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Interaction of Metronidazole with Nucleic Acids *in Vitro*

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SUMMARY

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The binding of metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] to nucleic acids was quantitated and characterized *in vitro*. [^{14}C]Metronidazole was incubated with nucleic acid under various conditions, the nucleic acid and associated ^{14}C were separated from lower molecular weight compounds by molecular sieve chromatography, and nucleic acid concentration and radioactivity were measured in the eluate. Maximum binding occurred when metronidazole was reduced by sodium dithionite in the presence of calf thymus DNA (0.73 molecule of drug per 10^3 nucleotides). Binding was significantly less if metronidazole was reduced prior to incubation with DNA, and did not occur with unreduced metronidazole. Binding of reductively activated metronidazole to bacterial and phage DNA and yeast tRNA was also demonstrated; it was 2 times greater to alkali-denatured than to native DNA, was less in the presence of $MgCl_2$ or NaH_2PO_4 and at higher pH, and did not affect the melting temperature or renaturation profile of calf thymus DNA. While 15-30% of bound ^{14}C was released by dialysis or rechromatography, the majority of the label remained complexed to nucleic acid under a variety of conditions known to dissociate noncovalent complexes. Studies with synthetic polynucleotides suggested binding specificity of reductively activated metronidazole for guanine and cytosine. Both unreduced and dithionite-reduced metronidazole bound to bovine serum albumin, and binding of the reduced drug was approximately 35% of binding to nucleic acids. The results indicate that reduction of metronidazole *in vitro* yields a short-lived, activated compound(s) which, while not cross-linking, binds largely in a covalent fashion primarily to guanine and cytosine of mammalian, bacterial, and phage DNA. This binding may have implications for the antimicrobial, mutagenic, and radiosensitizing actions of this drug.

INTRODUCTION

Metronidazole [Flagyl, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is an effective agent for the treatment of anaerobic protozoal and bacterial infections (1), increases the radiosensitivity of certain solid tumors (2), and induces mutations in bacteria (3, 4). The mechanism(s) of its cytotoxic, radiosensitizing, and mutagenic actions is not clear, although reduction of the nitro group is a common prerequisite for all three effects (2, 4).

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Interaction of metronidazole with nucleic acid was initially suggested as a possible action mechanism by Ings *et al.* (5), who demonstrated drug binding to DNA and impairment of macromolecular synthesis by metronidazole in sensitive organisms. Plant and Edwards (6) also demonstrated inhibition of DNA synthesis *in vivo*, while Willson *et al.* (7) showed binding to DNA of metronidazole activated by γ-irradiation.

In the studies reported here, we have quantified and characterized *in vitro* the binding of metronidazole to natural nucleic acids and synthetic polynucleotides. The results have been presented in part in abstract form (8).

MATERIALS AND METHODS

The following substances were obtained commercially: calf thymus DNA, *Escherichia coli* K-12 DNA, T2 phage DNA (General Biochemicals), and brewer's yeast tRNA (Schwarz BioResearch). Synthetic polynucleotides were obtained from P-L Biochemicals, and bovine serum albumin, from Miles Laboratories.

Calf thymus DNA was purified by precipitation and repeated deproteinization by chloroform (9). *E. coli* K-12 DNA was purified in a similar fashion, with the addition at 37° for two 15-min periods of ribonuclease T_1, approximately 20 units/mg of DNA, in a solution of saline citrate (15 mM sodium chloride and 2 mM citric acid brought to pH 7.4 with sodium hydroxide). The purified DNA preparations were sonicated in ice for 15 min total time (seven 2-min intervals and one 1-min interval with 1-min intervals between sonications) at 100 W power with a Branson Sonifier and centrifuged in a Sorvall Superspeed RC2-B centrifuge (10,000 rpm for 20 min), and the supernatant was dialyzed for 24 hr against 200 volumes of 17 mM sodium phosphate buffer, pH 7.4, the dialysate being changed twice. Analysis of the final DNA preparations indicated less than 0.5% protein by weight. Denatured DNA was prepared by raising the pH of the DNA solution to 12.7 with 3 N NaOH, stirring for 7 min, then neutralizing with 2 N HCl. The denatured DNA solution was then dialyzed as above. Stock solutions of all nucleic acids were stored frozen.

