Effects of Ionising Radiation on Deoxyribonucleic Acid. Part 2.[1] The Influence of Nitroimidazole Drugs on the Course of Radiation Damage to Aqueous Deoxyribonucleic Acid

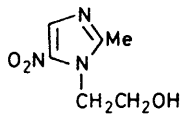
Philip J. Boon, Paul M. Cullis,* Martyn C. R. Symons,* and Brendan W. Wren
Department of Chemistry, The University, Leicester LE1 7RH

Exposure of frozen aqueous solutions of calf-thymus DNA to ^{60}Co γ-irradiation at 77 K gave electron-gain centres localised on thymine (T^-) and electron-loss centres localised on guanine (G^+), in approximately equal yields as judged from their e.s.r. spectra. The presence of metronidazole or misonidazole resulted in a marked reduction in the yield of T^-, the drug anions being detected in their place. The yield of G^+ was *not* enhanced, in contrast to the results of others. Similar treatment of frozen aqueous solutions of plasmid DNA (pBR 322) was shown by gel electrophoresis to result in extensive single- and double-strand breaks in the absence of drug. Inclusion of metronidazole significantly reduced the number of strand breaks, double-strand breaks being reduced by a proportionately greater amount. An explanation is offered for this. At the molecular level these drugs clearly *protect* DNA from radiation damage (at least in terms of radical yield and strand breaks), despite the fact that they are known to be efficient radiosensitisers at the cellular level. These observations are discussed with regard to the various proposals for the mechanism of action of these drugs.

In Part 1 we outlined a procedure for the study of primary radiation processes in DNA.[1] The method involves trapping primary radical products at 77 K for e.s.r. study, following their reactions on annealing, and estimating the resulting chemical damage, in the form of strand breaks, using an assay based on plasmid DNA. These conditions are designed to probe the direct effects, since the radiolysis products of water, especially hydroxyl radicals, are almost entirely trapped in the ice crystallites and cannot interact with DNA. Under these conditions it has been established by e.s.r. spectroscopy that the site of electron loss is guanine (giving G^+) and the site of electron gain is thymine (giving T^-).[2–4] We were able to demonstrate that single- and double-strand breaks occur at significant levels under the conditions where only G^+ and T^- are detectable. This strongly implies that these radicals can be the precursors of strand breaks; however, the reactions that convert G^+ and T^- ions into strand breaks are as yet unknown. Oxygen was shown to compete with DNA for electron gain thereby reducing T^-. In our system, oxygen, a powerful radiosensitiser *in vivo*, was shown to be only a modest sensitiser with respect to strand breaks. Since certain electron-affinic nitroimidazole drugs are known to be radiosensitisers *in vivo*, we have employed these techniques to determine whether this effect arises from modification of the damage to DNA resulting from direct ionisation.

Nitroimidazoles such as metronidazole (MET) (**1**) and misonidazole (MIS) (**2**) have been shown to be efficient radiosensitisers at the cellular level.[5,6] (Metronidazole is also used as an antimicrobial drug which is active against bacteria and protozoa.[7]) Since radiosensitising action appears to parallel the electron affinities of these compounds, the radical anions have been implicated as possibly significant intermediates.[8]

A previous report that the yield of G^+ is strongly enhanced by the presence of these nitroimidazoles[9] is of potential significance in terms of the radiosensitising action of these compounds, particularly if it could be established that G^+ centres lead to more significant damage than T^- centres. Indeed, these results can be taken as strongly supporting the first and most widely accepted theory for sensitising action.[10] According to this theory electrons and 'holes' can move along the DNA axis *via* charge transfer between the stacked bases, and by this process the quantum yield of such centres is normally



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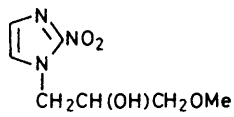

(2)

reduced by electron return. In the presence of an additive with high electron affinity, electrons are more deeply trapped, return is prevented, and hence the yield of centres is enhanced. This would nicely explain the greatly enhanced yield of G^+ reported by Graslund *et al.* For the above explanation to be consistent, we would have expected that the large increase in G^+ would be accompanied by a comparable amount of MET anion, since electron gain must equal electron loss. Thus there should be a correspondingly large increase in *total* radical yield. It is puzzling that this is apparently not the case.[11]

Having found little evidence for extensive electron return in our previous study on the effect of oxygen on radiation damage to DNA,[1] and because of the aforementioned anomaly, we planned in the present work to investigate the manner and the extent to which nitroimidazole drugs may modify the direct damage mechanism of γ-irradiation-induced damage to DNA in frozen aqueous systems.

