extensive studies on the mechanism of the gyrase-catalyzed DNA supercoiling process and genetic analysis of quinolone-resistant bacterial mutants lead to the conclusion that the functional target of quinolone drugs is the A subunit of the enzyme [for reviews, see Cozzarelli (1980), Gellert (1981), and Wang (1985)]. In a recent preliminary publication, however, we demonstrated that the direct binding site of the drug is the DNA molecule (Shen & Pernet, 1985). The drug was shown to bind preferentially to single-stranded rather than double-stranded DNA. We also observed a saturable drug binding phase with supercoiled DNA which was probably due to binding to a localized underwound region of the DNA molecule. Using an indirect competition method, we further demonstrated that the binding affinity of a number of quinolones correlates well with the drug's potency to inhibit DNA supercoiling catalyzed by DNA gyrase. This indicates that this type of saturable binding is specific and is an important determinant of the inhibitory potency. At this preliminary stage of investigation, no answer was obtained concerning the exact role of drug binding during gyrase inhibition, as the saturable form of binding was observed only with supercoiled DNA (the product) but not with the relaxed DNA (the substrate) or the substrate–enzyme complex.[1] In this paper, we present some additional binding data obtained with various DNA species and the result of cooperativity tests of the drug binding curve in an attempt to gain better insight into the binding specificity and modes of bindings. The information is crucial for proposing the cooperative drug–DNA binding model of DNA gyrase inhibition presented in the following paper (Shen et al., 1989b).

Experimental Procedures

Materials. Deoxynucleoside monophosphates and chromosomal DNA preparations from *Escherichia coli*, *Micrococcus lysodeikticus*, and *Clostridium perfringens* were pur-

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[1] The binding experiment was carried out in the absence of ATP or its analogue.

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chased from Sigma Chemical Co. (St. Louis, MO). Polydeoxynucleotides, polynucleotides, oligodeoxynucleotides, and *E. coli* tRNA were products of Pharmacia Inc. (Piscataway, NJ). ColE1 DNA was isolated from *E. coli* strain JC411. The isolated DNA contained approximately 95% supercoiled and 5% nicked forms. [^{3}H]Norfloxacin was the same material used previously (Shen & Pernet, 1985) but was repurified by a HPLC procedure (Shen et al., 1989a).

Binding Experiments. Procedures for equilibrium dialysis and membrane ultrafiltration binding methods were described previously (Shen & Pernet, 1985). Due to the highly irregular and thicker Amicon membrane discs manufactured in later batches, calculation of the data in all the experiments, except as otherwise mentioned, was modified. In the previous publication, we determined the amount of bound ligand directly from the radioactivity bound to DNA which was retained on the membrane. In this paper, the amount of ligand bound to the receptor was calculated by subtracting the free ligand concentration in the reaction mixture from the initial ligand concentration, both ligand concentrations being determined from the radioactivity counts of the corresponding filtrates with and without the receptors. The binding of nonradiolabeled oxolinic acid, pefloxacin, and nalidixic acid to ColE1 DNA was measured with a fluorescence method. In these experiments, the same membrane ultrafiltration procedures were used except that ligand concentrations were determined by measuring the drug's intrinsic fluorescence intensity instead of radioactivity. A Perkin-Elmer Model LS-5 fluorescence spectrophotometer was used for these experiments. The binding of [^{3}H]norfloxacin to oligo(dA)[2] species was determined with Amicon YM-2 Centrifree micropartition devices which have membranes with a molecular weight cutoff of 1000.

Data Analysis. All the binding saturation data were presented in the form of Klotz plots (Klotz, 1974). A semiempirical approach was employed to analyze the binding cooperativity (Cantor & Schimmel, 1980a). A nonlinear least-squares computer program, RS1 (Bolt, Beranek and Newman Software Products Corp., Cambridge, MA), was used to fit the binding data with the following basic binding equation:

$$R = R_m/[1 + (K_d/D)^n] \quad (1)$$

where R, R_m, K_d, and D represent molar binding ratio, maximum molar binding ratio, apparent dissociation constant, and free ligand concentration, respectively, while n is the Hill constant. When $n = 1$, the binding is purely noncooperative, and for large n, the binding is said to be increasingly cooperative. The R_m value, except as otherwise mentioned, was chosen manually as the R value at the apparent saturation plateau position, while other parameters were fitted by the computer program.

Results

Drug Binding of DNA: Equilibrium Dialysis versus Membrane Filtration Method. The time courses of [^{3}H]-norfloxacin binding to DNA gyrase and to supercoiled ColE1 DNA followed by equilibrium dialysis are shown in Figure 1A. The slow increase in the amount of drug bound as a function of time corresponds to the approach to equilibrium which required approximately 2 h. The level of drug binding to DNA gyrase at equilibrium was near the base-line value, while that to supercoiled DNA was much more appreciable. Addition of gyrase to supercoiled DNA did not change the binding quantitatively until a later stage (after 3 h) when the DNA relaxation process might have taken place in the absence of ATP, resulting in approximately 20% reduction in maximum binding. Addition of cold norfloxacin (to a final concentration of 130 μM) abolished the binding to the enzyme–DNA complex but could not entirely replace the drug bound to supercoiled DNA. This indicates that the binding of the radioligand to supercoiled DNA was tighter or more extensive than that to gyrase-relaxed DNA complex in the absence of ATP. The molar binding ratio obtained at equilibrium was between 4 and 6 mol of drug/mol of ColE1 DNA, comparable to our previously published values. The results of the membrane filtration assay for measuring the time course of drug binding to supercoiled DNA are shown in Figure 1B. The binding reached its maximum before the first measured point. Again the equilibrium binding plateau is equivalent to a molar binding ratio of about four drugs per DNA. Due to the simplicity of the technique, all subsequent experiments presented in this paper were performed with the membrane filtration method.

