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# EVIDENCE FOR THE DIRECT INTERACTION OF REDUCED METRONIDAZOLE DERIVATIVES WITH DNA BASES

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**Abstract**—The electrochemical behaviour of the bioreductive redox active nitroimidazole drug metronidazole has been examined in the presence and absence of the DNA bases using three electrochemical techniques, all of which indicate the capacity for interaction between reduced products and DNA bases. The 4-electron metronidazole ($RNO_2$) metronidazole-hydroxylamine (RNHOH) couple in an aqueous medium shows a positive shift in reduction potential upon addition of thymine, adenine and guanine, but a negative shift for cytosine. Interpretation of these results for an irreversible process is, however, inconclusive. In dimethylformamide/$H_2O$ the presence of DNA base on the one-electron addition product, the nitro radical anion, was examined by cyclic voltammetry. All except guanine resulted in interaction with the metronidazole nitro radical anion ($RNO_2^{\cdot-}$), as measured by the decrease in the return-to-forward peak current ratio, in the following order of increasing reactivity: cytosine, adenine and thymine (at a metronidazole : base ratio of 1 : 1). The increase in the stability of the radical anion by increasing the pH of the dimethylformamide/$H_2O$ medium resulted in a decreased reaction with thymine.

*Key words:* metronidazole; reduction products; DNA bases; voltammetry

The nitro-aromatics are an extremely important class of compound. They are extensively used in the treatment of anaerobic infections and are under continuing investigation regarding their use in cancer therapy, acting as specific cytotoxins, and more recently, as markers for hypoxic regions in tumours [1]. The reductive activation of the nitro group is a necessary initiation step in their biological function, in whatever capacity, although the precise identity of the active form is unknown [2]. Damage to DNA results, in the form of strand breaks and helix destabilisation [3]. The study of the initial interaction of nitroheterocyclic redox active drugs with DNA is particularly difficult because binding when it occurs is not related to DNA damage [4, 5]. Current opinion favours that there is an interaction involving a reduced free radical drug derivative and the DNA phosphodiester backbone, resulting in oxidation of the sugar and/or bases which lead to strand breaks. Since the interaction involves the passage of electrons from DNA to the free radical no binding occurs [6].

Electrochemical techniques are able to detect and quantify the interaction between a reduction product and its target (e.g. DNA bases) as interaction will result in modifications to the current–voltage response. In order to understand more fully the drug–DNA interaction, and consequently the overall biological action of this class of compound, we have compared the voltammetric behaviour of the 5-nitroimidazole, $RNO_2$†, in the presence and absence of DNA bases of varying concentrations. This has been analysed using three electrochemical techniques; d.c. polarography, differential pulse polarography and cyclic voltammetry. In all methods the redox active compound is subjected to a voltage scan. When a voltage is reached at which reduction is possible the transfer of electrons to the compound results in an increase in the current. In d.c. polarography the process is recorded as a sigmoid wave, the mid-point of which is the half-wave potential or $E_{\frac{1}{2}}$. In differential pulse polarography the sigmoid curve is differentiated so the mid-point of the reduction wave appears as a peak. In cyclic voltammetry after traversing the potential region of interest the direction of the scan is reversed, and the electrode reactions of intermediates and products formed on the forward scan can often be detected. Cyclic voltammetry is, therefore, extremely useful for measuring the stability or reactivity of reduction products, as reflected in the return-to-forward peak current ratio. Figure 1 shows the various types of behaviour recorded using each technique, the features of which are described in the legend. Addition of a target compound with which a reduced species interacts will induce changes to the voltammetric response, measured as shifts in redox potential, changes to peak height and, in particular, as alterations to the lifetime of the reduction product.

The reduction of a homocyclic or heterocyclic nitro compound is complex. Under appropriate conditions the nitro group can accept a single electron to yield the radical anion, which is most stable under alkaline conditions. Two- and four-electron additions form the nitroso and hydroxylamine derivatives, respectively; a further two-electron addition produces the amine, being the

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† Abbreviations: $RNO_2$, metronidazole; RNHOH, metronidazole-hydroxylamine; $RNO_2^{\cdot-}$, metronidazole nitro radical anion.