Experimental

Materials.—Plasmid DNA (pBR 322) was isolated according to the procedure of Birnboim and Doly.[12] Typically, pBR 322 DNA preparations contained *ca.* 95% of the superhelical Form I DNA. Tris buffer, ethylenediaminetetra-acetic acid (EDTA), ethidium bromide, metronidazole, and calf thymus DNA (for e.s.r. studies) were obtained from the Sigma Chemical Company. Agarose-ME was obtained from the Miles Laboratory. [$^2H_3$]Methyl alcohol (99.5 atom%) and 2-methyltetrahydrofuran were purchased from the Aldrich Chemical Company. Di-t-butyl nitroxide was obtained from Lancaster Synthesis Ltd. Misonidazole was a gift from Professor G. E. Adams.

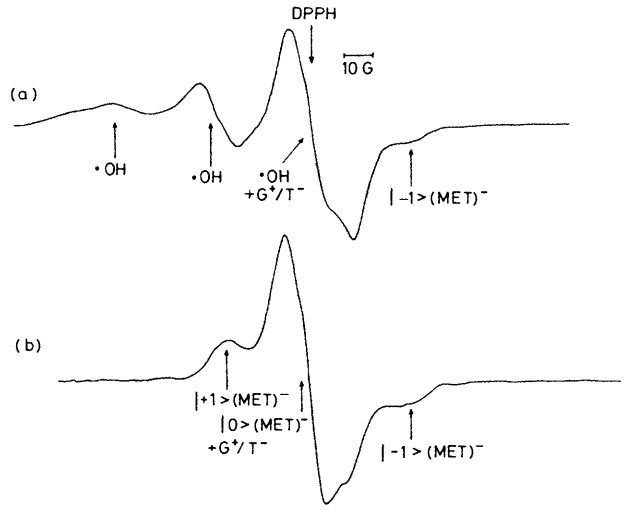

Figure 1. First-derivative *X*-band e.s.r. spectra for aqueous solutions of DNA (100 mg ml^{-1}) in the presence of metronidazole (5 mmol l^{-1}) (a) after exposure to ^{60}Co γ-rays at 77 K, and (b) after annealing the above sample to *ca.* 130 K to remove features due to OH·

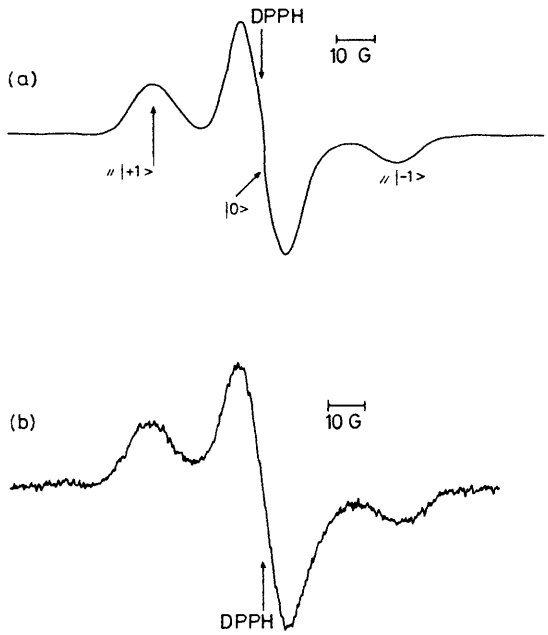

Figure 2. First-derivative *X*-band e.s.r. spectrum of metronidazole (a) in an aqueous [$^2H_3$]methyl alcohol glass after exposure to ^{60}Co γ-rays at 77 K, showing features for the metronidazole anion, and (b) metronidazole anion spectrum obtained by further annealing of the sample from Figure 1 to remove DNA radicals