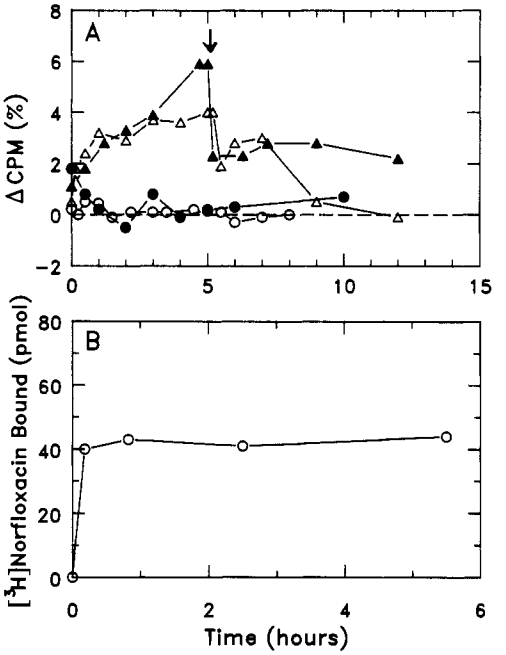

FIGURE 1: Time course of [^{3}H]norfloxacin binding followed by equilibrium dialysis (A) and membrane ultrafiltration (B). (Panel A) The amount of drug bound to supercoiled ColE1 DNA (▲), DNA gyrase holoenzyme (●), and DNA–enzyme mixture (△) is expressed by Δcpm (%) which is the difference in radioactivity count between the two compartments (1-mL capacity each) expressed as the percentage of total radioactivity used; Δcpm (%) is thus the percentage of the amount of drug bound to DNA. The position at Δcpm = 0% corresponds to a [^{3}H]norfloxacin concentration of 1.93 μM. Also shown is the control experiment with buffer only (○). Amounts of DNA and gyrase used are 37 and 64 pmol, respectively. Therefore, 1% of Δcpm is equivalent to a molar binding ratio of one drug per DNA. For the two binding experiments with DNA (△, ▲), proper amounts of nonlabeled norfloxacin (4 mM) were added to both compartments (at the time indicated by the arrow) to make the total concentration of norfloxacin in both compartments equal to 130 μM. (Panel B) Drug binding to supercoiled ColE1 DNA is followed by the membrane ultrafiltration technique. Concentration of free [^{3}H]norfloxacin is 1.9 μM. Amount of DNA used is 9 pmol in 400 μL.

[2] Abbreviations: R, molar binding ratio; R_m, maximum molar binding ratio; K_d, apparent dissociation constant; D, free ligand concentration; n, Hill constant; K_i, supercoiling inhibition constant; T_m, melting temperature; G, guanosine; C, cytidine; A, adenosine; dT, thymidine; I, inosine; d (when used with G, C, and A), deoxy; dNMP, deoxynucleoside 5′-monophosphates; dGMP, deoxyguanosine 5′-monophosphate; dAMP, deoxyadenosine 5′-monophosphate; dCMP, deoxycytidine 5′-monophosphate; dTMP, thymidine 5′-monophosphate.

Table I: Binding of [^{3}H]Norfloxacin to DNA with Different GC Contents[a]

DNA	GC content (%)[b]	T_m (°C)[b]	molar binding ratio ($\times 10^{-4}$ per NT) at drug concn of 2 μM	6 μM
M. lysodeikticus	72	99.5	3.0 ± 0.52	6.7 ± 0.5
E. coli	50	90.5	2.5 ± 0.28	6.7 ± 0.7
C. perfringens	26.5	80.5	2.4 ± 0.26	6.4 ± 0.6
linearized ColE1 DNA			1.8	6.9

[a] Abbreviations: G, guanine; C, cytidine; T_m, melting temperature; NT, nucleotide. [b] From Marmur and Doty (1962).

