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STUDIES ON THE ACTION OF NITROIMIDAZOLE DRUGS

THE PRODUCTS OF NITROIMIDAZOLE REDUCTION

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Abstract—The electron requirements for the electrolytic reduction of misonidazole, metronidazole and 4(5)-nitroimidazole have been measured using high-resolution coulometry. Eleven of the labelled final reduction products of metronidazole (a 5-nitroimidazole) have been separated by high-performance liquid chromatography and identified. These appear to be formed without the prior generation of a stable intermediate. In contrast, the reduction products of misonidazole (a 2-nitroimidazole) show little similarity to those of metronidazole but are likely to be formed via the four-electron hydroxylamine derivative. None of the final reduction products show toxicity towards *Clostridium bifermentans* or *Escherichia coli* suggesting that the short-lived cytotoxic agent of nitroimidazoles is a reduction product formed by the addition of not more than three electrons.

Nitroimidazole drugs are selectively toxic to anaerobic bacteria and protozoa and the basis of their activity lies in the necessity for the reduction of the nitro group because nitroimidazoles accept electrons at the level of pyruvate metabolism at potentials which are incapable of being generated in aerobic cells [1, 2]. Hypoxic mammalian cells, however, are more resistant to ionizing radiation than well-oxygenated ones and their presence in human tumours may lead to the failure of radiotherapy. Nitroimidazoles not only specifically radiosensitize hypoxic cells but are selectively cytotoxic to them [3–5], suggesting that the mechanism of cytotoxicity in bacteria, protozoa and mammalian hypoxic cells share a common if not identical pathway as proposed by Edwards *et al.* [6].

Cytotoxicity in all cells depends upon the reduction of the nitro group which produces a transient species which interacts with DNA resulting in damage characterized by helix destabilization and strand breakage [7–10], and the concomitant specific release of thymine nucleotides [11, 12].

Under anaerobic conditions or at low oxygen tensions (hypoxia) reduction is assumed to follow the same pathway as *p*-substituted nitrobenzenoids [13] and is known initially to involve the addition of a single electron [14, 15]. The subsequent formation of nitroso (R–NO) and hydroxylamino (R–NHOH) derivatives involves the addition of a total of four electrons as follows:

$$R\text{–}NO_2 + e \rightarrow R\text{–}NO_2^{\cdot -} \quad (1)$$

$$R\text{–}NO_2^{\cdot -} + H^+ \rightarrow R\text{–}NO_2H^{\cdot} \quad (2)$$

$$2R\text{–}NO_2H^{\cdot} \rightarrow R\text{–}NO_2 + R\text{–}N(OH)_2 \quad (3)$$

$$R\text{–}N(OH)_2 \rightarrow R\text{–}NO + H_2O \quad (4)$$

$$R\text{–}NO + e \rightarrow R\text{–}NO^{\cdot -} \quad (5)$$

$$R\text{–}NO^{\cdot -} + H^+ \rightarrow R\text{–}NOH^{\cdot} \quad (6)$$

$$R\text{–}NOH^{\cdot} + R\text{–}NO_2H^{\cdot} \rightarrow R\text{–}NHOH + R\text{–}NO_2 \quad (7)$$

These steps have been studied for the reduction of nitrobenzene to phenylhydroxylamine [16, 17] and a further addition of two electrons may yield the amine:

$$R\text{–}NHOH + 2e + 2H^+ \rightarrow R\text{–}NH_2 \quad (8)$$

In the presence of O_2 a "futile-cycle" reaction occurs whereby oxygen accepts an electron from the one-electron nitro-radical anion regenerating the original drug and forming superoxide [18, 19].

Electrolytic reduction of nitroimidazoles enables the transient cytotoxic agent to be generated at controlled rates and thus identification of the final reduction products is of importance in establishing not only the agent responsible for the selective cytotoxicity in microbial and human cells but also may be relevant to the toxic side effects of these drugs.

We report on the number of electrons involved in the reduction process and give details of the products of misonidazole and metronidazole after electrolytic reduction at constant potential in aqueous solution.

