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FERREDOXIN-DEPENDENT REDUCTION OF NITROIMIDAZOLE DERIVATIVES IN DRUG-RESISTANT AND SUSCEPTIBLE STRAINS OF *TRICHOMONAS VAGINALIS*

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Abstract—The inhibitory effect of a range of nitroimidazole-derivatives on H_2 production by metronidazole resistant (CDC-85) and susceptible (Cl-NIH) *Trichomonas vaginalis* strains was investigated. The 2-, 4-, and 5-nitro-derivatives used had one-electron reduction potentials within the range −250 to −525 mV. Nitroimidazole concentrations giving 50% inhibition of H_2 production (k_iH_2) for compounds with one-electron reduction potentials in the range −250 to −425 mV were found to be similar for both strains tested. Compounds with one-electron reduction potentials below −425 mV give 10-fold higher K_iH_2 values for the metronidazole resistant isolate. Both strains showed increased K_iH_2 for compounds with potentials lower than −500 mV. The addition of 2.1 kPa (0.02 atm) O_2 to the gas phase resulted in increasing the k_iH_2 values for all the compounds tested, but had the greater effect on results obtained with the resistant isolate using nitroimidazoles in the range −425 to −490 mV. The results enable the proposal that the resistant isolate CDC-85 has a ferredoxin with altered redox properties or reduced intracellular levels.

Nitroimidazole derivatives have received wide practical application in clinical situations both as radiosensitizers in cancer chemotherapy (2-nitroimidazoles, e.g. misonidazole) [1], and as antimicrobials in the chemotherapy of anaerobic infections (5-nitroimidazoles, e.g. metronidazole) [2]. The ease of reduction of these compounds is related to their electron affinity, expressed as a one-electron reduction potential, and various biological characteristics of these compounds have been assignable by this parameter [3–5]. Metronidazole (Flagyl) was developed for the treatment of vaginal infections due to *Trichomonas vaginalis* [6], however, recent clinical isolates exhibiting increased aerobic tolerance to the drug have been encountered [7, 8]. In several of these isolates O_2 has been shown to play an active part in resistance to the drug [9], and these strains have been shown to possess oxidase activity with lowered affinity for O_2 than that of susceptible strains [10]. Further, metronidazole resistant strains have been shown to be more sensitive to O_2 exposure than susceptible strains [11]. The direct interaction of O_2 with the reduced product(s) of the drug also produced preferential quenching of the metronidazole radical in the resistant strain CDC-85 as compared to susceptible strains [12]. In this resistant isolate it was also shown that a several-fold difference existed in the titre of metronidazole required to effect a 50% inhibition of hydrogen production as compared to a susceptible strain Cl-NIH [13] (previously designated ATCC 30001).

In this study we investigated the effects of a range of nitroimidazoles on H_2 production by a resistant isolate CDC-85, and a susceptible strain, Cl-NIH, by use of membrane-inlet mass spectrometry of continuously stirred cell suspensions.

MATERIALS AND METHODS

Cell cultures. T. vaginalis strains were cultured in Diamond's TYM medium [14] without agar and supplemented with 10% (v/v) horse serum. Stock cultures (5.5 ml) were maintained at 37° and subcultured daily. The *in vitro* minimum lethal concentration (MIC) to metronidazole by the strains are 2 $\mu g\ ml^{-1}$ aerobic and 0.5 $\mu g\ ml^{-1}$ anaerobic for the susceptible strain Cl-NIH [15]; 400 $\mu g\ ml^{-1}$ aerobic and 12.5 $\mu g\ ml^{-1}$ anaerobic for the resistant strain, CDC-85 (M. Müller, personal communication). Cells were harvested by centrifugation at 2000 rev min^{-1} at room temperature for 5 min in the swing out rotor of a MSE Centaur 2 centrifuge, washed in Doran's buffer [16], pH 7.0, and resuspended to a final vol. of 5 ml in this buffer. Cells were counted in a modified Fuch's-Rosenthal haemocytometer (depth 0.2 mm, 1/16 mm^2).

