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ELECTROCHEMICAL CHARACTERISTICS OF NITROHETEROCYCLIC COMPOUNDS OF BIOLOGICAL INTEREST VI. THE MISONIDAZOLE RADICAL ANION

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The addition of four aprotic solvents to misonidazole in an aqueous buffer system has been examined electrochemically. Qualitatively they all result in separation of the initial irreversible 4 electron reduction step into two stages, the $RNO_2/RNO_2^{\cdot-}$ and $RNO_2^{\cdot-}$/RNHOH couples respectively. Despite some difficulties in achieving measurements for the discrete $RNO_2/RNO_2^{\cdot-}$ without interference from the following reduction step, it was clear that the various aprotic solvents influenced the lifetime of the $RNO_2^{\cdot-}$ species to different degrees. Resolution of the two processes was best achieved using a water-acetone system and this has been employed to study the lifetimes of the misonidazole radical anion as a function of acetone content and drug concentration. Analysis of the cyclic voltammetric response showed a second order decay pathway, in line with the metronidazole system studied under similar conditions. This has been compared with results from pulse radiolysis work, which suggested a first order reaction of unknown pathway for 2-nitroimidazole radical anions.

KEY WORDS: Misonidazole, radical anion, cyclic voltammetry, aprotic solvents.

INTRODUCTION

Misonidazole and metronidazole are frequently taken as exhibiting properties which are generally typical of the 2- and 5-nitroimidazoles respectively. The selective hypoxic cytotoxicity displayed by these compounds is known to depend on biological reductive activation via the nitro group, causing DNA strand breaks and helix destablization.[1,2] The actual species responsible for causing biological damage has not been positively identified but is known to be a short-lived intermediate requiring the acceptance of fewer than 4 electrons.[3] The one-electron addition product, the nitro radical anion, has consequently attracted considerable attention, being an obligate intermediate in the nitro/hydroxylamine conversion.

Kinetic analysis of the metronidazole and misonidazole nitro radical anions, as generated by pulse radiolysis, has shown a second order disproportionation and a first order reaction respectively.[4,5] Using mixed aqueous/dimethylformamide (DMF) solvents, we have selectively generated the one-electron $RNO_2/RNO_2^{\cdot-}$ couple for metronidazole and other nitroaromatic compounds, including ring structures ranging from furan to imidazole, pyrazole and benzenoid.[6,7] Using the cyclic voltammetric

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display mode and using the theoretical approach of Olmstead *et al.*,[8] we have shown that the electrolytically generated metronidazole radical anion also reacts *via* a second order decay pathway. Although we have qualitatively observed that as the DMF content of the electrochemical medium was increased the lifetime of the misonidazole radical anion also increased, in line with observations on the metronidazole system, detailed analysis has to date not been feasible due to the poor resolution of the electrolytic response. We report, however, that the definition can be improved using acetone in place of DMF as the aprotic solvent, thereby allowing us to analyze fully the lifetime of the misonidazole radical anion, and its decay pathway.

MATERIALS AND METHODS

The 2-nitroimidazoles were supplied as follows and used as received without further purification. Misonidazole and benznidazole from Roche Products Ltd., and azomycin from the Sigma Chemical Co. Dimethylformamide and acetone were spectroscopic grade, acetone and dimethylsulphoxide (DMSO) were HPLC grade. All were purchased from the Aldrich Chemical Co.

Electrochemical measurements employed the cyclic voltammetric (CV) mode exclusively, using a PAR 264A polarographic analyzer interfaced with a PAR 303 cell stand with a 3-electrode cell configuration. A hanging drop mercury electrode was used as the working surface, and a Pt wire as the auxilliary electrode. Potentials were measured against an aqueous Ag/AgCl reference electrode.

The proportions of H_2O/aprotic solvent were varied between 10 and 50% (expressed at % v:v of the aprotic content) using 1.5×10^{-2} mol/dm^3 NaCl, 1.5×10^{-3} mol/dm^3 trisodium citrate buffer (0.1 SSC) as the supporting electrolyte. The CV response was routinely recorded between scan rates of $v = 10$ to 500 mVs^{-1} and the return-to-forward peak current ratio, ip_r/ip_f, for the reversible $RNO_2/RNO_2^{\cdot-}$ couple measured. The switching potential, E_λ, was chosen to be 100 mV more negative than the forward wave reduction peak. A routine drug concentration was 2×10^{-4} mol/dm^3, but the influence of misonidazole concentration on the ip_r/ip_f was examined at 33.3% acetone over a 3.3×10^{-5} to 3.5×10^{-2} mol/dm^3 range. For kinetic analysis the ip_r/ip_f data used were the average of at least three independent measurements. A variation in the ip_r/ip_f of $\pm$ 5% was considered to be the maximum acceptable otherwise the complete data set was rejected. For all linear relationships, correlation coefficients of 0.98 or better were found.

