# 4,5-Dihydro-4,5-dihydroxyimidazoles as products of the reduction of 2-nitroimidazoles. HPLC assay and demonstration of equilibrium transfer of glyoxal to guanine

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An HPLC method has been employed to study the electrochemical reduction (mercury cathode at −800 mV with respect to calomel electrode) of the 2-nitroimidazole benznidazole (*N*-benzyl-(2′-nitro-1′-imidazoyl)acetamide). The principal product of this reduction is the cyclic guanidinium ion **3***c* (protonated *N*-benzyl-(2′-amino-4′,5′-dihydro-4′,5′-dihydroxy-1′-imidazoyl)acetamide), which forms in a linear fashion as the nitroimidazole is reduced and accounts for 75% of the product upon completion of the reduction. To perform the HPLC analysis quantitatively an authentic sample of this product (isolated as *cis*:*trans* isomers) was prepared as the sulfate salt through the reaction of *N*-benzyl-2-guanidinoacetamide sulfate with aqueous glyoxal. The two isomers of **3***c* arise through the nonreductive decomposition of the 2-hydroxylaminoimidazole, which is the product of a four-electron reduction of the nitroimidazole. Analysis of high field $^1$H NMR spectra also showed that the two isomers of **3***c* were the principal products following electrochemical reduction, neutral aqueous zinc reduction, and radiation chemical reduction. Previous investigations using NMR of the reductions of misonidazole (3-methoxy-1-(2′-nitro-1′-imidazoyl)-2-propanol) and 1-methyl-2-nitroimidazole have shown that the corresponding dihydroimidazoles **3***a* and **3***b* are the major products. The agreement of these various NMR and HPLC results suggests that the formation of dihydroimidazoles **3** is a general phenomenon for model reductions of 2-nitroimidazoles in neutral aqueous solution. Previous workers have shown that 2-nitroimidazole reduction mixtures, when treated with guanine derivatives, form the adduct **4** derived from the guanine and glyoxal. This work demonstrates that this adduct is also formed when authentic samples of **3***a*, **3***b*, and **3***c* are reacted with 2′-deoxyguanosine. A quantitative HPLC analysis, however, demonstrates that the reaction does not proceed to completion, and in fact the equilibrium for formation of **4** is unfavorable. This suggests that guanines are not useful derivatizing agents for the quantitative assay of "glyoxal-like" products formed in chemical or biological reductions of 2-nitroimidazoles.

*Key words*: nitroimidazole, reduction of nitroimidazoles, glyoxal from nitroimidazoles.

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On a utilisé une méthode de CLHP pour étudier la réduction électrochimique (cathode de mercure à −800 mV par rapport à l'électrode de calomel) de la 2-nitroimidazole benznidazole (*N*-benzyl-(2′-nitro-1′-imidazoyl)acétamide). Le produit principal de cette réduction est l'ion guanidinium cyclique **3***c* (*N-benzyl-(2′*-amino-4′,5′-dihydro-4′,5′-dihydroxy-1′-imidazoyl)acétamide protoné) qui se forme d'une façon linéaire au fur et à mesure que le nitroimidazole est réduit et qui forme 75% du produit à la fin de la réduction. Afin de réaliser l'analyse quantitative par CLHP, on a préparé un échantillon authentique de ce produit (isolé sous la forme de sulfates des isomères *cis/trans*) par réaction du sulfate de *N*-benzyl-2-guanidinoacétamine avec du glyoxal en solution aqueuse. Les deux isomères du produit **3***c* proviennent de la décomposition non-réductive de la 2-hydroxyaminoimidazole, le produit d'une réduction de la nitroimidazole par quatre électrons. Une analyse des spectres RMN du $^1$H à haut champ a aussi démontré que les deux isomères du produit **3***c* sont les produits principaux de la réduction électrochimique, de la réduction par le zinc en milieu aqueux neutre et de la réduction chimique par des radiations. Des études antérieures utilisant la RMN ont aussi démontré que les réductions du misonidazole (3-méthoxy-1-(2′-nitro-1′-imidazoyl)-2-propanol) et du 1-méthyl-2-nitroimidazole conduisent principalement aux dihydroimidazoles **3***a* et **3***b* correspondants. La bonne correspondance entre les résultats de la RMN et de la CLHP suggère que la formation de dihydroimidazoles **3** est un phénomène général pour les réductions modèles de 2-nitroimidazoles, en solutions aqueuses neutres. D'autres chercheurs ont démontré antérieurement que les mélanges obtenus lors des réductions de nitroimidazoles réagissent avec des dérivés de la guanine pour former l'adduit **4** dérivé de la guanine et du glyoxal. Dans le présent travail, on démontre que ce genre de dérivé se forme aussi lorsqu'on fait réagit des échantillons authentiques des produits **3***a*, **3***b* et **3***c* avec de la 2′-désoxyguanosine. Une analyse quantitative par CLHP a toutefois permis de démontrer que cette réaction est incomplète et que, de fait, l'équilibre pour la formation de l'adduit **4** n'est pas favorable. Ce résultat suggère que les guanines ne sont pas des agents utiles pour former des dérivés permettant d'analyser quantitativement les produits «ressemblant au glyoxal» qui se forment lors des réductions chimiques ou biologiques des 2-nitroimidazoles.

