Effect of Reduction Potential on the Rate of Reduction of Nitroacridines by Xanthine Oxidase and by Dihydro-flavin Mononucleotide

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The cytotoxicity of many nitroaromatic compounds is mediated by bioreduction. There is a correlation between the one-electron-reduction potentials and cytotoxic potencies of simple nitroheterocycles such as nitroimidazoles, but no such correlation is observed with DNA-binding nitroacridines related to the drug nitracrine. A study of the reduction of 1-nitroacridines by the enzyme xanthine oxidase and by reduced flavin mononucleotide, $FMNH_2$ (as a model of the enzyme's active site) has been undertaken to explore the redox dependence in this series. The values of V_{max}/K_m for reduction by xanthine oxidase, XOD, and the second-order rate constants of reduction by $FMNH_2$, analysed using Marcus electron-transfer theory, indicate a correlation between reduction potential and the rate of reduction, but suggest that the potential-energy barrier for electron transfer is small relative to that imposed by requirements for association, orientation and desolvation of the reactants. The close similarity in Marcus parameters for reduction by $FMNH_2$ and by XOD suggests that reduction occurs by a similar mechanism in each case. The free-energy change for electron transfer, $\Delta G_o^{\ddagger}$, is only 1–2 kJ mol^{-1}. It appears that the rate of reduction is unlikely to be the only determinant of hypoxic cytotoxicity for the nitroacridines, although the applicability of findings with XOD to other nitroreductases is uncertain.

The physical events involved in a bimolecular collision in solution between two molecules A and B require a random diffusion of two molecules toward and away from each other, leading in some cases to the formation of a close-contact encounter complex, A · · · B, followed by their diffusion apart. We may write this process as eqn. (1). If electron transfer is to

$$A + B \underset{k_{-D}}{\overset{k_D}{\rightleftharpoons}} A \cdots B \qquad (1)$$

occur, it can occur at any stage in this process, with shorter distances being weighted more favourably.

For a reaction to occur, the reactants must approach each other to enhance the coupling of their electronic orbitals. At the same time there are fluctuations in other co-ordinates. The reaction system proceeds from co-ordinates in the vicinity of the equilibrium values for the reactants to those of the products. The electron transfer will only occur at or near nuclear configurations for which the total potential energy of the reactants and surrounding medium is equal to that of the prospective products and surrounding medium. A potential-energy diagram for a reaction involving electron transfer between A^+ and B^- is given in Fig. 1.

The Marcus equation[1] can be derived, recognising from Fig. 1 that eqn. (2) holds,[2] whence eqn. (3).

$$\Delta G_o^{\circ} = \Delta G_{obs}^{\circ} + w^p - w^r \qquad (2)$$

$$\Delta G_{obs}^{\ddagger} = w^r + \Delta G_o^{\ddagger}[1 + (\Delta G_{obs}^{\circ} - w^r + w^p)/4\Delta G_o^{\ddagger}]^2 \qquad (3)$$

In eqn. (3), w^r and w^p represent the chemical and solvational work required to bring reactants and products, respectively, to the mean separation distance for electron transfer. $\Delta G_o^{\ddagger}$ (sometimes symbolised as $\lambda/4$) is the value of

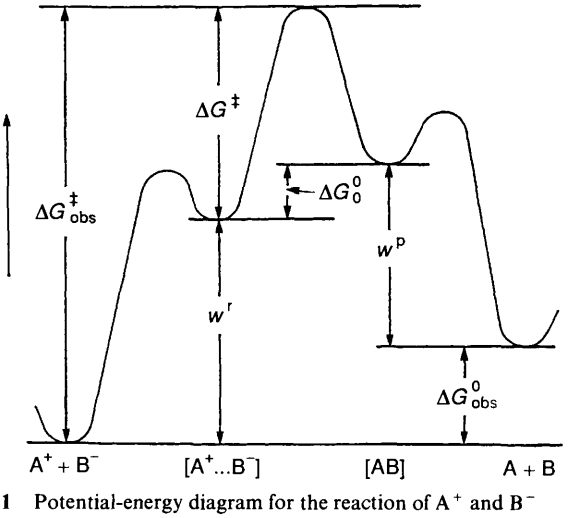


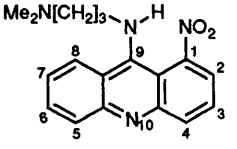
Fig. 1 Potential-energy diagram for the reaction of A^+ and B^-

$\Delta G^{\ddagger}$ for the electron transfer itself in the special case where there exists neither an overall thermodynamic driving force nor impediment ($\Delta G_o^{\circ} = 0$). There is thus predicted a parabolic relationship between the observables, $\Delta G_{obs}^{\ddagger}$ and ΔG_{obs}°, provided that $\Delta G_o^{\ddagger} \geqslant |4\Delta G_{obs}^{\circ}|$.