Binding Specificity. To reach a better understanding of the binding mechanism, the binding specificity of the drug was investigated. We first tested the binding of [^{3}H]norfloxacin to double-stranded DNA species with different GC contents. The three bacterial DNA species selected for the experiments had GC contents of 26.5, 50, and 72% as listed in Table I together with their published melting points (T_m). The molar binding ratios of these three DNA species, determined at the same drug concentration of 2 or 6 μM, were about the same and were roughly equal to that for relaxed ColE1 DNA. The results indicate that the drug binding does not exhibit base specificity when the DNA strands are paired. We have shown previously that quinolones prefer single-stranded to double-stranded DNA and that a simple thermal denaturation step done to double-stranded DNA enhances the drug binding by more than an order of magnitude (Shen & Pernet, 1985). Therefore, we next investigated the base specificity of the drug binding when DNA strands are separated. Employing a similar approach of using synthetic DNA model polymers to investigate the actinomycin–DNA interaction (Sobell, 1974), we chose the use of single-stranded homopolymer nucleic acids as our model system to investigate drug binding specificity. As shown in Figure 2A, binding of the drug to the single-stranded polydeoxyribonucleotides is evident at all three drug concentrations, and the level of the drug bound to poly(dA) is about twice that of the drug bound to poly(dT). The most intriguing result shown in Figure 2A is the observation that binding to poly(dA)·poly(dT) duplex was virtually nondetectable. The results again suggest that the basic requirement for greater drug binding is that the DNA double helix must be separated so as to allow the drug to bind to the unpaired bases. This presumably involves hydrogen bonding, as will be evidenced in later experiments. Also shown in Figure 2A are the amounts of the drug bound to poly(dG) and poly(dC). The results show a sequence of drug binding preference to poly(dG), poly(dA), poly(dT), and poly(dC) in decreasing order. The binding to poly(dG) is distinctively greater than that to the other three polydeoxyribonucleotides. One unique structural feature of the guanine base is that it has two common hydrogen-bond donors while other bases have only one; the results thus suggest the involvement of hydrogen bonds in drug binding. Also shown in Figure 2B is the drug binding to poly(dI) which shows a level of drug binding roughly equal to 20% of that to poly(dG). The structural difference between guanosine and inosine is again that the former has an extra amino group on the pyrimidine ring that is an important hydrogen-bond donor in pairing complementary strands. The result again suggests that the drug binds to unpaired DNA bases via hydrogen bonding. Binding studies of the drug with synthetic polyribonucleotides (Figure 2B) also support this conclusion. The binding preference was the same as that for polydeoxyribonucleotides: affinity being higher for poly(G)

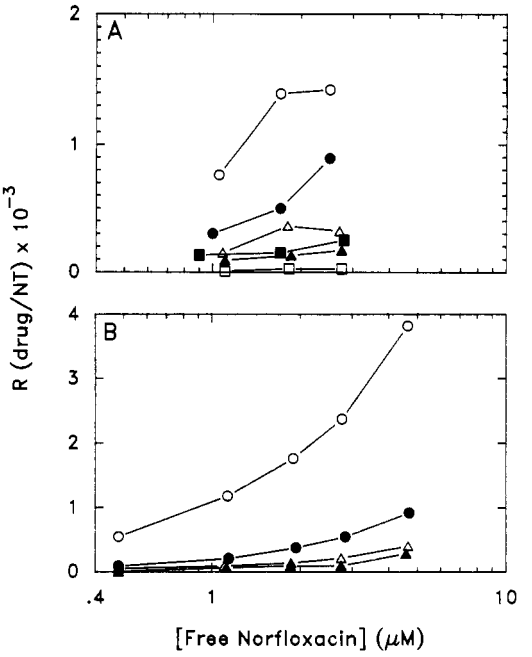

FIGURE 2: Binding of [^{3}H]norfloxacin to synthetic model homopolymers. (Panel A) Membrane filtration method was used to measure the drug binding to polydeoxyribonucleotides: poly(dG) (○), poly(dA) (●), poly(dT) (△), poly(dI) (■), poly(dC) (▲), and poly(dA)·poly(dT) duplex (□) at three drug concentrations. (Panel B) Same membrane filtration method was used to determine the drug binding to polyribonucleotides: poly(G) (○), poly(A) (●), poly(U) (△), and poly(C) (▲) at five drug concentrations. Amount of these synthetic homopolymers used was 20 μg in 400 μL.

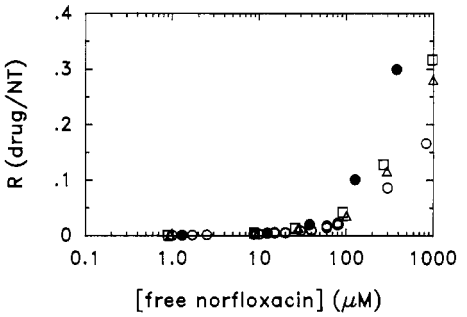

FIGURE 3: Binding of [^{3}H]norfloxacin to tRNA and oligo(dA) of different chain lengths. Results were obtained with the membrane filtration method and were presented in a Klotz plot: (□) oligo $(dA)_{20}$; (△) oligo $(dA)_{40-60}$; (○) oligo $(dA)_{360}$; (●) tRNA. Amount of oligo(dA) and tRNA used was 20 μg in 400 μL.

than for the other three homopolymers. The overall extent of bindings is slightly higher than that for the polydeoxyribonucleotides.

