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NITROIMIDAZOLES AS ANAEROBIC ELECTRON ACCEPTORS FOR XANTHINE OXIDASE

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Abstract—The rates of anaerobic nitroreduction of 15 nitroimidazoles by xanthine–xanthine oxidase were measured. The compounds studied were mainly 2-nitroimidazoles, including misonidazole and analogues of potential value in cancer therapy; most 5-nitroimidazoles such as metronidazole and nimorazole reacted too slowly for measurement under the conditions used. Using 0.1 mM nitroimidazole, reduction rates varied between *ca.* 4 and 680 nmole min^{-1} U^{-1} xanthine oxidase at 37° and pH 7.4. These rates were correlated with the reduction potential characterizing one-electron reduction to the nitro radical-anion, and compared with those for other nitroaryl compounds and with reduction by free flavin mononucleotide.

The rates of anaerobic reduction of several nitroimidazoles (probably to the hydroxylamines as initial product) by free, reduced flavin mononucleotide ($FMNH_2$)§ in water at pH 7.4 was recently reported to be predominantly dependent on the reduction potential E_7^1 for reduction to the nitro radical-anion [1]. The stoichiometry of anaerobic reduction of 5-cyano-1-methyl-2-nitroimidazole by xanthine–xanthine oxidase (EC 1.2.3.2, xanthine: O_2 oxidoreductase) was also shown to involve 4-electron stoichiometry corresponding to hydroxylamine formation. However, nitroimidazoles of current interest in cancer therapy, such as misonidazole and metronidazole [2–5], reacted too slowly under the conditions used in preliminary experiments [1] to permit measurement of relative rates of nitroreduction by xanthine–xanthine oxidase. By using a more active enzyme preparation and increasing the xanthine and nitroimidazole concentrations, it has now been possible to establish a redox relationship for the anaerobic reduction of nitroimidazoles with E_7^1 between −0.20 and −0.42 V (vs NHE) by xanthine–xanthine oxidase.

MATERIALS AND METHODS

Chemicals. Flavin mononucleotide, allopurinol, hypoxanthine, xanthine and xanthine oxidase, Grade III, from buttermilk (X 4500) were obtained from Sigma Chemical Co. (London, U.K.). The nitroaryl compounds (Table 1) were obtained as follows: **1, 3, 5, 8, 13** and **15** from Dr R. G. Wallace, Brunel University, Uxbridge, Middlesex; **2, 4** and **16** from Gruppo Lepetit S.p.A., Milan; **6, 7, 10, 11, 12** and **14** from Roche Products Ltd., Welwyn Garden City, Herts; **9** from Schering A.G., Berlin; **17** from Montedison Pharmaceuticals Ltd., Barnet, Herts; **18** from May & Baker Ltd., Dagenham, Essex; **19** from Aldrich Chemical Co., Gillingham, Dorset. All other chemicals were BDH AnalaR grade.

Anaerobic spectrophotometry. A Zeiss PMQ II spectrophotometer with thermostatted cell and digital printer output was used. A 10 mm silica cell with C10 socket was fitted with an 8 mm "Suba Seal" rubber stopper carrying a bubbling tube (22 G luer needle) and vent tube (1 mm i.d.). Nitrogen (B.O.C. Ltd., < 4 ppm O_2) was passed through 2 "Nil-Ox" deoxygenators (Jencons Ltd.) containing sodium dithionite (32 g l^{-1}) in 0.2 M Na_2HPO_4, washed with a solution of $NaHCO_3$ (10 g l^{-1}) and transferred to the cell via glass/metal tubing and a luer connecter (Omnifit). Solutions were deoxygenated by bubbling at *ca.* 15 ml min^{-1} for 15 min at 37°. Enzyme was added by microlitre syringe down the vent tube followed by immediate withdrawal of the vent tube and bubbling needle to form a sealed system. Anaerobicity was checked by measuring the stability of $FMNH_2$ (40 μM) at pH 7.4 and 37°; no oxidation could be detected spectrophotometrically (445 nm) after 30 min.