Biological electron transfers have some features in common with electron transfer between small molecules. For example, reactants in biological systems undergo vibrational and solvational changes during the reaction, and so the rate should depend on ΔG_{obs}° and $\Delta G_o^{\ddagger}$. However, there are also differences between electron transfers in biological and small-molecule systems. The environment for electron transfer is usually better understood in small-molecule systems than in biological systems. In biological systems, reactants may be held at spatially

distinct active sites and the mechanism of action often involves a series of intermediates and does not proceed in a single step. Additionally, protein conformational changes may proceed or follow electron transfer in enzyme systems. There may also be differences in binding affinities of the oxidised and reduced forms for an enzyme.[3] Marcus and Sutin[3] have extended electron-transfer theory for small molecules to electron transfers in biological systems.

It is well known that the biological properties of nitro-heterocyclic drugs are dominated by their one-electron-reduction potential (E^1) if this value is more positive than ~ -500 mV *versus* the standard hydrogen electrode.[4] For example, cytotoxic potency of the nitroimidazoles and nitrofurans in hypoxic mammalian cell cultures is logarithmically related to E^1.[5]



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Nitracrine,9-(3-dimethylaminopropylamino)-1-nitro-acridine, (**1**), is known to be a hypoxia-selective cytotoxin in mammalian cell culture and it is thought that reduction is the mechanism of metabolic activation of the compound.[6,7] However, for analogues of nitracrine no correlation between reduction potential and hypoxic cytotoxicity is observed.[7] For this reason we have examined the kinetics of reduction of both nitracrine and a series of analogues, using both xanthine oxidase (XOD) and dihydroflavin mononucleotide ($FMNH_2$) as a model of the flavin adenine dinucleotide-active site of XOD.

Experimental

Nitracrine and its analogues were synthesized by published methods.[8,9] Reduction of nitracrine and its analogues by $FMNH_2$ was undertaken in sodium formate buffer [0.1 mol dm^{-3}; pH 4; 20 °C containing ethylenediaminetetraacetic acid (EDTA) (1.8 μmol dm^{-3})]. Buffer (15 cm^3) was placed in a 5 cm pathlength cuvette and deoxygenated by bubbling for 30 min with nitrogen which had been passed through an oxy-trap (<0.1 ppm O_2). Flavin mononucleotide (5 μmol dm^{-3}) was reduced radiolytically by placing the cuvette in a ^{60}Co γ-radiation source and irradiating it at 268 Gy s^{-1} for 4.0 min, thus providing 1.98 electron reduction stoicheiometry. It is necessary to ensure that the reducing equivalents per flavin molecule do not exceed two as reduction beyond 1,5-dihydroflavin mononucleotide occurs very readily. The product of the initial reduction, $FMNH_2$, was stable for at least 30 min in a closed cuvette.

The deoxygenated nitro compound (2 mm^3; final concentration 10–25 μmol dm^{-3}) was then added through the septum of the cuvette using a syringe. The low reactant concentrations were used in order to provide rates low enough to measure with a Hewlett Packard HP8452A diode array spectrophotometer (first measurement 60 s after mixing, sampling frequency 60 s^{-1}). The change in absorbance at the maximum in the visible spectrum (400 nm) was monitored and second-order rate constants were evaluated by the method of Colter *et al.*[10]

Reduction of the nitroacridines using XOD was undertaken in 85 mmol dm^{-3} phosphate buffer; pH 7.4; 37 °C, containing xanthine (0.4 mmol dm^{-3}), EDTA (0.2 μmol dm^{-3}), and nitroacridine (40–400 μmol dm^{-3}). Anaerobicity was obtained by bubbling this solution with deoxygenated nitrogen for 15 min in a 1 cm pathlength sealed cuvette. The reaction was initiated by addition of enzyme (0.156 U; 20 mm^3) from a solution which had been deoxygenated by flushing with deoxygenated nitrogen for 45 min. The reaction was followed at λ_{max} in the visible spectrum (400 nm) using the HP8452A spectrophotometer. V_{max} (the extrapolated maximum velocity at saturating substrate concentrations) was calculated from at least seven zero-order rate constants collected in this way using the HP 89512 enzyme kinetics program. No reduction of the nitroacridines was observed under aerobic conditions.