We next examined the binding of [^{3}H]norfloxacin to tRNA (from *E. coli*) and oligo(dA) with different chain lengths. Results in Figure 3 show that the binding, in terms of the molar binding ratio (drug per nucleotide), is independent of chain length. There is no trace of saturable binding which can be detected up to a drug concentration of 1 mM, which is near the solubility limit of the drug. Similar results were obtained with tRNA. The results support the conclusion that the binding of the drug to single-stranded DNA is nonspecific and that the drug does not bind to specific sites on the single-stranded chain which may adopt certain intramolecular conformations in solution (Cantor & Schimmel, 1980b). In summary, the studies show that the drug binds poorly to double-stranded DNA and the binding has no base specificity. The drug binds better to single-stranded DNA presumably

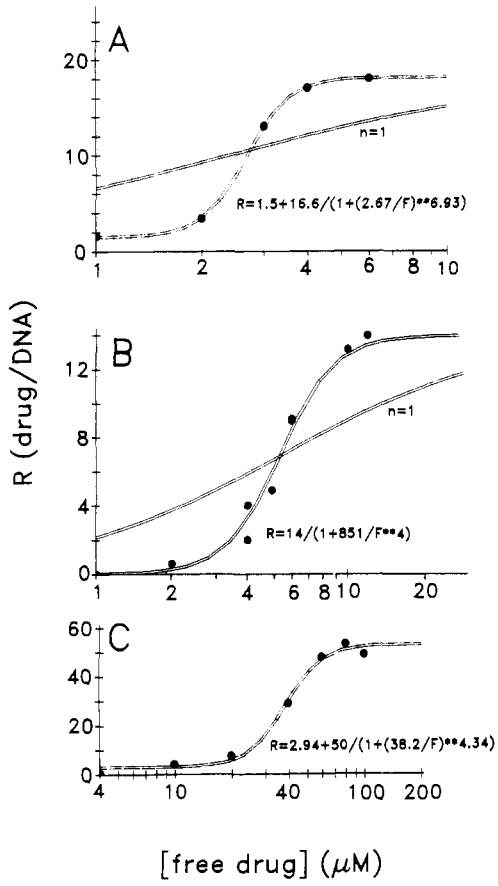

FIGURE 4: Binding of pefloxacin (A), oxolinic acid (B), and nalidixic acid (C) to supercoiled ColE1 DNA. The binding data were obtained with the membrane filtration technique with the use of the fluorescence measurement as the method of concentration determination. The binding curves were drawn by computer according to the Hill equations shown near each curve. Double asterisk (**) in the equations represents the exponentiation operator. Also shown in panels A and B are curves drawn with $n = 1$ while other parameters remained the same as the other curve in the same figure. DNA concentration used in these experiments were 9.5 pmol in 400 μL. Each data point represents the mean of three measurements. SE of the experiments is less than ±10%.

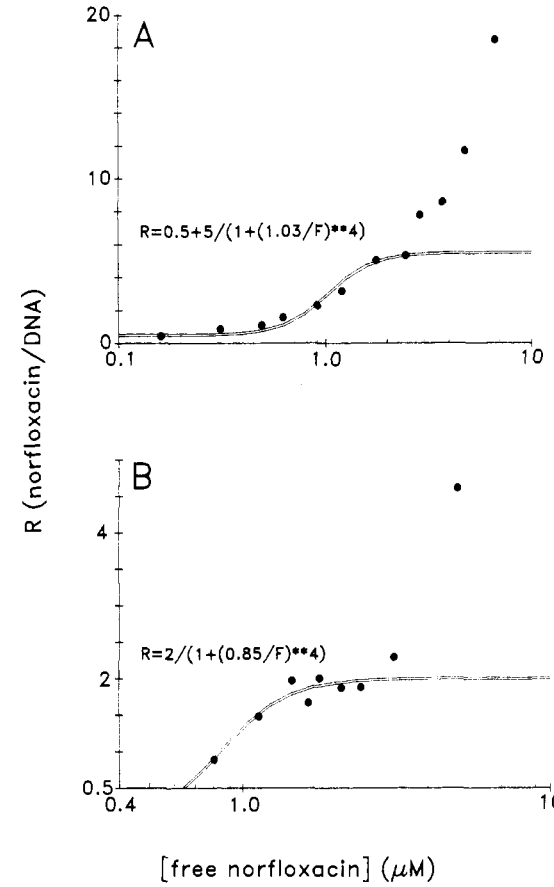

FIGURE 5: [^{3}H]Norfloxacin binding to supercoiled ColE1 DNA (A) and pBR322 DNA (B). Data were retrieved from Shen and Pernet (1985). Curves were drawn according to the Hill equations shown. Double asterisk (**) in the equations represents the exponentiation operator.

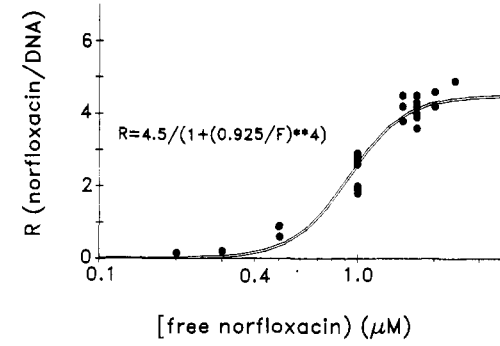

FIGURE 6: Binding of nonlabeled norfloxacin to ColE1 DNA using the fluorescence method. Curve was drawn according to the Hill equation shown. Double asterisk (**) in the equation represents the exponentiation operator. DNA concentration used in each experiment was 9.5 pmol in 400 μL.

through hydrogen binding. The binding to single-stranded DNA is not specific as it is nonsaturable and lacks cooperativity.