Anaerobic nitroreduction by xanthine oxidase. Solutions (2.5 ml) at 37° containing xanthine or hypoxanthine (0.06, 0.5 or 5 mM), nitroaryl compound (0.1 mM), potassium phosphate buffer (pH 7.4, 85 mM) were deoxygenated and xanthine oxidase (2–40 μl; 22.5–35.1 U ml^{-1}) added to initiate the reaction. Nitroreduction was measured by the decrease in extinction at 325 nm, i.e. near or at the absorption maximum of the nitro chromophore (except for **1**, where 368 nm was used); neither the product(s) of nitroreduction nor xanthine, hypoxanthine or uric acid show significant absorption at these wavelengths.

Physicochemical properties. Reduction potentials E_7^1 were measured from one-electron transfer equilibria with redox indicators [6, 7] using the pulse

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§ Abbreviations: $FMNH_2$, reduced flavin mononucleotide; E_7^1, one-electron reduction potential at pH 7 vs NHE, normal hydrogen electrode; ε, decadic molar extinction coefficient; *P*, octan-1-ol:water partition coefficient.

Table 1. The nitroaryl compounds studied

Compound	Name	Formula type	R^1	R^2
1	RGW-705	(8-nitro-caffeine)		
2	L8711	I	CH_3	CHO
3	RGW-801	I	CH_3	CN
4	L10333	I	CH_3	CO_2CH_3
5	RGW-806	I	CH_3	$CONH_2$
6	Ro 03-8799	I	$CH_2CH(OH)CH_2(NC_5H_{10})$*	H
7	Ro 03-9482	I	$CH_2CH(OH)CH_2N(CH_3)_2$	H
8	RGW-613	I	$CH_2CO_2CH_3$	H
9	ZK-28943	II	CH_3	CHO
10	Benznidazole	I	$CH_2CONHCH_2C_6H_5$	H
11	Misonidazole	I	$CH_2CH(OH)CH_2OCH_3$	H
12	Ro 05-9963	I	$CH_2CH(OH)CH_2OH$	H
13	RGW-609	I	$CH_2CH_2OC_6H_5$	H
14	Ro 03-9041	I	$CH_2CH(OH)CH_2OCH(CH_2Cl)CH_2OCH(CH_3)_2$	H
15	RGW-610	I	$CH_2CH_2CH_2OC_6H_5$	H
16	L6678	I	CH_2CH_2OH	CH_3
17	Nimorazole	II	$CH_2CH_2(NC_4H_8O)$†	H
18	Metronidazole	II	CH_2CH_2OH	CH_3
19	CMNI	III	CH_3	Cl

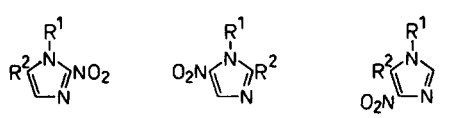
* -*N*-Piperidino group.
† -*N*-Morpholino group.

radiolysis technique [8]. Partition coefficients *P* (octan-1-ol: 0.1 M aqueous potassium phosphate, pH 7.4) were measured spectrophotometrically as described previously [9]. Molar extinction coefficients ε ($M^{-1}\,cm^{-1}$) were calculated from the extinction recorded for reaction solutions prior to addition of enzyme.

RESULTS

Chemical properties of the nitroaryl compounds studied

With the exception of compound **1** (Table 1) the compounds studied were 2-, 5- or 4-nitroimidazoles (Fig. 1). More electron-affinic compounds have more positive one-electron reduction potentials E_7^1; more lipophilic compounds have higher octan-1-ol: water partition coefficients *P* (Table 2). All except **6** and **7** (Table 1) are essentially unionized or unprotonated at pH 7.4; **6** has $pK_a = 8.7$ for the dissociation of the conjugate acid (piperidino function) and **7** has $pK_a = 8.5$ for the dimethylamino function [10]. Partition coefficients for **6** and **7** (Table 2) are for their unionized form (i.e. measured at $pH > pK_a + 2$) [10].



