process.[18a] This type of kinetic effect cannot be expected for the KIE in the ET process because the heavy atom reorganization occurs as a separate process from the transfer of electron; the KIE in the ET process is an equilibrium IE in this sense. A small IE would therefore be expected for an ET process even if a significant geometrical change is involved in the transition state.

In summary, the present study shows that carbon KIE, together with other mechanistic evidence, can be a useful means to distinguish an ET process from bond-forming processes. It was concluded that the reactions of ketones with organolithium reagents proceed via an initial ET and that the rate-determining step varies depending on the structure of ketones and the lithium reagents. The kinetic isotope effect study on ET has some precedent in deuterium IEs,[19] but we believe that carbon KIE is more useful and has wider applicability.

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Products of the Reductions of 2-Nitroimidazoles

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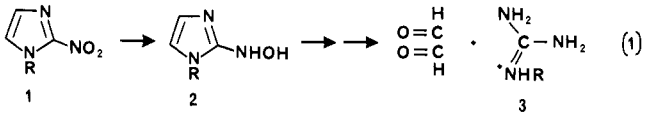
Abstract: Reductions under neutral conditions of misonidazole (1-(2′-hydroxy-3′-methoxypropyl)-2-nitroimidazole) and 1-methyl-2-nitroimidazole have been studied with radiation chemical, electrochemical, and chemical (zinc/ammonium chloride) techniques. Major products accounting for 70–85% of the reduction mixture have been identified as the cis:trans isomers of **4** (1-substituted 2-amino-4,5-dihydro-4,5-dihydroxyimidazolium ions). These have been independently synthesized by the reaction of glyoxal and the appropriate guanidinium ion. Their presence after nitroreduction has been established by ^{1}H NMR and by a spectroscopic analysis in which **4** is converted into glyoxal bis-oxime. The ability of misonidazole reduction mixtures to form glyoxal derivatives has been noted previously, even in vivo; the presence of the cyclic **4** accounts for this. The four-electron-reduced product, a 2-(hydroxylamino)imidazole, is the precursor of **4**. The hydroxylamine is unstable at pH 7, but it can be observed in acid where decomposition also gives **4** but in a much slower reaction. Nitroreduction or hydroxylamine decomposition in pH 7 phosphate gives two additional products which have been identified on the basis of their ^{1}H NMR spectra as cis:trans isomers of monophosphate esters of **4**. The reaction leading to these may model the DNA binding which is observed with reduced misonidazole. Azomycin (2-nitroimidazole) has been investigated by the radiation chemical technique. At pH 7 the isomers of **4** are formed, but they are minor products. The major product (70%) is 2-aminoimidazole.

Extensive biological and biochemical examinations of the radiation sensitizer misonidazole (**1a**) have shown a number of effects correlating with reductive metabolism.[1] These include a preferential toxicity toward hypoxic cells as compared to aerobic cells,[2] mutagenicity,[3] chemosensitization (the potentiation of the effect of other chemotherapeutic agents),[4] DNA binding,[5] and depletion of cellular thiols.[6] The implication that reduction is involved has



1 **2** **3** (1)

a, R = $CH_2CHOHCH_2OCH_3$
b, R = CH_3
c, R = H

led to an interest in the reduction chemistry. The six-electron product, a 2-aminoimidazole, is formed upon catalytic hydrogenation.[7] Other methods however have shown a four-electron stoichiometry,[8-15] implicating the (hydroxylamino)imidazole **2a**. Although there are indications of such a product after zinc reduction,[16] we have found that it is unstable, particularly at neutral pH.[11]

The subject of this paper is the nature of the decomposition products. Two groups have established that derivatives of glyoxal can be formed after misonidazole reduction.[17-19] This two-carbon dialdehyde presumably arises from C4,C5 of the imidazole ring, and a candidate for the other fragment, the guanidinium ion **3a**, has also been detected.[20] There is some question as to whether

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Table I. ^{1}H NMR Chemical Shifts (δ) in D_2O of 2-Amino-4,5-dihydro-4,5-dihydroxyimidazolinium Ions and of Monosubstituted Guanidinium Ions

1-substituent	isomer	dihydroxyimidazolinium ion H4/H5[a]		side chain	guanidinium ion
H	trans	5.22			
	cis	5.44			
CH_3	trans	5.18	5.04	3.03 (s)	2.81
	cis	5.37	5.24	2.94 (s)	
CH_2CH_2OH	trans	5.21	5.19	3.81 (2 H, t), 3.59 (2 H, t)	3.72 (2 H, t)
	cis	5.40		3.76, 3.57	3.34 (2 H, t)
$CH_2CH_2OCH_3$	trans	5.20	5.16	3.40 (3 H, s) } 3.6–3.7 (4 H, m)	3.62 (2 H, t), 3.34 (2 H, t)
	cis	5.39		3.39 (3 H, s) }	3.39 (3 H, s)
$CH_2CHOHCH_2OCH_3$	trans	5.215	5.16	3.40 (3 H, s)	3.40 (3 H, s)
	trans′	5.225	5.16	3.5–3.6 (4 H, m)	3.3–3.4 (2 H, m)
	cis	5.42	5.39	4.1–4.2 (1 H, m)	3.5–3.6 (2 H, m)
	cis′	5.40			4.1–4.2 (1 H, m)

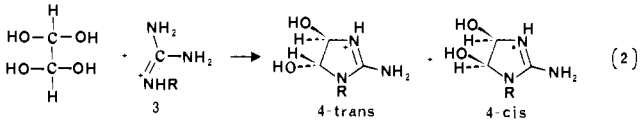
[a] See also Figure 1.

free glyoxal is actually present after reduction or a substance capable of releasing it.[17] This led us to explore the possibility of a glyoxal–guanidine adduct.[21] We report here the preparation and characterization of such substances and identify these as major products of reduction of three 2-nitroimidazoles: misonidazole (**1a**), the 1-methyl model **1b**, and azomycin (**1c**). NMR evidence for a phosphate adduct is also presented.