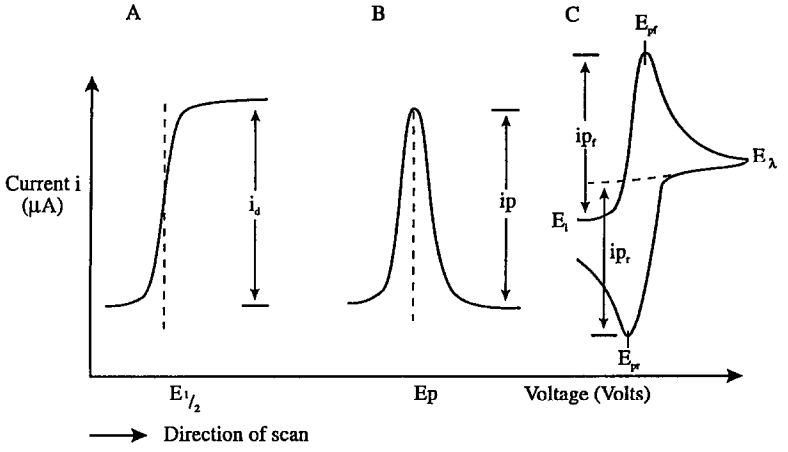


Fig. 1. Typical voltammetric response for a reversible charge-transfer reaction. (A) dc polarogram: $E_{\frac{1}{2}}$ mid-point potential of the wave; $i_d$, diffusion limiting current. (B) Differential pulse polarogram: Ep, peak potential; ip, peak current. (C) Cyclic voltammogram: $E_{\frac{1}{2}}$, mid-point between the forward ($Ep_f$) and return ($Ep_r$) peak potentials; Ei, initial or starting potential; Eλ, switching potential; $ip_f$ and $ip_r$, the forward and return peak current responses, respectively.

result of a total six-electron reduction. However, the reduction pathway is not straightforward. With 5-nitroimidazoles, for example, reduction produces the nitrite ion which is a decomposition product of the nitro radical anion or its protonated form. In addition, the reduction pathway is influenced by the proticity of the medium. Thus, the nature of the electrolytic medium influences the reduction pathway [3]. In an aqueous solvent a single four-electron step is observed, from the nitro to the hydroxylamine, the $RNO_2$/RNHOH couple. In a mixed dimethylformamide/$H_2O$ solvent, a two-stage reduction is found, involving first a one-electron addition, to the radical anion, the $RNO_2/RNO_2^-$ couple, followed, at more negative potentials, by an irreversible three-electron addition to give the hydroxylamine, the $RNO_2^-$/RNHOH couple. The one-electron addition step to give the nitro radical anion is chemically and electrochemically reversible, and kinetic and lifetime measurements on $RNO_2^-$ have been made [7, 8]. Consequently, the efficiency of and the direction of the reduction pathway of a redox-active nitroheterocyclic compound depends upon the pH of the medium, which feature determines the protonation and thus the possible reactivity of the free radical produced; the proticity or degree of aqueous or non-aqueous components in the medium, and finally, the presence of any other component with which the reduced species produced may interact.

We have made use of this feature of changing reduction pathway to study the effect of DNA base addition on different reduction products. All four DNA bases resulted in some alterations to the voltammetric characteristics of metronidazole indicating a direct interaction between the reduced drug derivatives and DNA bases. The effect of pH on the stability of $RNO_2^-$ and its interaction with the DNA base thymine have also been examined.

## MATERIALS AND METHODS

Metronidazole was obtained from Rhône-Poulenc Rorer (Dagenham, U.K.), and used as received. The DNA bases and dimethylformamide (DMF, spectroscopic grade) were purchased from the Aldrich Chemical Co. (Gillingham, U.K.).

Voltammetric measurements employed a PAR 264A polarographic analyser interfaced with a PAR 303A three-electrode cell stand and a Bausch and Lomb RE0088 x–y recorder. The working electrode was mercury, as either a dropping electrode (dme) for polarography, or as a hanging drop (hdme) for stationary electrode voltammetry. All potentials were measured against a Ag/AgCl aqueous reference electrode. A Pt wire was used as the counter-electrode. Polarography used an electronically controlled dme with a drop time of 1 sec and a scan rate of 5 mV/sec. Cyclic voltammetry used a varied scan rate from 10 to 500 mV/sec, but the typical value was 100 mV/sec.