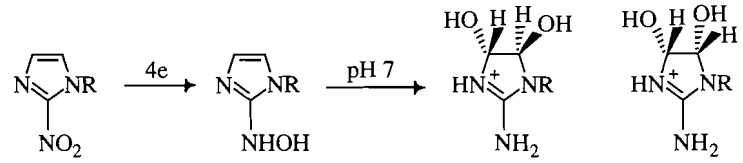
*Mots clés* : nitroimidazole, réduction des imidazoles, glyoxal dérivé des imidazoles.

[Traduit par la revue]

2-Nitroimidazoles **1** are currently undergoing clinical trials as radiosensitizers of hypoxic (oxygen-deficient) cells (1, 2), and may also have clinical application in chemotherapy since they show selective cytotoxicity towards hypoxic cells (1). A number of the important biological effects of these drugs are associated with reductive metabolism (3), and this has led to an interest in the reduction chemistry. A variety of reduction methods have been employed to investigate this chemistry, including radiation chemical reduction (4–7), electrochemical reduction (6, 8–10), zinc/ammonium chloride reduction (11, 12), and biochemical reduction (13–18). In general, these experiments have shown a stoichiometry of four electron equivalents per nitroimidazole reduced, for aqueous solutions at pH 7. The product that corresponds to such a stoichiometry is the 2-hydroxylaminoimidazole **2**. Work in our laboratory, however, has shown that such compounds are unstable at neutral pH (6, 10, 19, 20), with half-lives ranging from 2–15 min (21, 22). The major products of their further reaction have been identified as the *cis* and *trans* 2-amino-4,5-dihydro-4,5-dihydroxyimidazolium ions **3** (10, 20, 21).

Several other groups have carried out product analyses by adding to previously reduced solutions reagents that form deriva-

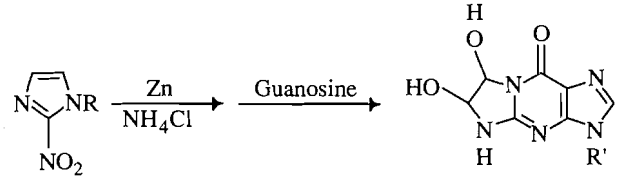
[1]Also, R. A. McClelland and R. Fuller. Unpublished results.

[1] 

1 2 *trans*-3 *cis*-3

*a* R = $CH_2CHOHCH_2OCH_3$ (Misonidazole)
*b* R = $CH_3$
*c* R = $CH_2CONHCH_2C_6H_5$ (Benznidazole)

tives of aldehydes and ketones. Such experiments have been found to result in the appropriate derivatives of the two-carbon dialdehyde glyoxal (7, 12, 17, 23–26). Of particular note in a biological context is the identification of **4**, the known adduct of guanosine and glyoxal (27, 28), by addition of guanosine to a solution obtained by the reduction of various 2-nitroimidazoles with zinc in aqueous ammonium chloride (12, 23). This same derivative has been observed following radiation chemical reduction (7, 26). This adduct, moreover, has been detected upon addition of guanosine to hypoxic cells treated with misonidazole (**1***a*) (29) and by addition of guanosine to the urine of a patient receiving misonidazole (30).