Results

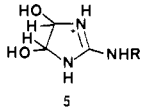
Glyoxal–Guanidine Adducts. Glyoxal as its bis-hydrate[22] in water was reacted with several guanidinium salts producing 1:1 adducts as established by analysis. Structural evidence was obtained by ^{1}H NMR spectroscopy (Table I and Figure 1). Upon reaction, the singlet at 4.80 ppm due to the glyoxal hydrate moves slightly downfield to give a set of peaks in the 5.0–5.5 ppm region with the two hydrogens now nonequivalent (except in the parent system). The signals for the guanidinium ion also disappear, being replaced by a new set of peaks. The methyl derivative is the most characteristic. Its spectrum clearly shows the presence of two species (Figure 1a): a major product (80%) with a small 0.7-Hz coupling and a minor product (20%) with a larger 5.4-Hz coupling. The two corresponding NCH_3 singlets are seen near 3.0 ppm. This spectrum suggests that the products are the cis:trans isomers of the cyclic adducts **4b**, with the trans isomer the major product, since J(cis) should be greater than J(trans) in this system.[23] These isomers are named as 2-amino-4,5-dihydro-4,5-dihydroxy-1-methylimidazolium ions.

The spectra of the products with the three other substituted guanidines of eq 2 can also be explained by a 1:4 mixture of cis:trans **4**, although the signals for H4 and H5 are closer, in some cases even overlapping (see Figure 1b). The chiral center of the



(2)

a, R = $CH_2CHOHCH_2OCH_3$
b, R = CH_3
c, R = H
d, R = CH_2CH_2OH
e, R = $CH_2CH_2OCH_3$



misonidazole side chain gives rise to two diasteriomeric trans adducts and two cis. Our analysis of the H4,H5 region (Figure 1c) is that the peak at 5.16 ppm represents overlapping doublets of the two trans isomers, with the mates for these near 5.22 ppm.

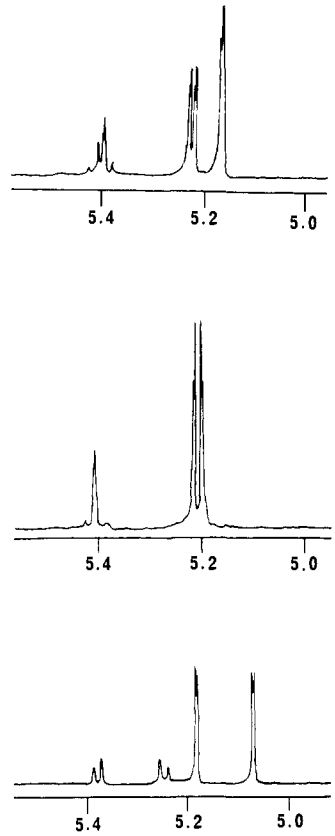


Figure 1. 360-MHz ^{1}H NMR spectra in D_2O of the H4,H5 region of the cyclic adducts **4**: (a, bottom) 1-methyl (**4b**); (b, middle) 1-(2′-hydroxyethyl) (**4d**); (c, top) 1-(2′-hydroxy-3′-methoxypropyl) (**4a**).

One of the cis isomers has H4 and H5 as a single peak at 5.40 ppm. In the other they are just separated and appear as two distorted doublets overlapping the 5.40 ppm signal. H4 and H5 are equivalent in the unsubstituted system and just two singlets are observed, at 5.44 (20%) and 5.22 ppm (80%). The major singlet is assigned to the trans isomer by analogy to the other compounds. This assignment also satisfies the general trend that the signals for H4,H5 in the trans isomer are upfield from the signals for the cis isomer.

The products have been assigned as the ring-substituted **4**, rather than as **5** where the substituents are on the external nitrogen. Cis:trans isomerization is also present in **5**, and H4 and H5 can be nonequivalent if there is restricted rotation about the C2–NHR bond. The situation in this respect with ions such as **5** is unknown, but there are literature reports showing restricted rotation in guanidinium ions near room temperature.[24,25] The more com-

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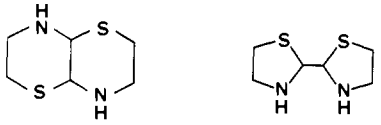
pelling evidence in favor of the ring-substitution products **4** lies in their alternative preparation from the nitroimidazoles, where the substituent necessarily starts on a ring nitrogen. The argument here breaks down, of course, if **4** can isomerize to **5** via a ring-opening–ring-closing sequence. There is some evidence in this regard which will be addressed in more detail later.

Although the reactions of glyoxal and the particular guanidines employed in this work appear not to have been investigated, there is some literature precedence. Glyoxal reacts with amidines to form 1:1 cyclic adducts directly analogous to those proposed here.[26-28] Similar products are found from amidines reacting with α-ketoaldehydes and α-diketones[27-29] and from guanidines reacting with α-diketones,[30] although further reactions initiated by dehydration also occur in these cases.

Reactions of 4b. These adducts are stable in D_2O of pD 1–10, in particular showing no indication in the NMR of reverting to glyoxal and methylguanidine. (The NCH_3 signal is diagnostic in this respect.) This implies that the addition equilibrium lies well to the adduct side. This was also seen in preparations carried out in D_2O and followed in the NMR. With just a 5% excess of glyoxal, the methylguanidine is entirely consumed.

Despite the unfavorable equilibrium, the adducts do react with various nucleophiles to give the same derivatives obtained from glyoxal itself, with the other product the methylguanidinium ion. Thus hydroxylamine reacts to form glyoxal bis-oxime, and 2,4-dinitrophenylhydrazine forms the bis-hydrazone. Reaction with the parent guanidinium ion results in the unsubstituted adducts **4c** releasing methylguanidinium ion, establishing that glyoxal can be transferred from guanidine to guanidine. An equilibrium is established; NMR indicates an equilibrium constant ([**3b**][**4c**]/[**3c**][**4b**]) of 0.1. Reaction of **4b** with 2-aminoethanethiol occurs readily, forming a 1:2 adduct. The NMR spectrum of this is consistent with either of the structures[31] below (one diasteriomer only).



