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2-HYDROXYLAMINOIMIDAZOLES—UNSTABLE INTERMEDIATES IN THE REDUCTION OF 2-NITROIMIDAZOLES

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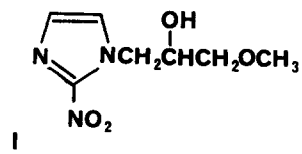
Abstract—An unstable 2-hydroxylaminoimidazole (2-hydroxylamino-1-methylimidazole) was prepared by the reaction of 2-fluoro-1-methylimidazole with hydroxylamine. This substance was sufficiently stable (half-life of 1–2 days) in acid solutions to be observed and characterized by NMR spectroscopy; decomposition at neutrality was, however, rapid (half-life of 1–10 min). Radiochemical and electrochemical reduction experiments were carried out at pH 4 and pH 7 with 2-nitro-1-methylimidazole and misonidazole [1-(3′-methoxy-2′-hydroxypropyl)-2-nitroimidazole]. A four electron stoichiometry was found in every case. The pH 4 reduced product was identified as the 2-hydroxylamino derivative (>80% yield). The pH 7 reduced solutions, on the other hand, showed no aromatic ^{1}H NMR signals, suggesting that a simple imidazole ring was no longer present. A shift to pH 7 of the hydroxylamine produced at pH 4, however, resulted in very similar NMR spectra. The conclusion, therefore, is that the hydroxylamine was produced initially on reduction of the nitroimidazole, but it was not stable.

Misonidazole (I) is currently undergoing clinical trials as a radiation sensitizer of hypoxic cells [1–4]. In addition, it exhibits marked preferential cytotoxicity toward hypoxic cells [5–8] and is mutagenic in the Ames assay system [9]. These properties are found in varying degrees with other nitroimidazoles, including metronidazole (2-methyl-5-nitroimidazole-1-ethanol), a drug widely used as an antimicrobial agent [10]. The differential toxicity of nitroimidazoles toward hypoxic cells as well as their mutagenic effects have been correlated with reductive metabolism. However, a detailed understanding of the effect has yet to emerge, and even the chemical behavior of the nitroimidazole class has not been fully characterized [11]. One widely cited mechanism involves anaerobic nitroreduction to a product or products which interact with critical cell targets [12–15] and, in the case of misonidazole, binding of reduced products to cellular macromolecules has been demonstrated [14, 15]. There is also evidence in experimental animal tumours for a diffusible product formed in hypoxic cells which is also toxic to surrounding cells [16, 17].

To clarify these proposed mechanisms and, in particular, to identify the cytotoxic species, attention has increasingly focused on the nature of the nitro-reduced species. Misonidazole reduction using a variety of techniques—radiolysis [18], electrolysis [19] and enzymatic reduction (xanthine oxidase) [20, 21]—proceeds with a four electron stoichiometry in nitroimidazole disappearance. Although this may mean that the products simply correspond to an average uptake of four electrons, the simple product of such a reduction, the hydroxylamine II, is obviously of considerable interest. In fact in the above reductions, and in a chemical (zinc–ammonia) reduction [22], a product is present with a mass spectral peak at $m/e = 187$ corresponding to the molecular ion of II. The zinc–ammonia reduction



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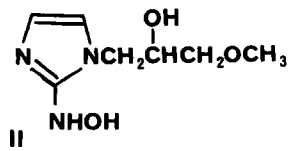

II

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also produces bimolecular azoxy, azo and hydrazo products which could be derived from the hydroxylamine. Despite this considerable evidence, however, other spectroscopic confirmation of the existence of the hydroxylamine under these reduction conditions is lacking, and there is one report [19] of the failure to detect an appropriate NMR spectrum under conditions where the product with *m/e* of 187 is present. This observation led to the suggestion that the hydroxylamine, while formed, may not be stable under the reducing conditions employed or may be present only in very small quantities. Furthermore, recent studies have demonstrated that ring fragmentation can occur. There is evidence for the presence of glyoxal in chemically reduced [23] or enzymatically reduced [24] misonidazole.