[2] 

**4**

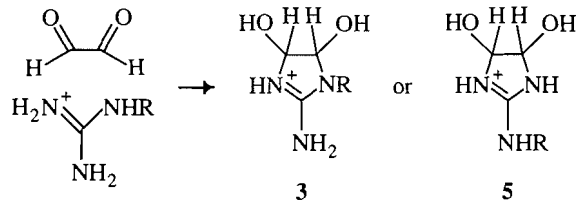
In general, yields of these glyoxal derivatives relative to the nitroimidazole reduced have been low, with reported numbers ranging from a fraction of a percent (7) to 25% (17). Moreover, there has been some discussion (17, 23) as to whether the reduction mixture actually contains free glyoxal or simply some species capable of reacting with the added reagent to form the glyoxal derivative. Our analyses have supported the latter viewpoint, the dihydroimidazoles **3** being able to react to give glyoxal derivatives (10). This species **3** is in fact the adduct of glyoxal and a monosubstituted guanidinium ion, with the equilibrium in aqueous solution lying well to the adduct side (10). We have moreover found that yields of **3** are high, the two isomers accounting for 80–90% of the initial nitroimidazole when reductions are carried out in the absence of other strong nucleophiles (10, 20). This result obviously contrasts with the situation for the glyoxal derivatives; we have argued that the derivatization procedures do not quantitatively produce the glyoxal adduct when **3** is the starting material.

That the two isomers of **3** constitute the major products of the reduction of 2-nitroimidazoles has come mainly from analyses of high field $^1H$ NMR spectra, following radiation chemical reduction, electrochemical reduction, and zinc reduction, principally of misonidazole and the model 1-methyl-2-nitroimidazole **1***b* (10). In this paper we report verification of this conclusion with an HPLC analysis conducted using the nitroimidazole benznidazole (**1***c*). This nitroimidazole is used extensively in South America for the treatment of the *Trypanosome cruzi* infection (Chaga's disease) (31), and has also been shown to function as a chemosensitizer of the antitumor agent chloroethylnitrosourea (32, 33). From the point of view of HPLC analysis, it has the advantage (18) of having a phenyl chromophore in its side chain, making the UV spectroscopic detection of **3***c* possible. The derivatives **3***a* and **3***b* are transparent down to 210 nm. We also report here experiments showing that pure samples of **3** can transfer glyoxal to guanine, but the equilibrium is unfavorable. We also provide unambiguous evidence that the structure of the **3** is the 1-substituted derivative, as written in eq. [1].

## Results

*Carbon-13 NMR analysis of* trans *3*b

The assignment of the structure of the products of the reduction as the isomers **3** was based upon a synthesis of analytically pure samples of these species by combining aqueous glyoxal and the appropriate monosubstituted guanidinium ion (10). The $^1H$ NMR spectra of these products were then shown to match the principal peaks in the spectra of the reduction mixtures. As we have discussed previously, some ambiguity exists as to whether the products are actually the 1-substituted **3** or the adducts **5** where the substituent is on the external nitrogen. In the case of the nitroimidazole reduction mixtures where the substituent starts on N-1, a situation where **5** arises via a ring opening – ring closing sequence could not be rule out (10).

[3] 

**3** **5**

We have now obtained unambiguous evidence for the structure **3** based upon an analysis of $^{13}C$ NMR spectra obtained with *trans* **3***b*. This species was obtained in pure *trans* form through the combination of glyoxal and methylguanidinium hydrochloride. This resulted in a 1:4 *cis*:*trans* ratio; recrystallization from methanol:ether gave pure (>98%) *trans*, as seen from the absence of the characteristic signal for the *cis* isomer in the $^1H$ NMR spectrum. The decoupled $^{13}C$ NMR spectrum of *trans* **3***b* (recorded in dilute DCl to suppress *trans*:*cis* equilibration (10)) had the expected four signals, at 161.0, 94.0, 86.8, and 31.8 ppm. The lowest field and highest field signals can be assigned to C2 and the methyl carbon respectively. The other two signals represent C4 and C5 but cannot be assigned from the decoupled spectrum. However, in a proton coupled spectrum (Fig. 1) these signals exhibited a different pattern. The signal at 86.8 ppm in the uncoupled spectrum appears as a doublet of doublets. This can be assigned to C4, with a large one-bond coupling to its directly attached hydrogen H4 ($^1J$(C4–H4) = 170 Hz) and a small two-bond coupling to H5 ($^2J$(C4–H5) = 2.4 Hz). The other signal at 94.0 ppm in the uncoupled spectrum, assigned to C5, is expected to have similar $^1J$ and $^2J$ couplings with H5 and H4 respectively. This signal, however, shows additional coupling, which must arise