Mass spectrometric determinations. Steady state H_2 and CO_2 production rates by intact organisms, were determined using a quadrupole mass spectrometer [17, 18], fitted with a Teflon membrane-covered inlet. The instrument used was a mass spectrometer type SX200 and associated DPP16 digital peak programmer (VG Gas Analysis, Aston Way, Middlewich, Cheshire CW10 0HS, U.K.) fitted with a turbo molecular pump (Balzers High Vacuum Ltd., Northbridge Road, Berhamstead, Herts., U.K.). The reaction vessel (5 ml) was maintained at 37° and the cell suspension was stirred by a tacho-controlled

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Table 1. Nitroimidazole derivatives used and their one-electron reduction potentials

Compound No.	Generic name or code number	—NO_2	Substitution on nitroimidazole ring (1—N—R_1)	(2—C—R_2 or 5—C—R_2)	E_7' (mV)
1	4-Nitroimidazole	4—C	—H	—H	−527
2	Me 108	5—C	—$CH_2CH_2NHC(S)OCH_3$	—CH_3	−498
3	Metronidazole	5—C	—CH_2CH_2OH	—CH_3	−486
4	Dimetridazole	5—C	—CH_3	—CH_3	−475
5	Ornidazole	5—C	—$CH_2CHOHCH_2Cl$	—CH_3	−467
6	RGW-614	2—C	—$CH_2CO_2Na.CH_3OH$	—H	−447
7	L-6678	2—C	—CH_2CH_2OH	—CH_3	−423
8	L-8580	2—C	—CH_3	—$CH{=}CH_2$	−392
9	Misonidazole	2—C	—$CH_2CHOHCH_2OCH_3$	—H	−389
10	Benznidazole	2—C	—$C_6H_5CH_2CONHCH_2$	—H	−380
11	ZK-28943	5—C	—CH_3	—CHO	−360
12	RGW-806	2—C	—CH_3	—$CONH_2$	−321
13	L-8711	2—C	—CH_3	—CHO	−243

motor at 1100 rev min^{-1}. Calibrations were performed with air/N_2 and H_2/N_2 mixtures from a digital gas mixer [19], and by using 0.5 M HCO_3^-. Half-times for equilibration of the gases in Doran's buffer at pH 7.0 were 4, 2.5 and 0.5 min respectively at m/z values of 32, 2 and 44. Solubilities of O_2 in air-saturated buffer and of H_2 at 37° were 220 and 705 μM, respectively [20]. The lower limit of sensitivity of the mass spectrometer for O_2 was 0.25 μM. Once a steady state of H_2 (T_L H_2 in μM) evolution has been reached a low concentration of nitroimidazole was added. A new (lower) steady state H_2 concentration resulted. Successive additions were made until approx. 80% inhibition of the original H_2 evolution was achieved. V_{H_2} (velocity of H_2 production) was calculated for each addition from K ($T_{L_{H_2}}$), where K is a constant $0.693/t_{\frac{1}{2}}$ and $t_{\frac{1}{2}}$ is the half-time for H_2 equilibration in the buffer in the absence of organisms at 37°. Straight lines were obtained by plotting data as reciprocal of V_{H_2} against the nitroimidazole concentration (mM).

Chemicals. Nitroimidazole derivatives tested are listed in Table 1; compounds will be referred to by number. Stock solutions were prepared in Me_2SO except for 6, which was dissolved in water. The compounds were obtained from Sigma Chem. Co., Dorset, U.K. 1, 3; May & Baker, Dagenham, Essex, U.K. 4; Dr. P. Wardman, Mount Vernon Hospital, Middx., U.K. 2, 11; F. Hoffmann-La Roche & Co. Ltd., Basel, Switzerland, 5, 9, 10; Dr R. G. Wallace, Brunel University, Uxbridge, Middx., U.K. 6, 12; Gruppo Le Petit Sp.A., Milan, Italy, 7, 8, 13. The one-electron reduction potentials quoted were obtained from Wardman and Clarke [20].

