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Anaerobic reduction of nitroimidazoles by reduced flavin mononucleotide and by xanthine oxidase

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Anaerobic reduction of nitroimidazoles and other nitroaromatic compounds used in medicine is thought to be responsible for both their therapeutic and their toxic properties [1]; these compounds may also have chemotherapeutic applications against hypoxic tumour cells [2]. Many of the known nitroreductases contain a flavin as coenzyme [3] and free flavins accelerate nitroreduction in isolated nitroreductases *in vitro* (e.g. Refs. 4 and 5). In a non-aqueous system the rate of reduction of substituted nitrobenzenes by free reduced flavins depends on the redox-related Hammett σ substituent parameter [6]. We compare here the anaerobic reduction of nitroimidazoles of varying electron affinity by a free reduced flavin and by the flavoenzyme xanthine oxidase.

Methods. $FMNH_2$* was produced in an anaerobic spectrophotometer cell by the addition of dithionite (BDH) to FMN (Sigma) using techniques similar to those described by Dixon [7, 8] and the nitro compound added to initiate the reaction. FMN production was monitored at 445 nm. Anaerobic reduction by xanthine oxidase (Sigma, from buttermilk X1875) used the Dixon anaerobic technique except that the N_2-bubbling needle was raised above the solution immediately after adding the enzyme. Other conditions were as used by Tatsumi *et al.* [9]. The liquid chromatographic (h.p.l.c.) equipment has been described [10]; a 10 μm Spherisorb ODS column and methanol (30–40%)/water eluent was used. Reduction potentials were derived from redox equilibria measured using the pulse radiolysis technique [11].

* Abbreviations used: FMN, FMNH·, $FMNH_2$: oxidized, semiquinone and reduced states of flavin mononucleotide; *Ar*, aryl or heteroaryl group; E_7^1 one-electron reduction potential at pH 7 vs NHE (normal hydrogen electrode); *t*, time in seconds.

Compounds. Misonidazole was provided by Roche Products Ltd., metronidazole by May & Baker Ltd., 4-nitrobenzoic acid and 4-nitroacetophenone by BDH, and nitrofurans by Koch-Light. Compounds prefixed 'RGW' were synthesized by Dr. R. G. Wallace at Brunel University from 2-nitroimidazole supplied by Roche Products Ltd. Buffers and other chemicals were BDH, A.R. grade.

Anaerobic reduction by $FMNH_2$ in water at pH 7.4 of some nitrobenzenes, nitrofurazone and all the nitroimidazoles investigated (Table 1) was shown to involve stoichiometry corresponding to reaction (1):

$$ArNO_2 + 2\ FMNH_2 \longrightarrow ArNHOH + 2FMN + H_2O. \quad (1)$$

4-nitroacetophenone was shown to yield 4-hydroxylaminoacetophenone by comparison with authentic material kindly supplied by Dr. J. W. Gorrod (λ_{max} = 312 nm; identical h.p.l.c. retention time); 4-electron stoichiometry has been reported previously for reduction of nitrobenzenes by reduced flavins in aqueous [12] and non-aqueous [6] solvents. Anaerobic reduction of 4-nitroacetophenone by xanthine–xanthine oxidase yielded only the hydroxylamine (no nitroso, no amine by h.p.l.c.), in agreement with the work of Tatsumi *et al.* [9]. Xanthine–xanthine oxidase reduction of most nitroimidazoles studied was inconveniently slow to permit measurement of the stoichiometry (see below), but with the highly electron-affinic nitroimidazole (*B*) (Table 1), complete removal of the nitro compound (30 μmoles. dm^{-3}) was accompanied by the formation of uric acid (60 μmoles.dm^{-3}), i.e. 4-electron stoichiometry.

Although the products of reduction of nitroimidazoles have not been indentified, experiments referred to in Ref. [1] and our own work (to be reported elsewhere) has shown that other reducing agents (dithionite, reduced viologen

Table 1. The compounds studied

Figure code	Name	E_7^1/V vs NHE
A	Nitrofurazone[5-nitro-2-furaldehyde-semicarbazone]	−0.257 *
B	RGW-801 [5-cyano-1-methyl-2-nitroimidazole]	−0.262 †
C	RGW-617 [1-(2-*N*-methylmorpholiniumethyl) 2-nitroimidazole iodide]	−0.318 †
D	4-Nitroacetophenone	−0.355 ‡
E	Misonidazole [1-(2-hydroxy-3-methoxypropyl) 2-nitroimidazole]	−0.389 §
F	4-nitrobenzoic acid	−0.425 †
G	RGW-614 [1-(1-carboxymethyl)-2-nitroimidazole, methanol sodium salt]	−0.443 †
H	Metronidazole [1-(2-hydroxethyl)-2-methyl-5-nitroimidazole]	−0.485 §
I	RGW-611[1-(2-morpholinoethyl)-4-nitroimidazole]	−0.554 †

* Ref [15].
† This work.
‡ Ref. [14].
§ Ref. [16].