[^{14}C]Metronidazole [1-(2-[*U*-^{14}C]hydroxyethyl)-2-methyl-5-nitroimidazole] was kindly supplied by Searle Laboratories and had a specific activity of 17 μCi/mg. Metronidazole has an extinction coefficient of 9310 at 320 nm (7) and a molecular weight of 171.

Experimental samples were incubated in a 10-ml pear-shaped flask. Anaerobic conditions were established by purging the flasks and the syringes used to transfer solutions with helium gas (chromatographic grade, Union Carbide) and by continuous bubbling of helium through the solutions. In a typical experiment, 1 μmole of nucleotide in nucleic acid or synthetic polynucleotide, or 300 μg of BSA,[2] was incubated with 2 μmoles of [^{14}C]metronidazole at room temperature for 45 min in 1.5 ml of 17 mM sodium phosphate buffer, pH 7.4. When desired, a reducing agent (a freshly prepared anaerobic solution of sodium dithionite or sodium borohydride) was added in single or multiple aliquots to the drug-macromolecule mixture or to the drug alone prior to addition of the macromolecule. The reaction mixture was then exposed to room air and chromatographed on a column (1.1 × 35 cm) of Sephadex G-100 with saline citrate as eluant. Forty 1-ml fractions were collected and immediately assayed for nucleic acid and ^{14}C.

Nucleic acid concentration was measured by absorption spectrometry and expressed as mononucleotide equivalents, i.e., moles of mononucleotide. The ϵ and λ_{max} values employed are listed in Table 2.

Radioactivity was measured by liquid scintillation counting of 0.5-ml aliquots in 10 ml of Aquasol (New England Nuclear). Counting efficiency for ^{14}C in the eluate was 56% and was unaffected by binding to DNA, as shown by comparison of samples counted before and after hydrolysis of nucleic acid (10).

The binding ratio, defined as molecules of drug per 10^3 nucleotide units, was calculated using data taken from the assay of a pooled sample of several fractions contain-

[2] The abbreviation used is: BSA, bovine serum albumin.

ing over 90% of the nucleic acid eluted from the column.

Binding of metronidazole to untreated and alkaline phosphatase-treated DNA was also tested: 2.5 μmoles of sonicated native calf thymus DNA in 0.55 ml of 20 mM Tris buffer, pH 8.5, were incubated with 25 units of bacterial alkaline phosphatase (Worthington, "phosphodiesterase-free") for 1 hr at 38°, and the mixture was dialyzed against 200 volumes of 17 mM phosphate, pH 7.4, overnight at 4°.

Similar binding experiments employing identical methodology were performed, using BSA in place of nucleic acid. Protein concentration in eluted fractions was determined chemically by the method of Lowry *et al.* (11). The binding ratio of [^{14}C]metronidazole to BSA was expressed as specific activity (counts per minute of ^{14}C per milligram of BSA), using data taken from the assay of a pooled sample of several fractions containing over 90% of the BSA eluted from the columns. For comparison, the specific activity of binding of metronidazole to DNA was expressed as counts per minute of ^{14}C per milligram of DNA.

Stability of binding of label to nucleic acid or synthetic polynucleotide was evaluated by comparing the binding ratios before and after dialysis (18 hr at 4° against 3000 volumes of saline citrate) or rechromatography of the DNA-^{14}C complex. Results were expressed as the percentage of label lost and were not different for dialysis or rechromatography of the same sample. In addition, the effects of a variety of strong conditions on the stability of the complex were tested. Stability to these agents was evaluated by first isolating a ^{14}C-native calf thymus DNA complex by dialysis, incubating it with a particular agent, then redialyzing it at 4° overnight against 1000 volumes of saline citrate. The binding ratios before and after redialysis of treated samples or an untreated control were then compared.

Finally, the melting curve reversibility method as described by Iyer and Szybalski (12), using a temperature-controlled Gilford recording spectrophotometer and saline citrate as buffer, was employed to study the drug-nucleic acid complex.

All binding, stability, and melting profile determinations were done in duplicate or triplicate except where indicated. The precision of the methodology was satisfactory, results of repeat experiments usually agreeing within 5% (mean coefficient of variation of all experiments, 5.1 ± 1.9% SD).

