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THE INTERACTION OF REDUCED METRONIDAZOLE WITH DNA

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Abstract—Electrolytic reduction of metronidazole was carried out at a controlled potential under anaerobic conditions in the presence of calf-thymus DNA. The DNA was subsequently examined during the reduction process by viscometry, hyperchromicity, melting and renaturation profiles and by hydrolysis using DNAase 1. The reduced drug decreased the viscosity and thermal hyperchromicity of DNA, inhibited its renaturation and caused a 50 per cent increase in the length of oligonucleotides produced from DNA as a consequence of DNAase action. The results indicate that reduced metronidazole causes a loss of DNA helix content, strand breakage, and a concomitant impairment of its function as an enzyme template which may be due to the presence of altered bases or a drug–base complex. These findings can be used to explain the action of the drug both as a cytocidal antimicrobial agent and as a hypoxic call tumour radiosensitizer.

Metronidazole (1-2′-hydroxyethyl-2-methyl-5-nitroimidazole) is an antimicrobial drug with a remarkable spectrum of activity. Its clinical usefulness encompasses protozoal disease, e.g. trichomoniasis, [1], giardiasis [2], amoebiasis [3] and balantidiasis [4]. Also nematode infections, e.g. dracunculiasis [5], anaerobic bacterial infections such as Vincent's disease [6], and post-operative surgical infections particularly gynaecology [7, 8], and is in experimental use as a radiosensitizer of hypoxic cell tumours [9, 10].

The wide spectrum of activity of metronidazole is reflected in its unusually selective toxicity towards anaerobic micro-organisms and anoxic or hypoxic cells. Consequently, it is of importance to establish the drug's mechanisms of action in order to explain both its properties as an antimicrobial agent and as a hypoxic cell tumour radiosensitizer.

Previous studies in our laboratory have shown that nitroimidazole drugs including metronidazole are reduced via the nitro group in susceptible cells by a ferredoxin-linked clostridial-type phosphoroclastic reaction which results in the selective inhibition of H_2 gas evolution but not CO_2 [11, 12]. This, however, is not the lethal event within the cell since the inhibition of the phosphoroclastic reaction is transient and functions normally once all the drug has been reduced [13, 14].

Consequently it has been tacitly assumed that it is the reduction product of metronidazole which is the agent lethal to the cell and in this respect nucleic acid has been suggested as a possible site of action [15, 16]. It has been established that the drug inhibits DNA synthesis in *Trichomonas vaginalis* [15], and inhibits the synthesis of and degrades existing DNA in *Clostridium bifermentans* [16]. Recent evidence from studies *in vitro*, indicating that the chemically reduced drug destabilises the DNA helix [17] further suggests an involvement of reduced drug with DNA. However, results indicating strand-breakage of DNA with dithionite-reduced metronidazole are open to criticism since dithionite itself induces strand breaks in DNA [18], (Muller—personal communication) and it is therefore difficult to assess unambiguously breakage due to the reduced drug. Consequently, since La Russo *et al.* [19] find no significant strand breakage with dithionite-reduced metronidazole we have examined the effect of the electrolytically reduced drug on DNA integrity and present evidence for its degradation involving loss of helix structure and strand-breakage.

MATERIALS AND METHODS

Chemicals. Metronidazole (1-2′ hydroxyethyl-2-methyl-5-nitroimidazole) was a gift from May and Baker Ltd., Dagenham, U.K. Adenosine, 2-deoxyadenosine, AMP, ADP, ATP, cytosine, CMP, guanine, GMP, thymine, TMP, calf-thymus DNA type 1 and DNAase 1 (deoxyribonuclease oligonucleotidohydrolase. E.C. 3.1.4.5.) were obtained from the Sigma Chemical Co., London U.K., and trisodium citrate, sodium hydroxide and sucrose were obtained from Fisons Scientific, Loughborough, U.K. All other chemicals were obtained from Hopkin and Williams Ltd., Chadwell Heath, U.K.