I II III

Fig. 1. Structural formulae of the compounds studied (see Table 1), which are 2-, 5- or 4-nitroimidazoles (I to III, respectively).

Anaerobic reduction of nitroimidazoles by xanthine–xanthine oxidase

Figure 2 shows typical data for the anaerobic reduction of nitroimidazoles (0.1 mM) by xanthine (0.5 mM) and xanthine oxidase. The initial, linear portion of the extinction/time curves enabled the rates of nitroreduction to be measured (Table 2), using the extinction coefficients ε listed. The rates of extinction change in the presence of the less electron-affinic nitroimidazoles **17–19** (Table 1) could not be distinguished from the very small change observed in the absence of any nitroimidazole over the convenient time period (up to 30–40 min), with the highest enzyme concentration used (*ca.* 1 U/2.5 ml). Progressively lower amounts of enzyme (down to *ca.* 0.1 U/2.5 ml) were convenient for the measurements with the more electron-affinic compounds.

In the previous study [1], we reported the rate of *uric acid formation* in the anaerobic xanthine–xanthine oxidase system with the 2-nitroimidazole **3** (Table 1) as 250 nmole min^{-1} mg^{-1} protein, using xanthine oxidase of activity 0.39 U mg^{-1} protein (i.e. rate 640 nmole min^{-1} U^{-1} xanthine oxidase). In the present work a rate *of nitroreduction* for this compound of 680 ± 147 (S.D.) nmole min^{-1} U^{-1} xanthine oxidase was measured from 17 experiments using three different batches of enzyme. Since the stoichiometry of the reaction involves 2 moles uric acid formed per mole nitro compound reduced [1], the present study corresponds to a *ca.* 2–3 times increased rate of nitroreduction than that calculated from the previous work. This increased rate reflects the *ca.* 3 times higher concentration of nitro compound used, as no difference in rate of reduction was observed for xanthine concentrations of 0.06 and 0.5 mM. Similarly, for misonidazole **11** (Table 1), no difference in rate of reduction was found using

Table 2. Physicochemical properties* and anaerobic reduction rates for nitroimidazoles

Compound	10^{-3} ε at 325 nm (M^{-1} cm^{-1})	E_7^1 (V)	P (oct/H_2O)	Reduction rate† (nmole min^{-1} U^{-1})
2	8.3	−0.243	0.80	565 ± 33 (2)
3	6.0	−0.262	0.79	680 ± 147 (17)
4	7.8	−0.300	3.2	244 ± 17 (2)
5	8.2	−0.321	0.56	189 ± 11 (3)
6	7.2	−0.346	8.5	8 ± 1 (4)
7	7.7	−0.351	0.72	9 ± 1 (2)
8	7.8	−0.355	0.82	21 ± 4 (2)
9	7.6	−0.360	0.45	33 ± 1 (2)
10	7.7	−0.380	8.2	18 ± 5 (5)
11	7.5	−0.389	0.43	8 ± 2 (6)
12	7.8	−0.389	0.11	7 ± 1 (2)
13	7.8	−0.391	77	33 ± 10 (6)
14	6.3	−0.395	21	8 ± 1 (2)
15	7.8	−0.409	120	18 ± 3 (6)
16	7.0	−0.423	1.5	4 ± 1 (2)
17	5.2	−0.457	1.4	<1
18	9.4	−0.486	0.96	<1
19	6.1	−0.517	2.6	<1

* Values of one-electron reduction potential [E_7^1 (V)] and octan-1-ol/aqueous buffer partition coefficient (P) not previously published in refs [7], [9], [10], [12] and [26] were measured as described in Materials and Methods.

† Values are means for number of determinations shown in parentheses (± S.D.) expressed as nmole nitroimidazole reduced (calculated from loss of 325 nm absorption using ε tabulated) per min per unit of xanthine oxidase at 37°, pH 7.4, under N_2.

xanthine concentrations of 0.06, 0.5 or 5 mM, or by substituting hypoxanthine for xanthine at 5 mM. The xanthine oxidase inhibitor, allopurinol (10 μM), completely prevented the reduction of misonidazole by the enzyme, but did not significantly alter the reduction rate for the more electron-affinic 2-nitroimidazole **3** (Table 1), 100 μM allopurinol being required for complete inhibition in this case.