RESULTS

Control experiments. Figure 1 shows the elution profiles for native, sonicated calf thymus DNA and [^{14}C]metronidazole when each compound, dissolved in 17 mM phosphate buffer, pH 7.4, was chromatographed separately. Similar experiments with BSA also showed complete chromato-

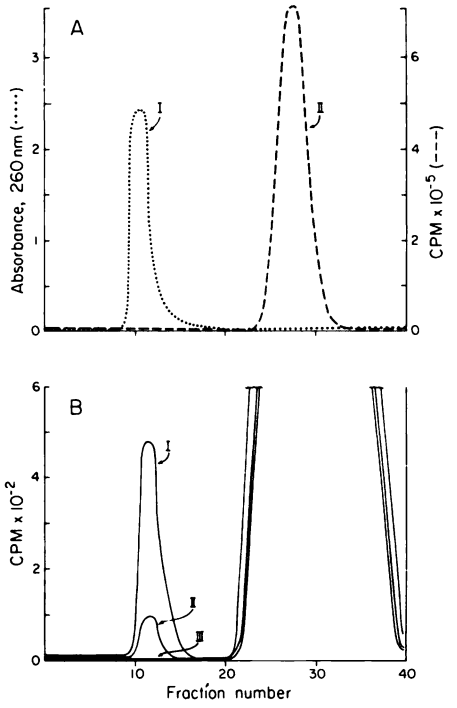

FIG. 1. *Elution profiles of DNA and [^{14}C]metronidazole on Sephadex G-100*

A. Elution profiles of 1 μmole of native calf thymus DNA (I) and 1 μmole of [^{14}C]metronidazole (II). B. Elution profiles of 1 μmole of native calf thymus DNA incubated with 2 μmoles of [^{14}C]metronidazole coreduced with 5 μmoles of dithionite (I), DNA and [^{14}C]metronidazole previously reduced with dithionite (II), and DNA and unreduced [^{14}C]metronidazole (III).

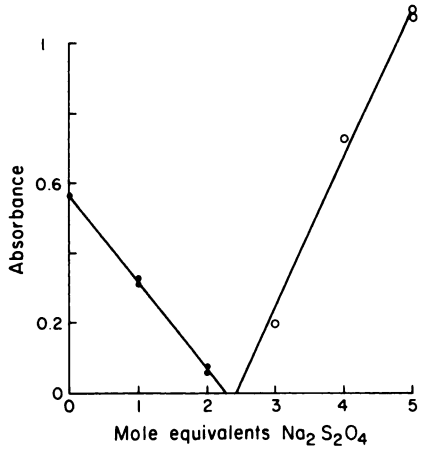

FIG. 2. *Reduction of metronidazole by sodium dithionite*

Metronidazole (60 μM) in 10 mM sodium phosphate buffer, pH 7.4, was titrated with 45 mM dithionite (on a weight basis) under constant bubbling of helium through the solutions. Aliquots for spectrophotometric readings were transferred with a helium-filled syringe into helium-purged cuvettes. Dithionite was added at 10-min intervals. The decrease in absorbance at 320 nm (●——●) was measured for the extent of disappearance of metronidazole. The increase in absorbance at 315 nm (○——○) was then measured for appearance of unconsumed dithionite. To ascertain that the A_{320} values measured in the 0–2 mole equivalent range of the titration were due to unreduced metronidazole without any contribution of unreacted dithionite (λ_{max} = 315 nm) possibly accumulating, the stability of A_{320} was tested by opening the cuvettes to air after the anaerobic reading. (The 315 nm absorption band of dithionite is air-sensitive.) Since no decrease in A_{320} was observed in this titration range, it is evident that dithionite was absent. In the 3–5 mole equivalent range, the A_{315} values were tested in a similar fashion. Here a decrease to zero occurred, proving that in this phase of the titration only the accumulating excess of dithionite was being measured.

graphic separation of this macromolecule from [^{14}C]metronidazole. Figure 2 shows reduction of metronidazole by sodium dithionite followed spectrophotometrically. Two moles of dithionite per mole of metronidazole were necessary for complete reduction of the drug, and reduction of metronidazole did not affect its elution profile on Sephadex G-100. Similar experiments indicated that a 100-fold excess of sodium borohydride completely reduced metronidazole but at a much slower rate, several hours being needed for complete reduction.

A single stock solution of sonicated native calf thymus DNA was employed in all quantitative studies, since results with unsonicated DNA were not different from those employing the sonicated material.