$\Delta G^{\ddagger}_{obs}$ can be calculated from a second-order rate constant, k_2, using a form [eqn. (4)] of the Eyring equation.

$$\ln k_2 = -\Delta G^{\ddagger}_{obs}/RT + 23.760 + \ln T \qquad (4)$$

In the case of XOD, $\Delta G^{\ddagger}_{obs}$ was calculated from the measured V_{max}- and K_m-values, using the assumption that V_{max} represented a first-order rate constant for decomposition of the substituted nitroacridines bound to the enzyme. In all cases Michaelis–Menten kinetics were observed and V_{max} was evaluated by extrapolation to high substrate concentration. In the simplest kinetic case, eqns. (5) and (6) hold, where $[E]_o$ is the total

$$V_{max} = k_2[E]_o \qquad (5)$$

$$K_m = (k_{-1} + k_2)/k_1 \qquad (6)$$

enzyme concentration (constant in this case) and k_2 is the rate constant for the reaction occurring within the enzyme–substrate complex. Hence, although K_m ([S] for $V_{max}/2$) does vary between substrates as a result of substituent effects on binding to the active site, the quotient V_{max}/K_m for strongly bound systems ($k_{-1}/k_2 \gg 1$) is directly proportional to the second-order rate constant k_1 for substrate–enzyme interaction and the first-order rate constant k_2 for reaction within the enzyme–substrate complex. In our Marcus treatment the quotient V_{max}/K_m has been used as a pseudo-first-order rate constant in the absence of information on the molar concentration of the enzyme.

In an outer-sphere bimolecular electron-transfer reaction, ΔG°_{obs}, the standard free-energy change of reaction, is related to ΔE^1 by eqn. (7), where eqn. (8) holds.

$$\Delta G^{\circ}_{obs} = -nF\Delta E^1 \qquad (7)$$

$$\Delta E^1 = E^1(ArNO_2) - E^1(FMNH_2) \qquad (8)$$

One-electron-reduction potentials of the nitroacridines at pH 4 (E^1_4) and pH 7.4 ($E^1_{7.4}$) were calculated by the method of Wardman[4] from those measured[8] at pH 7 (E^1_7), using the pK_a-data of Wilson *et al.*[8]

ΔG°_{obs} at 37 °C for XOD was calculated by assuming that $E^1_{7.4}$ for the flavin adenine dinucleotide (FAD) in XOD at pH 7.4 was −0.329 V.[11] ΔE^1_4 for $FMNH_2$ at pH 4 has been determined by Anderson[12] to be −0.142 V.

Results and Discussion

Table 1 lists the results for the second-order rate constants for the reduction of nitracrine analogues by $FMNH_2$. ΔG°_{obs} is calculated from ΔE^1, and $\Delta G^{\ddagger}_{obs}$ is calculated from k_2. Table 2 lists similar parameters for the reduction of the nitracrine analogues by XOD where $\Delta G^{\ddagger}_{obs}$ is calculated from V_{max}/K_m. Also shown are the values of K_m which show substantial variation between substrates. In all cases (with the possible exception of the 4-F-substituted nitracrine) Michaelis–Menten kinetics were observed and K_m and V_{max} were well defined, *i.e.* saturation was approached.

Fig. 2 shows the relationship between $\Delta G^{\ddagger}_{obs}$ and ΔG°_{obs} for the reduction by $FMNH_2$ and Fig. 3 shows the relationship for reduction by XOD. Parabolic curves of best fit are shown in both cases, and are seen to correlate the data satisfactorily in the

Table 1 Observed values of k_2 and ΔE, values of $\Delta G^{\ddagger}_{obs}$ and ΔG°_{obs} calculated from these data, and the values of $\Delta G^{\ddagger}_{calc}$ for the reaction of nitroacridines with $FMNH_2$ at pH 4

Substituent	k_2/dm^3 mol^{-1} s^{-1}	$\Delta G^{\ddagger}_{obs}$/kJ mol^{-1}	ΔE^{1}_{4}/V	ΔG°_{obs}/kJ mol^{-1}	$\Delta G^{\ddagger}_{calc}$/kJ mol^{-1} [a]
4-H	5 200 ± 500	50.8	−0.128	12.3	50.8
4-CO_2Me	13 000 ± 2 000	48.6	−0.022	2.1	48.6
4-F	2 900 ± 300	52.3	−0.135	13.0	51.2
4-Me	5 100 ± 300	50.9	−0.150	14.5	52.2
4-NMe_2	700 ± 100	55.8	−0.172	16.6	54.1
4-OMe	540 ± 30	56.4	−0.204	19.7	57.4
2-NO_2	100 ± 20	60.5	−0.226	21.8	60.2
9-NH[CH_2]$_3$NHMe side-chain	8 300 ± 300	49.7	−0.124	12.0	50.5