The reaction with hydroxylamine was developed as a spectroscopic analytic method. Glyoxal bis-oxime has a UV absorbance with λ_{max} at 272 nm, where solutions of glyoxal, the adducts, hydroxylamine, and the chemically reduced nitroimidazoles are transparent. In a pH 10.3 solution containing 0.25 M hydroxylamine, glyoxal (25–100 μM) forms the 272-nm absorbance upon mixing, while the adducts (25–100 μM) react much more slowly (half-life of about 10 min). Extinction coefficients at 272 nm were obtained with authentic bis-oxime, $(1.76 \pm 0.02) \times 10^4$; glyoxal, $(1.68 \pm 0.05) \times 10^4$; and **4b**, $(1.72 \pm 0.04) \times 10^4$. A period of 90 min was required with **4b** for full development of the absorbance, but within experimental uncertainty, bis-oxime has quantitatively formed after this delay. This procedure therefore not only quantitates the adducts but also it distinguishes these from free glyoxal through the rate of oxime formation.

Neutral Reduction of 1-Substituted 2-Nitroimidazoles. This section summarizes results with misonidazole and 1-methyl-2-nitroimidazole and three reduction methods: A, chemical (zinc/ammonium chloride);[16] B, electrochemical (mercury cathode);[12] and C, radiation chemical.[8] Method C involved irradiations in a ^{60}Co γ cell of aqueous solutions containing formate and N_2O to convert the primary water radicals e_{aq}^-, $^{\bullet}OH$, and $^{\bullet}H$ into $CO_2^{\bullet-}$, the actual reductant. Oxygen was excluded in each case. Methods B and C had phosphate present to maintain a pH near neutrality. The pH in A was initially 5–6, but this rose to 8–8.5 during reduction. Methods B and C had a stoichiometry of 4.0 ± 0.2 equiv/electron equivalents per RNO_2 reduced, in agreement with previous studies.[8-13]

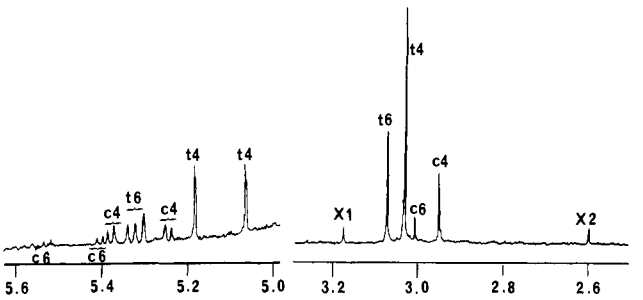


Figure 2. 360-MHz ^1H NMR spectra in D_2O after radiation chemical reduction (N_2O-saturated D_2O) of 1-methyl-2-nitroimidazole (1 mM), phosphate (pD 7, 20 mM), and formate (0.1 M). There are no other signals in this spectrum, except those due to HOD (4.80 ppm) and HCOONa (8.40 ppm). Relative NCH_3 integration t4, 53%; c4, 14%; t6, 22%, c6, 4%; X1, 3%; and X2, 4%. (Note that the two regions were not recorded at the same attenuation.)

Reductions were run directly in D_2O (C) or the product mixture taken up in D_2O after lyophilization (A, B), and 360-MHz ^1H NMR spectra were recorded. In every case the 1:4 ratio of cis:trans **4** accounts for 70–90% (by integration) of the product. Other minor products are also seen. These are particularly apparent with the methyl compound where the NCH_3 singlets provide at high field a "chromatogram" of the product mixture. With misonidazole, the complexity of the spectra, particularly in the side-chain region, makes the observation of minor products more difficult. The following focuses therefore on the methyl compound.

Figure 2 shows the result of reduction by method C. The CH_3 region implies a total of six products, barring overlap. Two of these are the isomers of **4b** (signals t4, c4). Two others, with peaks assigned as t6 and c6, will be tentatively identified later. The minor products producing peaks X1 and X2 are unknown. The intensity ratios of the signals for 4 and 6 are dependent on the phosphate concentration, while X1 and X2 are always present in very small amounts.

With method B the spectra are very similar, with **4** as the major product, and the signals for **6**, X1, and X2 are also present in about the same proportion. Peaks accounting for 3–5% of the starting material are also observed at 7–8 and 3.5–4.0 ppm. These correspond to products retaining the imidazole functionality and are probably due to azo and azoxy compounds.[32] These solutions are yellow in accord with the formation of such products.

The zinc reductions were carried out in dilute RNO_2 solutions in order to avoid dimeric products,[16] and sufficient time was allowed for decomposition of the hydroxylamine before workup. These reductions are particularly clean, with **4b** accounting for 80–90% of the signals and the only other peaks present corresponding to those of 1-methyl-2-aminoimidazole. Misonidazole also forms the adducts (**4a**) as the major product, with its amine derivative as the only other observable species. The 2-aminoimidazoles are characterized by a pair of doublets at 6.7–6.85 ppm; the 1-methyl compound also has a characteristic singlet at 3.49 ppm.

The formation of **4** in high yields was verified with the glyoxal bis-oxime assay described in the previous section. With each

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reduction mixture there is very little initial burst in the 272-nm absorbance, as would be characteristic of free glyoxal. However, the oxime absorbance does appear, at the rate characteristic of the reaction with **4**. The 90-min absorbance accounts for the following percentages of the starting RNO_2–misonidazole: C, 86%; 1-methyl, C, 77%; 1-methyl, B, 73%; and 1-methyl, A, 84%. Misonidazole was not studied with the last two techniques. Based upon replicate experiments, the error in these numbers is about 5%.