MATERIALS AND METHODS

The nitroheterocyclic compounds used in this study are shown with their relevant physicochemical data in Table 1. All other chemicals used were of the highest purity available and were used without further purification.

Misonidazole was obtained from Roche Products Ltd (Welwyn Garden City, U.K.), metronidazole from May & Baker Ltd (Dagenham, U.K.) and 4(5)-nitroimidazole from Aldrich Chemical Co. (Gillingham, Dorset, U.K.).

The amine derivative of misonidazole [1-(2-amino-1-imidazolyl)-3-methoxy-2-propanol] as the

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Table 1. The nitro compounds used in this study

Drug	Formula	E_7^1 (mV)	E_2^1 (mV)	Reduction voltage (mV)	pH dependence (V)	n
Misonidazole	1-(2-Nitro-1-imidazolyl)-3-methoxy-2-propanol	−389	−272	−800	$E_2^1 = 0.22–0.07pH$	4.07
Metronidazole	1,2′-Hydroxyethyl-2-methyl-5-nitroimidazole	−486	−382	−900	$E_2^1 = 0.07–0.065pH$	3.60
4(5)-Nitroimidazole	As column 1	−527	−540	−1000	$E_2^1 = 0.05–0.07pH$	6.00

E_7^1 values are quoted relative to the normal hydrogen electrode.
E_2^1 values are relative to the Ag/AgCl electrode.
n is the number of electrons involved in the reduction process.

hydrochloride was kindly donated by Dr C. Smithen (Roche Products Ltd, Welwyn Garden City, U.K.).

Coulometry and electrolytic reduction

Details of the polarographic determination of half-wave potentials (E_2^1) and electrolytic reduction of the drugs have been published previously [7–10] but generally drugs are reduced at redox potentials about −500 mV below the E_2^1 value as indicated in Table 1. Reduction was carried out on 67 ml of 300 μM aqueous solutions using a Hg pool cathode and Ag/AgCl anode at pH 7 ± 1 under N_2, the pH being adjusted by addition of HCl through a septum.

One mole of electrons, i.e. 1 Faraday (=96,460 C) is required to perform a one-electron reduction of 1 mole of substance. For an n-electron reduction, n Faradays will be required; therefore for any reduction it follows that:

$$n = \frac{q}{(M_i - M_f)F}$$

where M_i = initial concentration (moles/l.), M_f = final concentration, q = charge exchanged during reduction (C) and F = Faraday. Thus, n represents the statistical average number of electrons added to each molecule during reduction.

The coulomb is the product of current and time (i.e. amps × seconds). Given Ohm's law, $I = V/R$, current can be measured as a voltage in parallel with a known resistance and coulombs measured with a TSM integrating millivoltmeter (Time Electronics, Tonbridge, U.K.).

The method is sensitive to the amount of substance present: at a concentration of 20 μmoles in 67 cm^3, a one-electron reduction would produce 57,900 counts on the coulometer and reproducibility such that n could be calculated to two decimal places. Reduction was carried out at an initial current density of 30 μA and proceeded until zero current flowed.

Determination of nitrite

Nitrite was analysed by the sulphanilamide/*N*-(1-naphthyl)ethylenediamine method [20] using Wardman's modification (P. Wardman, personal communication).