Binding of Norfloxacin, Pefloxacin, Oxolinic Acid, and Nalidixic Acid to Supercoiled DNA. We examined the binding of several representative nonradiolabeled quinolones to supercoiled ColE1 DNA. Utilizing the intrinsic fluorescence property of the drug as an alternative method for determining drug concentration, we were able to determine binding to DNA of pefloxacin, oxolinic acid, and nalidixic acid. These drugs were selected as they cover a range of supercoiling inhibition potency (with K_i values of 4, 12, and 110 μM, respectively). Results are shown in Figure 4. The binding curves resemble the binding pattern of norfloxacin to supercoiled ColE1 DNA (Shen & Pernet, 1985; also see Figure 5A) and demonstrate similar partial saturation phases. We employed a semiempirical approach (see Experimental Procedures) to estimate the K_d values and to analyze the cooperativity of the ligand binding according to eq 1. K_d values of 2.7, 5.4, and 38 μM are obtained for pefloxacin, oxolinic acid, and nalidixic acid, respectively. These values are lower than the values previously obtained with a competition method (10, 12, and 280 μM, respectively); but they are still in reasonable agreement with the K_i values when compared in the log–log plot as presented previously (Shen & Pernet, 1985). All binding curves show high values of Hill constants, indicating a high degree of binding cooperativity of the drug to the DNA receptor site. The observed high R_m values and a much higher n value (6.93) for oxolinic acid remain to be investigated. Our previous binding data obtained with [^{3}H]norfloxacin (Shen & Pernet, 1985) were reexamined for binding cooperativity. As shown in Figure 5, those previous binding curves are also best fitted by eq 1 with $n = 4$. Furthermore, we have reinvestigated the binding of norfloxacin to ColE1 DNA using the fluorescence method with nonlabeled norfloxacin, and the results are shown in Figure 6. A partial saturation phase similar to the one observed previously (Figure 5A) was demonstrated, and again

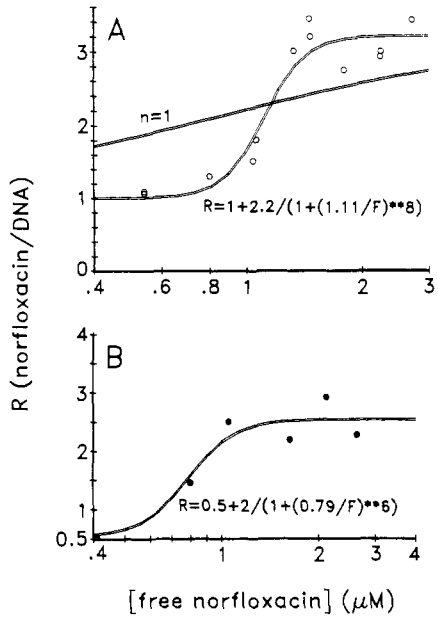

FIGURE 7: Binding of [^{3}H]norfloxacin to gyrase–DNA complex. Data were retrieved from Figure 3 of Shen et al. (1989a). Curves were drawn according to the Hill equations shown. Double asterisk (**) in the equations represents the exponentiation operator. Panel A shows the concentration dependence of norfloxacin binding to complex formed by adding 3.8 pmol of DNA gyrase to an equimolar amount of linear ColE1 DNA. A total of 1 mM ATP was present. Panel B shows the binding to the same complex formed in the absence of ATP or its analogue. Also shown in panel A is the curve drawn with $n = 1$ with the other parameters remaining the same as for the other curve.

the curve was fit according to eq 1 with $n = 4$. The similarity of the results in terms of binding cooperativity and binding affinity obtained with nonlabeled norfloxacin and that obtained with [^{3}H]norfloxacin (with K_d equal to 1.03 and 0.925, respectively) indicates that the fluorescence method is a reliable technique in determining the drug binding.

Binding of [^{3}H]Norfloxacin to Gyrase–Linear DNA Complex. Recently, we have obtained results using membrane filtration and a spin-column technique to study the binding of [^{3}H]norfloxacin to complexes formed by adding gyrase to different forms of relaxed DNA (Shen et al., 1989a). It was found that drug binding may be enhanced by the addition of gyrase to relaxed forms of DNA in the presence of ATP or a nonhydrolyzable ATP analogue. These data indicate that a drug binding site is induced in the enzyme–DNA complex. We have tested the cooperativity of these binding curves. Two such curves, obtained with linear DNA in the presence and in the absence of ATP, are shown in Figure 7. It is demonstrated that such complex-dependent bindings of norfloxacin are highly cooperative (with n greater than 6) and the saturations are taking place at drug concentrations corresponding to those observed with the supercoiled DNA (Figures 5 and 6).