To evaluate the stability of 2-hydroxylaminoimidazoles, we sought a method of synthesizing such a species in a non-reductive manner. We were successful in this synthesis and found that the 2-hydroxylamine so obtained, though relatively stable at acid pH, was converted to as yet unidentified products of neutral pH with a half-life of the order of minutes. We have, moreover, demonstrated that the 2-hydroxylamine derivative was formed using radiolytic and electrolytic reduction. This product was sufficiently stable in acid solutions to be observed after these reductions, but at neutrality it was rapidly converted to other products and was not observed.

METHODS

Misonidazole was provided by Roche Products Ltd. Literature procedures were used to prepare the following compounds: 1-(2-aminoimidazol-1-yl)-3-methoxypropane-2-ol [25]; 1-methyl-2-nitroimidazole, from 2-nitroimidazole [26, 27]; 1-methyl-2-aminoimidazole [28]; and 1-methyl-2-fluoroimidazole [29].

The reaction of 1-methyl-2-fluoroimidazole (III) with hydroxylamine was carried out by dissolving the reagents as their hydrochloric acid salts in D_2O (typically 2 mmoles of III, 3 mmoles of $NH_2OH \cdot HCl$ in 2 ml of D_2O), followed by the addition of sufficient concentrated NaOD in D_2O to bring the pD to 3.5–4.0. The pD decreases during the course of the reaction and base was added at intervals to maintain it within the above range. NMR spectra were recorded directly on the reacting solution.

Irradiation experiments were carried out in pH and pD 7 solutions prepared with 10 mM phosphate buffer and 100 mM sodium formate and in pH and pD 4 solutions prepared with 100 mM sodium formate and sufficient HCl or DCl to give the desired pH or pD. The nitroimidazoles (0.5 to 1.0 mM) were added to these and the solutions degassed by saturating with N_2O. Irradiations were carried out using a ^{60}Co γ cell (Atomic Energy of Canada Ltd.), with a dose rate of 5.35 ± 0.15 Krad/min. The four reducing equivalent stoichiometry was determined by following the disappearance of the parent nitroimidazole u.v. peak at 326 nm as a function of irradiation time. Full details of these procedures can be found in Whillans and Whitmore [18].

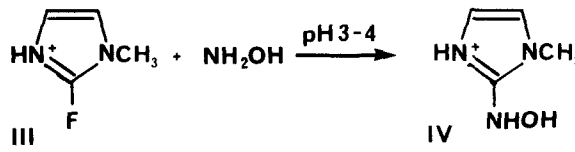
The NMR spectra in Figs. 1 and 2 were obtained on solutions of drug in D_2O within 1 hr of completion of irradiation. Spectrum 2d was obtained by adding K_3PO_4 in D_2O to the solution responsible for spectrum 2c. Spectra were recorded at 360 MHz at the Toronto Biomedical NMR Centre, University of Toronto, with the assistance of Dr. Arthur Gray and Mr. Alan Lee. Full details of the NMR peaks are indicated in the figure captions with chemical shift assignments based on an acetone reference at δ 2.225.

Electrolytic reduction experiments were conducted at pH 7 in 10 mM phosphate buffer and 100 mM formate, at pH 4 in 100 mM formate buffer, and in 0.1 M HCl solutions. The technique used was essentially that described previously [19]. Reductions were carried out on deoxygenated solutions (N_2 bubbler) over a mercury pool operating at a constant potential of −0.8 V with reference to a Calomel electrode. Current flow varied but was of the order of 20 mA for the most concentrated solutions (40 mM). The usual work-up procedure involved lyophilization of the reduced solution followed by re-dissolving in D_2O for recording of NMR spectra. In the hydrogenation experiments described below the reduced solutions were used directly, after the appropriate time or adjustment of pH.

Hydrogenation experiments were carried out over 5% palladium on charcoal at room temperature and one atmosphere. Hydrogen uptake was measured with a pressure-equalized burette. Work-up procedures involved filtration of the catalyst, lyophilization, and re-dissolving in D_2O.