Quantitation of binding of [^{14}C]metronidazole to nucleic acid. Figure 1 and Table 1 show the binding of reduced and unreduced [^{14}C]metronidazole to native calf thymus DNA. Maximum binding occurred when [^{14}C]metronidazole was reduced with sodium dithionite in the presence of DNA ("coreduction"). Significantly less binding of [^{14}C]metronidazole to DNA occurred when [^{14}C]metronidazole had been completely reduced by dithionite prior to incubation with DNA ("previous reduction"), whether the reducing agent was added 3, 30, or 300 min earlier. Unreduced metronidazole did not bind to DNA.

When [^{14}C]metronidazole was completely reduced by sodium borohydride in the presence of DNA, binding was approximately 5% of that with dithionite as reducing agent. No change in binding was observed if the same total amount of dithionite required for complete reduction was added to the DNA-drug mixture in one, two, or five steps. Hence metronidazole was reduced in the presence of DNA in all subsequent experiments by addition of a single amount of dithionite (2.5 moles of dithionite per mole of metronidazole). [^{14}C]Metronidazole reductively activated by dithionite in this fashion was shown to bind to a variety of natural nucleic acids

TABLE 1

Effect of reduction of [^{14}C]metronidazole on binding to calf thymus DNA

Reaction conditions are given in MATERIALS AND METHODS and the legend to Fig. 1B.

Drug reduction	Binding ratio
	molecule/10^3 nucleotides
Coreduced with sodium dithionite	0.733
Previously reduced with sodium dithionite	0.050
Unreduced	<0.001
Coreduced with sodium borohydride	0.040

(Table 2). Binding to calf thymus DNA could be increased by increasing the molar ratio of metronidazole to DNA in the reaction mixture (Fig. 3). Drug-DNA binding could also be increased by repeatedly adding metronidazole and dithionite to the same solution of calf thymus DNA (Fig. 4).

Characterization of binding of [^{14}C]-metronidazole to nucleic acid. Binding of reductively activated metronidazole to DNA was shown to be affected by the ionic composition and pH of the reaction buffer, and by the secondary structure or base composition of the nucleic acid or synthetic polynucleotide employed. Increasing amounts of magnesium chloride or sodium phosphate inhibited binding (Fig. 5), while decreased pH promoted binding (Table 3). Alkali denaturation of calf thymus or *E. coli* DNA, which produces predominantly

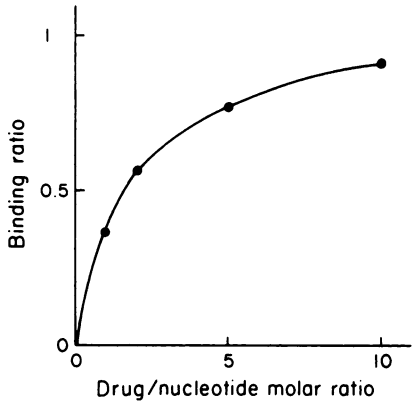

FIG. 3. *DNA-[^{14}C]metronidazole binding*
Native calf thymus DNA (1 μmole) was incubated with different amounts of [^{14}C]metronidazole coreduced with 2.5 μmoles of dithionite per micromole of [^{14}C]metronidazole. Binding ratio is defined as molecules of drug per 10^3 nucleotide units.

TABLE 2

Binding of reductively activated [^{14}C]metronidazole to polynucleotides

Substrate (1 μmole) was incubated with 2 μmoles of [^{14}C]metronidazole, and 5 μmoles of dithionite were added as described in MATERIALS AND METHODS.

Substrate	ϵ[a] (ref.)	λ_{max}	Binding ratio[b]	Label lost after dialysis or rechromatography
	$M^{-1}\,cm^{-1}$	*nm*		%
Calf thymus DNA				
Native	6,412 (13)	260	0.73	14.0
Alkaline phosphatase-treated	6,412 (13)	260	0.71[c]	
Denatured	7,308[d]	260	1.23	13.7
E. coli K-12 DNA				
Native	7,000 (14)	260	0.56	21.4
Denatured	8,000[d]	260	1.91	36.9
T2 phage DNA, denatured	7,342 (15)	260	0.79	29.5
Yeast tRNA	7,220[e]	260	0.86[c]	18.1
Synthetic polynucleotides				
Poly(dA)	8,600 (16)	257	0.46	9.7
Poly(dT)	8,520 (17)	264	0.21[c]	—[f]
Poly(dG)	10,100 (18)	253	1.01	35.0
Poly(dC)	7,400 (17)	274	1.19	7.7
Poly(dA):poly(dT)	6,000 (19)	260	0.08	—[f]
Poly(dG):poly(dC)	7,400 (19)	253	1.33	12.7
Poly(dA·dT):poly(dA·dT)	6,600 (20)	262	0.18[c]	—[f]
Poly(dG·dC):poly(dG·dC)	8,400 (19)	254	1.30[c]	3.1