Two Types of Drug Binding on Supercoiled DNA. We have demonstrated that there are at least two types of drug binding to DNA: (i) the nonspecific, noncooperative binding and (ii) the specific, cooperative binding to a unique single-stranded pocket either in supercoiled DNA or in an enzyme–DNA complex. In fact, both types of drug binding may be observed with supercoiled DNA at a wider drug concentration range. Figure 8 shows the binding of [^{3}H]norfloxacin to supercoiled ColE1 DNA at higher drug concentrations. The binding observed in this range is primarily of the nonspecific type as it is best fitted by eq 1 with $n = 1$. It should be pointed out that the observed high level of nonspecific binding shown in

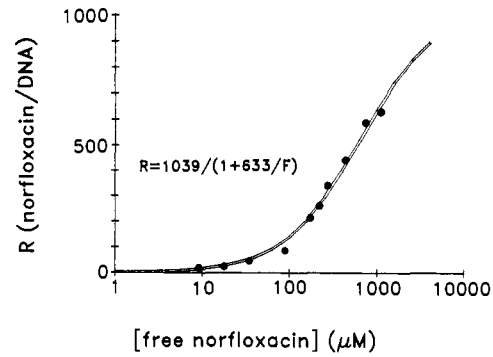

FIGURE 8: Binding of [^{3}H]norfloxacin to supercoiled DNA at high drug concentration. Curve was drawn according to the Hill equation shown (with $n = 1$). R_m value (1039) was also obtained by computer fitting. The solubility of norfloxacin at pH 7.4 was 1100 μM. DNA concentration was 4.6 pmol in 400 μL.

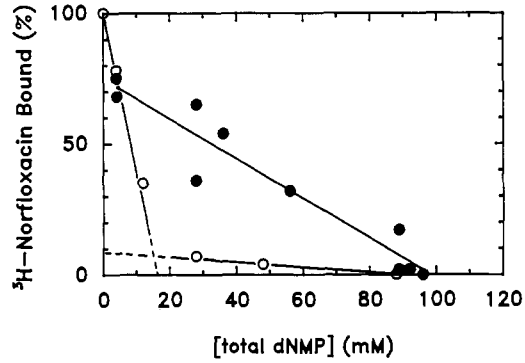

FIGURE 9: Competition of specific and nonspecific forms of [^{3}H]-norfloxacin binding with deoxynucleoside monophosphates (dNMP). The antagonizing effect of dNMP on [^{3}H]norfloxacin binding to supercoiled ColE1 DNA was tested at two different [^{3}H]norfloxacin concentrations: 50 (○) and 1.7 μM (●). Abscissa is the total concentration of the four dNMPs (dGMP, dAMP, dCMP, TMP) in equal proportions. Amount of DNA used was 4.3 pmol/400 μL. Ordinate is the percentage of drug remaining bound to DNA in the presence of competing dNMPs; 100% position corresponds to 18 and 294 pmol of bound drug for experiments at 1.7 and 50 μM, respectively.

Figure 8 covers the lower level specific type of binding which is saturated at a much lower drug concentration (near 1 μM; see Figures 4–6). The existence of these two classes of binding site of different binding affinities may be demonstrated by a simple competition test with monomeric deoxynucleotides. To simplify the experiment, we used a mixture of four deoxynucleoside monophosphates (dGMP, dAMP, dCMP, dTMP) as the competing molecules. Figure 9 shows the results of two competition experiments: one at a radioligand concentration of 50 μM at which the binding to supercoiled ColE1 DNA is primarily nonspecific and one at a radioligand concentration of 1.5 μM at which binding was essentially of the specific type. As expected, both competition curves are biphasic, indicating the coexistence of the two types of binding. At 50 μM, 90% of the ligand binding was easily inhibited due to the existence of a major portion of nonspecific binding; the remaining 10% is more resistant to the competition and requires a much higher concentration of the competing deoxynucleotides. Fifty percent inhibition for phase 1 and phase 2 takes place at the competing monophosphate nucleoside concentrations of 8.4 and 58 mM, respectively. At 1.5 μM, the extents of the first (nonspecific) and second (specific) phases are reversed as expected. At this lower ligand concentration, the specific type of binding prevails, and we indeed observed that a majority of the binding (80%) is more resistant to the competition with 50% inhibition taking place at 50 mM. It is conceivable that competition may result

from the interaction between deoxynucleotides and [^{3}H]norfloxacin, thus preventing the latter from binding to DNA. Such interactions, however, cannot contribute to the difference between the two competition curves.

Discussion

As shown in our previous publication (Shen & Pernet, 1985), the binding of norfloxacin to relaxed DNA, with or without the addition of gyrase (in the absence of ATP), was low and did not shown any trace of saturable drug binding near the drug's K_i value, whereas a distinct saturable binding was shown with the supercoiled DNA without the participation of the enzyme. Such an observation was puzzling as the results suggested the binding of an inhibitor to the product of a catalytic reaction, but not to the substrate. The seemingly controversial observations have been understood through our further investigations on the drug binding specificity shown in this paper as well as our studies with the binding to enzyme–DNA complex shown in a recent paper (Shen et al., 1989a). As a first and important step to the understanding of the mode of drug binding, we have concentrated on the studies of drug binding specificity. In an early attempt to gain such understanding, we found that thermal denaturation of DNA drastically enhanced the drug binding, suggesting that the DNA structure was a major determinant of drug binding specificity. It is evident that the basic requirement for the drug to bind strongly at low drug concentrations is the separation of the double-helical strands so that the hydrogen-bond donors thus become available for pairing with the acceptors on the drug molecules (carbonyl or fluoro groups). Experiments with synthetic poly(deoxy)ribonucleotides support such a proposition. The involvement of hydrogen bonding in quinolone binding to DNA is suggested in Figure 2, which demonstrates a drug binding preference to poly(dG) and poly(G) over other homopolymers. The drug is likely to bind to DNA bases through hydrogen bonding rather than through stacking of the quinolone ring with the purine ring, since drug binding appears to be dependent on the number of the available hydrogen-bond acceptors rather than on ring types. For example, one observes that drug binding to poly(dG) is 5-fold greater than that to poly(dI), although both have purine rings. It is known that synthetic homopolyribonucleotides may adopt certain ordered structures in solution such as the single-stranded helix (Cantor & Schimmel, 1980b). However, poly(G) and poly(I) only have been shown to have similar structures (Arnott et al., 1974); the differences in structure, if any, between poly(G) and poly(I) do not appear large enough to account for the 5-fold difference in drug binding.