RESULTS

One method which appeared particularly attractive for the preparation of a 2-hydroxylaminoimidazole involves the substitution of the fluorine of a 2-fluorimidazole [29–31] with hydroxylamine (Eqn. 1). We therefore prepared 1-methyl-2-fluoroimidazole III [29] and mixed this with an excess of hydroxylamine, maintaining the pH or pD in the



region 3–4. Reaction occurred over a period of about 1 day and was monitored directly using NMR spectroscopy by carrying out the reaction in D_2O. As the fluoroimidazole disappeared, its peaks in the spectrum, a 2H multiplet at δ 7.1 and a 3H singlet at δ 3.75, were replaced by a 1.1 pair of doublets (J = 2) at 7.00 and 6.95 ppm and a 3 hydrogen singlet at 3.49 ppm. This spectrum, particularly the pair of doublets, suggests a 2-substituted imidazole system consistent with the formation of the 2-hydroxylaminoimidazole IV. One other possible product which could form is 2-hydroxy-1-methylimidazole derived from nucleophilic substitution with solvent; we have prepared such a compound [32], and its NMR spectrum in acids has the imidazole hydrogens near 6.5 ppm. A comparison is also available with 2-amino-1-methylimidazole. In acid solutions, this

has a pair of doublets ($J = 2$) at 6.82 and 6.79 ppm, and a methyl singlet at 3.49 ppm. This spectrum is also different, as was verified with solutions where both amine and hydroxylamine were present. The amine, however, served as a model of the hydroxylamine, and the similarities in the spectra provide further support for the structural assignment of the latter.

The key feature of the hydroxylamine produced in the synthesis is that it was indeed not stable. In fact, its instability has thus far prevented our obtaining an analytically pure sample, since a significant amount of its further reaction occurred in the time required for the preparation. The reaction or reactions which occurred were characterized by the disappearance of the doublet near 7.0 ppm, with no new signals appearing in the imidazole proton region above 6 ppm. A set of peaks did appear at 5–5.5 ppm beside the solvent (HOD) signal. The N-methyl signal at δ 3.49 simultaneously disappeared and was replaced by a number of peaks around 3 ppm. The time required for this reaction to be complete was of the order of 2–3 days in acids, but what is of particular interest is the observation that, when the acid solution containing the hydroxylamine was neutralized, the reaction was complete within 5 min. The previous studies of 2-nitroimidazole reduction were conducted in neutral or basic solutions, and this finding would suggest that, even if formed, the 2-hydroxylamine is not likely to be observed. The enhanced stability in acid, however, implies that such a species might be observed under acidic conditions.

One of the techniques with which we chose to test this was radiation reduction, since that seemed easily adapted to acid conditions. As substrates, both misonidazole and 1-methyl-2-nitroimidazole were employed, the latter being chosen since its hydroxylamine derivative is available for comparison. Radiation reduction of the two compounds was performed at both pH 4 and, for comparison, pH 7. The technique was identical to that used by Whillans and Whitmore [18] in their previous study with misonidazole at pH 7. In brief, irradiations were carried out using a ^{60}Co γ cell on dilute solutions of the nitroimidazoles in the presence of excess formate and N_2O. Under these conditions the primary water radiolysis species e^-_{aq} and $\cdot OH$ are converted to CO_2^-, the actual reducing species. At both pH 4 and 7, radiation dosimetry showed that the disappearance of the nitroimidazoles was accompanied by the consumption of 4.0 ± 0.2 reducing equivalents per molecule, as found by Whillans and Whitmore for misonidazole at pH 7.

By the use of D_2O solutions, the NMR spectra shown in Figs. 1 and 2 were obtained directly on the irradiated solutions after completion of the reduction. (At these concentration levels and dose rates, this required 1–2 hr.) NMR spectra were also recorded for solutions which had received half the radiation dose required by the four electron stoichiometry. These showed only a 50/50 mixture of the spectra of a fully reduced sample and the spectra of unreacted nitroimidazole, indicative that no intermediate accumulates during reduction. The reductions at pD 7 shown in Figs 1b and 2b were accompanied by the complete disappearance of characterizable imidazole ring protons and the appearance of a complex set of peaks at 5–5.5 ppm beside the HOD peak. This absence of ring protons is reminiscent of the result of Whillans and Whitmore [18] for misonidazole reduction at pH 7, although these workers had gone through an HPLC separation before recording the NMR spectra. The degree of complexity of the product mixture is more clearly indicated with the methylimidazole by the set of N-methyl signals observed near 3 ppm after reduction (Fig. 2b).

In marked contrast, however, were the spectra obtained after pD 4 reduction, since now a product which retains imidazole signals was clearly still present (Figs. 1c and 2c). That this must be the hydroxylaminoimidazole can be demonstrated with the 1-methyl system by the complete superimposition of this spectrum on that obtained in the nucleophilic substitution. We assume by analogy that the corresponding species II was being formed in the misonidazole reduction.