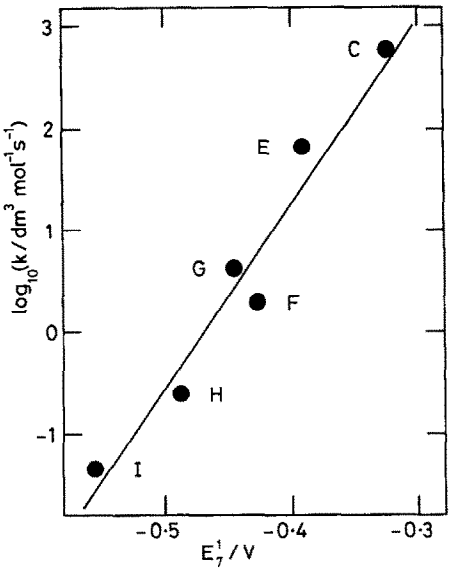

Fig. 1. Dependence of the second-order rate constant, k (see text) for reduction of nitroaromatic compounds by reduced flavin mononucleotide at pH 7.4 and 25° upon the one-electron reduction potential E_7^1. See Table 1 for key to compounds.

radical-cations, $CO_2^{\cdot-}$ (see Ref. 11), hydroquinones) generally reduce nitroimidazoles with 4-electron stoichiometry. Bray [13] has reviewed reduction of oxygen and quinones by xanthine oxidase and has noted the importance of flavin sites in reduction via one-electron transfers to produce superoxide or semiquinone free radicals. There are many similarities in the one-electron transfer reactions of oxygen, quinones and nitroaromatic compounds [11]. We suggest, therefore, that flavin sites are the route of reduction of nitroaromatic compounds by xanthine–xanthine oxidase, although the analogy with nitrate reductase and the involvement of molybdenum sites cannot be ruled out. We conclude that further reduction of hydroxylaminoimidazoles to the amines is relatively difficult using $FMNH_2$ and most other reductants (including xanthine oxidase) and suggest that reduction of nitroimidazoles by both $FMNH_2$ and xanthine oxidase can be represented by equation (1).

Using e.g. 20–40 μmoles. dm^{-3} $ArNO_2$, reaction (1) was found to proceed on a time-scale of minutes using most of the nitroimidazoles listed in Table 1. In agreement with studies with nitrobenzenes [6, 12] we found the rate to be first-order in $[ArNO_2]$. Oxidized flavin (FMN) inhibited the reaction (initial rate $\propto[FMN]^{-0.5}$) and the order with respect to $[FMNH_2]$ varied during the reaction, apparently increasing from ~1 to ~2 as the reaction progressed; these observations were not reported by the earlier workers. Reactions could be accelerated ~10-fold by adding Fe(II) salts (~5 μmoles. dm^{-3}); EDTA (~20 μmoles. dm^{-3}) was therefore added to all solutions to prevent the noticeable and varied effects of Fe impurity in the buffers used. Experiments with 4-nitrobenzoate (*F*) using EDTA, analysed according to Voshall and Carr [12], gave a rate constant > 10-fold lower than they reported, possibly because of Fe impurity in the earlier work.

Analysis of the rate data, assuming rate $\propto[FMNH_2][ArNO_2]$[6], was useful for the initial half of the reactions where $[FMN] < [FMNH_2]$, and for comparison with rates for nitrobenzenes in non-aqueous solvents[6]. Overall second-order rate constants k for reaction (1) were derived using this simplified rate expression. These data are plotted in Fig. 1 for 4-nitrobenzoate (*F*) and for the nitroimidazoles listed in Table 1. The rate constants span four orders of magnitude and reaction (1) is faster the higher the reduction potential of $ArNO_2$; nitrofurans and other nitroimidazoles with $E_7^1 > -0.3$ V reacted too fast for study, but are expected to follow the relationship indicated in Fig, 1. Data for 4-nitroacetophenone (*D*) did not fit the overall second-order analysis; the reaction was ~three times faster than that with the 2-nitroimidazole (*C*) (Table 1) and ~two times slower than with nitrofurazone (*A*). Hence, 4-nitroacetophenone is more reactive towards $FMNH_2$ than one would predict from Fig. 1 and the value $E_7^1 = -0.36$ V [14]; we have previously noted that the disproportionation rate of $ArNO_2^{\cdot-}$ is orders of magnitude more rapid with 4-nitroacetophenone than with many other nitroaromatic compounds at pH 7.4 [15].