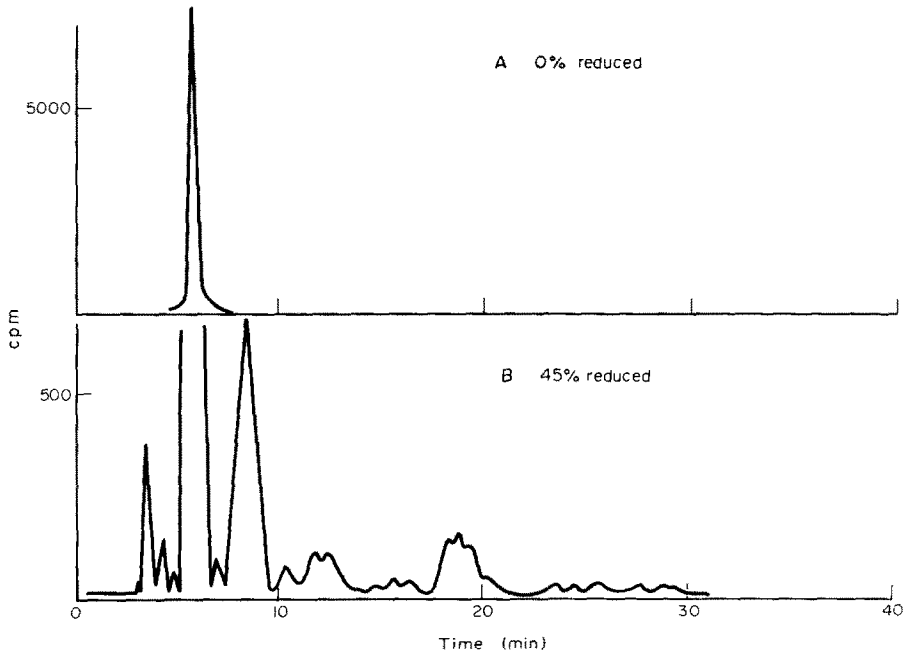

Fig. 1A and B.

Two reagent solutions were prepared. Reagent A consisted of 0.5% (w/v) sulphanilamide in 20% (v/v) HCl [57% (v/v) of 35% HCl] and reagent B of 0.3% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride in 1% (v/v) HCl.

For analysis 0.5 cm^3 of A was added to 25 cm^3 of sample (diluted when necessary) and after 5 min 0.5 cm^3 of B was added and the purple colouration indicating the presence of nitrite measured at 540 nm after a further 10 min using a 4-cm path length cell, against a reagent blank. The concentration of nitrite was calculated from a calibration curve constructed from a standard solution of $NaNO_2$. The sensitivity of this assay is such that the minimum detectable concentration of nitrite is about 25 pM, which corresponds to an absorbance reading (A_{540}, 4-cm path length) of 0.005.