[a] Per phosphorus.
[b] Molecules of drug per 10^3 nucleotides.
[c] Single experiment.
[d] Value determined experimentally by absorbance difference before and after denaturation of native DNA.
[e] Value provided by manufacturer.
[f] Not tested.

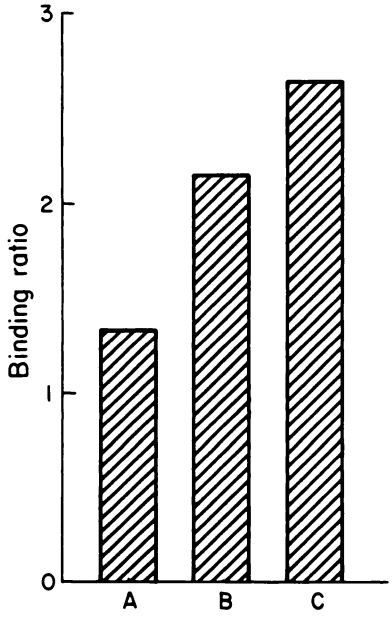

FIG. 4. *DNA-[^{14}C]metronidazole binding after repeat exposure of DNA to coreduced metronidazole*

A. Alkali-denatured calf thymus DNA (3 μmoles) was incubated with 15 μmoles of [^{14}C]metronidazole coreduced with 37.5 μmoles of dithionite, and an aliquot was removed for determination of binding ratio (molecules of drug per 10^3 nucleotide units). B. [^{14}C]Metronidazole (10 μmoles) and dithionite (25 μmoles) were added to the same reaction flask and incubated with the 2 μmoles of DNA remaining, and an aliquot was again removed for determination of binding ratio. C. The procedure was repeated, maintaining constant the ratio of the drug to DNA to dithionite.

single-stranded DNA, increased the binding of metronidazole as compared with the native, double-stranded forms (Table 2). Prior removal of monophosphate end groups from native calf thymus DNA by alkaline phosphatase had no effect on the binding ratio (Table 2).

Figure 6 shows the melting curves of native calf thymus DNA alone and after exposure to reductively activated metronidazole. Reduced metronidazole did not affect the transition profile, melting temperature, or renaturation curve of DNA.

Binding to a variety of single- and double-stranded synthetic polynucleotides was also demonstrated for reduced metronidazole, and was 3–15 times greater for compounds containing guanine and/or cytosine than for those containing adenine and/or thymine (Table 2). This apparent binding specificity of metronidazole for guanine and cytosine was also evident in studies employing denatured natural nu-

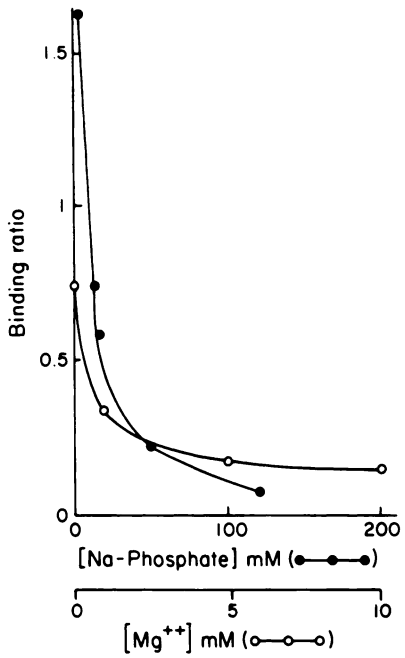

FIG. 5. *DNA-[^{14}C]metronidazole binding and ionic composition of reaction solution*

Native calf thymus DNA (1 μmole) was incubated with 2 μmoles of [^{14}C]metronidazole coreduced with 5 μmoles of dithionite in solutions with different amounts of sodium phosphate or $MgCl_2$. Binding ratio is defined in the legend to Fig. 3.