Previous identifications of glyoxal after misonidazole reduction have suggested much lower yields.[17-19] We believe, however, that these did not pick up all the potential glyoxal. As in this study, the quantitative determination was based upon derivatization, as the guanosine cyclic adduct[17] or the bis[2,4-dinitrophenylhydrazone].[18,19] We have investigated the formation of the latter from **4**. The hydrazone does form, but the reaction under the published acid conditions is extremely slow. We have not looked into the guanosine reaction, but it is unlikely to be quantitative since even the reaction with free glyoxal is known to be reversible.[33]

We can also comment upon a proposal that the products of radiation reduction of these same two nitroimidazoles are the oxime tautomers of the (hydroxylamino)imidazoles.[10] This assignment was based only upon NMR spectra, which were in fact the same as those observed in our work (although the spectra of minor products were not reported). Thus this previous study was forming the same products. Our preparation from the glyoxal–guanidine reaction, however, establishes that these are **4** and not the oximes.

Finally, a comment can be made regarding mass spectral analysis. The products **4** did exhibit weak electron-impact mass spectra with the ion of highest m/e corresponding to **4**–H_3O^+ (m/e 187 from **4a**, 113 from **4b**). These products are of course salts, and we suspect that the mass spectral ions arise from decomposition in the heated probe of the mass spectrometer. The nitroimidazole reduction mixtures had very similar mass spectra (including the fragmentation pattern). The ion at m/e 187 from misonidazole corresponds to the molecular weight of the hydroxylamine. The observation of this ion in the mass spectra of reduced misonidazole has been taken as evidence for such a product.[15,16] The same mass spectrum is, however, found with the glyoxal–guanidine adduct where NMR and elemental analysis establish a different structure.

Acid Reduction. We have previously described 2-(hydroxylamino)imidazoles **2a** and **2b** as the products after radiation chemical reduction at pH 4.[11] These were characterized in the NMR by the retention of imidazole proton signals near 7 ppm. The structure was supported by the observation with the methyl system that a product with the same NMR spectrum is produced by the reaction of 2-fluoro-1-methylimidazole and hydroxylamine. Varghese has also discussed chemical evidence for such compounds.[34]

The result of a pH 3–4 electrochemical reduction of 1-methyl-2-nitroimidazole (3.7 ± 0.1 electrons per RNO_2) is shown in Figure 3a. The pair of doublets near 7 ppm and the singlet at 3.50 ppm correspond to the hydroxylamine **2b**, and this is the major product. There are four other NCH_3 signals near 3 ppm, and, as seen in the insert, these apparently arise from two pairs of cis:trans dihydroimidazoles. The pair present in the lesser amount corresponds to **4**, while the other pair identified as **7** are of an unknown structure. The small quantity of **4** arises from decomposition of the hydroxylamine during the time required for reduction, as will be apparent. The other products do not come from the hydroxylamine, as shown below, and are not seen in pH 7 reductions.

An immediate change to pH 7 at this stage results in the disappearance of the hydroxylamine. The unknown **7** are unchanged. The major products are **4** (1:4 cis:trans), with new

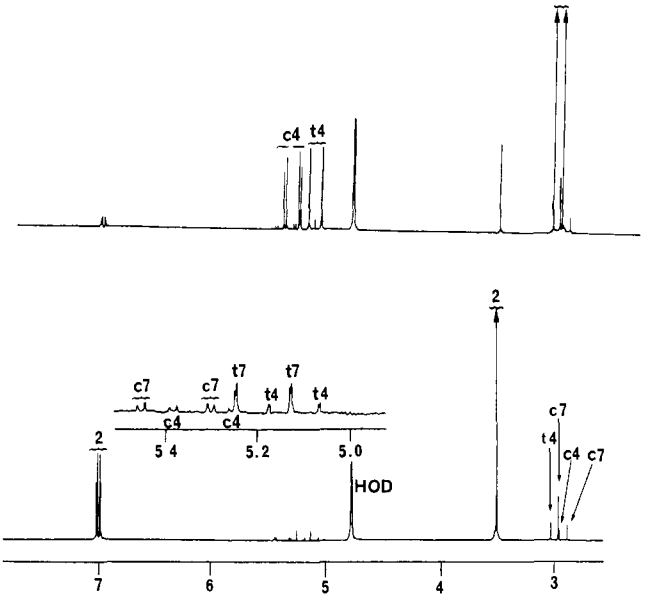
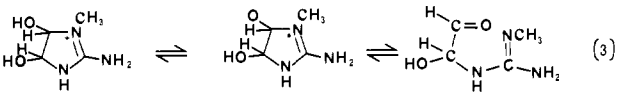


Figure 3. 360-MHz ^1H NMR spectra in D_2O after electrochemical reduction of 1-methyl-2-nitroimidazole at pH 3–4: (a, bottom) initial spectrum recorded at pD 1–2; (b, top) after standing 1 week. (Peaks indicated with arrows are off-scale.)

signals matching those previously assigned as **6** (see Figure 2). The hydroxylamine is more stable in acid, but not indefinitely so, disappearing over a period of a week (Figure 3b). The signals which grow correspond to **4**, and there are no new products. The two isomers of **4** are now seen in an almost 1:1 cis:trans ratio. A change to pH 7, however, results in an immediate adjustment to the 1:4 ratio. This is unchanged upon reacidification.

These observations have important implications regarding the dynamic behavior of the dihydroxyimidazolium ion products. The 1:4 cis:trans mixture observed in all experiments at higher pH apparently represents the equilibrium situation. The observation of a nonequilibrium 1:1 mixture in acid which converts to the 1:4 mixture at higher pH suggests that the isomerization equilibration requires base. One possible mechanism involving ring opening is shown below. Such a reaction also permits nucleophiles such as hydroxylamine to react with the free carbonyl of the ring-opened form and eventually form glyoxal derivatives. We are currently in the process of a kinetic investigation of these derivatization processes and will defer a detailed mechanistic discussion until a later date.