Polarography and electrolytic reduction. Polarography of 100 μM metronidazole in 0.2 M Na_2HPO_4, NaH_2PO_4 buffer pH 7.0 was carried out under N_2 using a dropping Hg cathode (drop time was 3 sec) and Ag/AgCl anode at 25°. Voltage scanning was recorded from a digital multivoltmeter (Advance Instruments Ltd., London, U.K.) with sensitivity of ± 1 mV and current was recorded on a microammeter (Pye, Cambridge, U.K.).

Electrolytic reduction of metronidazole in the presence of DNA was carried out under N_2 using a Ag/AgCl anode and Hg pool cathode at a constant voltage of −900 mV and current 30 μA ± 5 μA

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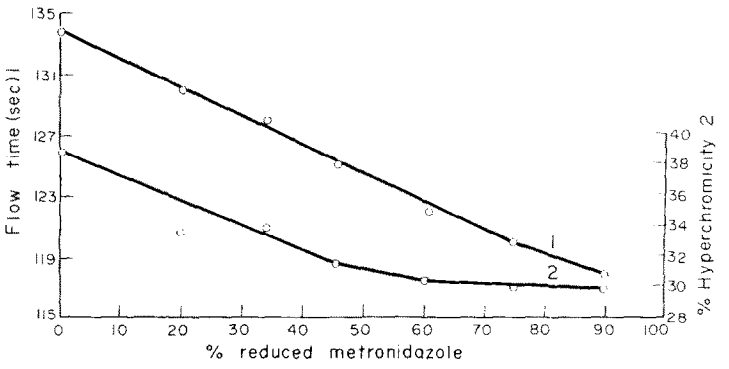

Fig. 1. The effect of reduced metronidazole on the viscosity flow time and thermal hyperchromicity of calf-thymus DNA. Viscosity was measured in an Ubbelohde-type miniature suspended level viscometer at 30 ± 0.01°. All flow time values are averages of triplicate readings recorded to an accuracy of 0.1 sec. Hyperchromicity was measured as the percentage increase in absorbance at 260 nm from 25° to 95°.

in 15 mM NaCl, 1.5 mM trisodium citrate, 1 mM EDTA pH 7.2. The reduction cell contained 15.0 mg calf-thymus DNA and 30 μmoles metronidazole in 100 ml buffer (drug–nucleotide ratio 1 : 0) at 25°. Samples were removed at intervals to determine the extent of drug reduction and for DNA analysis.

For the determination of the effects of reduced metronidazole on DNAase 1 activity the drug was reduced in 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.5 under the conditions specified above.

Viscometry. Viscometry measurements were performed with an Ubbelohde-type miniature suspended level viscometer (Poulton, Selfe and Lee, Wickford, U.K.). All measurements were made in a thermostatted viscometer bath (Grant Instruments, Cambridge, U.K.) at 30° ± 0.01°C. Flow times were averages of triplicate readings to an accuracy of 0.1 sec.

Spectrophotometry. Concentrations of DNA and metronidazole were measured spectrophotometrically using E^{M}_{260} (nucleotides) = 6600 for calf-thymus DNA [20], and E^{M}_{320} = 9310 for metronidazole [21].

Reduction of metronidazole was followed by measuring the decrease of absorbance at 320 nm with a Pye-Unicam SP-800 Series B recording scanning spectrophotometer.

Interactions of metronidazole and adenosine, 2-deoxy-adenosine, AMP, ADP, ATP, cytosine, CMP, guanine, GMP, thymine and TMP were measured spectrophotometrically in a Pye-Unicam SP-800 Series B recording scanning spectrophotometer (Pye-Unicam, Cambridge, U.K.).