Cell solutions were purged with solvent-saturated $N_2$ prior to all measurements, with a positive pressure of $N_2$ being maintained throughout. The supporting electrolyte in aqueous media was $1.5 \times 10^{-1}$ mol/dm$^3$ NaCl and $1.5 \times 10^{-2}$ mol/dm$^3$ trisodium citrate (1.0 SSC buffer). For DMF/$H_2O$ solutions, the DMF content was 33.3% by volume, with 1.0 SSC as the supporting electrolyte. In DMF only the supporting electrolyte was 0.1 mol/dm$^3$ tetra-*n*-propylammonium tetrafluoroborate ($TPABF_4$). The concentration of metronidazole was $2 \times 10^{-4}$ mol/dm$^3$. The DNA bases were added directly to the electrochemical cell as a pre-weighed solid to give a metronidazole:DNA base ratio ranging from 1:0.2 to 1:300 (i.e. $4 \times 10^{-5}$ to $6 \times 10^{-2}$ mol/dm$^3$) depending on DNA base solubility. The small amount of dissolved $O_2$ was displaced by $N_2$ gas while the bases dissolved. The pH of the electrolytic

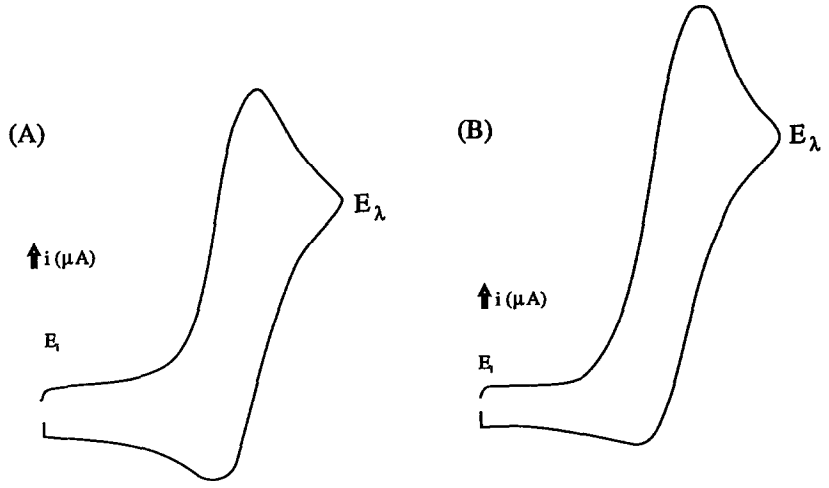


Fig. 2. The cyclic voltammetric response for metronidazole (A) alone, and (B) in the presence of thymine (at a metronidazole:thymine of 1:10). Ei = − 0.60; Eλ = − 1.01 V at a scan rate of $\nu$ = 100 mV/sec. $ip_r/ip_f$ for (a) = 0.71; for (b) = 0.55 giving a %Δ of 22.5.

Table 1. The effect of DNA base addition on the voltammetry of metronidazole in aqueous and mixed dimethylformamide/$H_2O$ media

| Base | Aqueous* CV($Ep_f$) | Aqueous* d.c.($E_{1/2}$) | DMF/$H_2O$ %Δ† | DMF/$H_2O$ [base]‡ |
|---|---|---|---|---|
| Thymine | +10 | +40 | 5.0 | $2.14 \times 10^{-5}$ |
| Adenine | +45 | +60 | 3.0 | $3.55 \times 10^{-5}$ |
| Cytosine | −30 | −10 | | $1.38 \times 10^{-3}$ |
| Guanine | +35 | +50 | | |

* The shift (in mV) in the $NO_2$ reduction potential ($RNO_2$/RNHOH) upon base addition at a drug:base ratio of 1:10, except guanine at 1:1.

† The % change in $ip_r/ip_f$ for the $RNO_2/RNO_2^-$ couple at a drug:base ratio of 1:1.

‡ The concentration of DNA base (mol/dm$^3$) required to give a 10% decrease in $ip_r/ip_f$.

Table 2. The effect of thymine addition on the $ip_r/ip_f$ ratio for the metronidazole $RNO_2/RNO_2^-$ couple as a function of pH

| | pH 8.5 | pH 9.7 | pH 10.5 | pH 11.2 |
|---|---|---|---|---|
| $ip_r/ip_f$ | 0.706 | 0.846 | 0.861 | 0.872 |
| +thymine | 0.636 | 0.784 | 0.886 | 0.893 |
| %Δ | −9.92 | −7.33 | +2.9 | +2.4 |

All comparisons at $\nu$ = 100 mV/sec.
Metronidazole:thymine ratio of 1:6.6.

medium was monitored by a Whatman PHA 250 pH probe.

The reproducibility of the results was confirmed by independent experiments. This was particularly important where small changes to the voltammetry were produced by DNA base addition. Repeat voltage scans were correct to ±1%; repeat current measurements from individual experiments were accurate to ±2%.