γ-Irradiation and Assays for DNA Breaks.—The assay used has been described in our previous publication.[1] Slight variations were introduced in order to optimise the separation of the linear Form III DNA. A solution of the appropriate nitroimidazole (10 μl; 50 mmol l^{-1}) was added to the plasmid DNA solution (90 μl; 80 μg ml^{-1}) in Tris HCl buffer (10 mmol l^{-1}; pH 7.6) containing EDTA (1 mmol l^{-1}). Samples with and without nitroimidazole additive were γ-irradiated in a ^{60}Co source under the appropriate conditions. Oxygenated and deoxygenated samples were prepared by purging with oxygen and oxygen-free nitrogen for 60 min, then sealed and irradiated. Following irradiation a dye–EDTA mixture (6 μl; 56% glycerol v/v; 50 mmol l^{-1} EDTA; 0.05% Bromophenol Blue w/v) was added and portions were removed for analysis by agarose gel electrophoresis.

Gel Electrophoresis.—Samples of irradiated DNA (0.7—1 μg) were layered on agarose slab gels (1.3%) and subjected to electrophoresis in a horizontal slab gel apparatus for *ca.* 16 h at room temperature, using Tris buffer (90 mmol l^{-1}; pH 8.3) containing boric acid (90 mmol l^{-1}) and EDTA (2.5 mmol l^{-1}). After electrophoresis the gels were stained with ethidium bromide (2.5 μg ml^{-1}) in electrophoresis buffer for at least 15 min. The stained gels were then excited with a transilluminator and photographed with a Polaroid MP-4 Land Camera using a red filter (Kodak Wratten No. 9) and Polaroid type 55 film. The negative films of gels were used for densitometric scanning.

Quantitation of Single- and Double-strand Breaks.[13]—The negative films of the ethidium bromide-stained gels were scanned using a u.v.–visible spectrophotometer with gel-scanning attachment (SP 8-100 Pye Unicam). The superhelical Form I of the plasmid takes up less ethidium bromide than the other two forms. We have used a staining efficiency of 81% (as demonstrated for pBR 322 DNA[14]) to normalise our data. No further uptake of ethidium bromide was observed on prolonged incubations. All samples were analysed in duplicate and average values are reported in the Results section.

γ-Irradiation and E.s.r. Measurements.—Frozen samples were prepared by cooling in liquid nitrogen a Pyrex tube containing a solution of DNA (50 mg ml^{-1}; Type I sodium salt DNA, Sigma) with and without nitroimidazole (2 mmol l^{-1} additive unless otherwise stated). Samples were irradiated as already described, and the e.s.r. spectra recorded (*X*-band Varian E-109 spectrometer, 100 kHz field modulation). Spectra were stored and used for computer simulations and subtractions as outlined previously. *G*-Values were estimated by comparison with the double integral value taken from a spectrum of di-t-butyl nitroxide of known spin concentration.

Results

Radical Yields.—Irradiation of frozen aqueous solutions of DNA at 77 K gives rise to an e.s.r. spectrum that comprises features for the OH· radicals in ice crystallites together with features from DNA radicals (T^- and G^+). In oxygenated solutions, O_2^- features are also present.[1] In the presence of MET or MIS, at drug : base ratios in the neighbourhood of 1 : 80, the high-field $M_1 = -1$ parallel feature of the drug anion is also clearly defined [Figure 1(a)]. On annealing to *ca.* 130 K the OH· radicals in ice are lost irreversibly. This event is confined to the ice crystallites and does not affect the signals of the organic radicals [Figure 1(b)]. To confirm that the remaining spectrum comprises the drug anion together with T^- and G^+ we have prepared MET^- radicals by irradiating dilute solutions of MET both in methyltetrahydrofuran and in aqueous deuteriated methanol [Figure 2(a)]. The resulting spectra are very similar to that which is found in the DNA systems after annealing to *ca.* 185 K, where the DNA radicals have all been lost [Figure 2(b)]. This confirms that we do indeed generate the drug anion. The e.s.r. parameters for the MET^- are given in the Table.

We have used the spectrum of the drug anion [Figure 2(b)] for computer subtraction in order to determine the effect of the drug on the DNA radicals; the result is shown in Figure 3(a). The spectrum after subtraction of the features for MET^- apparently still comprises just those for T^- and G^+, with G^+ centres being in excess over T^- centres as would be expected if MET competes with T for electron capture. A reasonable

Table. ^{14}N Hyperfine coupling and estimated orbital population for the NO_2 group in MET^- and nitrobenzene anions.