TABLE 3

Effect of pH on binding of reductively activated [^{14}C]metronidazole to calf thymus DNA

DNA (1 μmole) was incubated with 2 μmoles of [^{14}C]metronidazole at different pH values, and 5 μmoles of dithionite were added as described in MATERIALS AND METHODS. The ionic strength of the reaction solution was maintained constant for different pH levels by adjusting the NaH_2PO_4 concentration, and melting curve determinations indicated no denaturation of DNA at any pH employed.

pH	Binding ratio
	molecules/10^3 nucleotides
4.6	1.22
5.5	1.21
6.6	0.82
7.4	0.73

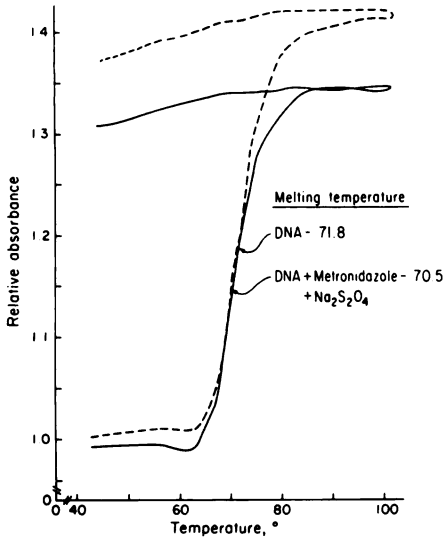

FIG. 6. *Melting curve of DNA alone* (——) *and after exposure to reductively activated metronidazole* (- - -)

Native, unsonicated calf thymus DNA (1 μmole), alone or incubated with 2 μmoles of metronidazole and 5 μmoles of dithionite added as described in MATERIALS AND METHODS, was chromatographed. Fractions containing DNA were dialyzed against 200 volumes of saline citrate at 4° for 16 hr before melting curve measurements were performed.

cleic acids with different amounts of guanine plus cytosine (Fig. 7).

Stability of binding of [^{14}C]metronidazole to nucleic acid and synthetic polynucleotides. An average of about 20% of bound ^{14}C was released after prolonged dialysis or repeat chromatography of the drug-macromolecule complex (Table 2). No additional label could be dissociated from a ^{14}C-native calf thymus DNA complex by the treatment for 4 hr at room temperature with either 1 M NaCl, 100 mM $MgCl_2$, 5% sodium dodecyl sulfate, 20% formamide, or 7 M urea. Heating this complex to 100° for 30 min or treating it with NaOH, pH 12, dissociated an additional 78% and 38% of the label, respectively, compared with the control.

Quantitation of binding of [^{14}C]metronidazole to BSA. Table 4 gives results for binding of metronidazole to both BSA and DNA. Relatively stable binding of unreduced metronidazole occurred to BSA but not to DNA. Coreduction with dithionite approximately doubled the binding of metronidazole to BSA, compared with results with the unreduced drug. Finally,

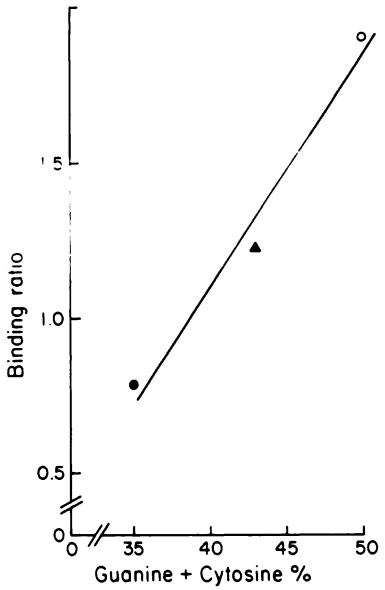

FIG. 7. *DNA-[^{14}C]metronidazole binding and guanine plus cytosine content of DNA*

One micromole of denatured T2 phage (●), calf thymus (▲), or *E. coli* (○) DNA was incubated with 2 μmoles of [^{14}C]metronidazole coreduced with 5 μmoles of dithionite. Binding ratio is defined in the legend to Fig. 3.

TABLE 4

Binding of [^{14}C]metronidazole to macromolecules

BSA (300 μg) or DNA (1 μmole) was incubated with 2 μmoles of [^{14}C]metronidazole, and 5 μmoles of dithionite were added as described in MATERIALS AND METHODS.