Assuming the overall rate of (1) to depend on the formation of a nitroso intermediate which is reduced more rapidly than the parent nitro compound, a reaction scheme for (1) can be postulated [6] in which the first step is production of the flavosemiquinone FMNH· and the nitro radical $ArNO_2^{\cdot-}$ in a one-electron transfer equilibrium involving $ArNO_2$ and $FMNH_2$:

$$FMN + FMNH_2 \rightleftharpoons 2\ FMNH\cdot \quad (2)$$

$$FMNH_2 + ArNO_2 \rightleftharpoons FMNH\cdot + ArNO_2^{\cdot-} \quad (3)$$

$$FMNH_2 + ArNO_2^{\cdot-} \rightarrow FMNH\cdot + ArNO + OH^- \quad (4a)$$

$$FMNH\cdot + ArNO_2^{\cdot-} \rightarrow FMN + ArNO + OH^-. \quad (4b)$$

Mason's studies [17] have suggested that $ArNO_2^{\cdot-}$ is formed whenever reduced flavoenzymes are present. The dependence of $\log_{10} k$ upon E_7^1 (Fig. 1: least-square slope = 18.4 ± 2.1 V^{-1}) is in good agreement with the value 16.9 V^{-1} (i.e. *F*/2.3RT) calculable from such a model. The full rate equation (5) derived from a modification of Gibian and Baumstark's scheme [6] may involve an initial inverse square root dependence upon [FMN], an apparently varying order for $[FMNH_2]$ as the reaction proceeds and a first-order dependence on $[ArNO_2]$:

$$d[FMN]/dt = 2(K_3/K_2^{1/2})\ [FMNH_2]\ [ArNO_2] \quad (5)$$

$$\times \{k_{4a}([FMNH_2]/[FMN])^{1/2} + k_{4b}K_2^{(1/2)}\}. \quad (5)$$

Since the values of the one-electron transfer equilibria in the scheme are now calculable, the rate equation has only two unknown parameters. These are the rate constants k_{4a} and k_{4b} for one-electron reduction of $ArNO_2^{\cdot-}$ to $ArNO$ by either $FMNH_2$ or FMNH·, respectively. Estimates of these rate constants were obtained by taking tangents of the extinction–time curve, converting to rate (d[FMN]/d*t*) and calculating $[FMNH_2]$, and [FMN] and $[ArNO_2]$ via the known extinction coefficients and reaction stoichiometry, and solving the resulting pairs of simultaneous equations after insertion of the individual values in equation (5). Draper and Ingraham [18] give $K_2 = 0.0071$ and $E_{7.4}^1$ (FMNH·/$FMNH_2$) = −0.194 V. Using the relationship

$$E_{7.4}^1(ArNO_2/ArNO_2^{\cdot-}) - E_{7.4}^1\,(FMNH\cdot/FMNH_2) = 0.059 \log K_3 \quad (6)$$

where the potentials E^1 (in V) are given in Table 1, we calculate $K_3 = 5.0 \times 10^{-4}$ and 1.1×10^{-5} for misonidazole and metronidazole, respectively. (The nitro compounds have virtually identical values of E^1 at pH 7.0 and 7.4.)

In Fig. 2 we compare observed and calculated extinctions during reduction by $FMNH_2$ of misonidazole (with and without excess FMN) or metronidazole. Numerical integration of the rate equation (5) was performed using a modified Euler or fourth-order Runge–Kutta procedure with rate constants derived from the variation of rate during