	^{14}N Hyperfine coupling (G)[a]			Orbital populations[b]	
Anions	$A_{\parallel}$	$A_{\perp}$	A_{iso}	a_s^2 (%)	a_p^2 (%)
MET^-	32	5.3	4.2[c]	2.6	54
$PhNO_2$[d]	27.6	6.5	13.5	2.4	43

[a] 1 G = 10^{-4} T. [b] Estimated as described in 'Chemical and Biochemical Aspects of Electron Spin Resonance Spectroscopy,' M. C. R. Symons, Van Nostrand Reinhold, New York, 1978. [c] From ref. 18. [d] From ref. 21.

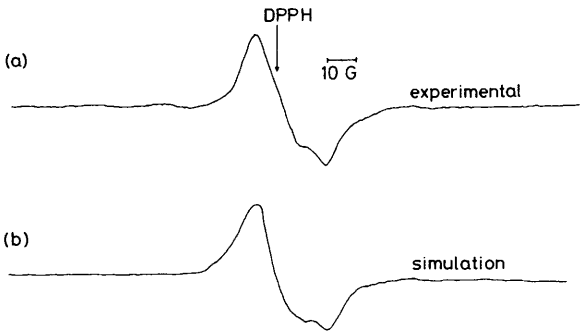

Figure 3. (a) First-derivative *X*-band e.s.r. spectrum derived by subtraction of Figure 2(b) from Figure 1(b). The residual spectrum is composed of the DNA radicals, G^+ and T^-. (b) Computer simulation of spectrum 3(a) by addition of G^+ and T^- in the ratio *ca.* 2:1, respectively

simulation of this spectrum was obtained by addition of authentic spectra of G^+ and T^- in a ratio of *ca.* 2:1, respectively [Figure 3(b)]. This therefore indicated a substantial loss of T^- and little change in the yield of G^+. The charges are balanced by production of MET^- in place of T^-. These results are repeatable and we are satisfied, in particular, that there is *no* substantial gain in G^+. Double integration of the initial spectrum indicated that the total radical yield increased owing to the presence of MET. We estimate that the total radical yield is enhanced by a factor of 1.5, which is in good agreement with previous studies.[11]

MET and MIS behaved comparably in our systems, despite the differences in their electron affinities. Both drugs were observed to affect the e.s.r. spectra at a critical concentration of *ca.* 2 mmol l^{-1}; concentrations below this produced little effect and for example at 0.2 mmol l^{-1} MET the drug anion was not detected.

In oxygenated solutions in the presence of nitroimidazole drugs, the results were initially unchanged. However, on annealing, the normal growth of signals from RO_2· radicals was greatly reduced by the presence of drug. Also the temperature at which the drug anions were lost was reduced from *ca.* 223 to *ca.* 203 K by the presence of oxygen.

In the absence of oxygen and additive, on annealing, we detected the protonation of T^- to give the characteristic octet of the thymin-5-yl radical TH·. However, under anaerobic conditions in the presence of MET we did not observe TH· formation on annealing.

Strand Breaks.—Our assay for strand breaks (both single and double) has been described in detail elsewhere.[1] Plasmid DNA can be extracted as the superhelically coiled Form I. The introduction of a 'nick' (a single-strand break) allows the superhelical twists to unwind to give the 'relaxed' Form II of the

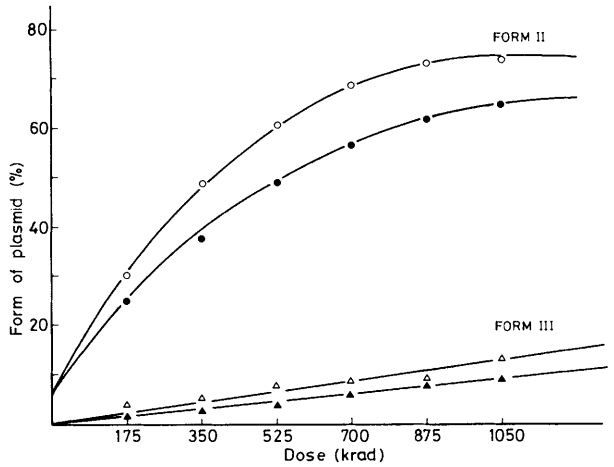

Figure 4. The effect of metronidazole on strand-breaks induced by γ-irradiation of plasmid DNA (pBR 322) at 77 K. The percentage of Form II indicates single-strand breaks produced in the presence (●) and absence (○) of metronidazole. Double-strand breaks formed in the presence (▲) and absence (△) of metronidazole are indicated by Form III