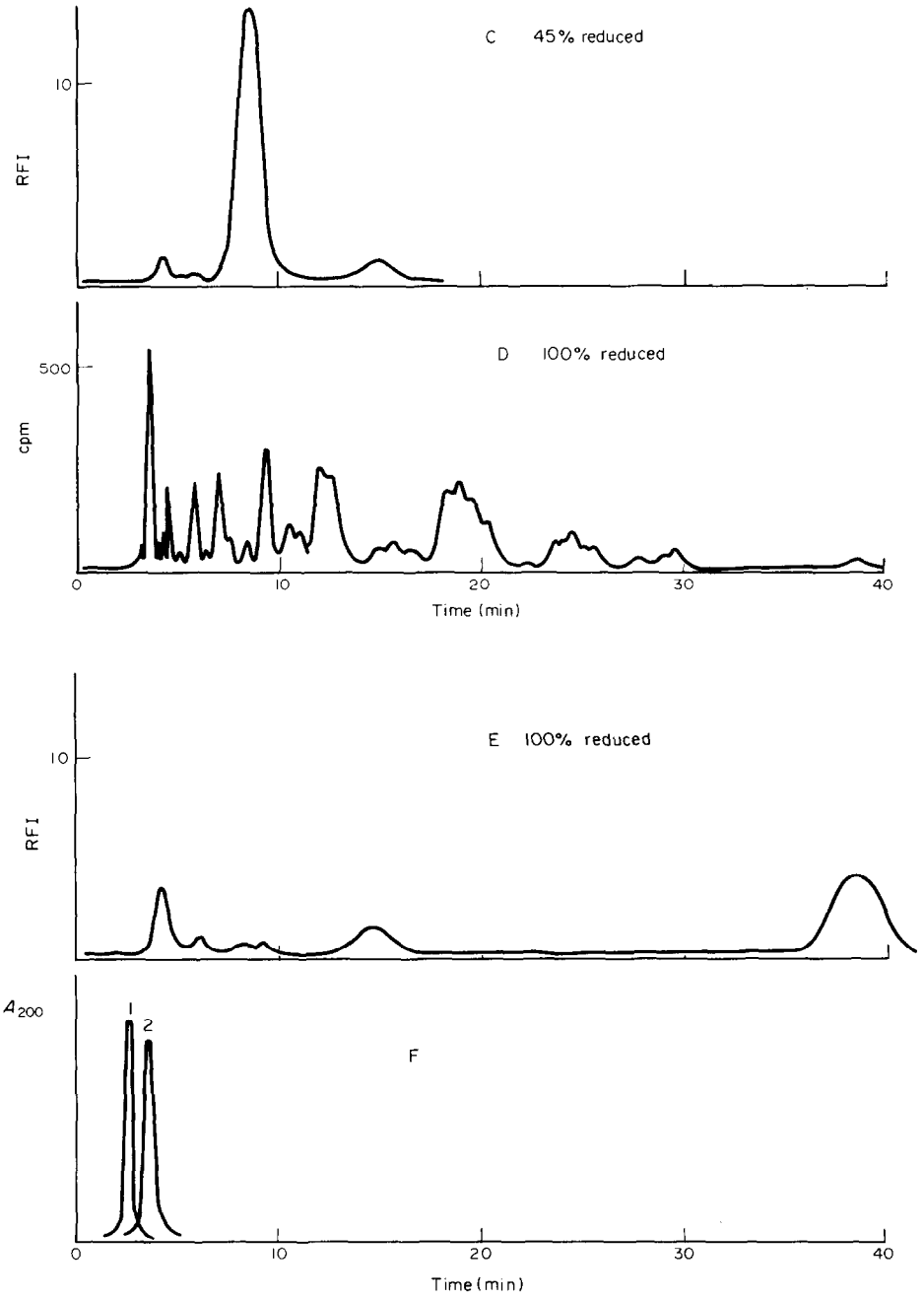

Fig. 1. HPLC profiles of electrolytically reduced misonidazole monitored by 2-^{14}C activity (A, B and D) and fluorescence (C and E). F is the profile of the amine derivative of misonidazole, 1-(2-amino-1-imidazolyl)-3-methoxypropanol (peak 1) and hydroxyurea (peak 2) measured at 200 nm. The column was a Partisil SCX, 25 × 0.4 cm eluted isocratically with 0.05 M NaH_2PO_4 at 1 cm^3/min. RF1 refers to the relative fluorescence intensity (C and E).

Spectrophotometry

U.v. and visible spectrophotometry of reduced and unreduced drugs was carried out using a Pye–Unicam SP-800 Series B or SP-8150 scanning spectrophotometer. Fluorescence spectra were measured using an Aminco–Bowman 8911 spectrofluorimeter and a Phillips PM-1820 XY recorder. I.r. spectra were recorded using a Pye–Unicam SP-200G i.r. spectrophotometer.

Liquid scintillation photometry

Radioactivity was measured in vials containing 0.3% (w/v) 2,5-diphenyloxazole, 25% (v/v) Triton X-114 and 75% (v/v) xylene—a scintillant developed because of its high solubilising power with aqueous samples [21]—in a Model 3390 Tri-Carb Liquid Scintillation Spectrometer (Packard Instruments Ltd, Berks, U.K.).

HPLC. Reduction products of misonidazole and metronidazole were chromatographed by HPLC using a Pye–Unicam LC-XPD dual piston pump, LC-UV variable wavelength absorption detector with a 1 cm path length, 8 μl quartz flow-cell and a LC-FL filter fluorimeter with a 2 mm path length, 25 μl quartz flow cell coupled to a Phillips PM 8252 dual pen recorder. Samples were injected on to a Partisil SCX column (25 × 0.4 cm) with a Rheodyne 7120 valve and eluted isocratically at a flow rate of 1 cm^3/min with 0.05 M NaH_2PO_4 in 5% (v/v) aqueous methanol formed by mixing 5% (v/v) aqueous methanol and 0.1 M NaH_2PO_4 in 5% (v/v) aqueous methanol in a Pye–Unicam LC-XP gradient programmer and solvent mixer. All solvents were HPLC grade and were continually degassed with helium. Samples of reduced drug (10 or 100 μl) were injected directly on to the column. Samples which were radioactive were collected (200-μl fractions) for further analysis.