To ensure that the alterations to the voltammetry of metronidazole upon DNA base addition are due to reaction between electrochemically generated intermediates and the target, and not due to an electrode adsorption phenomenon, 150 μL of cyclohexanol was added to both aqueous and DMF/$H_2O$ solvent systems to prevent electrode adsorption effects.

## RESULTS

For the studies in aqueous media the results are expressed in terms of a positive or negative shift in the $Ep_f$ or $E_{\frac{1}{4}}$ values in mV for cyclic voltammetry and d.c. polarography, respectively. For studies in DMF/$H_2O$ the percentage change in the $ip_r/ip_f$ ratio was used. If the species formed during reduction (i.e. the forward scan) is stable on the electrochemical time-scale then the return and forward current responses will be equal, giving an $ip_r/ip_f$ ratio of unity. As current response is proportional to concentration, if the species formed by reduction is capable of reacting with another molecule or is unstable on the electrochemical time-scale, there will be a decreased concentration available for reoxidation, giving an $ip_r/ip_f$ ratio of less than 1. Because such changes in numerical terms are small, but highly reproducible, the results are expressed as the percentage change in the $ip_r/ip_f$ ratio. For the pH study the $ip_r/ip_f$ ratios and the percentage change in the ratios are given for ease of comparison. Figure 2 shows a typical CV for metronidazole alone and in the presence of thymine. All data are given in Tables 1 and 2.

### *In aqueous media*

The effect of DNA base addition on the electrochemical response was monitored by d.c. and differential pulse polarographies, and by cyclic voltammetry (CV) using a metronidazole:base ratio

of 1:10 (±0.75), except for guanine where the ratio was 1:1 (±0.1). Metronidazole itself showed behaviour characteristic of many nitro-aromatic compounds with a single-step four-electron reduction, with no return wave by CV and a pronounced streaming effect on the d.c. polarogram [3]. Thymine, guanine and adenine resulted in an increasing shift to more positive reduction potentials (10, 30 and 45 mV, respectively, as measured by CV; by d.c. polarography potential shifts were 15 mV greater, Table 1). A decrease in the current response by all techniques was observed, ranging from 5 to 30%. The general features of the differential pulse and the CV methods remained unaltered. By d.c. polarography the streaming effect was significantly diminished with guanine or completely absent. Increasing the concentration of thymine gave no further changes until a ratio of 1:40 was reached, when a negative shift in reduction potential was observed. Increasing the adenine concentration caused the nitro reduction to become increasingly complex in nature, with the development of additional redox steps. The addition of cytosine (1:10) resulted in a negative shift in reduction potential, with a small decrease in current response. This trend was continued up to a ratio of 1:100, although the changes were most marked after the initial addition of base.

### *In* 33.3% *dimethylformamide*/$H_2O$

The effect of base addition on the one- and three-electron addition steps, the $RNO_2/RNO_2^{\cdot-}$ and $RNO_2^{\cdot-}$/RNHOH couples, respectively, were examined by the same methods employed for the purely aqueous system. To examine specifically the effect of DNA base addition on the lifetime of $RNO_2^{\cdot-}$ a switching potential of 100 mV negative of the forward wave peak potential for the one-electron couple in the CV mode was chosen, and the return-to-forward peak current ratio, $ip_r/ip_f$, measured.

By CV the $E_{\frac{1}{4}}$ of the $RNO_2RNO_2^{\cdot-}$ and the $Ep_f$ of the $RNO_2^{\cdot-}$/RNHOH couples showed no significant alteration with addition of base. Adenine, cytosine and thymine resulted in a decrease in $ip_r/ip_f$, with the difference from the control value increasing as the concentration of base was increased. The $RNO_2^{\cdot-}$/RNHOH couple showed a complementary decrease in CV current response. For a given base the decrease in $ip_r/ip_f$ from the control measurement was found to vary with scan rate, $\nu$, with a maximum between 100 and 200 mV/sec. This behaviour was observed throughout the complete base concentration range examined.

To allow comparison between the effect produced by the bases, the $ip_r/ip_f$ ratios were best expressed as the percentage change, %Δ, from the control value. The variation found between the various bases was in some cases considerable (Table 1). A plot of %Δ vs log of base concentration (Fig. 3) gave an approximately straight line relationship for adenine and thymine, while cytosine gave a curved response. The slope of this curve indicates the relative sensitivity of the nitro radical anion for interaction with the base.