As noted before with the product of the nonreductive synthesis, the hydroxylaminoimidazoles were not stable at pD 4, the NMR spectra 1c and 2c slowly changing with time, with the signals near 7 ppm disappearing and being replaced by signals near 5–5.5 ppm. The signals associated with the side chain of misonidazole also changed, but their complexity makes analysis difficult. With the methyl compound, the disappearance of the 7 ppm signals was accompanied by the disappearance of the N-methyl singlet at 3.5 ppm and the appearance of a set of peaks near 3.0 ppm. This decomposition had, in fact, occurred to some extent in the time required to complete the irradiation experiment. This can be seen in the weak signals near 3.0 ppm in Fig. 2c.

The half-lives of the 2-hydroxylaminoimidazoles were of the order of a day at pD 4 and ambient temperature. When, however, the pD was adjusted to neutrality, reaction was complete within 5–10 min. These results were again expected on the basis of the synthetic sample. What is more important, however, is that the solutions, when shifted from pD 4 to pD 7, exhibited spectra that were very similar to those obtained by direct reduction at this pD. This can be seen most clearly in spectrum 2d of the 1-methyl system. This is the NMR spectrum obtained on the sample responsible for the spectrum in 2c after a shift to pD 7 and a wait of 30 min. The same set of N-methyl signals in approximately the same ratio was present as observed in spectrum 2b, which was obtained after direct reduction of 1-methyl-2-nitroimidazole at pD 7. There are two small additional peaks in 2d near 2.8 ppm, but these appear to have arisen from reaction at pD 4 prior to the change. Preliminary results indicate that the breakdown products of the 2-hydroxylamine at pH 4 were not the same as the breakdown products at pH 7.

Studies of the electrochemical reduction of the two nitroimidazoles produced results which were exactly the same as those observed in the radiolysis. Four electrons were consumed in both neutral and acid solutions. The acid reductions, however, yielded the hydroxylamines while the neutral reductions gave only the products of their further reaction. These reductions could also be carried out on a larger scale