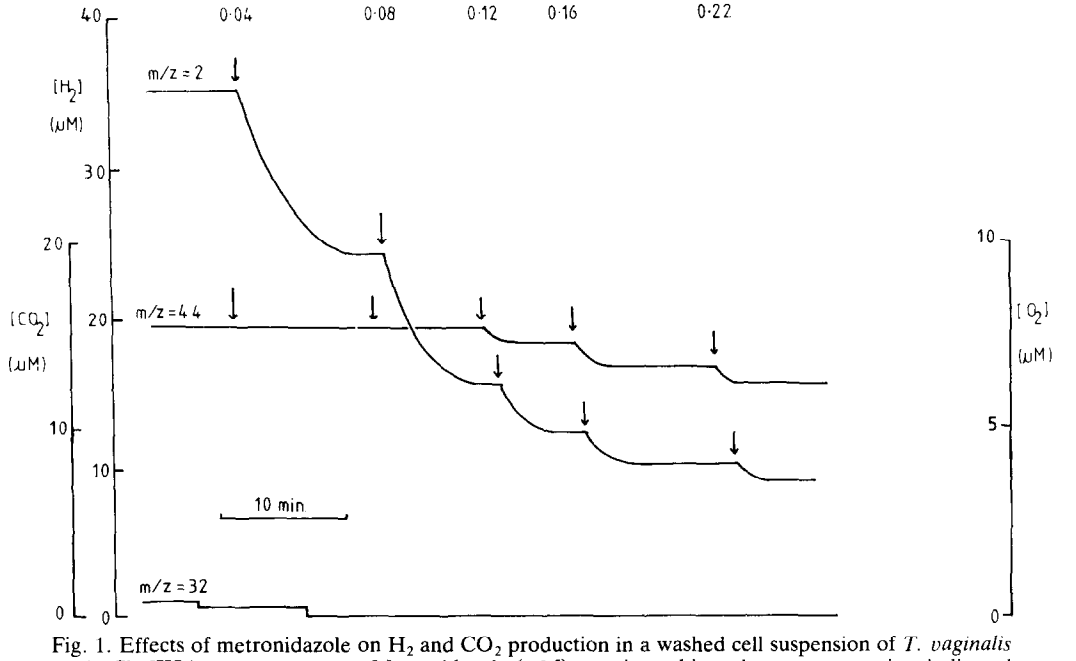


Fig. 1. Effects of metronidazole on H_2 and CO_2 production in a washed cell suspension of *T. vaginalis* strain Cl-NIH in an open system. Metronidazole (mM) was titrated into the system at points indicated by the arrows. Dissolved O_2 concentration (m/z = 32), dissolved H_2 concentration (m/z = 2), dissolved CO_2 concentration (m/z = 44). Cell density was 4.9×10^6 organisms ml^{-1}.

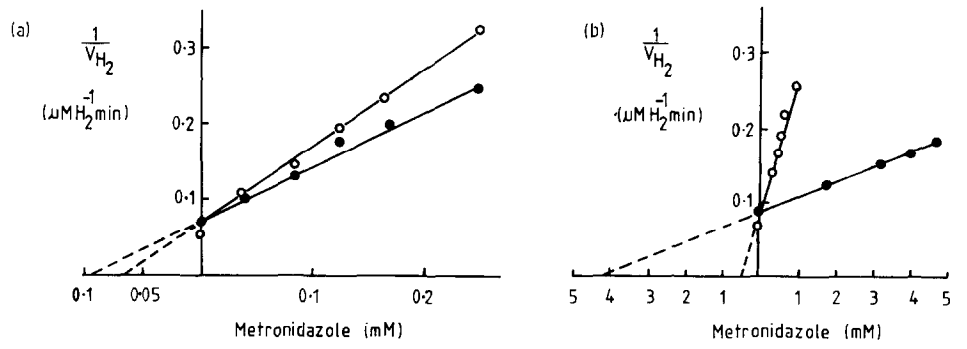


Fig. 2. Metronidazole inhibition of H_2 generation by (a) metronidazole-susceptible strain (Cl-NIH) and (b) metronidazole-resistant strain (CDC-85) of *T. vaginalis*. Anaerobic titrations were performed under a gas phase of N_2 (○); aerobic conditions were established under 2.1 kPa O_2 (●). The V_{max} H_2 production was (a) 2.5 μM H_2 min^{-1} per 10^6 organisms, cell density was 5.7×10^6 ml^{-1}; (b) 1.3 μM H_2 min^{-1} per 10^6 organisms, cell density was 8.5×10^6 ml^{-1}.