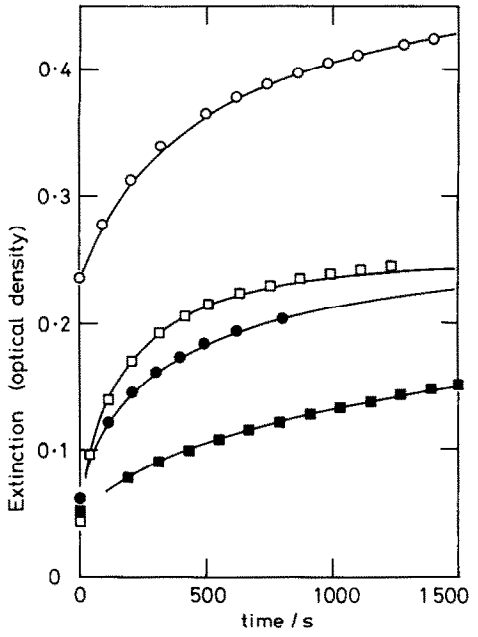

Fig. 2. Extinction changes observed at 445 nm (pathlength 10 mm) during reaction of nitroimidazoles with reduced flavin mononucleotide at pH 7.4 and 25°. All solutions initially contained 17–20 μmoles. dm^{-3} $FMNH_2$ together with: (●) 20 μmoles. dm^{-3} misonidazole (first half-life 230 sec); (○) 20 μmoles. dm^{-3} misonidazole and 14 μmoles. dm^{-3} FMN (400 sec); (□) 40 μmoles. dm^{-3} misonidazole (130 sec); (■) 1 mmole. dm^{-3} metronidazole (1450 sec).

the reaction, as described above. The solid lines are computed simulations using $k_{4a} = 1.6 \times 10^4$ or 3.0×10^3 $dm^3.mol^{-1}.sec^{-1}$, and $k_{4b} = 4.3 \times 10^4$ or 2.6×10^3 $dm^3.mol^{-1}.sec^{-1}$ for misonidazole or metronidazole, respectively. This analysis suggests that the two alternative one-electron reductions of $ArNO_2^-$ noted above have rate constants k_{4a} and k_{4b} of about the same order, although the absolute values obtained by these procedures are susceptible to errors in K_2 and/or K_3.

Tatsumi *et al.* [9] measured the rates of reduction of many nitroaromatic compounds in the anaerobic xanthine–xanthine oxidase system; we obtained similar reduction rates for 4-nitroacetophenone and nitrofurazone (690 and 540 nmoles uric acid formed.min^{-1}.mg $protein^{-1}$, respectively). Our value for 4-nitrobenzoate (~24 nmoles.$min.^{-1}mg^{-1}$) was somewhat lower than that reported by Tatsumi *et al.* (80 nmoles.$min.^{-1}mg^{-1}$). For 5-cyano-1-methyl-2-nitroimidazole (*B*) we measured a reduction rate of 250 nmoles.$min.^{-1}mg^{-1}$, but the rates of reduction of the less electron-affinic nitroimidazoles listed in Table 1 were very much slower; e.g. no reaction was detected after 30 min at 37° when misonidazole (75 nmoles), xanthine (150 nmoles) and xanthine oxidase (0.08–0.06 mg protein) in 2.5 ml were incubated (rate ≤ 1 nmole.$min.^{-1}mg^{-1}$). If the xanthine–xanthine oxidase system had a similar redox dependence to that observed with free $FMNH_2$, a 200-fold slower rate would be expected for misonidazole compared to 5-cyano-1-methyl-2-nitroimidazole. Tatsumi *et al.* [9] found more electron-affinic nitrobenzenes were reduced most rapidly; comparison of the rates with those of nitroimidazoles of similar E_7^1 shows that the redox relationship in the xanthine–xanthine oxidase system is not as independent of aromatic ring structure or other chemical properties (lipophilicity, configuration) as is likely to be the case with the free flavin.

In conclusion, the stoichiometry of reduction of nitroimidazoles by both free and a protein-bound flavin has provided evidence for the production of hydroxylamines during biochemical nitroreduction, as has been discussed for nitrobenzenes [6, 9, 12], 5-nitrofurans [19] and a nitrothiazole [20]. Thermodynamically-reversible reduction potentials, E_7^1, have enable us to provide quantitative support of a reaction scheme for the anaerobic reduction of nitroaromatic compounds by reduced flavin mononucleotides. The scheme includes the production of nitro free-radicals in a one-electron transfer reaction; these radicals are known to be produced from isolated nitroreductases [17] and we have shown that the nitro radicals transfer their electrons to O_2 (to give O_2^-) at a redox-controlled rate [15]. Quantification of the importance of redox properties in controlling the enzymatic reduction of the medically-important nitroimidazole group of compounds should facilitate the identification of other chemical properties which influence nitroreduction *in vitro*.