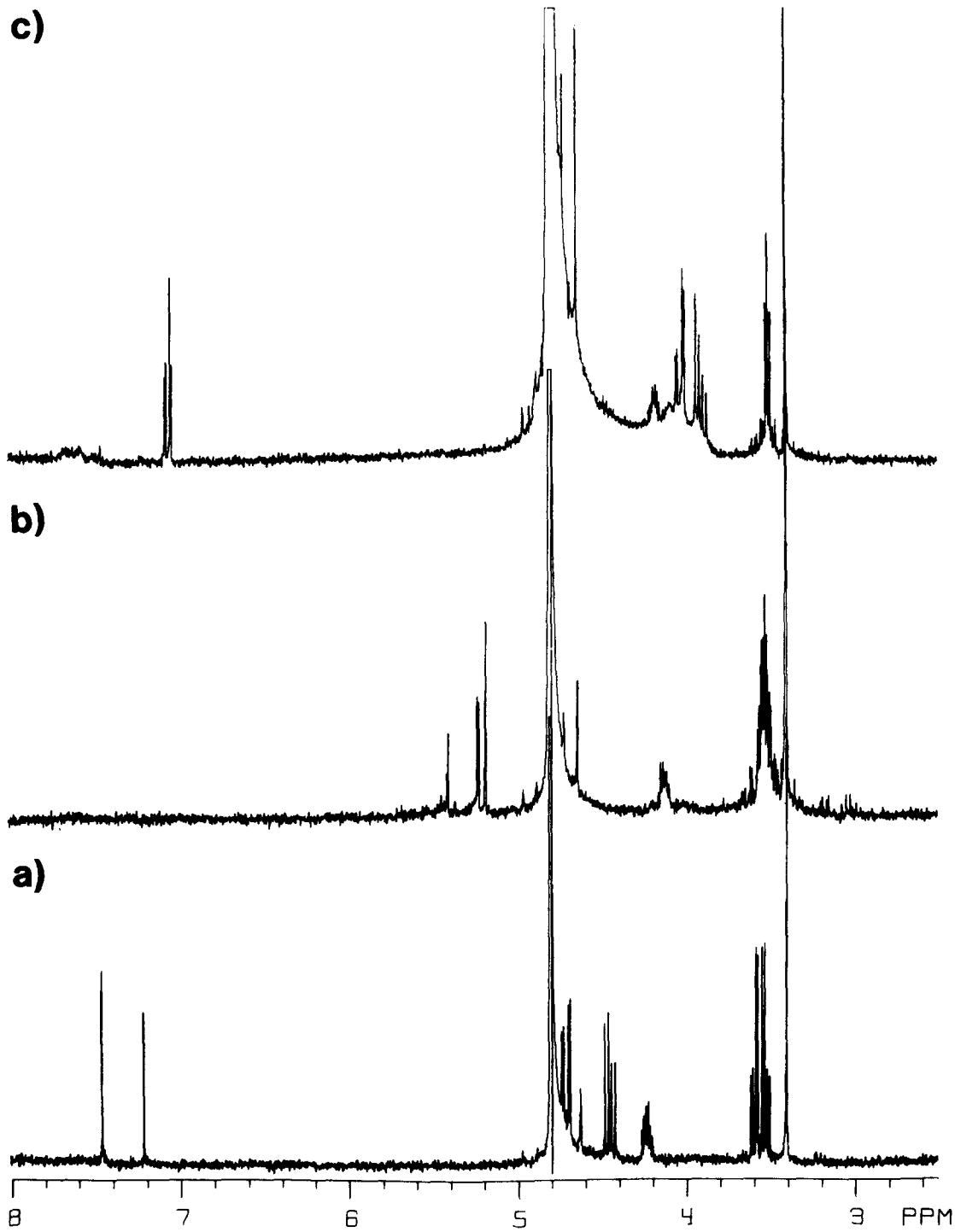


Fig. 1. 360 MHz ^{1}H NMR spectra of misonidazole: (a) in D_2O; (b) after radiolytic reduction at pD 7; and (c) after radiolytic reduction at pD 4. Chemical shifts were as follows: Spectrum 1a—misonidazole: δ 7.445 (1H, d, J = 1.5), 7.205 (1H, d, J = 1.5), 4.70 (1H, dd, J = 14.2 and 4.2), 4.44 (1H, dd, J = 14.2 and 8.2), 4.225 (1H, m), 3.585 (1H, dd, J = 11.2 and 4.0), 3.515 (1H, J = 11.2 and 6.0), and 3.395 (3H, s). Spectrum 1c—1-(2-hydroxylaminoimidazole-1-yl)-3-methoxypropane-2-ol: δ 7.06 (1H, d, J = 2.2), 7.03 (1H, d, J = 2.2), 4.175 (1H, m), 4.025 (1H, dd, J = 15.2 and 3.0), 3.90 (1H, dd, J = 15.2 and 8.9), 3.51 (2H, m), and 3.40 (3H, s).