Determinations of DNA T_m values, melting, and renaturation profiles were carried out using a Pye-Unicam 1750 Spectrophotometer fitted with a SP-1805 Programme controller. The cuvette chamber was heated electrically from a SP 876 Series 2 temperature programme controller at a heating rate of 0.25°/min. Cooling rates were uncontrolled but averaged 1.0°/min. Temperature and absorbance at 260 nm were automatically recorded on a Phillips PM 1820 X–Y recorder. All measurements of DNA hyperchromicities, melting and renaturation profiles were corrected for the presence of unreduced drug. No corrections in absorbance were made for the expansion of cuvette contents during heating.

DNA-ase 1 activity. Hydrolysis of calf-thymus DNA by DNAase 1 was carried out in 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.5 The reaction was followed spectrophotometrically at 260 nm and assessed as hyperchromicity. The hyperchromicity was related to oligonucleotide length following the method of Bernardi *et al.* [22]. Enzyme concentration was 50 μl of a freshly prepared solution containing 1 mg/ml DNAase 1. DNA concentration was 0.45 mg in a total reaction volume of 3.0 ml.

RESULTS AND DISCUSSION

The nature of reduced metronidazole is not known and speculation exists as to the active reduced species. The polarographic half-wave potential $E_{\frac{1}{2}}$ of metronidazole is −382 mV at pH 7.0 and this increases to a more negative potential with increasing pH—a behaviour characterized by the equation $E_{\frac{1}{2}}$ = 0.07–0.065 pH. At pH 7.0 a single polarographic wave occurs and by choosing a potential of −900 mV for electrolytic reduction it ensures that complete reduction occurs irrespective of slight pH increases during the reduction process.

The electrolytic reduction of metronidazole was followed spectrophotometrically where the absorbance at 320 nm due to the nitro group decreases making possible a quantitative measurement of reduction. During the reduction process samples were removed and the DNA examined viscometrically and by hyperchromicity as shown in Fig. 1. Here the decrease in flow time from 134 to 118 sec is precisely the difference in flow times between double stranded and single stranded DNA. However, the decrease is not due to the drug-induced strand separation to this extent because the decrease in hyperchromicity from 39 to 30 per cent is only about one quarter of what would be expected if total strand separation or helix–coil transition occurred. The decrease in viscosity thus represents a destabilization of the DNA helix only part of which involves strand separation. These effects are not observed with the unreduced drug, nor with DNA exposed to the reducing voltage for 5 days, nor with increases of pH up to 11.0.

There are two possible mechanisms which may explain the large decrease in flow-times—strand

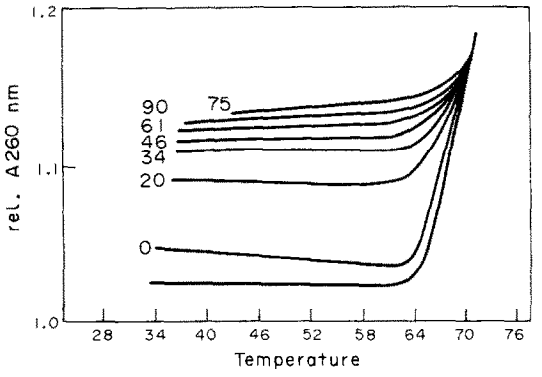

Fig. 2. Renaturation profiles of reduced metronidazole-treated DNA. Absorbancies of samples were measured at 260 nm after correction for unreduced drug absorbance. All samples were heated to the T_m value of the helix–coil transition (71°). Figures alongside each renaturation curve indicate the reduced drug–nucleotide ratio × 100. The unlabelled curve is the baseline heating curve for all samples, the remainder are cooling curves.