RESULTS

Mass-spectrometry

The inhibitory effect of nitroimidazole-derivatives on hydrogen production (expressed as 50% inhibition of the observed H_2 production), (k_iH_2) by *T. vaginalis* strains were measured using membrane-inlet mass-spectrometry. Figure 1 shows simultaneous monitoring of H_2, CO_2 and O_2 dissolved in a suspension of *T. vaginalis* Cl-NIH. Addition of glucose (50 mM) stimulated H_2 and CO_2 production under anaerobiosis. Addition of small aliquots of the 5-nitroimidazole, metronidazole, resulted in inhibition of H_2 production and with metronidazole levels of 0.12 mM, CO_2 was also inhibited. Addition of 2.1 kPa (0.02 atm) O_2 gave 30% inhibition of the observed H_2 production at dissolved O_2 levels below the limit of detectability by the mass-spectrometer system (0.25 μM). Under these conditions the drug was less inhibitory; the k_iH_2 for metronidazole was increased from 0.07 mM (anaerobically) to 0.10 mM (Fig. 2a). In comparison H_2 production by CDC-85 was found to be less sensitive to metronidazole inhibition, with an anaerobic k_iH_2 of 0.5 mM,

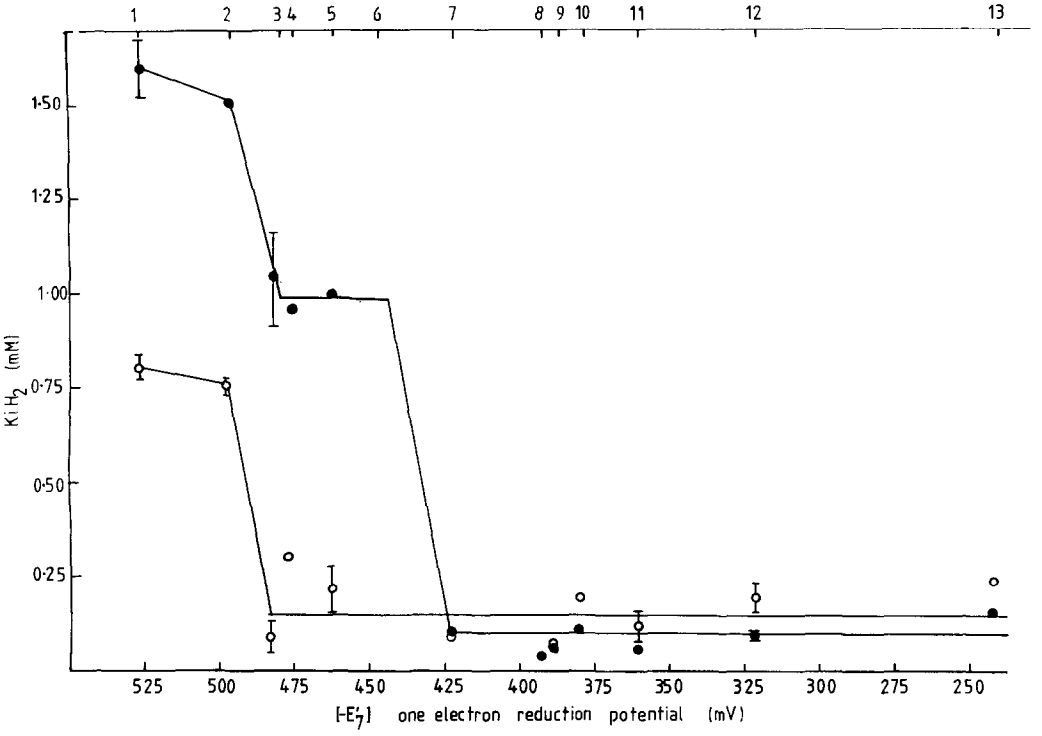


Fig. 3. Values for k_i50 of various nitroimidazole derivatives on H_2 generation in metronidazole-susceptible, Cl-NIH (○), and -resistant, CDC-85 (●), strains of *T. vaginalis*. Data were obtained by membrane-inlet mass spectrometry measurements of H_2 production as described in the methods. The compounds used, numbered 1 to 13, are listed in Table 1.