Misonidazole amine. The amine derivative of misonidazole, i.e. 1-(2-amino)-1-imidazolyl-3-methoxy-2-propanol HCl, was prepared from its picrate derivative by passing 100 mg of the latter in 20 cm^3 50% (v/v) aqueous ethanol through an ion-exchange column (20 × 1.5 cm Bio-Rad AG-1X8 in the chloride form) and eluting with 100 cm^3 aqueous ethanol. Evaporation of the eluate *in vacuo* yielded the amine HCl as a semi-crystalline gum which was stored in ethanol.

Antimicrobial activity

The activity of reduced metronidazole and misonidazole was tested against *E. coli* under anaerobic and aerobic conditions, and against *C. bifermentans* anaerobically. *E. coli* (NCIB 86) was grown on nutrient agar (Oxoid Ltd, Hants, U.K.) at 37° and inoculated from a 17-hr culture (0.1 cm^3) grown on nutrient broth (Oxoid Ltd.)

C. bifermentans (NCIB 506) was grown at 37° on reinforced clostridial agar (Oxoid) inoculated with 0.1 cm^3 of a 40-hr culture grown in reinforced clostridial medium (Oxoid). Anaerobic conditions were maintained in jars using a CO_2 and H_2 gas phase Gas-Pak system (Baltimore Biological Laboratories, MD).

Samples of reduced drugs (100 μl) were absorbed on to sterile filter discs (Whatman No. 1, 1.2 cm diameter) dried in air and placed on to Petri dishes previously inoculated with either *E. coli* or *C. bifermentans*. The dishes were incubated at 37° for 40 hr and examined for zones of inhibition at 24 and 48 hr.

Carbon dioxide formation. Fragmentation of the imidazole ring may lead to CO_2 formation [22]. The generation of CO_2 from the C_2-position of the imidazole ring was detected using ^{14}C-labelled metronidazole and misonidazole labelled in the C_2-position. A 10-cm^3 solution of the drug (10 mM) containing 10 μCi[2-^{14}C]metronidazole or misonidazole was reduced as previously described. The nitrogen exhaust containing any CO_2 released was passed through a solution of 100 cm^3 0.1 M KOH and the potassium carbonate formed measured as ^{14}C activity.

RESULTS AND DISCUSSION

The value *n* represents the statistical distribution of the electron requirements of the reduced species. These electron requirements are shown in Table 1 and show marked differences between the *n* values for typical 5-substituted nitroimidazoles and 2- or 4-substituted compounds. The integral value for misonidazole indicates reduction to the four-electron hydroxylamine derivative in approximately a 100% yield. These results substantiate the work of Clarke *et al.* [23] who postulated a reduction of misonidazole to the hydroxylamine based on the reaction stoichiometry of such drugs with xanthine oxidase.

In contrast metronidazole gives non-integral values of *n* between 3 and 4, indicating a distribution of products of differing reduction levels. The parent compound 4(5)-nitroimidazole is readily reduced to the six-electron amine derivative and this process suggests that substitution of the N_1-position of the imidazole ring limits reduction to the hydroxylamine.

The reductions of nitroimidazole drugs are accompanied by a loss of absorption in the u.v. region characteristic of the charge transfer band ascribed to the nitro group and an appearance of fluorescence. None of the unreduced drugs shows any detectable fluorescence characteristics nor do reduced drugs give any characteristic u.v. absorption peaks above 210 nm. In general it did not prove possible to crystallize any reduced component from aqueous or ethanolic solutions, but reduced drugs could be precipitated from ethanol with diethylether. The precipitate from reduced misonidazole, recovered by centrifugation and dried *in vacuo*, was a tan brown powder which was extremely hygroscopic in air and turned to a brown viscous liquid in 0.5 min. Reduced metronidazole behaved in a similar fashion. No picrate derivative could be prepared from reduced misonidazole or metronidazole. Reduced 4(5)-nitroimidazole on exposure to air yielded a blue-purple solution from which a deep purple pigment precipitated. Such a compound had previously been identified as 5,5′-diimidazole-4-one [24] which contained the characteristic indigoid group.