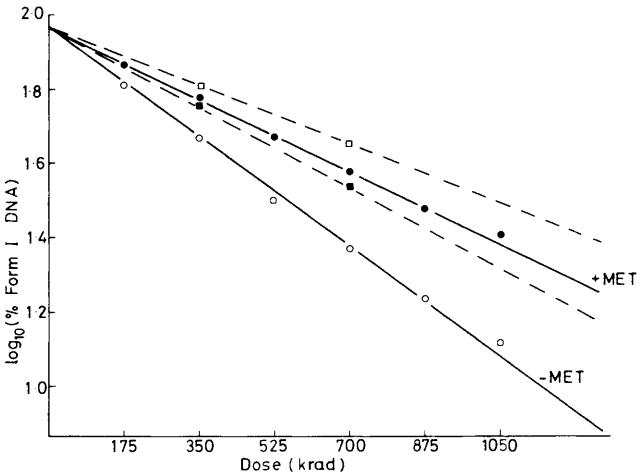

Figure 5. Semi-log plot of single-strand breaks induced in plasmid DNA (pBR 322) following irradiation with ^{60}Co γ-rays at 77 K in the absence (○) and in the presence of metronidazole under ambient (●), oxygenated (■) and deoxygenated (□) atmosphere

plasmid. If both strands are broken at a coincident site (a double-strand break) the linear form of the plasmid, Form III, is obtained. The easy separation and quantitation of these three forms of the plasmid by gel electrophoresis offers a convenient and accurate assay for damage introduced by ionising radiation.

The overall results are summarised in Figure 4. Using concentrations in the mmolar range, we have found that MET and MIS both *reduce* the number of single- and double-strand breaks in comparison with controls in which the drugs have been omitted. The magnitude of the effect is dependent on the drug concentration. However, the effect reaches saturation in the range 5—10 mmol l^{-1} in terms of both strand breaks and e.s.r. studies. Estimating the numbers of double-strand breaks is somewhat inaccurate because of the relatively small amounts of the linear form of the plasmid generated under these irradiation conditions. However, there does appear to be a greater

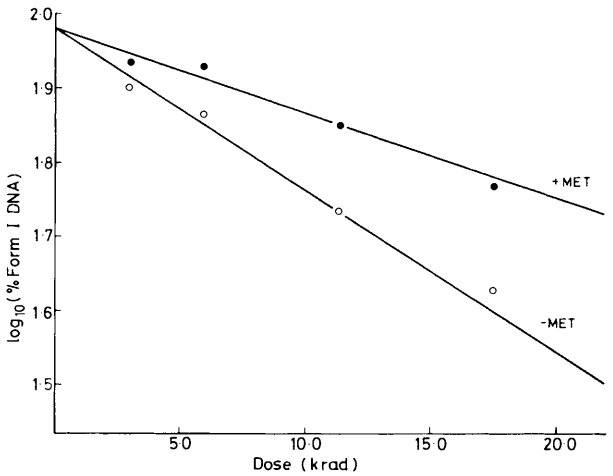

Figure 6. Semi-log plot of single-strand breaks induced in plasmid DNA (pBR 322) following irradiation at room temperature in the presence (●) and absence (○) of metronidazole

proportional decrease in the number of double-strand breaks as compared with single-strand breaks.

The ability of metronidazole to *reduce* the numbers of strand breaks could be demonstrated both in the absence and in the presence of oxygen (Figure 5). The effects of oxygen and nitroimidazole are apparently opposite and approximately additive. The effect of nitroimidazole on strand breaks is obviously more pronounced under anaerobic conditions.

Although not directly related to our e.s.r. studies, we have also investigated the effect of electron-affinic nitroimidazoles on the extent of damage to DNA brought about by irradiation of a dilute aqueous solution of plasmid DNA at room temperature. Here the principal mode of damage is *indirect*, mediated through hydroxyl radicals. Under these conditions we find that metronidazole still *reduces* the numbers of strand breaks (Figure 6), in agreement with the results of Lohman and his co-workers.[15] Thus its *in vitro* effect on DNA appears to be in the opposite sense to its *in vivo* action. It has been suggested that MET acts as a hydroxyl-radical scavenger under these conditions.