Judging from the structures of DNA and quinolones, hydrogen bonding and ring stacking are the two possible modes of interaction between these two similarly charged molecules. Between these two, the former is favored. The suggestion of the involvement of hydrogen bonding is based not only on the difference in binding affinity of homopolymers but also on evidence showing that the binding is unlikely due to the ring stacking. The exclusion of ring stacking as a possible mode of quinolone–DNA interaction was derived from the observation that the drug binds preferentially to single-stranded DNA rather than the double-stranded DNA and that the drug lacks a DNA intercalation effect (Shen et al., 1988; Tornaletti & Pedrini, 1988). Furthermore, the absolute requirement for inhibitory activity of the C4 carbonyl group on the quinolone ring and the C3 carboxylic acid group, both of which are potential hydrogen-bond receptors, is another strong implication of the hydrogen-bonding specificity in the interaction. The issue concerning how the formation of some limited hydrogen bonds can render the drug a high binding affinity to the specific DNA site will be elucidated in the model proposed in the following paper (Shen et al., 1989b).

We have observed three different modes of quinolone binding to DNA of different structural forms. First is the binding of the drug to native double-stranded DNA, which is weak and noncooperative (Figure 8). Results in Figure 2A suggest that strand pairing effectively prevents the drug binding. Second is the noncooperative form of binding to single-stranded DNA; this simple form of binding may represent the prototype of specific binding to the DNA site due to its greatly enhanced binding affinity. The third form is the specific binding such as the saturable binding to supercoiled ColE1 DNA or to complexes formed between DNA gyrase and DNA; this binding mode is observed at concentrations near the quinolones' K_i against *E. coli* DNA gyrase and is highly cooperative. The fact that the high-affinity drug binding site exists only in supercoiled DNA but not in other topological forms of DNA is consistent with our finding of the drug's binding preference to single-stranded DNA and the topological principle in DNA supercoiling (Bauer et al., 1980; Wang, 1982): negative supercoiling promotes DNA strand separation. It is known that the supercoiled form is the topological form of lowest energy for an underwound DNA. Although such an underwound region is compensated by the supertwisting, it is still possible that the drug binds to a region which is prone to form a localized bubble. The size of the denatured bubble need not be large. We have shown previously that there are only four drugs bound per molecule of supercoiled ColE1 DNA of 6646 base pairs (Chan et al., 1985). This represents only $4/6646 = 0.06\%$ of the entire DNA molecule assuming that only four base pairs are involved in the binding. The topological change of the supercoiled DNA (presumably with a difference of less than one superhelical turn) subject to the stabilization of such a small region upon drug binding is less likely to be detected by any physical method.

Another possible candidate for the specific drug binding site in supercoiled DNA is the cruciform (or hairpin) DNA structure that forms in the region with palindromic sequence; this structure is known to be promoted by negative supercoiling (Gellert et al., 1978; Lilley, 1980; Mizuuchi et al., 1982). The cruciform is characterized by single-stranded DNA regions at the center and at the branch terminals which have been demonstrated by cleavage with S1 nuclease, which has specificity toward single-stranded DNA (Panayotatos & Wells, 1981; Singleton & Wells, 1982). Such hairpin structure, featured with single-stranded DNA properties, does exist in supercoiled ColE1 and pBR322 DNA. More specifically, the site of hairpin formation in ColE1, demonstrated by S1 nuclease digestion, is an inverted repeat of 13 nucleotides separated by a central nonrepetitious segment of 4 nucleotides. pBR322 DNA demonstrates multiple sites; the major site is an inverted repeat of 11 nucleotides with 3 central nonrepeated nucleotides. The possible size difference in the unpaired terminal bubbles of the cruciform between ColE1 and pBR322 DNA may account for the difference in R_m shown in Figure 5, if the binding site is indeed located at the terminal loop.

The capability of the drug to stabilize a denatured region or to bind to a cruciform in supercoiled DNA may require DNA unwinding to provide a driving force for the entry. The recent finding by Tornaletti and Pedrini (1988) showed that quinolones do have the capability to unwind DNA in the presence of magnesium ions in a nonintercalative manner. The unwinding effects of quinolones are found to be proportional to their inhibitory potencies. Whether the observed DNA

unwinding effect results from the stabilization of a denatured region or from other forms of drug binding remains to be answered.