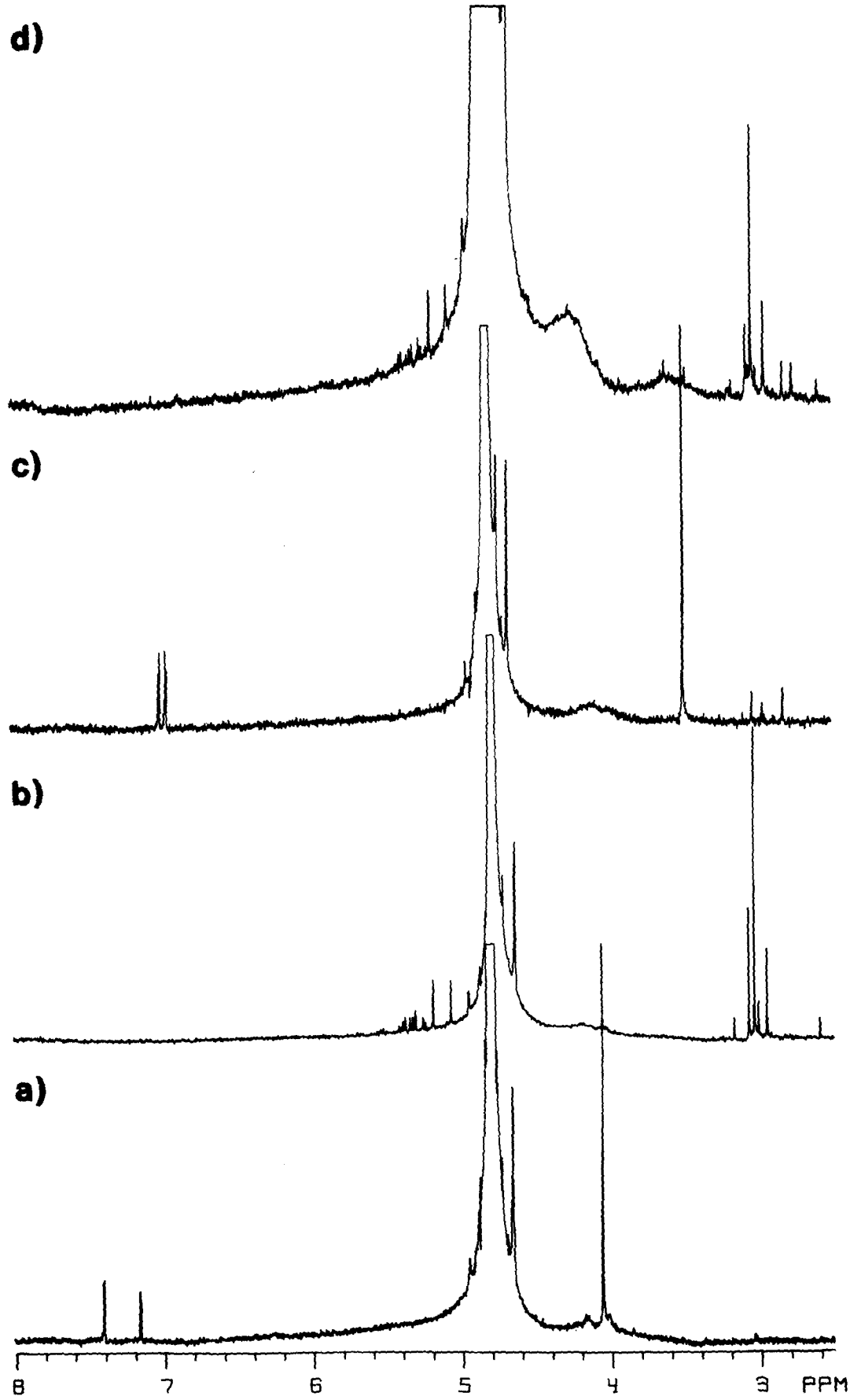


Fig. 2. 360 MHz ^{1}H NMR spectra of 1-methyl-2-nitroimidazole: (a) in D_2O; (b) after radiolytic reduction at pD 7; (c) after radiolytic reduction at pD 4; and (d) after radiolytic reduction at pD 4, and the addition of sufficient K_3PO_4 to change the pD to 7. Chemical shifts were as follows: Spectrum 2a—1-methyl-2-nitromidazole: δ 7.395 (1H, d, $J = 1$), 7.155 (1H, d, $J = 1$), and 4.058 (3H, s). Spectrum 2c—1-methyl-2-hydroxylaminoimidazole: δ 7.00 (1H, d, $J = 2.2$), 6.95 (1H, d, $J = 2.2$), and 3.49 (3H, s).

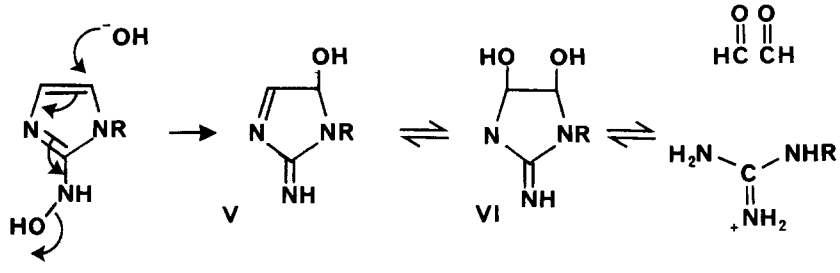
and permitted the following experiments which further characterize the hydroxylamine. Since the amine derivative of misonidazole has been produced from the nitro compound by catalytic hydrogenation [25], the hydroxylamine should also be capable of being further reduced in this way. Indeed, subsequent hydrogenation of solutions of misonidazole electrolytically reduced in formate buffer at pH 4 or in HCl solutions resulted in the uptake of slightly less than one equivalent of the hydrogen with the production of the amine of misonidazole, as characterized by comparison of its NMR spectrum with that of an authentic sample [25]. Attempts to hydrogenate solutions of misonidazole electrolytically reduced at pH 7, reduced at pH 4 and shifted to pH 7, and reduced at pH 4 and allowed to stand for 2 weeks revealed no uptake of hydrogen and no evidence of amine formation. The failure to observe an exactly one equivalent hydrogen uptake directly after pH 4 reduction is also explained by the presence of small amounts (10–30%) of further reaction products which had formed during the time required for electrolysis.