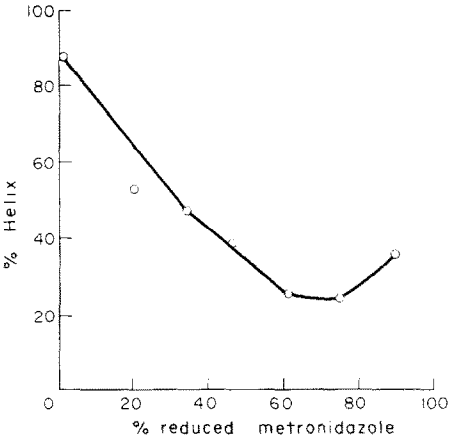

Fig. 3. Reduced metronidazole-induced loss of helix formation in calf-thymus DNA. Percentage losses of helix were calculated from the renaturation data taking a maximum thermal hyperchromicity of 40 per cent as equivalent to the loss of 100 per cent helix formation and a hypochromicity of 40 per cent on renaturation as a 100 per cent reformation of helix 30. The inhibition of renaturation as shown in Fig. 2 were thus directly converted into terms of helix loss at each concentration of reduced metronidazole. To obtain reduced drug–nucleotide ratios the percentage of reduced drug is divided by 100.

breakage and 'bending' of the helix [23]. Should strand breakage be the mechanism then DNA heated to partially unwind the strands would not renature as completely as untreated native DNA since strand fragments would be released from the molecule during heating. The results of such renaturation experiments are shown in Fig. 2 where the DNA has been heated to the mid-point of its helix–coil transition (the T_m value) and subsequently cooled. It is clear that as the concentration of the reduced drug increases, the ability of the DNA to renature is impaired or inhibited indicating a loss of helix formation at least up to 75 per cent reduced drug or a drug–nucleotide ratio of 0.75. The unreduced drug has no effect on the renaturation profiles.

A complete helix–coil–helix transition which occurs during the heating and renaturation process produces a maximum possible hyperchromicity of 40 per cent (our DNA exhibits a 38.8 per cent hyperchromicity) when the molecule is in the random coil conformation which disappears on renaturation. An increase in hyperchromicity on renaturation therefore indicates a loss of helix the extent of which can be calculated from the renaturation data as shown in Fig. 3. Here it can be observed that there is a 68 per cent loss of helix when DNA is exposed to 75 per cent reduced drug (a reduced drug–nucleotide ratio of 0.75). Further reduction to 90 per cent (reduced drug–nucleotide ratio 0.9) increases helix formation—an event which we assume to be due to the random renaturation of small released strand fragments. Thus reduced metronidazole induces extensive helix loss due to strand breakage—an observation which could easily explain the large decrease in viscosity flow time of drug-treated DNA. For these reasons we have not invoked the possibility of DNA 'bending' to explain the viscosity effects.

That helix loss is due to extensive strand breakage is shown by the passage of dialysable material from drug-treated DNA indicating the presence of small fragmented molecules. This degradation product or products has an absorption maximum of 275 nm—a peak which does not correspond to the spectral characteristics of any DNA component. It may thus represent a base which has been chemically altered by the drug, or a drug–base complex. The identification of this material is in progress.

No indications of specific interactions between the unreduced drug and adenosine, 2-deoxyadenosine, AMP, ADP, ATP, cytosine, CMP, guanine, GMP, thymine or TMP were observed spectrophotometrically in terms of hyper-or hypochromic, bathy-or bathochromic shifts. Such drastic changes in the structure of the DNA molecule may well alter its function as a template for DNA-specific enzymes, and experiments with DNAase 1 illustrate this. The enzyme is an endonuclease which preferentially hydrolyses between adjacent purines and pyrimidines and, using the conditions of Bernardi *et al.* [22], the enzyme activity can be measured as an increase in absorbance at 260 nm and this hyperchromicity related to the length of oligonucleotides produced as a result of the hydrolysis.

As is shown in Fig. 4 the effect of reduced metronidazole is to increase the average length of nucleotides produced from about 10 to 15 as the reduced drug concentration is increased. There is no apparent effect on the initial rate of the enzyme and the unreduced drug does not show these effects except at drug–nucleotide ratios exceeding five. Because the initial rate is not affected the increased oligonucleotide length would be expected if chemically altered bases or drug–base complexes existed which the enzyme was unable to recognise. The presence of existing strand breaks would tend to decrease the average nucleotide length and since this does not occur there may also be a direct effect on the enzyme itself. Further work to distinguish between these possibilities is in progress.