RESULTS

Detailed analysis of the ip_r/ip_f ratio of the $RNO_2/RNO_2^{\cdot-}$ couple by CV as a function of scan rate and aprotic content of the electrochemical medium has allowed us to determine the lifetime of $RNO_2^{\cdot-}$ for a range of nitroaromatic species. Generally, we have used DMF as the aprotic solvent, primarily because this presents no evaporation problems. For misonidazole, however, although qualitatively the same overall trend in ip_r/ip_f towards unity with increasing % DMF was observed illustrating the extended lifetime of $RNO_2^{\cdot-}$, measurement of ip_r/ip_f was impossible due to interference from the irreversible 3 electron $RNO_2^{\cdot-}$/RNHOH couple. Our studies were therefore extended to benznidazole and azomycin. Although benznidazole showed an improve-

ment in resolution permitting measurement of ip_r/ip_f, insufficient data were available to allow full analysis of the trend in ip_r/ip_f with changing solvent, as the first evidence of return wave character was only found above 40 % DMF content. For azomycin no measurements were possible as the forward wave of the $RNO_2/RNO_2^{\cdot-}$ couple was observed only as a shoulder on the dominant $RNO_2^{\cdot-}$/RNHOH couple.

In place of pursuing the benznidazole system further with its very limited data set, we have turned our attention to the influence of different aprotic solvents on the more widely studied misonidazole. Using DMSO the resolution of the two reduction steps further deteriorated in comparison to DMF. Both acetone and acetonitrile showed considerable improvements, particularly the former, where the reduction steps were well separated even at $v = 10\,mVs^{-1}$. It would also appear that the different aprotic solvents influence the stability of $RNO_2^{\cdot-}$ to varying degrees. For example, considering the data at 33.3 % aprotic content, DMF gave consistently greater ip_r/ip_f values, signifying more stable $RNO_2^{\cdot-}$ ($ip_f/ip_r = 0.924$ at $v = 100\,mVs^{-1}$) than either acetone or acetonitrile ($ip_r/ip_f = 0.701$ and 0.759 at $100\,mVs^{-1}$ respectively). Due to the very poor resolution with the H_2O/DMSO system, the absolute ip_r/ip_f values must be treated with caution, but it does appear that this mixture yields a misonidazole radical anion of intermediate lifetime, with an estimated $ip_r/ip_f = 0.88$ at $v = 100\,mVs^{-1}$.

We have therefore chosen to concentrate our studies on variation in the acetone content, as this combination presents the best resolution of the two reduction processes at slow scan rates. We have analyzed the results by both a "half-life" time constant approach and by kinetic treatment based on theoretical calculations, which have been successfully employed for other nitroaromatic compounds.[7]

FIRST ORDER ANALYSIS

Using the theoretical approach of Nicholson and Shain[19] for a first order reaction following a reversible charge-transfer step, the experimentally derived $ip_r ip_f$ values were fitted to a working curve which related ip_r/ip_f, to log $k_f\tau$, where k_f = first order rate constant and τ = the time constant defined as $\tau = (E_{1/2} - E_i)/v$. At any particular % acetone content, therefore, if first order kinetics apply the k_f value should be constant and independent of the scan rate employed. This was found not to hold. Over the complete range of acetone concentrations examined, the k_f value increased with v, then (at 33.3 % acetone content) at $v = 200\,mVs^{-1}$ or greater, decreased markedly.

SECOND ORDER ANALYSIS

Using the same approach as previously for metronidazole[6] (the method of Olmstead *et al.*[8]) the ip_r/ip_f values at each scan rate were inserted into a working curve to determine the ω parameter, which incorporates the effects of rate constant, drug concentration and scan rate. A plot of ω *vs* τ resulted in a straight line relationship, with a slope given by equation (1)

$$\text{slope} = k_2 C^* \exp[0.078(a\tau - 4)] \qquad (1)$$

where k_2 = second order rate constant ($= 2k_{obs}$ for a disproportionation reaction $2A \rightarrow B + C$ with rate equation $-d[A]/dt = 2k_{obs}[A]^2$)