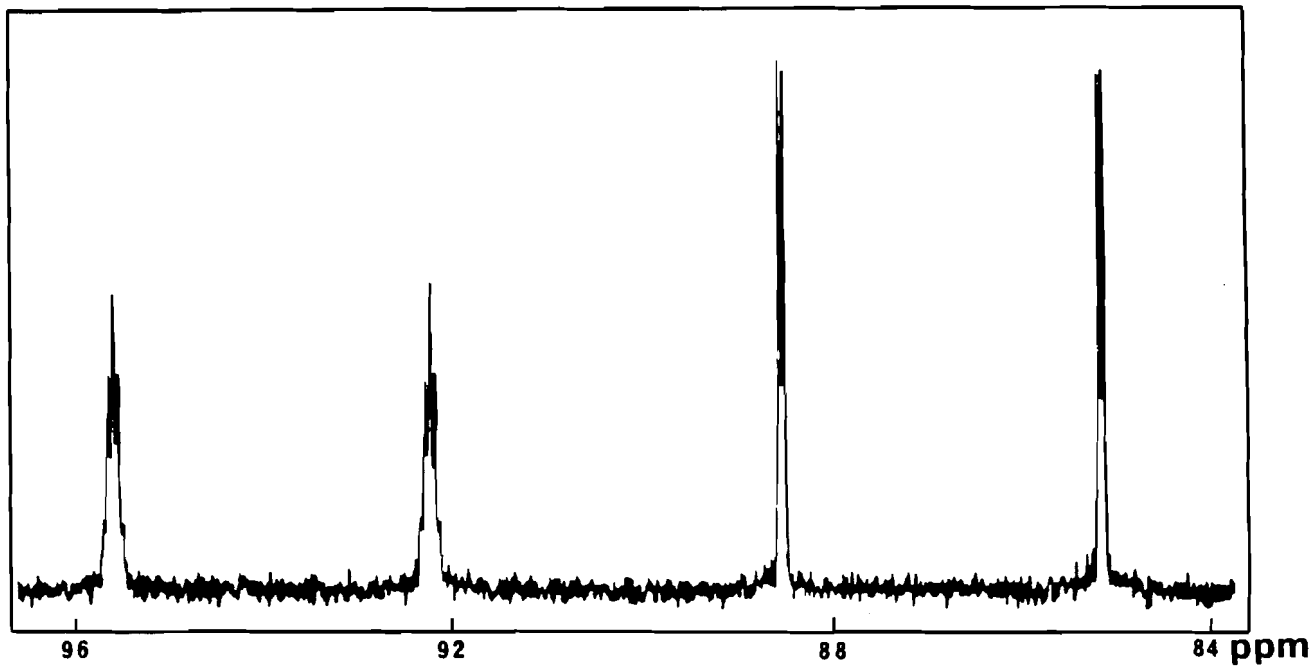


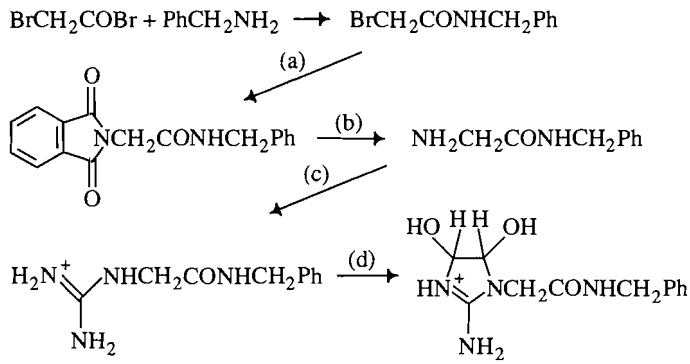

FIG. 1. Proton-coupled 50.4 MHz $^{13}C$ NMR spectrum (85–95 ppm region) of *trans*-**3***b* in dilute DCl.