A mechanism such as that of eq 3 would also permit the N1 substituent to move out of the ring, through rotation and ring closure on the NH_2 group. The key observation here, however,



(3)

is that there is no interconversion of the cis:trans isomers in acid solution. Thus, when formed from the nitroimidazole in such solutions, the products *must* have the substituent on the ring where it started. Since there is no change other than in their proportion, they must be ring substituted at higher pH as well.

Phosphate Esters. The products **6** are observed in the reductions and hydroxylamine decomposition when phosphate is present but are otherwise absent. This suggests that they are arising from the buffer, and indeed as the phosphate concentration is increased, the signals for **6** increase at the expense of those for **4**, with **6** becoming the major product in, for example, 1 M phosphate (Figure 4). The patterns of signals at 5.0–5.6 ppm due to the new products are now much more evident than in reductions where they are formed in lesser quantity. Coupled with the dependency on phosphate, those spectra suggest that **6** are monophosphate esters.

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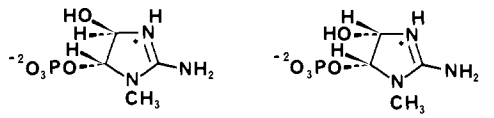
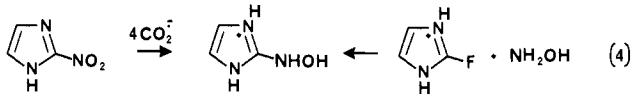


t6 c6

In the trans isomer (CH_3 at 3.07 ppm) the H4–H5 coupling is not observed, and H4 is a single peak (5.30 ppm) with H5 a doublet (5.315 ppm) because of the coupling to phosphorus (J = 7.2 Hz). The cis isomer (CH_3 at 2.99 ppm) does show H4–H5 coupling (J = 5.4 Hz), and H4 is a doublet (5.39 ppm) with H5 a doublet of doublets (5.51 ppm) because of the additional phosphorus coupling (J = 7.6 Hz). Further indication of these structures is that unlike for **4** the NMR peak positions are somewhat pH dependent. Those with the phosphorus couplings in particular move 0.05 ppm downfield at pD 3. Protonation of the phosphate would be responsible for this.

The NMR is, of course, also consistent with a 4-phosphate ester, although bis-phosphates and a cyclic phosphate can be ruled out. Our preference for the 5-ester is based upon a proposed mechanism (see Discussion). One other important observation can be noted, namely, that the phosphate esters do *not* form when **4** is dissolved in phosphate buffers.

Azomycin. Reduction of the parent 2-nitroimidazole was examined with the radiation technique. At pH 4 of the stoichiometry is 4 electrons per RNO_2, and a single peak is observed at 6.99 ppm in the NMR spectrum. A superimposable singlet is produced in the reaction at pH 3–4 of 2-fluoroimidazole and hydroxylamine. We propose that this corresponds to the unsubstituted 2-(hydroxylamino)imidazole **2c**.



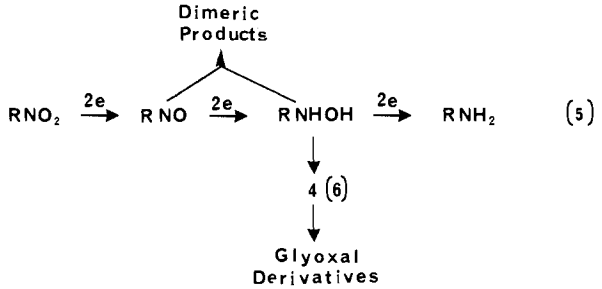
(4)

As with its 1-substituted analogues, **2c** is not stable, decomposing slowly (days) in acid and much more rapidly (minutes) at pH 7. The products are the cis:trans isomers of **4c**, which appear as singlets at 5.44 and 5.22 ppm, respectively, in the NMR. Interestingly, in the phosphate-buffered pH 7 reaction, there is no indication of other products, in particular phosphate esters.

The acid reduction behavior is thus no different from that of the 1-substituted RNO_2. However, at pH 6.6, the stoichiometry is closer to 6 electrons per RNO_2, with the major product (70%) being 2-aminoimidazole. This gives a singlet at 6.79 ppm, and, in contrast to the hydroxylamine, is stable. The production of amine by pH 7 radiation reduction of azomycin has been noted.[10] What was not reported in the previous study is that there are minor products, the two isomers of **4c**, which are observed in about a 30% yield.

Discussion

A scheme summarizing the anaerobic reduction of 2-nitroimidazoles is presented below. In general 4 electron equivalents



(5)

go into the system forming the hydroxylamine. This can be observed after radiation or electrochemical reduction at acid pH, although a slow reaction[35] forming **4** is occurring. This reaction

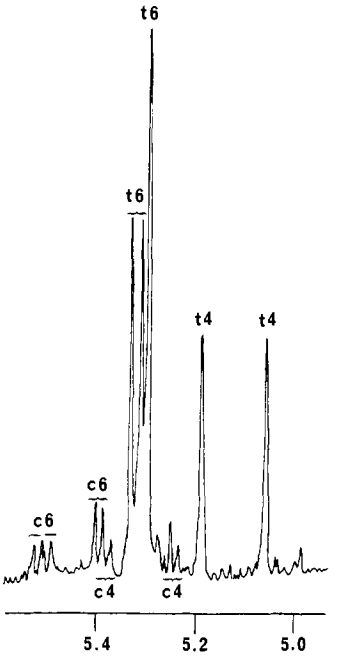


Figure 4. 360-MHz ^1H NMR spectrum in D_2O after electrochemical reduction of 1-methyl-2-nitroimidazole in 1 M phosphate buffer, pH 7–8.