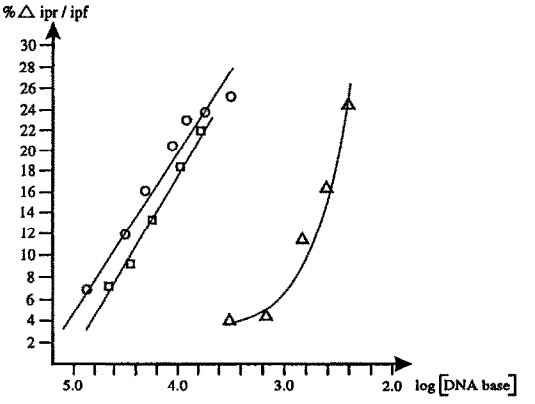


Fig. 3. Plot of %Δ $ip_r/ip_f$ vs log [DNA base]. Thymine (○), adenine (□) and cytosine (Δ) at a scan rate of $\nu$ = 100 mV/sec.

### *In dimethylformamide*

In DMF the $RNO_2/RNO_2^{\cdot-}$ couple is uncomplicated, with the classic features for a fully reversible charge-transfer process, i.e. $ip_r/ip_f = 1.0$ at all scan rates, $\Delta Ep = 60$ mV. No other reduction features were observed. Addition of all four DNA bases resulted in a marked decrease in the $ip_r/ip_f$ ratio from the control. A metronidazole:thymine ratio of 1:4 resulted in a 37% decrease in $ip_r/ip_f$ and a distinct increase in forward wave response. In the DMF/$H_2O$ system, the same thymine concentration caused a 16% decrease in $ip_r/ip_f$. The %Δ continued to increase slightly as the thymine concentration was increased, so that at a metronidazole:thymine ratio of 1:28, the %Δ was 46.3. The addition of cytosine resulted in a percentage decrease in the $ip_r/ip_f$ approximately four times greater than that found in DMF/$H_2O$. A metronidazole:thymine ratio of 1:1 produced the complete removal of all return wave characteristics. The addition of guanine to give a saturated solution gave a 14% decrease in $ip_r/ip_f$.

### *Cyclohexanol addition*

In aqueous media cyclohexanol addition produced a change to the redox mechanism from a single four-electron step to a two-stage process, directly comparable with the DMF/$H_2O$ system, i.e. $RNO_2/RNO_2^{\cdot-}$ and $RNO_2^{\cdot-}$/RNHOH couples identified, with appropriate changes in current response. A 150 mV negative potential shift was observed from the original four-electron couple to the reversible one-electron addition step.

The addition of cyclohexanol to a DMF/$H_2O$ medium did not cause any significant changes in the voltammetric response.

### *Changes in pH*

Increasing the pH of the DMF/$H_2O$ medium resulted in greater stability for the $RNO_2^{\cdot-}$ species, as illustrated by an increase in the $ip_r/ip_f$ ratio (Table 2). Addition of thymine at a metronidazole:thymine ratio of 1:6.6 decreased the $ip_r/ip_f$ compared with

the control which became progressively less as the pH was increased. At higher pH, thymine addition appeared to have little effect on $ip_r/ip_f$. No change in potential was observed by CV on increasing the pH or by thymine addition.

## DISCUSSION

The use of the DMF/$H_2O$ electrolytic medium has enabled us to examine the effect of DNA base addition on the $RNO_2/RNO_2^-$ and the $RNO_2^-$/RNHOH couples. Changes to the voltammetric response were greatest using CV, where the addition of DNA base (except guanine) resulted in a decrease in $ip_r/ip_f$ ratio for the one-electron couple (Table 1) dependent on the nature and concentration of the biological target. The $ip_r/ip_f$ value reflects the quantity of $RNO_2^-$ available for oxidation to the starting material; therefore, a decrease from the control upon addition of base indicates interaction between $RNO_2^-$ and the DNA base. The decrease in current of the $RNO_2^-$/RNHOH couple upon base addition is compatible with less $RNO_2^-$ being available to undergo the further reduction step.

Thymine followed by adenine were the most effective at removal of $RNO_2^-$, which is in line with previous studies using bulk electrolytic reduction of metronidazole in the presence of DNA of varying compositions. The greatest damage was found to result in DNA with the highest A + T ratio [9]. In addition, metronidazole is most effective against those anaerobic organisms with a high A + T content [6].