Discussion

If, under conditions of direct damage to DNA, G^+ and T^- are the primary source of damage then a reduced yield of these radicals would naively be expected to reduce the extent of the damage. We have already shown that in systems where only G^+ and T^- are initially detected by e.s.r. spectroscopy, strand breaks persist at significant levels. This suggests that these radicals are capable of producing strand breaks by a pathway as yet undefined. Using our plasmid DNA assay we have demonstrated in this study that both single- and double-strand breaks are significantly reduced by the presence of metronidazole. Previous failures to detect any effects of the drug on yields of strand breaks are undoubtedly associated with the large errors involved in the analytical methods. The use of plasmid DNA to assay for strand breaks is considerably more accurate.

From our e.s.r. studies we conclude that the major effect of these drugs is to capture electrons, thereby reducing the concentration of T^- in a 1:1 manner. This was indeed the expected behaviour for these highly electron-affinic molecules. The concentration of G^+ is largely unaffected. From our observations it appears that additives capable of reducing the yield of DNA radicals should also reduce the yields of strand breaks; this is indeed the case with these nitroimidazoles. There is an important caveat to this: when the radicals derived from the additive itself are highly reactive then other pathways involving for example hydrogen-atom abstraction from the sugar moiety by the drug radicals may make a significant contribution. MET^- is expected to be a relatively unreactive radical, and in our e.s.r. studies it persists at significantly higher temperatures than the DNA radicals. The pathway by which it decays is unknown.

The most important aspect of this study is that we find no evidence for the previously reported[9] 2—3 fold increase in yield of G^+. In the study by Graslund *et al.*[9] of partially orientated DNA ribbons doped with MET, they conclude that in addition to the formation of the drug anions, there is a reduction in the yield of T^- and a major increase in the yields of G^+, by factors as large as 2—3. In a subsequent study Graslund found that the total radical yield in non-orientated solid DNA was only enhanced by a factor of 1.4 by the addition of MIS.[11] She also observed that there was no detectable change in numbers of single-strand breaks on addition of MIS. Washino *et al.*[16] also found an increase of 30—40% in the yield of radicals formed in DNA in the presence of MIS. Outer parallel features assigned to the drug anion were detected in both studies but no attempts were made to establish this by deriving the complete spectrum of the anions. Our systems do differ, and it is possible that with dry, orientated DNA, the conduction process is more important, thus explaining the enhancement in G^+. However, if that were so what should really have been observed in the orientated systems is a markedly lower yield of T^- and G^+ in the absence of electron acceptors, which apparently is not the case. In terms of total radical yield we estimate that the presence of MET results in a small enhancement. Our enhancement factor of 1.5 is in good agreement with those reported by others,[11,16] particularly in view of the large errors involved in double integrations. Any substantial increase in G^+ concentration would have to be mirrored by a similar gain in anionic centres. Thus, unless there is some form of spin pairing confined to the anionic centres, which is improbable, there should be a corresponding large gain in total radical yield. This is clearly not the case.

The small increase in total radical yield is somewhat puzzling. One possible explanation for this increase is that the inclusion of a large concentration of solute (nitroimidazole) increases the glassy regions surrounding the DNA which would effectively increase the irradiation volume. Alternatively, there may be some of the drug cation produced in addition to the major MET^- component. Unfortunately we have been unable to generate authentic drug cations using our recently developed methodology[17] because of the lack of solubility of these polar molecules in Freon solvent. The successful computer subtractions using MET^- alone (Figure 3) suggest that no MET^+ is present, but we have no definitive evidence on this point. We consider that our estimates of the effect of MET on the concentration of G^+ and the total radical yield are self-consistent, and do not accord with extensive electron return.