[a] Calculated using equation (3) and the fitted Marcus parameters for the entire data set: $\Delta G^{\ddagger}_{o}$ = 1.57 kJ mol^{-1}, w^{r} = 48.4 kJ mol^{-1} and w^{p} = 37.5 kJ mol^{-1}.

Table 2 Observed values of k_2 and ΔE. Values of $\Delta G^{\ddagger}_{obs}$ and ΔG°_{obs} calculated from these data and the values of $\Delta G^{\ddagger}_{calc}$ for the reduction of nitroacridines by XOD at pH 7.4

Substituent	K_m/mmol dm^{-3}	V_{max}/μmol dm^{-3} s^{-1}	$\Delta G^{\ddagger}_{obs}$/kJ mol^{-1}	$\Delta E^{1}_{7.4}$/V	ΔG°_{obs}/kJ mol^{-1}	$\Delta G^{\ddagger}_{calc}$/kJ mol^{-1} [a]
4-H	0.20	1.31 ± 0.03	89.0	+0.046	−0.44	88.5
4-OMe	0.12	0.098 ± 0.003	94.4	−0.051	4.92	96.8
4-Cl	0.32	0.60 ± 0.01	92.3	+0.019	1.83	91.4
4-F	1.9	0.11 ± 0.01	101.2	−0.048	4.63	96.2
4-NMe_2	0.71	0.42 ± 0.01	95.2	−0.025	2.41	92.3
4-CO_2Me	0.32	1.57 ± 0.03	89.8	+0.062	−5.98	85.5
4-Me	0.10	0.77 ± 0.01	88.6	−0.013	1.25	90.6
2-NO_2	0.39	0.30 ± 0.02	94.6	−0.056	5.40	97.8
3-NO_2	0.14	6.8 ± 0.1	83.9	+0.059	−5.69	85.5
4-NO_2	0.05	5.8 ± 0.4	81.6	+0.042	−4.05	85.9

[a] Calculated using equation (3) and the fitted Marcus parameters for the entire data set: $\Delta G^{\ddagger}_{o}$ = 0.68 kJ mol^{-1}, w^{r} = 85.5 kJ mol^{-1} and w^{p} = 88.9 kJ mol^{-1}.

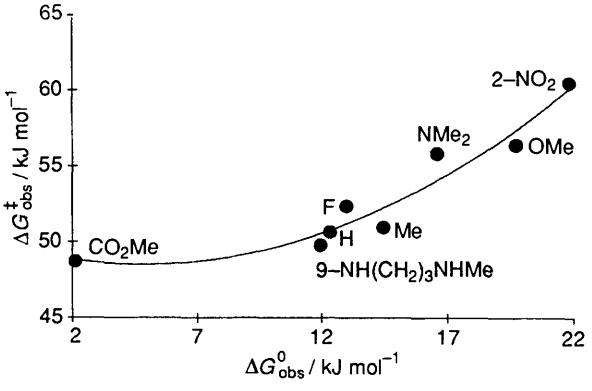

Fig. 2 $\Delta G^{\ddagger}_{obs}$ *versus* ΔG°_{obs} for the reduction of nitroacridines with $FMNH_2$ at pH 4 and 20 °C. The solid line represents the parabola of best fit: $\Delta G^{\ddagger}_{obs} = 49.19 - (3.64 \times 10^{-4})\Delta G^{\circ}_{obs} + (3.98 \times 10^{-8})(\Delta G^{\circ}_{obs})^2$. The compounds are 4-substituted analogues of 9-[3-(*N*,*N*-dimethylamino)propylamino]-1-nitroacridine unless otherwise indicated.