The demonstration of the saturable drug binding phase with enzyme–DNA complex (Figure 7) provides key evidence showing that the drug binds to a similar denatured DNA site created by *E. coli* DNA gyrase, but not a site on the enzyme created by the bound DNA. The high degree of similarity in binding cooperativity and in apparent dissociation constant (K_d) between the binding curves obtained with the complex (Figure 7) and with supercoiled DNA (Figures 5 and 6) strongly favors such a conclusion.

Since the binding saturation is partial, a Scatchard analysis of the data is not feasible. We present the binding data in Klotz plots and use a semiempirical approach to analyze the data by use of the Hill test with the aid of computer fitting. The results clearly demonstrate the high cooperativity of the binding. The principle of binding cooperativity is that the binding of the first ligand to the receptor site makes the binding of the next ligand easier until the site is saturated. We envision this to occur with strong intermolecular interactions among the drug molecules assembled at the drug binding site. Details of a proposed cooperative drug–DNA binding model are described in the following paper (Shen et al., 1989b).

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Registry No. Poly(dG), 25656-92-2; poly(dA), 25191-20-2; poly(dT), 25086-81-1; poly(dC), 25609-92-1; poly(dA)·poly(dT), 24939-09-1; poly(dI), 27732-54-3; poly(G), 25191-14-4; poly(A), 24937-83-5; poly(U), 27416-86-0; poly(C), 30811-80-4; norfloxacin, 70458-96-7; pefloxacin, 70458-92-3; oxolinic acid, 14698-29-4; nalidixic acid, 389-08-2.

REFERENCES

Arnott, S., Chandrasekaran, R., & Marttila, C. M. (1974) *Biochem. J. 141*, 537–543.

Bauer, W. R., Crick, F. H. C., & White, J. H. (1980) *Sci. Am. 243*, 118–133.

Cantor, C. R., & Schimmel, P. R. (1980a) in *Biophysical Chemistry*, Part III, pp 863–864, W. H. Freeman, New York.

Cantor, C. R., & Schimmel, P. R. (1980b) in *Biophysical Chemistry*, Part III, Chapter 22, W. H. Freeman, New York.

Chan, P. T., Ohmori, H., Tomizawa, J.-I., & Lebowitz, J. (1985) *J. Biol. Chem. 260*, 8925–8935.

Cozzarelli, N. R. (1980) *Science 207*, 953–960.

Fernandes, P. B. (1988) *J. Clin. Pharmacol. 28*, 156–168.

Gellert, M. (1981) *Annu. Rev. Biochem. 50*, 879–910.

Gellert, M., Mizuuchi, K., O'Dea, M. H., & Nash, H. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3872–3876.

Gellert, M., Mizuuchi, K., O'Dea, M. H., Ohmori, H., & Tomizawa, J. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 35–40.

Hooper, D. C. (1986) in *Microbiology-1986* (Leive, L., Ed.) pp 226, American Society of Microbiology, Washington, DC.

Hooper, D. C., & Wolfson, J. S. (1985) *Antimicrob. Agents Chemother. 28*, 716–721.

Klotz, I. M. (1974) *Acc. Chem. Res. 7*, 162–168.

Lesher, G. Y., Froelich, E. J., Gruett, M. D., Bailey, J. H., & Brundage, R. P. (1962) *J. Med. Chem. 5*, 1063–1065.

Lilley, D. M. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6468–6472.

Marmur, J., & Doty, P. (1962) *J. Mol. Biol. 5*, 109–118.

Mizuuchi, K., Mizuuchi, M., & Gellert, M. (1982) *J. Mol. Biol. 156*, 229–243.

Neu, H. C. (1987) *Antimicrob. Newsl. 4*, 9–14.

Panayotatos, N., & Wells, R. D. (1981) *Nature 289*, 466–470.

Shen, L. L., & Pernet, A. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 307–311.

Shen, L. L., Baranowski, J., Wai, T., Chu, D. T. W., & Pernet, A. G. (1988) in *The International Telesymposium on Quinolones* (Fernandes, P., Ed.) Prous Science, Barcelona, Spain (in press).

Shen, L. L., Kohlbrenner, W. E., Weigl, D., & Baranowski, J. (1989a) *J. Biol. Chem.* (in press).

Shen, L. L., Mitscher, L. A., Sharma, P. N., O'Donnell, T. J., Chu, D. W. T., Cooper, C. S., Rosen, T., & Pernet, A. G. (1989b) *Biochemistry* (following paper in this issue).

Singleton, C. K., & Wells, R. D. (1982) *J. Biol. Chem. 257*, 6292–6295.

Sobell, H. M. (1974) *Cancer Chemother. Rep., Part 1 58*, 101–116.

Tornaletti, S., & Pedrini, A. M. (1988) *Biochim. Biophys. Acta 949*, 279–287.

Wang, J. C. (1982) *Sci. Am. 247*, 94–109.

Wang, J. C. (1985) *Annu. Rev. Biochem. 54*, 665–697.

Wolfson, J. S., & Hooper, D. C. (1985) *Antimicrob. Agents Chemother. 28*, 581–586.