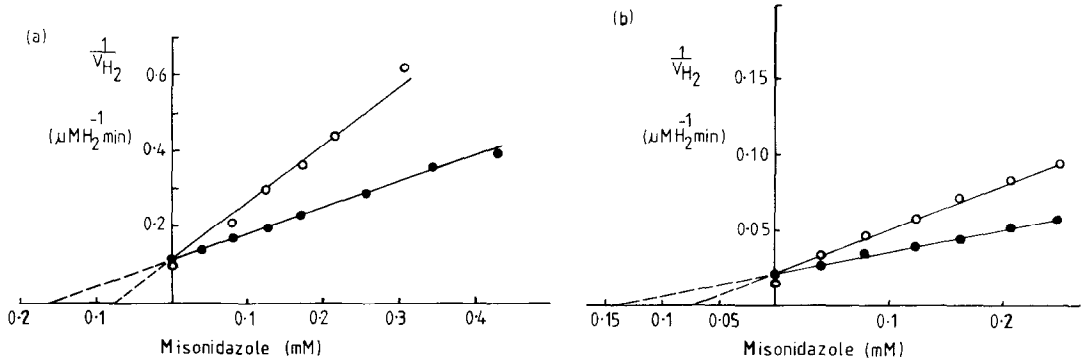


Fig. 4. Misonidazole inhibition of H_2 generation by (a) metronidazole-resistant strain (CDC-85), and (b) metronidazole-susceptible strain (Cl-NIH) of *T. vaginalis*. Anaerobic titrations were performed under a gas phase of N_2 (○); aerobic conditions were established under 2.1 kPa O_2 (●). The V_{max} H_2 production was (a) 1.2 μM H_2 min^{-1} per 10^6 organisms, cell density was 8.3×10^6 organisms ml^{-1}; (b) 2.8 μM H_2 min^{-1} per 10^6 organisms. Cell density was 1.7×10^7 organisms ml^{-1}.

increasing to 4.3 mM under an atmosphere of 2.1 kPa O_2 in N_2 (Fig. 2b). The 5-nitroimidazoles, ornidazole and dimetridazole with one-electron reduction potentials lower than −425 mV gave similar results (Fig. 3). In contrast to the 5-nitroimidazoles, inhibition of H_2 production by CDC-85 was found to be approximately 10-fold more sensitive to the 2-nitroimidazole, misonidazole, with a k_iH_2 of 0.08 mM (Fig. 4a); a similar k_iH_2 value was obtained for the metronidazole susceptible strain, Cl-NIH (Fig. 4b). The addition of 2.1 kPa O_2 to the gas phase resulted in similar increases in titre levels of misonidazole to effect a 50% inhibition in H_2 production by both CDC-85 and Cl-NIH strains, 0.15 mM (Figs. 4a, b). Other 2-nitroimidazole derivatives tested, L-8711, RGW 806, benznidazole, L-8580 and L-6678, gave similar k_iH_2 values with both metronidazole-susceptible and -resistant strains (Fig. 3). Nitroimidazole-derivatives with one-electron reduction potentials below −495 mV (4-nitroimidazole and Me 108) were found to have the least effect on H_2 production by both strains, supporting previous data which showed that this class of compound is not easily reduced by trichomonads. Including 2.1 kPa O_2 in the gas phase increased the value of k_iH_2 of 4-nitroimidazole from 1.2 to 1.6 mM for CDC-85, and from 0.8 to 1.1 mM for Cl-NIH (Figs. 5a, b). The 5-nitroimidazole, ZK-28943, with one-electron reduction potential of −360 mV, gave similar results to those obtained for 2-nitroimidazoles for both strains tested (Fig. 3). The 2-nitroimidazole RGW-614, with a one-electron reduction potential of −447 mV gave very high k_iH_2 values for both metronidazole-susceptible and -resistant strains, 13 mM and 20 mM, respectively (not shown); the acidic ionisation (negative charge) of carboxylate-substituted nitroimidazoles has previously been shown to be responsible for inhibiting drug uptake in mammalian cells [21].

The V_{max} values obtained for H_2 production by the metronidazole-resistant isolate, CDC-85, were found to be about half of that for the susceptible isolate Cl-NIH (1.0 ± 0.3 μM H_2 min^{-1} per 10^6 cells and 2.5 ± 0.4 μM H_2 min^{-1} per 10^6 cells, respectively).