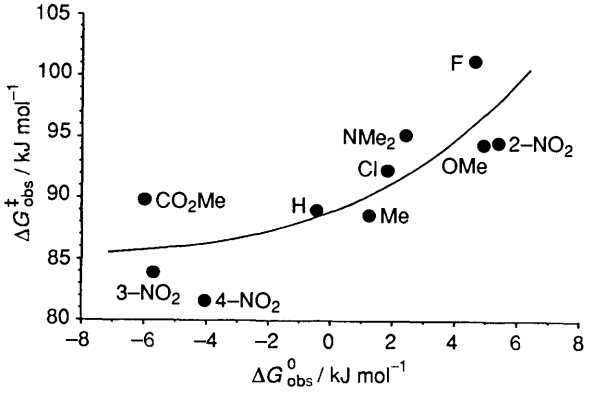

Fig. 3 $\Delta G^{\ddagger}_{obs}$ *versus* ΔG°_{obs} for the reduction of nitroacridines catalysed by XOD at pH 7.4 and 37 °C. The solid line represents the parabola of best fit: $\Delta G^{\ddagger}_{obs} = 89.00 + (1.14 \times 10^{-3})\Delta G^{\circ}_{obs} + (9.16 \times 10^{-8})(\Delta G^{\circ}_{obs})^2$. The compounds are 4-substituted analogues of 9-[3-(*N*,*N*-dimethylamino)propylamino]-1-nitroacridine unless otherwise indicated.

case of Fig. 2. In the $FMNH_2$ case (Fig. 2) the best-fit parabola provides a correlation coefficient r = 0.966, whilst r = 0.838 for attempted linear regression. Hence, inclusion of the quadratic term is real. A minor problem arises in that the parabolic minimum occurs within the ΔG°_{obs} data range instead of beyond it as required by Marcus theory. However, the apparent minimum is so close to the CO_2Me point that the onset of limiting encounter control may be assumed to have occurred; *i.e.*, $\Delta G^{\ddagger}_{obs} = w^{r}$, with no further change of $\Delta G^{\ddagger}_{obs}$ as ΔG°_{obs} becomes increasingly negative. Indeed, analysis of the best-fit parabola in terms of the Marcus equation, equation (3), yields the following Marcus parameters for $FMNH_2$ reduction: $\Delta G^{\ddagger}_{obs}$ = 1.57 kJ mol^{-1}, w^{r} = 48.4 kJ mol^{-1} and w^{p} = 37.5 kJ mol^{-1}. Therefore $\Delta G^{\ddagger}_{obs}$ for the CO_2Me compound (Table 1) is virtually identical with w^{r}. In Marcus terms the condition $\Delta G^{\ddagger}_{o} \geqslant 4|\Delta G^{\circ}_{obs}|$ has been violated, but inspection of Fig. 2 shows that the discrepancy is not serious.

If the CO_2Me compound is removed from the data set to avoid the problem of the parabolic minimum the quantitative conclusion is but little affected. The Marcus parameters for the restricted data set are: $\Delta G^{\ddagger}_{o}$ = 2.6 kJ mol^{-1}, w^{r} = 44.6 kJ mol^{-1} and w^{p} = 37.7 kJ mol^{-1}. The salient point, that both w^{r} and w^{p} are much greater than $\Delta G^{\ddagger}_{o}$, is maintained.

In the case of reduction of $ArNO_2$ by XOD, Marcus equation analysis of the parabola of best fit yields the parameters $\Delta G^{\ddagger}_{o}$ = 0.68 kJ mol^{-1}, w^{r} = 85.5 kJ mol^{-1} and w^{p} = 89.0 kJ mol^{-1}. The correlation coefficient r = 0.827 is significantly greater than r = 0.800 for attempted linear regression of $\Delta G^{\ddagger}_{obs}$ against ΔG°_{obs}. Neither of these correlations is a particularly good fit of

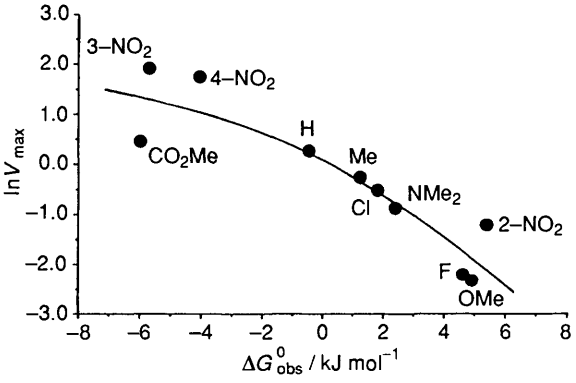

Fig. 4 ln V_{max} *versus* ΔG°_{obs} for the reduction of nitroacridines catalysed by XOD at pH 7.4 and 37 °C. The solid line represents the parabola of best fit: ln $V_{max} = 0.178 - (3.20 \times 10^{-4})\Delta G^{\circ}_{obs} - (2.00 \times 10^{-8})(\Delta G^{\circ}_{obs})^2$. The compounds are 4-substituted analogues of 9-[3-(*N*,*N*-dimethylamino)propylamino]-1-nitroacridine unless otherwise indicated.