from the protons of the methyl group. This requires the methyl group to be attached to the ring nitrogen, three bonds away, rather than to the external nitrogen, which is five bonds away, too far to show significant coupling. A five-line pattern actually results for C5 since $^2J$(C5–H4) and $^3J$(C5–$CH_3$) are similar.

*Reduction of benznidazole. NMR analysis*

Figure 2 shows the $^1H$ NMR spectrum obtained upon the crude mixture following radiation chemical reduction (4, 6) of benznidazole in phosphate-buffered neutral aqueous solution. Very similar spectra were also obtained following electrochemical reduction and reduction with zinc in aqueous ammonium chloride. The radiation chemical and electrochemical reductions also showed the expected stoichiometries of $4.0 \pm 0.2$ electron equivalents per nitroimidazole. The products that are consistent with the NMR spectrum and the stoichiometry are the *cis* and *trans* isomers of **3***c*, arising from the further reaction of the 2-hydroxylaminoimidazole, as in eq. [1]. A detailed discussion of the NMR pattern has been presented previously in the context of the methyl derivative **3***b*. In the present case the two pairs of doublets at 5.0–5.5 ppm (see insert) represent H4 and H5 of the two isomers of **3***c*, the major isomer (80%) with the smaller coupling constant being assigned the *trans* geometry. The set of signals at 4.15–4.3 ppm can be assigned to the $NCH_2CO$ group of the side chain, with the two diastereotopic protons of this methylene group being nonequivalent. Again two sets of signals for the two isomers can be recognized (see insert). The remaining signals at 7.4 ppm and 4.42 ppm arise from the phenyl groups and the benzylic methylenes respectively, the latter appearing as a single peak in each isomer with, in fact, almost the same chemical shifts for the two isomers.

Verification of the structure of **3***c* was obtained by the alternate preparation from the reaction of aqueous glyoxal and the appropriate guanidinium ion. The latter was prepared as the sulfate salt, described as follows.




(a) Potassium phthalimide. (b) Hydrazine.
(c) 2-Methyl-2-thiopseudourea sulfate. (d) Aqueous glyoxal, pH 8

This sequence of reactions resulted in an analytically pure sample of the two isomers of **3***c*, whose NMR spectrum in $D_2O$ matched that obtained from the various reductions of the nitroimidazole.

*Reduction of benznidazole. HPLC analysis*

The previous set of experiments, involving the unambiguous synthesis of the dihydroguanidinium ion and the demonstration of identical NMR patterns with those of reduction mixtures, are the same as those carried out earlier in characterizing the products of misonidazole and 1-methyl-2-nitroimidazole (10). As in those experiments, the two isomers of **3***c* account for virtually all the peaks in the NMR spectra of the benznidazole reduced by the three techniques. Even recognizing that the NMR limits of detection may be as high as 10–20%, particularly for complex systems, the conclusion must be that **3***c* is the major product of these reductions of benznidazole.

As noted above, the phenyl group in the side chain provides a handle for HPLC experiments with spectroscopic detection. This group also has the feature of imparting some hydrophobic