Microscopy

Microscopic examination of both resistant and susceptible strains at the end of the experiment revealed 60% of the cells to be motile after k_iH_2 deter-

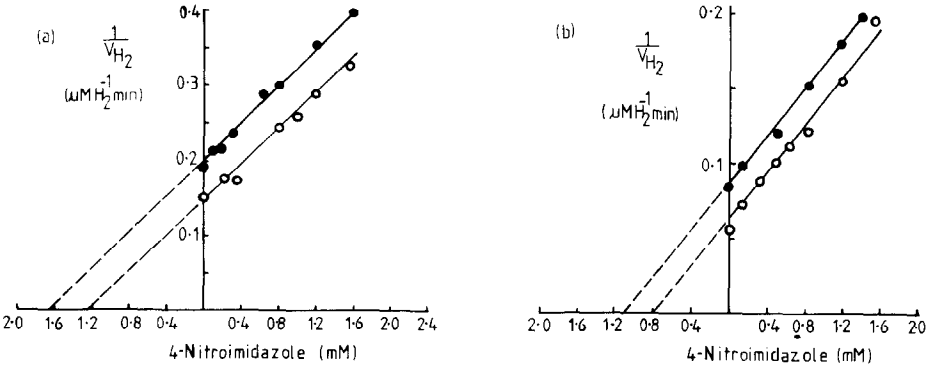


Fig. 5. 4-Nitroimidazole inhibition of H_2 generation by (a) metronidazole-resistant strain (CDC-85), and (b) metronidazole-susceptible strain (Cl-NIH) of *T. vaginalis*. Anaerobic titrations were performed under a gas phase of N_2 (○); aerobic conditions were established under 2.1 kPa O_2 (●). The V_{max} H_2 production was (a) 1.3 μM H_2 min^{-1} per 10^{-6} organisms, cell density was 4.9×10^6 organisms ml^{-1}; (b) 2.4 μM H_2 min^{-1} per 10^6 organisms, cell density was 7.6×10^6 organisms ml^{-1}.

Table 2. Inhibition of motility in *T. vaginalis* metronidazole susceptible (Cl-NIH) and resistant (CDC-85) isolates by nitroimidazole-derivatives used in this study

Nitroimidazole one-electron reduction potential (mV)	Cl-NIH			CDC-85		
	Time of exposure (min)	Final concn. (mM)	Motility (%)	Time of exposure (min)	Final concn. (mM)	Motility (%)
−250 to −425	50	0.25	60	50	0.21	60
−425 to −498	60	0.10	10	50	1.0	10
−498 to −525	60	1.17	90	60	1.17	90

minations with compounds in the range −250 to −425 mV even though 90% inhibition of H_2 production had been achieved (Table 2). In contrast, microscopic examination of cells after k_iH_2 determinations with compounds in the range −425 mV to −495 mV, revealed less than 10% motile organisms, the majority of cells were rounded and some lysis was evident. The appearance of cells after treatment with compounds having one-electron reduction potentials lower than −495 mV reveled 90% motile for both strains (Table 2).

DISCUSSION

Several aspects of the biological properties of nitroimidazoles have been shown to be dependent upon their one-electron reduction potential values; these include antimicrobial activity [4, 22], mutagenicity [23], *in vitro* inhibition of DNA synthesis [3], hypoxic and radiosensitizers [24] and anaerobic reduction by reduced flavins and xanthine oxidase [25]. In this study we examined the inhibitory effect of a number of nitroimidazoles (2-, 4-, and 5-derivatives) on H_2 production by a metronidazole susceptible isolate of *T. vaginalis* (Cl-NIH) and a metronidazole resistant isolate (CDC-85). Inhibition of H_2 production by *T. vaginalis* Cl-NIH, expressed as k_iH_2 for the nitroimidazole tested, was found to be independent of the one-electron reduction potential down to −486 mV; a mean value of 0.2 ± 0.1 (16 determinations) was obtained for 9 different derivatives. With one-electron reduction potentials below −486 mV a sixfold increase in the k_iH_2 was obtained, supporting previous evidence that compounds of low electron affinity are not easily reduced [5].