DISCUSSION

Our results therefore provide strong evidence for the formation of 2-hydroxylaminoimidazoles in the radiation and electrolytic reduction of 2-nitroimidazoles. This product was sufficiently stable in acid solutions to be observed and, at least in the case of misonidazole, it could be further reduced to the amine form using catalytic hydrogenation. 2-Hydroxylaminoimidazoles were also initially formed at pH 7, but here they were too unstable to be observed. Using samples of the hydroxylaminoimidazole prepared in acids, we are currently conducting kinetic studies on this decomposition, following the disappearance of the hydroxylamine using u.v., NMR and HPLC. Our initial results revealed that the reaction at constant pH is first-order in hydroxylamine and, as expected, that there is a pronounced pH dependency on the observed first-order rate constants, the rate increasing significantly with increasing pH. This pH dependency stands in marked contrast to the behavior of benzenoid hydroxylamines, which are generally stable in neutral solutions but undergo an acid-induced rearrangement [33, 34].

Our kinetic studies show that the half-life of the hydroxylamine from misonidazole is 2.5 min in a dilute phosphate buffer at pH 7. On this basis we feel that the *m/e* peak of 187 observed in pH 7 reduced misonidazole is not due to the simple hydroxylamine II. We have obtained mass spectral data of pH 4 reduced misonidazole which shows an *m/e* peak at 187 with a slightly different fragmentation pattern from that of samples reduced at pH 7. We have, however, reservations regarding mass spectra of reduced misonidazole; we are, for example, uncertain as to the state of protonation of the material being analyzed, particularly at pH 7. Thus far, any attempt on our part to obtain the neutral hydroxylamine has resulted in decomposition. This instability of the neutral form has also frustrated our attempts at derivitization. We have established that the 2-hydroxylamine formed at pH 4, shifted to pH 7, and then back to pH 4 has undergone irreversible changes. This would appear to rule out the possibility that the pH 7 material is the oxime tautomer of the neutral hydroxylamine.

With metronidazole, products derived from ring fragmentation are usually found after reduction [35–39]. This normally requires three or four electrons per molecule depending on the reduction technique used, although hydrogenation to the amine has been reported recently [40]. We are currently attempting to identify the stable products of reduction of the 2-nitroimidazoles at pH 7. The following speculative scheme, similar to one proposed by Raleigh and Liu [24], accounts for our present understanding of this system. The initial reaction, which explains the pH dependency of the hydroxylamine stability, is a bimolecular version of the Bamberger rearrangement. This type of nucleophilic reaction on an arylhydroxylamine is without precedent with hydroxide ion as the nucleophile, although a similar mechanism has been proposed recently for the reaction between phenylhydroxylamine and sulfite or bisulfite [41]. The product of this rearrangement V is an isomer of the hydroxylamine and may well account for the *m/e* 187 peak. This compound and its hydrated form (VI) serve then as precursors for the glyoxal [23, 24] and guanidine [42] fragments which are observed. There are several examples in the chemical literature of compounds of the general structural type V and VI, these being formed in a reversible reaction of a 1,2-dicarbonyl compound and an amidine or quanidine [43–46].



Experiments are also underway to test for the biological activity of 2-hydroxylaminoimidazoles and products derived from them. It is interesting to note in this regard that the amine form of misonidazole is a major excretory product [47], implying that under biological reduction conditions the hydroxylamine is sufficiently long lived that further reduction can

occur. This may, of course, mean that the results of our model studies may be limited to the degree with which they can be extrapolated to biological systems. However, they would appear to represent an important source of basic information which will aid in the overall understanding of bioreductive processes involving 2-nitroimidazoles.

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