As illustrated by an increase in the $ip_r/ip_f$ ratio, the lifetime of $RNO_2^-$ was increased at alkaline pH, in line with pulse radiolysis studies [10]. The natural decay of $RNO_2^-$ is by a disproportionation reaction *via* the protonated radical, $RNO_2H\cdot$, therefore in alkali the unprotonated radical is more stable. We have proposed previously that DNA damage induced by nitroimidazoles and benzotriazine-*N*-oxides is due to the protonated radical anion. This is based on evidence which shows that DNA damage is pH dependent and increased at acid pH [11, 12]. This illustrates that the possible rate-determining events of DNA damage are a combination of reduction to the radical anion and its protonation to yield the damaging radical, $RNO_2H\cdot$. Voltammetrically, the interaction between $RNO_2^-$ and thymine decreased as the lifetime of $RNO_2^-$ was extended (see Table 2). The influence of pH is therefore apparent in modifying both radical anion stability and reduced drug interactions with DNA bases.

We might predict that in an aprotic medium where $RNO_2^-$ shows no tendency to undergo further reaction, addition of a DNA base would not influence the one-electron couple. This was not observed, with all four DNA bases causing a decrease in the $ip_r/ip_f$ ratio, by a considerably greater amount than produced by the same base concentration in DMF/$H_2O$ solvent. For example, at a metronidazole : thymine ratio of 1 : 4 the $ip_r/ip_f$ ratio decreased by 37% in DMF compared with a 14% decrease in DMF/$H_2O$. Guanine, which produced no alteration in the $RNO_2/RNO_2^-$ couple in DMF/$H_2O$, caused a 14% decrease in the $ip_r/ip_f$ ratio in DMF. In the aprotic medium, with its deficiency of $H^+$ ions, the DNA base may act as the proton donor, forming $RNO_2H\cdot$, leading to subsequent disproportionation (Eqn 1).

$$2RNO_2^- + 2H^+(\text{from DNA base}) \rightarrow 2RNO_2H\cdot \rightarrow RNO_2 + RNO + H_2O. \quad (1)$$

However, in the DMF/$H_2O$ system, with a 67% water content and consequently a ready supply of $H^+$ ions in the medium, proton donation from the DNA base is unlikely. The influence of base concentration and the sensitivity of radical interaction with the bases are presently under further investigation.

Previous d.c. polarographic studies by Declerck and Ranter [13, 14] on a number of nitroimidazoles in $H_2O$ showed a positive shift in $E_{\frac{1}{2}}$ upon addition of adenine, guanine and cytosine (in decreasing order) dependent on base concentration. This was assigned to reaction between electrochemically generated species and the added target. The negative shift in $E_{\frac{1}{2}}$ upon thymine addition was assigned to base adsorption onto the Hg electrode by comparison with the effect of known adsorption agents, e.g. cyclohexanol. Under our experimental conditions the results do not correlate with those from earlier studies. Thymine was found to give a positive potential shift in $E_{\frac{1}{2}}$, with a negative shift only being observed at a metronidazole : thymine ratio exceeding 1 : 40. Cytosine was found to give a negative potential shift. Addition of the adsorption agent cyclohexanol resulted in a negative potential shift, accompanied by a change in the reduction pathway to a two-step process involving the sequential addition of one and three electrons. This is analogous to the effect produced by addition of aprotic solvents. The changes observed to the voltammetric response of metronidazole in an aqueous medium upon base addition, for example loss of electrode streaming, are in line with those found upon addition of a surfactant, e.g. gelatin [3].

The irreversible nature of the four-electron $RNO_2$/RNHOH couple in $H_2O$ makes potential shift data very difficult to interpret. Theoretically a reduction product–base interaction may yield either a positive or a negative potential shift [15, 16]. In addition, no information was obtained on which of the reduction intermediates is responsible for changes to the voltammetry. We have used the data from the aqueous system to compare with the results from the mixed solvent medium. From an examination of Table 1 it can be seen that the results do not correlate, indicating that interaction of other reduction intermediates with DNA bases occurs in addition to that found with $RNO_2^-$. Note that the concentration of thymine and adenine used in $H_2O$ resulted in a complete removal of reversibility for the $RNO_2/RNO_2^-$ couple in DMF/$H_2O$.

In conclusion, interaction between the metronidazole nitro radical anion with the bases thymine, adenine and to a lesser extent cytosine, was clearly shown. In DMF where $RNO_2^-$ is chemically stable, rapid interaction with thymine was observed suggesting that a different reaction mechanism may be in operation under aprotic conditions. In an aqueous media the ordering of interaction with base

did not correlate with that found in a mixed solvent, indicating that other (unidentified) reduction products also interact with the DNA bases.

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