This structure, however, could not be confirmed. I.r. spectra showed a large absorbance band in the region 2500–3500 cm^{-1}, characteristic of a large number of hydroxy groups. Elemental analysis of the pigment did not give a simple empirical formula but

was consistent with a carbon–nitrogen ratio of $C_3:N_2$ and suggests a polymeric structure containing an intact imidazole ring.

The coulometric data suggest reduction of misonidazole to a four-electron hydroxylamine derivative in 100% yield, but the overall process is far more complex as illustrated in Fig. 1 where the HPLC profile of misonidazole shows 32 reduction products containing ^{14}C activity from the C_2-position of the imidazole ring, the greatest yield of any product being 5.7%. The amine was not detected, in contrast to the report of Born *et al.* [25] who prepared the amine by catalytic hydrogenation. Nevertheless, reduction proceeded by way of an intermediate characteristic fluorescence. This product was identified as the hydroxylamine derivative by characteristic

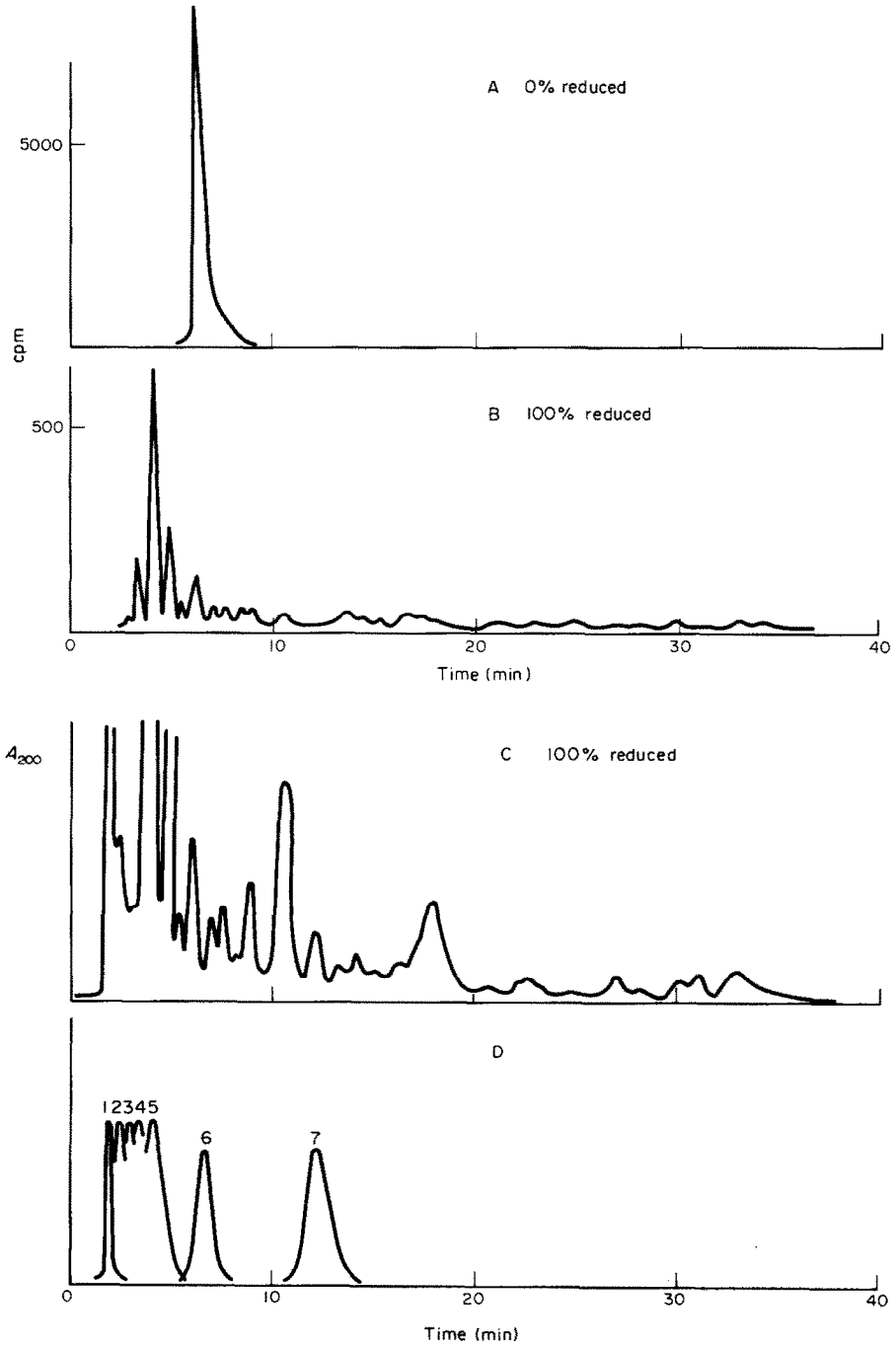

Fig. 2. HPLC profile of electrolytically reduced metronidazole monitored by 2-^{14}C activity (A and B) and absorbance at 200 nm (C and D). The seven components in D correspond to oxamate (**1**), glycine (**2**), *N*-acetylglycine (**3**), acetate (**4**), acetamide (**5**), *N*-acetylethanolamine (**6**) and ethanolamine (**7**). The column details are as for Fig. 1.