In Part 1 we suggested that the remarkably high percentage of double-strand breaks detected in these studies arises because electron-loss and electron-gain centres are formed initially close together.[1] Electron loss is indiscriminate, but loss from sugar or phosphate moieties or solvating water molecules is evidently followed by rapid electron-transfer from adjacent bases, and the loss-centres end up as G^+. If G^+ and T^- centres are too close, electron return would be expected. Furthermore, if they were trapped close together they would constitute triplet state radical pairs which were not detected by e.s.r. To account for the high incidence of double-strand breaks we postulated that a significant proportion of G^+/T^- pairs are trapped between *ca.* 15 and 30 Å and that these, when present in opposite strands,

are responsible for most of the double-strand breaks. This, of course, requires that both G^+ and T^- centres can ultimately lead to strand breaks. If this postulate is correct, when MET^- is formed in place of T^- we would expect a greater percentage decrease in the yield of DSB relative to that for SSB. A random 50% reduction in T^- would lead also to the same percentage reduction in double-strand breaks; however, since G^+ is largely unchanged and providing T^- and G^+ form strand breaks with comparable efficiency, a 50% reduction in T^- reduces single-strand breaks by only 25%. From the results summarised in Figure 4 this appears to be the case.

In the presence of oxygen, electron capture by MET or MIS dominates. On annealing, oxygen becomes mobile at *ca.* 193 K and reacts with localised radicals such as TH• to give RO_2•, with characteristic spectra. Whilst we do not expect the delocalised radicals T^- and G^+ to give RO_2• directly, our previous results[1] indicated that G^+ radicals are indeed converted into RO_2•, presumably *via* a precursor such as •GOH, which immediately reacts with O_2. Unfortunately we have not been able to detect •GOH even in oxygen-free systems. The reduced yield of RO_2• in oxygenated systems obviously reflects the reduced yields of T^- and G^+.

The enhanced rate of loss of MET^- in the presence of oxygen is probably due to the charge-transfer reaction (1). It has been

$$MET^- + O_2 \longrightarrow MET + O_2^- \qquad (1)$$

pointed out to us that the reduction potentials for oxygen (−150 mV) and for MET (−486 mV) at pH 7 indicate that the charge transfer should be favourable, which nicely explains the enhanced rate of loss of MET^- under aerobic conditions. The failure to detect TH• formation from T^- under anaerobic conditions in the presence of MET may indicate a thermal charge transfer between T^- and MET (2). The alternative

$$T^- + MET \longrightarrow T + MET^- \qquad (2)$$

explanation would be that the presence of MET prevents protonation of T^-; however, it is difficult to imagine a mechanism by which this would occur.

Structure of Nitroimidazole Anions.—The radical anion of MET in aqueous solution has been studied by several workers using e.s.r. spectroscopy and pulse radiolysis techniques.[18–20] Two species with slightly different e.s.r. parameters were detected for MET. Ayscough *et al.*[20] suggested that the species studied by Willson *et al.*[18] is really a radical adduct rather than the anion. If this is correct, it is surprising that the ^{14}N coupling for the nitro group is smaller for the adduct than for the anion, whereas the reverse is normally expected. For MET there was also a well defined proton coupling of *ca.* 5.3 G whereas for MIS the only large coupling was to the nitrogen nucleus of the nitro group. We know of no direct solid-state e.s.r. studies of these anions which are pertinent to the present work.

The foregoing results for MET^- give incidental, useful structural information which should be reported. From our data and the liquid-phase results[20] we can estimate the anisotopic hyperfine coupling to ^{14}N (NO_2) and hence obtain approximate orbital populations (Table). The results show that the spin-density on nitrogen is *ca.* 56.6%, which is remarkably high relative to that for other nitro-anions. Thus, for example, that for nitrobenzene anions, also listed in the Table, is only *ca.* 45.4%.[21]

Conclusions

Our results appear to indicate that metronidazole does not increase damage to DNA either in terms of DNA radicals or in terms of strand breaks. As far as *in vitro* systems are concerned these highly electron-affinic additives appear to function as protection agents. Since these nitroimidazoles are efficient radiosensitisers *in vivo*, our results suggest that this clinical property does not arise from simple direct enhancement of radiation damage to DNA. Alternative suggestions for the mode of action of these drugs have been put forward; in particular there is a suggestion that one of the reduction products (reduction of the nitro group) is responsible for its radiosensitising action.[22] This suggestion is appealing since it may also be this reduction product that is responsible for the radiation-independent antimicrobial activity of these drugs. It is active against anaerobic microbes where reduction of the drug would be favoured.

Acknowledgements

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