Substrate	Specific activity	Label lost after dialysis or rechromatography
	cpm/mg BSA or DNA	%
Serum albumin		
Unreduced metronidazole	1426	29.2
Coreduced metronidazole	2649	30.2
DNA		
Unreduced metronidazole	0	
Coreduced metronidazole	7000	14.0

binding of reductively activated metronidazole was approximately 3 times greater to DNA than to BSA.

DISCUSSION

Quantitation of binding of metronidazole to nucleic acid. The major finding of these studies is that metronidazole binds to nucleic acid when the drug has been activated by reduction. Presumably reduction of the nitro group produces a derivative or derivatives of metronidazole which can associate with DNA. The necessity for reductive activation is particularly significant since reduction appears to be a requirement for the cytotoxic, radiosensitizing, and mutagenic effects of this compound (2, 4). These results are in agreement with those of Willson *et al.* (7), who observed binding to DNA of metronidazole activated by γ-irradiation. Since these authors did not calculate a binding ratio, we cannot compare the extent of binding to DNA of metronidazole activated by different means.

While a large number of chemical compounds, especially environmental carcinogens and mutagens, have been shown to interact *in vivo* and *in vitro* with nucleic acids after oxidative activation (21), reductive activation is less common. Certain quinone-type antibiotics, such as streptonigrin (22–24), and mitomycin C (12, 14), as well as nitrofurans (25, 26), have been recognized in this category. Our studies now add metronidazole to this class of reductively activated, nucleic acid-modifying drugs.

Although the maximum binding between activated metronidazole and native calf thymus DNA was not great (approximately 1 molecule of drug per 1000 mononucleotide units), a low binding ratio is not an uncommon finding when the interaction of an unstable, activated drug with nucleic acid is studied *in vitro* [e.g., mitomycin C (14)]. In our particular system, the relatively low binding may reflect saturation of DNA binding sites, limited supply of activated drug, or limitations of the method. We tested the possibility that the low binding ratio might reflect saturable binding of activated metronidazole to terminal secondary phosphate groups of DNA. Removal of these groups from native calf thymus DNA by alkaline phosphatase had no effect on the binding ratio. This result, as well as the significant increase in binding after exposure of the same DNA to repeated additions of activated metronidazole, suggests that the relatively low binding of activated metronidazole to DNA in our system reflects primarily a limited concentration of activated compound rather than saturation of DNA binding sites.

Characteristics of binding of metronidazole to nucleic acid. The results of experiments testing the stability of the drug-nucleic acid complex suggest that the binding is predominantly covalent. Approximately 15–30% of the bound label was lost from the complex upon prolonged dialysis or rechromatography. However, no additional label was released upon exposure to a variety of conditions known to dissociate various noncovalent drug-DNA complexes. High salt (1 M NaCl) or Mg^{++} concentrations, for instance, break electrostatic bonds, while urea, sodium dodecyl sulfate, and formamide dissociate hydrophobic associations (see ref. 14 for review). The stability of the ^{14}C-DNA complex under all these conditions strongly supports a covalent linkage. The loss of the majority of label from the complex under the extreme condition of heating to 100° does not exclude the existence of a covalent bond, since covalently alkylated DNA is known to lose alkyl groups upon heating (27).

The inhibition of binding of metronidazole to DNA observed with Mg^{++} or increasing salt concentrations suggests that some electrostatic forces may also be involved in the binding process, implying that the active species may have a net positive charge. The greater binding at lower pH is consistent with this interpretation, suggesting that the active species is the protonated form of a weak base present to an extent inversely proportional to the pH. While other interpretations of these data are possible, we favor the proposed explanation, since it is entirely analogous to that reported for nitrogen mustards, another class of compounds which bind to DNA (28).

Failure of reductively activated metro-

nidazole to affect the renaturation curve of calf thymus DNA provides strong evidence against interstrand cross-linking as a mode of binding. Even at the relatively low binding ratios achieved in our system, one would expect evidence of renaturation of DNA upon cooling if cross-links were present (29).