Table 2. Reduction products of misonidazole and metronidazole

Product	Formula	Formula weight	Molar yield (%)	
			Misonidazole	Metronidazole
Carbon dioxide	CO_2	44	0.21	0.35
Nitrite	NO_2^-	46	0.5	16.1
Hydroxyurea	$HONHCO_2NH_2$	76	5.7	0
Glycine	NH_2CH_2COOH	75	0	5*
Acetamide	CH_3CONH_2	59	0	28.3
Ethanolamine	$NH_2(CH_2)_2OH$	61	0	10*
Oxamate	$NH_2COCOOH$	89	0	14*
Acetate	CH_3COOH	60	0	3.8
N-Acetylglycine	$CH_3CONHCH_2COOH$	117	0	0.5
N-Acetylethanolamine	$CH_3CONH(CH_2)_2OH$	103	0	0
Azo or azoxy dimer			—	5.5

* Indicates that the compound contains no radioactivity from the C-2 position of the imidazole ring.

reactions with picryl chloride [26, 27] and sodium aminoprusside [28, 29] and confirmed by the presence of ^{14}C-labelled hydroxyurea (5.7%) formed by decomposition of the hydroxylamine.

The hydroxylamine derivative has a half-life of 12–15 hr under anaerobic conditions at pH 7 and 25° but is much less stable at acid pH—observations which are consistent with previous observations. Koch *et al.* [22] postulated reduction of misonidazole to the amine based on urea formation by urease. Urease, however, can also use hydroxyurea as a substrate [30] and this interpretation corroborates the results of Josephy *et al.* [31, 32] Clarke *et al.* [23] and Whillans and Whitmore [14] using xanthine oxidase stoichiometry and radiation-induced reduction systems respectively, all of which are consistent with hydroxylamine formation. The reduction of misonidazole thus proceeds via a four-electron pro-