the data when compared with the fit of the $FMNH_2$ data in Fig. 2. It is not possible, for instance, to state with certainty that inclusion of a quadratic term is justified. The fact that a pseudo-first-order rate constant (V_{max}/K_m) rather than a second-order rate constant is used to calculate $\Delta G^{\ddagger}_{obs}$ is one factor contributing to uncertainty, and neglect of enzyme concentration (although constant) is another. The relative scatter may well result from the fact that *both* V_{max} and K_m are derived from the same Michaelis–Menten plot. It may be significant that the values for the 4-F derivative were obtained in a concentration region far from saturation: the 4-F point is the most deviant. Nevertheless, there is clearly evident an overall trend in that the rate of enzymatic reduction is related to E^1 and, for what the Marcus parameters are worth, the pattern is the same as in the $FMNH_2$ system: very small $\Delta G^{\ddagger}_{o}$ and much larger w^r- and w^p-values.

In the limit of saturation kinetics, V_{max} represents the rate of the chemical reaction within the enzyme–substrate complex, with the influence of K_m having been factored out. It is therefore significant that a plot of ln V_{max} (proportional to k_2) *versus* ΔG°_{obs} for the XOD data, shown in Fig. 4, is more clearly parabolic than is the plot in Fig. 3. The correlation coefficient for the parabolic fit in Fig. 4 is 0.921, which signifies that the reduction potential is more closely related to reaction rate itself than it is to V_{max}/K_m, where binding parameters unrelated to electron transfer are incorporated. Marcus parameters cannot be calculated from data used to generate Fig. 4, but the low curvature of the best-fit parabola suggests a low value of $\Delta G^{\ddagger}_{o}$[2] in accord with the tentative conclusion reached earlier. In this case encounter control is apparently not achieved within the range of substituents employed, and the CO_2Me compound is significantly less reactive than predicted by the best-fit parabola. Also, the approximate $\Delta G^{\ddagger}_{obs}$-values appear to be approaching the asymptotic limiting value corresponding to encounter control for the most reactive compounds.

The conclusion that $\Delta G^{\ddagger}_{o}$ is very small in comparison with w^r and w^p stands in the case of reduction by both $FMNH_2$ and XOD. In the former case the very low value of $\Delta G^{\ddagger}_{o}$ (1.6 kJ mol^{-1}) shows that there is only a very small potential-energy barrier to transfer of the electron from a reactant- to a product-configuration. This result implies that the major part of the overall activation barrier originates from the energy required for association, orientation and desolvation of the reactants. $\Delta G^{\ddagger}_{obs}$ for 4-methoxycarbonylnitracrine, the most reactive compound, for which electron transfer is most favoured in the thermodynamic sense, is thus very close in magnitude to w^r. This near equality means that the reaction of this compound with $FMNH_2$ is close to being association and orientation controlled.

The similar, very low values of $\Delta G^{\ddagger}_{o}$ (1–2 kJ mol^{-1}) obtained for reduction of the substituted nitracrines by both $FMNH_2$ and XOD suggests that $FMNH_2$ models the active site of XOD. However, this interpretation should be treated with caution as the reaction of $FMNH_2$ with the nitroacridines was examined at a lower pH and lower temperature than was reduction by XOD in order to decrease the rate of the former reaction to measurable levels.

Conclusions.—The original aim of this study was to determine whether the variations in cytotoxic potency in this series of compounds might reflect differences in rates of metabolic nitroreduction. The similarity in redox dependence of the kinetics of nitroreduction by $FMNH_2$ and by XOD suggests a similar mechanism of reduction in both cases and supports the view that the FAD centre of XOD is the active site for electron transfer to nitroacridines. This study shows that the rate of XOD-catalysed reduction is dependent on the one-electron-reduction potential. However, it is unclear to what extent XOD can be viewed as a general model for nitroreductases since it is a partially degraded enzyme, derived from the native xanthine dehydrogenase enzyme, and has a low substrate specificity. Even for XOD there is a 40-fold range in K_m-values for closely related nitracrine derivatives, indicating significant substrate specificity beyond that imposed by reduction potential. Such variations may be even more pronounced for other (native) nitroreductases. The present study therefore does not exclude the possibility that the critical cellular nitroreductases responsible for activation of nitroacridines may impose redox-independent substrate selectivity at sub-saturating intracellular drug concentrations.

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