Figure 3 shows the dependence of the logarithm

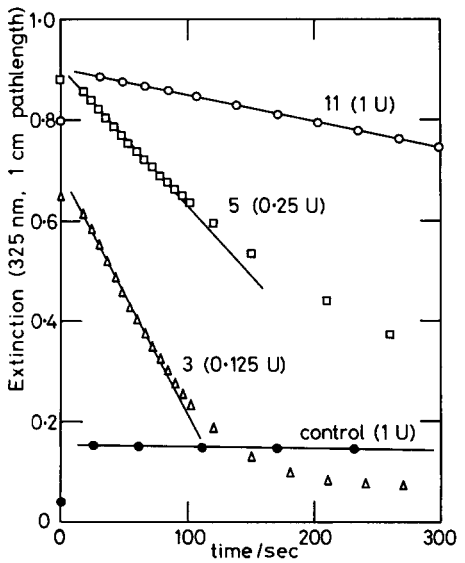


Fig. 2. Extinction vs. time plots for reduction of nitroimidazoles (0.1 mM) with xanthine (0.5 mM) and xanthine oxidase. ○: misonidazole (**11**), 1 U enzyme; □: RGW-806 (**5**), 0.25 U; △: RGW-801 (**3**), 0.125 U; ●: no nitroimidazole, 1 U.

of the rate of anaerobic nitroreduction upon the one-electron potential E_7^1 for 11 neutral nitroimidazoles of low to moderate lipophilicities (see below). The rates varied by over two orders of magnitude over the range of E_7^1 investigated. The free energy relationship correlating rate with E_7^1 is described by the least squares equation:

$$\log \text{rate (nmole min}^{-1}\text{ U}^{-1}) = (6.37 \pm 0.35) + (13.75 \pm 1.00)E_7^1(\text{V}), \quad (1)$$

where the uncertainties are standard errors.

Values of lipophilicity P are included in Table 2 because of the possibility that the nitroreduction rate could be dependent upon factors which influence binding of the nitro compound to the enzyme. It has been shown [11] that only neutral nitroimidazoles of high lipophilicity (the study included compounds **6**, **10**, **11** and **13**) bind significantly to the protein, bovine serum albumin. Compounds **10**–**15** were therefore included in the present study since they have similar values of E_7^1 but vary in P over three orders of magnitude (Table 2). An apparent effect of lipophilicity was only seen with compounds **13** and **15**, the two compounds of highest lipophilicity, their nitroreduction rates being *ca.* 2–3 times higher than expected from their E_7^1 using equation (1), which describes the behaviour of compounds with $P < 21$. Figure 3 shows that the rates for compounds **13** and **15** are outside the 95% confidence limits for the predicted values of rate for an individual compound, but the exclusion of these compounds from the regression analysis is nevertheless somewhat arbitrary.

An examination of the effect of molecular charge was made using the 2-nitroimidazoles **6** and **7** which are >95% protonated at pH 7.4. For both compounds a 5-fold lower rate of nitroreduction was

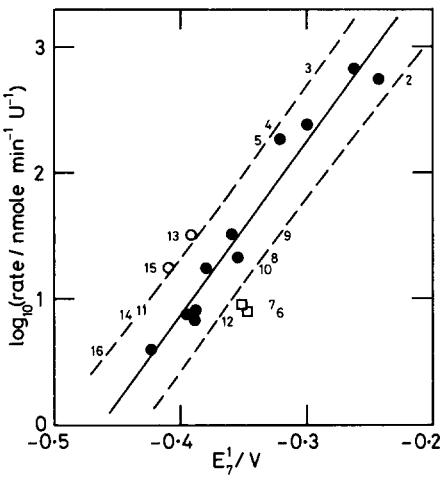


Fig. 3. Redox dependence of the initial rates of anaerobic nitroreduction of nitroimidazoles (0.1 mM) by xanthine (0.5 mM) and xanthine oxidase. The individual compounds are labelled as in Table 1. ●: neutral nitroimidazoles with $P < 21$ (see text); ○: neutral nitroimidazoles of higher P; □: nitroimidazoles with basic substituents. The solid line is that represented by equation (1) and the dashed lines are the 95% confidence limits on the predicted value of rate of an individual compound.