Note added in proof—Using a more active enzyme preparation (Sigma X4500), it has since been possible, by monitoring at 325 nm, to measure the rates of nitroreduction by xanthine oxidase of seven 2-nitroimidazoles (including misonidazole), with reduction potentials between −0.42 and −0.24 V. Preliminary analysis of the over 100-fold variation in rate showed a plot of log(initial rate) vs reduction potential had a least-squares slope and correlation coefficient of 13.0 ± 1.3 V^{-1} and 0.976, respectively.

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Effects of propranolol on the biochemical modifications induced by a β-adrenergic drug in ischemic hearts

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It has already been demonstrated that the control by propranolol of the cardiac activity stimulated by a β-adrenergic drug [1, 2] was accompanied by the return to basal values of the cellular oxidations supplying the myocardium with the major part of the additional energy produced: the increase in oxygen consumption determined from coronary flow and arteriovenous difference in oxygen content is suppressed by propranolol [2, 3]. The excess substrates oxidized in these circumstances are circulating nutritive substances, glucose, lactate and free fatty acids (FFA), but chiefly FFA in hearts *in situ* [4, 5, 6] or similar substances originating in the cardiac tissue itself from the hydrolysis of glycogen [3–7] and triglycerides [8–10].

But the anaerobic breakdown of glycogen also represents a source of additional energy which is certainly the most rapidly available under any conditions and is the most important when the oxygen supply is restricted by obstructive lesions of the coronary vessels. The predominance of anaerobic processes in supplying energy, when catecholamine release and ischemia combine their effects, may cause sufficient alterations in metabolite concentrations to promote disorders such as thoracic pain. Consequently, it was of interest to investigate whether propranolol attenuated these alterations as well as the stimulation of the oxidative metabolism.

The study was carried out on hearts *in situ*, in dogs weighing 20–28 kg, that had not been given any food for 12 hr. The dogs were anesthetized with barbiturates (25 mg/kg of sodium thiopental intravenously injected to which 5 mg/kg of sodium pentobarbital were added 10 min before the beginning of the test period). All were submitted to ischemia for 45 min. In addition to the ischemia, from the 15th min, six received a perfusion of isoproterenol ($1\ \mu g \cdot kg^{-1} \cdot min^{-1}$) and six the same perfusion associated with the administration of propranolol (0.2 mg/kg intravenously injected and 0.2 mg/kg perfused over 30 min).

The ischemia desired was an incomplete one, analogous to that responsible for angina pectoris, i.e. eliciting a relative deprivation of oxygen and nutritive substances as compared with requirements. Considering the difficulty of initiating an ischemia stable at a given level, which had, furthermore, to be strictly identical in both groups of animals, the blood flow was alternately totally interrupted and left totally free for 2 min by means of a thread placed loosely round the left coronary artery just before its division into two branches, interventricular and circumflex arteries.

Sampling of the myocardium, required for the determination of the main substrate content, planned to be carried out several times in the course of an experiment, 10 min before and 15, 20, 30 and 45 min after the beginning of the test, was made possible by an extracorporal circulation system [11]. After opening and removing a large part of the right thoracic wall, the venae cavae blood flow was diverted to an extracorporal circuit including oxygenator, peristaltic pump and heat exchanger and returned to the animal via the abdominal aorta centrally cannulated. In this way, it was possible to take samples of the left ventricle external wall from its total thickness using the 'drill biopsy' technique [12], the quantitative determinations of lactate [13], glycogen [14], triglycerides [15] and FFA [16] being subsequently performed on the subendocardial and subepicardial layers after separation [11]. With regard to FFA, the analysis was made according to Duncombe's method [16], which is unspecific, because the aim was only to investigate the modification of the overall FFA concentration. But it was made on 200 μl of tissue extract after neutralization instead of being made on 500 μl of plasma. Between sampling and homogenizing, the myocardial fragments were kept in liquid nitrogen in which they had been immersed as soon as obtained (in less than 10 sec, in order to avoid glycogenolysis and lipolysis especially).

The statistical study was made between control and test values using Student's *t*-test.

Effects of isoproterenol on ischemic hearts. The heart rate acceleration due to isoproterenol was notably less considerable under the ischemic than normal conditions: the maximal increase was from 142 ± 12 (S.E.M.) to 163 ± 8 beats per min (N.S.) in the former case and from 123 ±