is more rapid[35] at neutral pH, and the hydroxylamine is usually[36] not observed. Further reduction to the amine competes with the decomposition at neutral pH,[37] being observed to a small extent in the zinc reduction and to a larger extent with azomycin. This further reduction presumably also occurs in biological systems since the amine of misonidazole is observed as a metabolite.[7] The hydroxylamine can also couple with the nitroso intermediate, giving rise to an azoxy compound and other dimeric products. Indications are that this reaction is more important at basic pH[32] or in more concentrated RNO_2 solutions.[16] The hydroxylamine can also react with phosphate and other nucleophiles. An example of the latter is the glutathione adduct observed with misonidazole.[38] The hydroxylamine can also react with oxygen, forming other products.[34,39]

Equation 5 accounts for greater than 90% of the nitro compound reduced by the three techniques employed here. The overall simplicity contrasts significantly with the current picture for reduction of 5-nitroimidazoles. Although conditions for reduction to the amine have been established,[40] the latter are generally characterized by extensive fragmentation of the imidazole ring, with a number of products in quite low yield being found.[41] In

(35) A rate–pH profile for this reaction with **2b** has been published;[21] the behavior of **2a** is similar (unpublished observations). These profiles show a slow pH-independent reaction at pH 1–3.5, with half-lives at 25 °C of 7 h for **2a** and 13 h for **2b**. The rate increases from pH 4 to pH 7 and then levels again by pH 8. Half-lives at pH 7 are 5 min for **2a** and 2 min for **2b**.

(36) Unless the reduction and workup are fast, as can be the case in the zinc reduction.[34]

(37) We are uncertain why the radiation and electrochemical reductions stop at the hydroxylamine stage in acid. Experiments have been carried out with the former technique where irradiations were continued beyond the 4-electron stage and these simply result in extensive decomposition of the system and no amine. The amine, however, is produced quantitatively by zinc/hydrochloric acid[16] and also by hydrogenation, not only of the parent nitro compound[7] but also of the hydroxylamine.[11]

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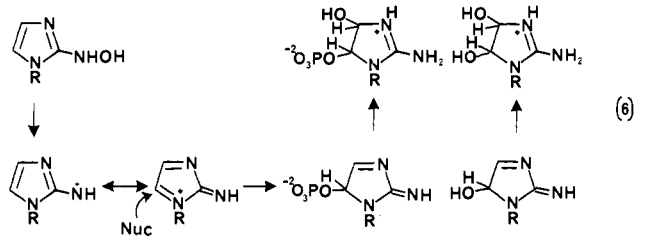
terms of the glyoxal derivatives, fragmentation can occur with 2-nitroimidazoles. However, the initially formed products are ring intact.

The observations that the nitroreduction products are also formed starting with the hydroxylamine imply that the latter is formed as an intermediate. A mechanism for its decomposition to the stable final products is summarized below. This mechanism



(6)

is directly analogous to that of the Bamberger rearrangement whereby phenylhydroxylamines are converted to *p*-hydroxyanilines and other ring-substituted anilines.[42] A major difference with the imidazoles is the formation of ring-saturated products, although analogous species have now been observed with phenyl derivatives.[43] The hydroxylamine owes its stability in acid to the fact that it is protonated (on the ring). The neutral hydroxylamine is the reactive species, and in contrast to phenylhydroxylamines, cation formation is rapid because of the resonance stabilization.[44]

The formation of products able to release glyoxal may be important biologically, since this reactive dialdehyde is well established to interact with cellular components.[45] Varghese and Whitmore have shown that the urine of patients receiving misonidazole will form the glyoxal derivative,[46] although whether or not the adducts **4** are responsible is unknown. The observation does imply, however, that whatever the structure of the glyoxal precursor in vivo the substance can diffuse from the cell of its production. There has been some evidence in animals for a diffusable product formed in hypoxic cells which is toxic to surrounding cells.[47] The observation of a phosphate-derived product[48] may also have some biological significance. [2-^{14}C]-Misonidazole binds DNA to a small extent upon reduction, the binding being detected by the radiolabel.[5] Interaction with glyoxal obviously cannot explain this, since C4 and C5 are unlabeled. Formation of a product such as **6** with the DNA phosphate, however, would.

Experimental Section

General Methods. High-field NMR spectra were recorded on a Nicolet 360-MHz spectrometer at the Toronto Biomedical NMR Centre, University of Toronto, with the assistance of Dr. Arthur Gray and Alan Lee. Chemical shifts are based on an acetone reference assigned a value of 2.225 ppm. UV spectra were recorded on Cary 219 or 2390 spectrophotometers. Electrochemical reductions were conducted with an Eco Instruments Model 550 potentiostat equipped with a Model 721 integrator. Radiation chemical reductions were carried out by using a ^{60}Co γ cell (Atomic Energy of Canada Ltd.), with a dose rate of 5.35 ± 0.15 krad/min. Analyses were provided by Microanalysis Laboratories, Markham, Ontario.

Materials. Imidazoles. Misonidazole was provided by Roche Products Ltd. Literature procedures were used to prepare the following: 1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol;[8] 2-aminoimidazole,[49] from this 2-nitroimidazole;[50] 2-amino-1-methylimidazole,[49] from this 1-methyl-2-nitroimidazole;[50] and 2-fluoroimidazole, from 2-aminoimidazole.[51]

Glyoxal. The 40% aqueous solution (Aldrich) was used directly, or aqueous solutions were prepared by heating to 110 °C the solid glyoxal trimer dihydrate (Sigma) with P_2O_5, with a stream of dry nitrogen to carry the monomeric glyoxal so released to an H_2O or D_2O trap.[52] The concentration of glyoxal in aqueous solutions was determined by addition of hydrogen peroxide to a basic solution, followed by titration with HCl.[53]