measured compared to the values calculated using equation (1). The nitro compound of highest electron affinity (−0.205 V) so far measured in the Gray Laboratory radiosensitizer development programme [12] is 8-nitrocaffeine **1** (Table 1). Its nitroreduction rate (979 ± 100 nmole $min^{-1} U^{-1}$ xanthine oxidase), measured under the same conditions used for the nitroimidazoles, was *ca.* 4-fold lower than expected for a nitroimidazole of the same E_7^1.

DISCUSSION

Tatsumi *et al.* [13] measured the rates of anaerobic reduction at 37°, pH 7.4, of many 4-substituted nitrobenzenes (30 μM) using xanthine (60 μM) and xanthine oxidase (0.05 U/2.5 ml), and reported a trend of higher rates of uric acid formation with increasing electron-withdrawing power (Hammett sigma) of the substituent. Wardman [14] has shown that the Hammett relationship:

$$E_7^1(\mathrm{V}) = (-0.484 \pm 0.011) + (0.168 \pm 0.014)\sigma_p^- \qquad (2)$$

can be used to relate E_7^1 with σ_p^-. Using values of σ_p^- [15] for the substituents studied by Tatsumi *et al.*, together with equation (2), for 16 uncharged 4-nitrobenzenes, an equation can be derived relating the reduction rates reported by Tatsumi *et al.* [13] to E_7^1 similar to equation (1), which has a least-squares slope of 6.5 ± 0.7 V^{-1}. This is about half the redox dependence of reduction rate found in the present work for nitroimidazoles, equation (1). In our earlier work [1] we reported a similar reduction rate for 4-nitroacetophenone to that found by Tatsumi *et al.* under the same conditions.

It is difficult to explain this apparent difference in the relative importance of redox properties in the nitroreduction of nitroimidazoles and nitrobenzenes without much more extensive studies. Chrystal *et al.* [16] report a K_m value of 3.6 mM for metronidazole when reduced by hypoxanthine–xanthine oxidase at 37°, pH 7.4. Raleigh *et al.* [17] present data from which an approximate nitroreduction rate of 70 nmole $min^{-1} U^{-1}$ xanthine oxidase for 1 mM misonidazole reduced by xanthine–xanthine oxidase at 37°, pH 9, can be estimated. As the rate of electron transfer to nitro compounds from xanthine oxidase using xanthine or hypoxanthine as electron donor can be expected to be similar at pH 7.4 and pH 9 [18, 19], it appears that the rate of nitroreduction for misonidazole is a function of concentration of nitro compound up to at least 1 mM (c.f. nitroreduction rate of 8 ± 2 nmole $min^{-1} U^{-1}$ for 0.1 mM misonidazole, this work). Thus, in the present work, and very probably in that of Tatsumi *et al.* [13], using concentrations of nitro compound of 0.1 and 0.03 mM, respectively, the rates of nitroreduction observed are well below those expected at concentrations where V_{max} would be measured. Under such conditions, if the K_m values for the generally more lipophilic nitrobenzenes differ from those for nitroimidazoles, then the slopes of redox relationships such as equation (1) can be expected to vary.