Since DNAase 1 is involved both in degradation

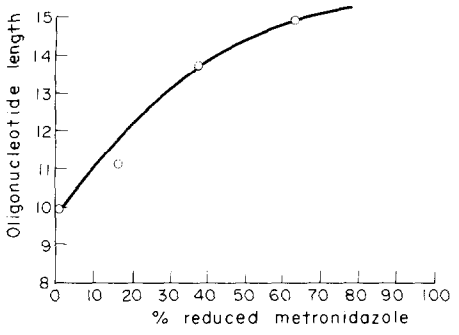

Fig. 4. The effect of reduced metronidazole on oligonucleotide length produced from the hydrolysis of calf-thymus DNA by DNAase 1. Hydrolysis was measured at 25° as an increase in hyperchromicity at 260 nm. The reaction mixture contained 0.45 mg DNA exposed to reduced drug and 50 μl freshly prepared 1 mg/ml DNAase 1 in 3.0 ml 50 nM Tris–HCl, 10 nM $MgCl_2$, pH 7.5. Hyperchromicity was converted into average oligonucleotide length using the Bernardi equation $22\bar{P}\ n^{-1} = 0.0028 \times HS + 0.025$, where $\bar{P}n^{-1}$ is the reciprocal average oligonucleotide length and HS the hyperchromicity measured as a percentage increase of the initial absorbance value at 260 nm. Reduced drug–nucleotide ratios may be obtained by dividing the drug concentration by 100.

and repair of DNA, the effect of reduced metronidazole may have important consequences for the cell in that it may be unable to repair lesions produced by the drug. It seems clear, however, that reduced metronidazole produces sufficient damage to DNA by loss of helix, strand breakage and a concomitant impairment of its function as an enzyme template possibly involving altered bases, to account for its microbicidal action and the observed degradation of DNA *in vivo* [16]. In this respect we have preliminary evidence for significant drug-induced single strand breakage in *Clostridium bifermentans* as evinced by alkaline sucrose density gradient centrifugation of labelled DNA, further details of which will be published elsewhere. These findings do, however, indicate that the events we have observed *in vitro* are relevant to the *in vivo* situation.

More significant is the application of these results to explain a possible general mechanism of radiosensitization. The nature of reduced metronidazole has not been established but pulse radiolysis experiments which generate the 1-electron radical anion have half-wave potentials which are close to the polarographic half-wave potentials for a 4 or 6 electron process. This suggests that a 1-electron reduction is the rate limiting step and the further steps (2-electron nitroso-, 4-electron hydroxylamino-, or 6-electron amino derivative) spontaneously occur in a cascade manner. Whatever the nature of the biologically active reduced species a recently proposed theory [17] suggests that because nitroimidazoles, nitrofurans [25] and bleomycin [25, 26] are all radiosensitizers, are all known DNA strand-breakers, and all require reduction before they are able to exert a toxic effect, these three parameters must be related. It would seem logical therefore, that since radiation itself causes DNA strand breaks [27], the combined action of radiation and drug-induced strand-breakage would potentiate the effect which is observed as radiosensitization.

The major pre-requisite for this theory is that nitroheterocyclic drugs should be reduced within anoxic or hypoxic tumour cells. That this situation exists is suggested by the work of Willson *et al.* [28] who showed that radiation not only reduces metronidazole and induces binding to DNA but only does so under anoxia or hypoxic conditions. Verification comes from Flockhart *et al.* [29] who has demonstrated that misonidazole (Ro-07-0582) a 2-nitroimidazole radiosensitizer is reduced in hypoxic tumour cells. Thus it would appear that the cytotoxic action of metronidazole both as an antimicrobial agent and as a radiosensitizer are closely related.

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