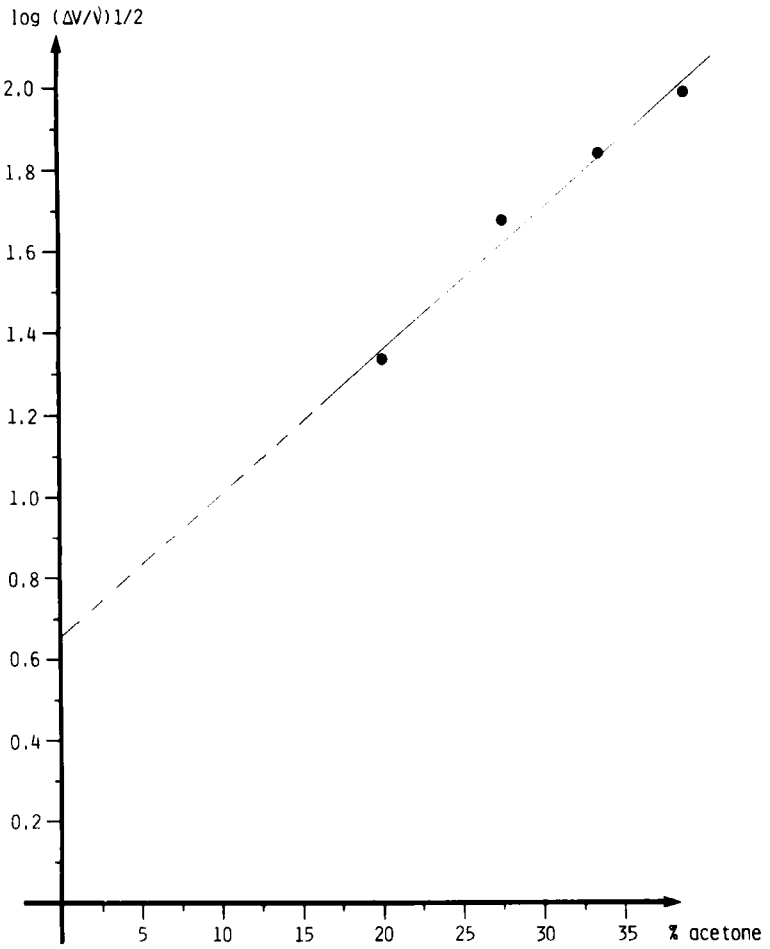

FIGURE 1 Plot of $\log k_2$ *vs* % acetone content for the misonidazole radical anion, illustrating extrapolation to aqueous conditions (zero % acetone).

C^* = analytical drug concentration

$a = nFv/RT$, where n = number of electrons involved in charge-transfer step, and other terms have their usual meanings.

As all other variables are known, k_2 can be calculated. This procedure was repeated for each % acetone content between 10 and 40, beyond which no further increases in ip_r/ip_f were observed. The resulting k_2 values showed a decrease as % acetone was increased, as would be expected for successively slower reactions. Plotting $\log k_2$ *vs* % acetone gave a good straight line relationship (Figure 1) from which extrapolation to zero % acetone allowed us to determine the k_2 value for the misonidazole radical anion in purely aqueous media of $k_2 = 3.89 \times 10^4 \text{dm}^3/\text{mol-sec}$ ($\pm$ 10 %). This in turn gave a half-life $t_{1/2}$ of 25.7 ($\pm$ 3) seconds for a free radical concentration of $1 \times 10^{-6} \text{mol/dm}^3$.

EFFECT OF MISONIDAZOLE CONCENTRATION

Maintaining the % acetone constant at 33.3, the drug concentration was varied from 3.3×10^{-5} to 3.5×10^{-2} mol/dm^3. As the drug concentration was increased stepwise from 3.3×10^{-5} to 1.0×10^{-3} mol/dm^3, a steady decrease was observed in the ip_r/ip_f ratio, from 1.0 to 0.79 at $v = 100\,\text{mVs}^{-1}$. Above this drug concentration, the ip_r/ip_f level remained virtually constant. It was also noted that quite severe distortions to the voltammograms were evident as the misonidazole concentration increased beyond 1.0×10^{-3} mol/dm^3. This was initially observed in the form of a slight increase in the peak-to-peak separation from 80 to 100 mV. However, at a concentration of 5.2×10^{-3} mol/dm^3, the response was very noticeably distended ($\Delta Ep = 160$ mV) and at 1.9×10^{-2} mol/dm^3, distortion was so severe that measurement was no longer possible.

DISCUSSION

The use of a variety of aprotic solvents produced the same general change in the redox mechanism of misonidazole on addition to an aqueous electrolyte system from a single irreversible 4 electron step ($RNO_2/RNHOH$) to a two stage process, giving discrete 1 electron ($RNO_2/RNO_2^{\cdot-}$) followed by a 3 electron ($RNO_2^{\cdot-}/RNHOH$) addition steps. The resolution of the electrode response was also influenced by the nature of the aprotic solvent. Using DMF, and more particularly DMSO, the separation between the two reduction steps at slower scan rates was not well defined, making analysis of the $RNO_2/RNO_2^{\cdot-}$ couple in isolation difficult. Acetone and acetonitrile showed a considerable improvement and therefore the former was used to study the lifetime of the misonidazole radical anion. By comparison of the ip_r/ip_f values measured under comparable conditions, it would appear that different aprotic solvents stabilize the radical anion to varying degrees. We have previously found that a range of electrochemically generated $RNO_2^{\cdot-}$ species of different ring structures, show different sensitivities to the dimethylformamide content of the media,[7] and preliminary studies indicate the order of stability to be dependent on both solvent and radical anion.[10] These differences observed in the misonidazole radical anion stability depending on the aprotic solvent employed make comparison of the actual lifetimes obtained in the present study with those derived for other nitroaromatic compounds using a H_2O/DMSO medium, of little value. Studies are in progress to further evaluate the role of the different aprotic solvents in the redox mechanism.