In view of the above difficulties in the quantitative evaluation of redox relationships involving enzymatic reduction at a single concentration of nitro compound, it may be inappropriate to make comparisons with non-enzymatic model systems, for example, to help identify the site of electron transfer from the reduced enzyme to the nitro compound. Thus, in the previous work [1] we demonstrated a free-energy relationship for the reduction of a similar series of compounds by free reduced flavin mononucleotide ($FMNH_2$), involving a redox dependence of $\log_{10}$ (reduction rate) of 18.4 ± 2.1 V^{-1}. We suggested that this coefficient, similar to the factor 16.9 V^{-1} (F/2.3 RT at 25°) was evidence in support of a rate-determining electron-transfer reaction involving a single electron transfer. The 95% confidence limits on the redox coefficient in equation (1) is 11.5–16.0 V^{-1} and F/2.3 RT at 37° = 16.3 V^{-1}. However, as it is not practicable in the present work (because of spectrophotometric and solubility limitations) to measure optimal nitroreduction rates (V_{max}) or to determine K_m values, it would be premature to conclude that the present results for the reduction of nitroimidazoles by xanthine–xanthine oxidase are evidence that nitroreduction involves one-electron transfer reactions at the flavin site by analogy with our free flavin work [1].

In spite of these reservations, these results again demonstrate the overwhelming importance of the reduction potential in controlling the chemical reactions and biological effects of nitroaryl compounds. Watts *et al.* [11] have summarized some redox relationships involving effects on cell survival and other endpoints *in vitro*. In particular, Olive [20] has shown that the rates of nitroreduction of some nitroheterocyclics by L-929 cells, *E. coli* B/r or mouse liver microsomes *in vitro* all involve a redox dependence analogous to equation (1) with a coefficient of *ca.*

11 V^{-1}. Raleigh *et al.* [17, 18] have previously found that the efficiency of electron transfer from aerobic xanthine–xanthine oxidase to the 5-nitrofuran *cis* AF-2 is reduced by the presence of other nitroheterocyclic compounds in a redox-related manner. However, measurements of nitroreduction rates in their system are likely to be complicated by the occurrence of other one-electron transfer reactions, involving the two nitro compounds and oxygen. In particular, the radical-anion of the competing nitro compound will transfer its electron to *cis* AF-2 and oxygen on the same timescale, before isomerization (*cis* to *trans* AF-2) (Clarke and Wardman, unpublished work).

We reported earlier [1] that the stoichiometry of nitroreduction of the 2-nitroimidazole **3** (Table 1), by xanthine–xanthine oxidase involved the transfer of 4 electrons, as indirect evidence for hydroxylamine formation with nitroimidazoles. Josephy *et al.* [21] reported 4-electron stoichiometry for the reduction of misonidazole (0.1 mM) in a similar system, and, using hypoxanthine as electron donor measure, a nitroreduction rate of 6 nmole $min^{-1} U^{-1}$ xanthine oxidase (cf. 8 ± 2 nmole $min^{-1} U^{-1}$ enzyme measured in this work for misonidazole). Chrystal *et al.* [16] observed anaerobic reduction of metronidazole (5.85 mM) by hypoxanthine–xanthine oxidase over *ca.* 25 hr, from which a very approximate average nitroreduction rate of *ca.* 4 nmole $min^{-1} U^{-1}$ enzyme can be derived. Equation (1) predicts a reduction rate for metronidzole of *ca.* 0.5 nmole $min^{-1} U^{-1}$ when the concentration of nitro compound is 0.1 mM. This dependence of rate on concentration of nitro compound is consistent with the K_m reported (3.6 ± 0.5 mM) [16].

CONCLUSION

There is considerable evidence for the involvement of the nitro-radical anion as an obligate intermediate in the biochemical reduction of nitroaryl compounds [22, 23] and this work has again demonstrated the usefulness of measurements of the energetics of this one-electron reduction process. Xanthine oxidase is widely distributed in humans [24], but there are many other known flavin-containing enzymes which can function as nitroreductases [13, 19, 22, 25]. Although xanthine oxidase may be only one nitroreductase functioning *in vivo*, it may well model the importance of reduction potential in controlling the reduction of nitroaryl compounds by these other flavoenzymes. Nitroaryl compounds, like quinones and viologens, yield relatively stable free radicals and span a wide range of one-electron reduction potentials [2–12, 26, 27]. They may be more generally useful as redox probes and mediators of electron transport in investigating other aspects of enzyme function.

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