Our studies using synthetic polynucleotides suggest specificity of drug binding for guanine and cytosine. Experiments employing the double-stranded duplex polynucleotides indicate that both guanine-cytosine polymers [poly(dG):poly(dC) and poly(dG·dC):poly(dG·dC)] bind by at least an order of magnitude more than the adenine-thymine polymers. Furthermore, the extent of binding to these double-stranded synthetic guanine-cytosine polynucleotides is approximately twice that found with the native, naturally occurring DNAs. This finding fits remarkably well with the guanine plus cytosine content being approximately twice as great in these synthetic polymers as in the natural DNAs employed. The specificity for guanine and/or cytosine suggested by these data is further supported by the experiments with single-stranded polymers, as well as by those employing natural DNAs containing different amounts of guanine plus cytosine.

The greater binding of metronidazole to denatured than to native DNA has been observed with other DNA-binding drugs (24, 30, 31). Presumably, loss of the hydrogen bond structures of the double helix facilitates binding in some fashion.

Additional studies appear warranted to clarify further the precise nature of the drug–nucleic acid interaction, as well as to investigate the effect of metronidazole on nucleic acid integrity.

Binding of [^{14}C]metronidazole to protein. Binding studies employing BSA were performed both to clarify the effect of reduction on the binding of metronidazole to protein, and to permit direct quantitative and qualitative comparisons of drug binding to protein and nucleic acid measured by the same technique. The binding of unreduced metronidazole to BSA in our system is quantitatively consistent with published data (32). After reduction by dithionite, binding to BSA approximately doubled, suggesting that modification of the nitro group may affect the binding of metronidazole to protein. Others have reported that alterations of the alkyl groups also may affect binding of unreduced metronidazole to plasma proteins (32). In our studies, binding of reductively activated metronidazole to DNA was approximately 3 times greater on a weight basis than to BSA under identical conditions. Thus our data suggest that metronidazole binds to DNA in a manner which is both qualitatively (i.e., reduction is necessary) and quantitatively different from the manner in which its binds to protein. These differences *in vitro* may be relevant to the biological effects of the drug.

Nature of active derivative of metronidazole. While the aim of these studies was not to characterize the active derivative of metronidazole which binds to nucleic acid, our work provides certain indirect information regarding its chemical nature. Reduction of metronidazole by dithionite is analogous to reduction by ferredoxin, which has been implicated in the reduction of metronidazole by sensitive organisms (4, 5, 33–35). Both agents transfer the same number of electrons (four) to metronidazole in similarly rapid fashion (4, 7). Complete reduction involving a 4-electron transfer by either agent should result in an end product or products on the over-all reduction level of hydroxylamine. Since these end products have never been isolated, their chemical nature remains conjectural. However, it is assumed that the primary reduction products are unstable and rapidly decompose (36). Our demonstration that binding is greater by a factor of 15 if reduction occurs in the presence of nucleic acid, rather than prior to exposing DNA to the fully reduced drug, is consistent with the major active derivative being short-lived and unstable.

Our data further suggest that the compound(s) which actually binds to DNA is an unstable derivative of the end product(s) of reduction rather than a partially reduced intermediate [i.e., a nitroso compound or the 1-electron addition product

observed in pulse radiolysis experiments (7)]. Indeed, if a partially reduced intermediate were the active derivative, one might anticipate increased binding when the drug was reduced by small portions of the reducing agent, as with mitomycin C (14). We were unable to demonstrate such an enhancement. Clearly, additional work will be necessary to isolate and characterize this active compound.

Finally, significantly less binding of metronidazole to DNA occurred if reduction was accomplished with sodium borohydride rather than sodium dithionite. This difference may reflect generation of different reduction products of metronidazole by these agents. While both compounds abolished the 320 nm absorption peak of metronidazole, indicating reduction of the nitro group, no information exists as to whether borohydride stops at the 2-electron reduction stage or proceeds further, as does dithionite, to the 4-electron stage. Preliminary paper electrophoresis experiments by us indicate qualitative and quantitative differences between the borohydride and dithionite reduction products of metronidazole.

In summary, our results suggest that reduction of metronidazole *in vitro* yields an unstable, positively charged breakdown product or products of the fully reduced compound which binds to mammalian, bacterial, and phage DNA, RNA, and synthetic polynucleotides. Binding is predominantly covalent, does not involve interstrand cross-links, and appears specific for guanine and cytosine. These data, as well as those of others indicating interaction of metronidazole with DNA (5-7), may be relevant to the cytotoxic, mutagenic, and radiosensitizing actions of this compound.

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