Guanidinium Salts. Guanidinium (**3c**) chloride and methylguanidinium (**3b**) chloride were commercially available. (2-Hydroxyethyl)guanidinium (**3d**), (2-methoxyethyl)guanidinium (**3e**), and (2-hydroxy-3-methoxypropyl)guanidinium (**3a**) ions were obtained as sulfate salts by the reaction in water of 2-methyl-2-thiopseudourea sulfate and, respectively, ethanolamine, (2-methoxyethyl)amine, and (2-hydroxy-3-methoxypropyl)amine, following a general procedure for the preparation of guanidinium ions.[49] The last amine was obtained by addition of aqueous ammonia to 1,2-epoxy-3-methoxypropane.[50] This in turn was prepared from epichlorhydrin and methanol.[51]

1-Substituted 2-Amino-4,5-dihydro-4,5-dihydroxyimidazolium Salts (4). The preparation of **4a** illustrates the general procedure. (2-Hydroxy-3-methoxypropyl)guanidinium sulfate (0.02 mol) and aqueous glyoxal (0.021 mol) were mixed in a total volume of 500 mL of water, and the pH was adjusted to 8. After 5 days the water was removed (rotary evaporator followed by lyophilization). The residual oil was taken up in anhydrous methanol and the salt precipitated as a solid by the addition of anhydrous diethyl ether. These salts, particularly those bearing hydroxy groups, were extremely hygroscopic and were kept in a desiccator over P_2O_5. Their NMR spectral data are summarized in Table I. Analytical data establish the 1:1 stoichiometry.

4a Sulfate. Calcd for $(C_7H_{16}N_3O_4)_2{\cdot}SO_4$: C, 33.07; H, 6.30; N, 16.54; S, 6.30. Found: C, 32.81; H, 6.31; N, 16.15; S, 6.54.

4b Chloride. Calcd for $C_4H_{10}N_3O_2Cl$: C, 28.67; H, 5.97; N, 25.08; Cl, 21.17. Found: C, 28.42; H, 6.12; N, 25.62; Cl, 20.37.

4c Chloride. Calcd for $C_3H_8N_3O_2Cl$: C, 23.46; H, 5.21. Found: C, 23.28; H, 5.16.

4d Sulfate. Calcd for $(C_5H_{12}N_3O_3)_2{\cdot}SO_4$: C, 28.57; H, 5.24. Found: C, 28.66; H, 5.31.

4e Sulfate. Calcd for $(C_6H_{14}N_3O_3)_2{\cdot}SO_4$: C, 32.14; H, 5.80. Found: C, 32.30; H, 5.90.

Reactions of 4b. With the Guanidinium Ion. 4b chloride (0.01 M) and guanidinium chloride (0.01–0.50 M) were dissolved in D_2O, and the pH was adjusted to 8. NMR spectra (360 MHz) were recorded after 1 week at ambient temperature (and were unchanged thereafter). Reaction was characterized by the decrease in the signals due to **4b**, with the appearance of signals for **4c** at 5.22 and 5.44 ppm and for the methylguanidinium ion at 2.81 ppm. The extent of reaction was determined from the integration of the *N*-methyl signals.

With 2-Aminoethanethiol. The thiol (0.5 M) and **4b** chloride (0.2 M) were mixed in H_2O and D_2O, and the pH was adjusted to 8–9. The disappearance of **4b** and formation of the methylguanidinium ion could be observed in the NMR in D_2O. A solid precipitates during this reaction. An identical solid is obtained from glyoxal and the thiol at this pH: ^{1}H NMR ($CDCl_3$) δ 4.864 (1 H, s), 3.63 (1 H, ddd, J = 12.3, 5.8, 3.4 Hz), 3.07 (1 H, ddd, J = 12.3, 8.2, 6.1 Hz), 2.97 (1 H, ddd, J = 9.9, 6.1, 3.4 Hz), 2.87 (1 H, ddd, J = 9.9, 8.2, 5.8 Hz), 2.20 (1 H, br s). Anal. Calcd for $C_6H_{12}N_2S_2$: C, 40.87; H, 6.86; N, 15.89; S, 36.37. Found: C, 40.80; H, 6.81; N, 15.92; S, 36.42.

With Hydroxylamine (NMR Analysis). A solution of **4b** (0.1 M) and hydroxylamine hydrochloride (0.25 M) was prepared in D_2O and the pH adjusted and held in the range 8–9. The NMR spectrum after 3 days indicated the complete formation of *N*-methylguanidine (2.81 ppm) and glyoxal bis-oxime (singlet at 7.8 ppm).

Spectroscopic Analysis of Glyoxal Bis-Oxime. A stock solution was prepared containing 0.50 M hydroxylamine hydrochloride, 0.50 M sodium hydroxide, and 0.10 M pH 10.3 carbonate buffer (1:1 $NaHCO_3$:Na_2CO_3). This was mixed with an equal volume of the solution to be tested, and the optical density at 272 nm was recorded immediately and after 90 min standing at ambient temperature. Standardizations

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were carried out with 20-100 μM (final concentration) solutions of authentic bis-oxime, glyoxal, and **4b**. Nitroreduction mixtures were added such that the concentration of initial RNO_2 would have corresponded to 50-100 μM. The yield of glyoxal bis-oxime was calculated as 100% (OD(272)/1.72 × 10^4[RNO_2]) where OD(272) was the 90-min reading.

Radiation Chemical Reduction.[8,11] Solutions at pH 7 were prepared with 20 mM phosphate and 100 mM sodium formate. Solutions at pH 4 were prepared with 100 mM sodium formate and the appropriate quantity of HCl. For experiments in D_2O, 99.96% D_2O (Aldrich), sodium deuterioformate (Merck), and either DCl/D_2O or deuteriated phosphate were employed. The latter was obtained by lyophilization (twice) of D_2O/phosphate solutions. Nitroimidazole concentrations were 0.5-1.0 mM. Solutions were degassed by saturation with N_2O and were then placed in sealed vials for irradiations in the γ cell. The reduction stoichiometry was determined as previously described,[8] by following the disappearance of the nitroimidazole UV peak at 326 nm as a function of irradiation time. NMR spectra were obtained on irradiated solutions in D_2O, generally within 1 h of completion. Control experiments established that there is no difference in the spectra obtained at half and full reductions, other than the peaks for unreacted RNO_2. The spectra of pH 7 reduced solutions are unchanged after 4 days.