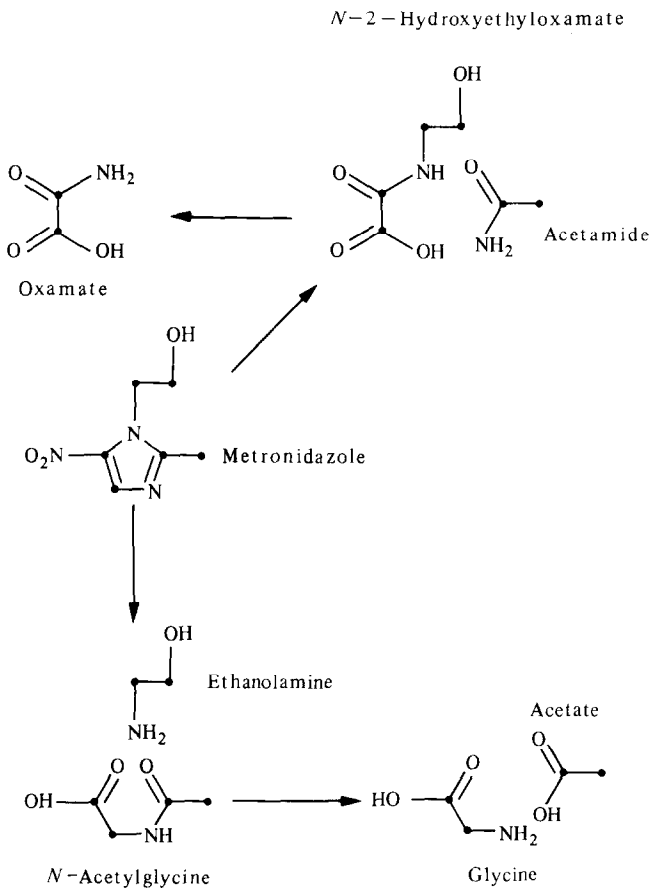

Fig. 3. Fragmentation pathway of reduced metronidazole during electrolytic reduction.

cess to the hydroxylamine. The numerous other products arise from its subsequent decomposition and various interreactions.

Reduced metronidazole yields 26 labelled reduction products as shown in Fig. 2 and others which are not radioactive. No major intermediate occurs but a number of final reduction products have been identified as indicated in Table 2. The presence of most of these compounds was confirmed by mass spectrometry and co-chromatography with authentic reference compounds. The results are broadly similar to those of Chrystal *et al.* [33] who reduced metronidazole with xanthine oxidase. However, *N*-acetylethanolamine was not detected and is not formed in the electrolytic reduction system. The products formed are consistent with fragmentation of the imidazole ring at C_2 and C_4 as shown in Fig. 3. Koch *et al.* [34] and Chrystal *et al.* [33] suggested that ring fragmentation occurs of a labile intermediate which they suggest as the nitroso derivative. Metronidazole reduction leads to significant yields of nitrite which is indicative of the decomposition of the one-electron nitro radical anion [41] and we suggest this latter product is a more plausible candidate for the unstable intermediate. One other major ^{14}C-labelled product (a yellow compound with λ_{max} = 375 nm at pH 4) was tentatively identified as the azo or azoxy dimer believed to be formed by condensation of the nitroso and hydroxylamine derivatives.

Dimers of this type may be comparable to those isolated from misonidazole [31] during the reduction of other nitroaromatic compounds [35, 36]. Such dimers may account for the colour observed during the reduction of metronidazole with xanthine oxidase [33] or chemical reductants [37] and in the urine of patients taking the drug [38]. Reduction of metronidazole also yields many other compounds whose nature could not be identified, but their relatively high absorbance above 200 nm and fluorescence (data not shown) suggest that the imidazole ring remains intact.

There was no observed antimicrobial activity of reduced misonidazole nor metronidazole against *C. bifermentans* anaerobically or *E. coli* aerobically or anaerobically at concentrations up to 10 mM. This lack of activity may be due to the reduction products being too polar to enter the bacterial cell or that toxicity is due to a short-lived intermediate of reduction. Given that there are marked contrasts in the reduction process and the nature of the final products, the latter would seem the most probable.

Reduction of nitroimidazoles involves a complex system of reduction events, decomposition and product interreactions which give rise to marked differences between 5-nitroimidazoles and 2-nitroimidazoles. Generally, the 5-nitroimidazoles are less stable, rapidly decomposing during reduction. The 2-nitroimidazoles undergo a four-electron reduction to the hydroxylamine derivative which decomposes after formation. Thus, both types of nitroimidazoles yield a myriad of final reduction products.

Whilst these reduction products would not seem to cause the primary cytotoxic effect of these drugs, they could be involved in their side effects such as neurotoxicity or mutagenicity which become apparent in mammalian cells at very high doses. In this respect the formation of acetamide, a weak hepatic carcinogen in rats [39], from metronidazole and ornidazole [40] may be relevant as discussed by Koch *et al.* [34], but the nature of the cytotoxic agent would appear to be a reduction product of less than four electrons.

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