Examination of the misonidazole-acetone system strongly suggested that the misonidazole radical anion undergoes a second order decay pathway, in line with metronidazole, and not by a first order mechanism as found from pulse radiolysis studies.[5] The second order decay may be simply viewed as the disproportionation reaction illustrated in equation (2)

$$2RNO_2^{\cdot-} + H^+ \rightarrow RNO_2 + RNO + H_2O \qquad (2)$$

The highly linear ω *vs* τ relationships found by second order kinetic analysis for all % acetone concentrations indicate a second order reaction, together with the complete failure of the first order analysis. To help confirm the second order nature we also examined the ip_r/ip_f ratio at 33.3 % acetone content, varying the misonidazole concentration over three orders of magnitude. For a second order process the lifetime of the radical should increase (the ip_r/ip_f ratio should tend towards unity) as the

concentration is decreased. This was indeed found up to a 1.0×10^{-3} mol/dm^3 concentration. Above this drug level essentially no change in ip_r/ip_f was observed. Kinetic analysis was carried out at 2×10^{-4} mol/dm^3, and therefore well within the region where second order behaviour would be expected. The constancy of the ip_r/ip_f ratios found at drug concentrations of 1×10^{-3} mol/dm and above are more indicative of first order kinetic behaviour. Kinetic analysis, however, was not feasible due to the increasingly severe distortions of the CV response under these conditions. Our observations are in contrast with pulse radiolysis data, where the misonidazole radical anion, with an initial concentration of 2.5×10^{-6} mol/dm^3, decayed with a reasonable first order fit, although the decay was somewhat concentration dependent.[5] The reason for this discrepancy is unknown and it is unfortunate that an electrochemical kinetic analysis was not feasible for a misonidazole concentration greater than 1×10^{-3} mol/dm^3 where behaviour may be comparable. Further investigations into the electrochemical concentration effect of 2-nitroimidazoles are in progress. It is clear, however, that at the drug concentrations generally employed in electrochemical studies, second order disproportionation of the misonidazole radical anion takes place, in line with metronidazole and other nitroaromatic compounds.

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References

1. Edwards, D.I. Reduction of nitroimidazoles *in vitro* and DNA damage. *Biochem. Pharmacol.*, **35**, 53–58, (1986).
2. Olive, P.L. Mechanism of the *in vitro* toxicity of nitroheterocycles including Flagyl and misonidazole. In: *Radiation Sensitizers* (ed L.W. Brady), Masson, New York, pp. 39–44, (1980).
3. Knox, R.J., Edwards, D.I. and Knight, R.C. The mechanism of nitroimidazole damage to DNA: coulometric evidence. *Int. J. Rad. Oncol. Biol. Phys.*, **10**, 1315–1318, (1984).
4. Wardman, P. Some reaction and properties of nitro radical anions important in biology and medicine. *Environ. Health Perspec.*, **64**, 309–320, (1985).
5. Wardman, P. Lifetimes of the radical anions of medically important nitroaryl compounds in aqueous solution. *Life Chem. Rep.*, **3**, 22–28, (1985).
6. Tocher, J.H. and Edwards, D.I. Electrochemical characteristics of nitroheterocyclic compounds of biological interest. IV. Lifetime of the metronidazole radical anion. *Free Rad. Res. Commun.*, **6**, 39–45, (1989).
7. Tocher, J.H. and Edwards, D.I. Electrochemical characteristics of nitroheterocyclic compounds of biological interest. V. Measurement and comparison of nitro radical lifetimes. *Int. J. Rad. Biol.*, (in press).
8. Olmstead, M.L., Hamilton, R.G. and Nicholson, R.S. Theory of cyclic voltammetry for a dimerization reaction initiated electrochemically. *Anal. Chem.*, **41**, 260–267, (1969).
9. Nicholson, R.S. and Shain, I. Theory of stationary electrode polarography. single scan and cyclic methods applied to reversible, irreversible, and kinetic systems. *Anal. Chem.*, **36**, 706–723, (1964).
10. Tocher, J.H. Unpublished work.

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