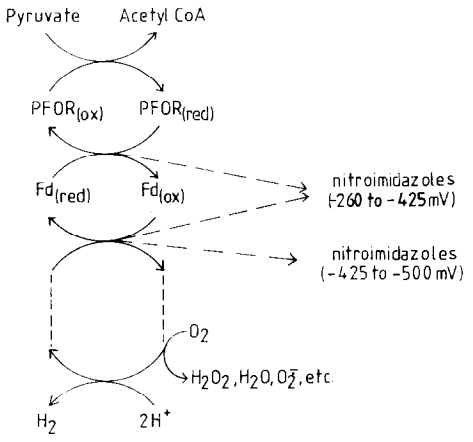
Inclusion of 2.1 kPa O_2 in the gas phase had a greater effect on the observed k_iH_2 by those compounds with more positive one-electron reduction potentials; e.g. the k_iH_2 for misonidazole (−389 mV) was increased by 87%, whereas those for metronidazole (−486 mV) and 4-nitroimidazole (−572 mV) were only increased by 43% and 38% respectively. The secondary plots obtained from these results suggest that O_2 acts as a competitive inhibitor of nitroimidazole reduction for those compounds with one-electron reduction potentials down to −500 mV. That very different plots were obtained for 4-nitroimidazole (−527 mV) indicates uncompetitive inhibition by O_2, and are suggestive therefore of a different mechanism in the reduction of this compound.

In contrast to *T. vaginalis* Cl-NIH inhibition of H_2 production by the metronidazole-resistant strain CDC-85, was found to be dependent upon the one-electron reduction potential of the nitroimidazole used for all those compounds with one electron potentials below −425 mV. The k_iH_2 for compounds with one-electron reduction potentials from −243 mV to −425 mV were of the same order of magnitude as observed for strain Cl-NIH, with a mean value of 0.10 mM (± 0.05 for 10 determinations). However, a marked increase (about 10-fold) in k_iH_2 values was observed with compounds tested with one-electron reduction potentials below −425 mV.

Further differences between the two strains were observed when 2.1 *k*Pa O_2 was included in the gas phase. Whereas results obtained for compounds with one-electron reduction potentials from −243 mV to −425 mV are identical for both strains at potentials below this (−425 to −500 mV) approx. a 40-fold increase in k_iH_2 was obtained for the resistant strain and a further increase in k_iH_2 was observed for compounds with one-electron reduction potentials below −500 mV. Inclusion of 2.1 kPa O_2 in the gas phase had a similar effect for both resistant and susceptible strains with compounds having one-electron reduction potentials below −500 mV.

Microscopic observation of both resistant and susceptible strains after treatment with the nitroimidazole-derivatives revealed that those compounds with one-electron reduction potentials in the range −425 to −495 mV gave a 90% reduction of motility when 90% inhibition of H_2 production had been reached. In contrast, those derivatives with potentials of −250 to −425 mV achieved 40% reduction of motility and derivatives with potentials lower than −495 mV achieved only 10% reduction of motility. It is suggested that the mechanisms of action of these three groups of compounds are different.

It has previously been shown [5] that for 5-nitroimidazoles (one-electron reduction potentials −425 to −500 mV) reduction is a ferredoxin-dependent event, but that considerable 2-nitroimidazole (one-electron reduction potential range −200 mV to −425 mV) reduction occurs in the absence of ferredoxin. It is therefore proposed that reduction of those compounds with potentials in the range −260 mV to −425 mV occurs by an enzymically dependent process (Scheme 1); levels of pyruvate:-ferredoxin oxidoreductase, the main enzyme for drug activation in trichomonads, have been shown to be similar for both resistant and susceptible strains (M. Müller, personal communication) explaining the k_iH_2 results obtained with both isolates for this class of compound. In contrast reduction of those com-



Scheme 1. Interaction of nitroimidazole derivatives with the electron transport components of *Trichomonas vaginalis*. (PFOR, pyruvate ferredoxin oxidoreductase; Fd, ferredoxin).

pounds with potentials below −425 mV occurs solely by chemical interaction with ferredoxin that has first been reduced by pyruvate:ferredoxin oxidoreductase [5], hence in this case these compounds do not interact directly with the enzyme but rather are chemically reduced by ferredoxin. The large difference that is observed between these strains in terms of k_iH_2 with these compounds would therefore be explained by intracellular decrease in content of ferredoxin or a change in the redox-properties of this component in the resistant isolate, CDC-85. That this relationship is dependent upon the one-electron reduction potential of the compound and not the position of the nitro group, is shown by the observation that compound ZK-28943 (a 5-nitroimidazole with a one-electron reduction potential of −360 mV), has a k_iH_2 value typical of high potential compounds.

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