Electrochemical Reduction. These were carried out in a standard H cell with an agar plug separator. The anode was a 5 cm^2 sheet of platinum foil; this compartment contained 0.5 M NaCl. A mercury pool cathode (12 cm^2 surface area) was used; this was set at a constant potential of -800 mV with reference to a Calomel electrode. Both compartments were continually bubbled with nitrogen during electrolysis. A phosphate buffer was used to maintain a pH in the cathode compartment near neutrality (pH 6.5-7.5), while an acetic acid-acetate (20:1) buffer was used for pH 3.5-4.0. In both cases the pH was continually monitored during the electrolysis and 1 M HCl added where necessary. Current flow depended upon RNO_2 concentration; for solutions of 50 mM RNO_2 the initial current was 50-100 mA. The current drops to the background level (0.1-0.2 mA) on complete RNO_2 consumption. For determination of the reduction stoichiometry, samples were periodically withdrawn, and after the appropriate dilution the RNO_2 concentration determined spectroscopically at 326 nm. Plots of [RNO_2] vs. the number of coulombs passed were linear. NMR spectra of reduced products were recorded after lyophilization, followed by redissolving in D_2O. For the acetate-buffered reductions the pH was adjusted to 1 by the addition of concentrated HCl before lyophilization.

Zinc Reduction. The nitroimidazole (0.2 mM) and ammonium chloride (2 mM) were dissolved in water, and after 15 min of N_2 bubbling, an excess of zinc dust was added. The N_2 bubbling was continued and the mixture stirred. After 30 min the mixture was filtered. The filtrate was lyophilized and taken up in D_2O for NMR analysis.

Reaction of 2-Fluoroimidazole with Hydroxylamine. 2-Fluoroimidazole, as its HCl salt (2 mmol), and hydroxylamine hydrochloride (5 mmol) were dissolved in 5 mL of H_2O, and the pH was adjusted and maintained in the range 3.5-4.0 over a 6-h period. After lyophilization the NMR spectrum was recorded in D_2O. Approximately 10% unreacted fluoroimidazole remains, with 60-70% of the product being represented by the 6.99 ppm signal assigned to the 2-(hydroxylamino)imidazole. Several peaks at 5.0-5.5 ppm (including those for **4c**) account for the remainder. Attempts to separate the hydroxylamine by recrystallization or ion-exchange chromatography failed to improve the purity.

Acknowledgment. This work was supported by grants from the National Cancer Institute of Canada, Medical Research Council of Canada, and Ontario Cancer Treatment Research Foundation.

Calixarenes. 20. The Interaction of Calixarenes and Amines

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Abstract: The interaction of calixarenes and amines in CH_3CN solution is postulated to involve a two-step process, viz., proton transfer from the calixarene to the amine to form the amine cation and the calixarene anion followed by association of the ions to form a complex. The overall association complex for *p*-allylcalix[4]arene and *tert*-butylamine, as a typical example, is about 10^6, the larger part of this arising from the proton transfer, as measured from UV spectral observations, and the smaller part arising from the association of the ions, as measured from ^1H NMR observations. A 2D NOE spectrum of this complex indicates that the methyl groups of the amine and the allyl groups of the calixarene are proximate, in agreement with an *endo*-calix structure for the complex.

The interaction of calixarenes (**1**) and amines is discussed in an earlier paper in this series[1] wherein it is suggested that a proton transfer from calixarene to amine occurs, followed by association of the calixarene anion with the ammonium cation to form an *endo*-calix complex (**3**), as shown in Figure 1. The present paper describes a more detailed study of this phenomenon.

In the present discussion the proton transfer step (K_1) and the "complexation" step (K_2) are treated as discrete processes, as depicted in eq 1, although it is recognized that these might be merged into a single step. Whereas UV spectral measurements, discussed in a later section of this paper, appear to arise primarily as a result of the first step, NMR measurements assess the net

$$ArOH + R_3N \overset{K_1}{\rightleftharpoons} ArO^- + R_3NH^+ \overset{K_2}{\rightleftharpoons} ArO^-\cdots{}^+HNR_3 \quad (1)$$

result of both steps and will be discussed first. The calixarene of choice for this investigation was *p*-allylcalix[4]arene (**1a**), because it is more soluble than the more easily accessible *p*-*tert*-butylcalix[4]arene (**1b**). The amines of choice for particular attention were *tert*-butylamine and neopentylamine, because the earlier work[1] had demonstrated a small but reproducible difference in their behavior in the presence of *p*-allylcalix[4]arene. Table I presents the proton chemical shift and relaxation rate values of the amine component that are observed in the ^1H NMR spectra of *tert*-butylamine and neopentylamine in CD_3CN solutions containing equimolar amounts of *p*-allylcalix[4]arene at 16 and -30 °C. Tables II and III show similar data for the calixarene component of these complexes.

Inspection of the data in Table I shows that both *p*-allylcalix[4]arene and trifluoroacetic acid exert a downfield shift on the methyl resonances of *tert*-butylamine and methyl and methylene resonances of neopentylamine. At 16 °C the calixarene is about 90% as effective as trifluoroacetic acid in its influence on *tert*-butylamine and about 55% as effective in its influence on neopentylamine, as judged from the relative values of the chemical shifts. At -30 °C, however, these values come closer to one another, diminishing in the case of *tert*-butylamine to 82% and increasing in the case of neopentylamine to 74-85%. These values might be interpreted as a measure of the extent of proton transfer from the phenol to the amine (assuming 100% proton transfer

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