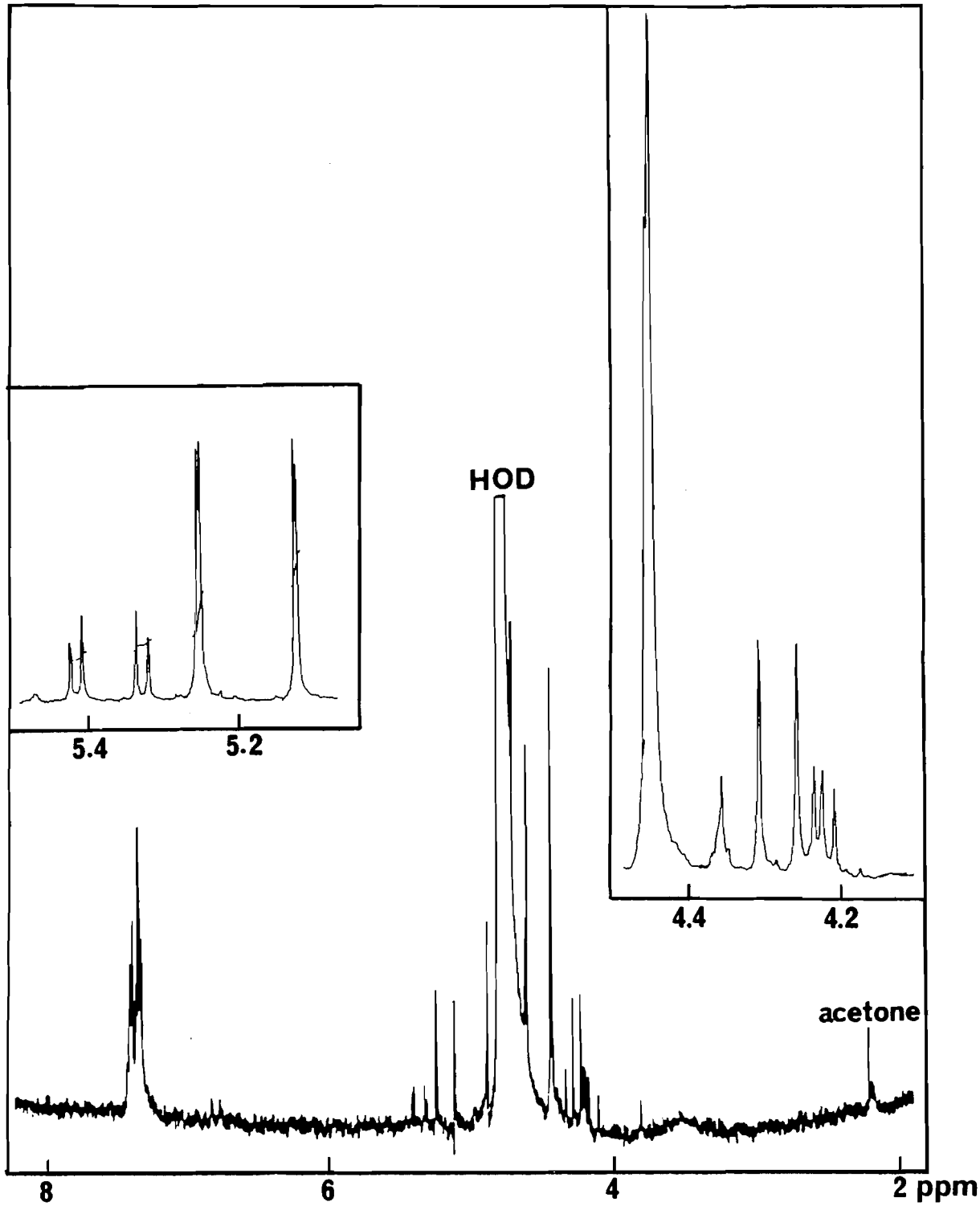



FIG. 2. The 400 MHz $^1$H NMR spectrum of benznidazole after radiation chemical reduction in phosphate buffered $D_2O$ at pD 7. This spectrum was obtained directly on the irradiated $D_2O$ solution.

character so that the highly polar product ions do not migrate with the solvent front in reversed-phase HPLC. We employed HPLC conditions similar to those previously used to analyze benznidazole and its amine derivative (18), and found that authentic samples of the nitroimidazole and **3***c*, the latter prepared as described above, eluted with retention times of 8.7 min and 4.6 min respectively (the two isomers of **3***c* not separating). Figure 3 shows chromatograms obtained from the analysis of the electrochemical reduction at pH 7 of benznidazole. It is clear that as the nitroimidazole disappears, it is replaced by **3***c*. The situation is shown quantitatively in Figure 4. As expected, the nitroimidazole disappears in a linear fashion with the number of reducing equivalents. In the experiment represented in the figure a total of 9.9 coulombs was required to completely

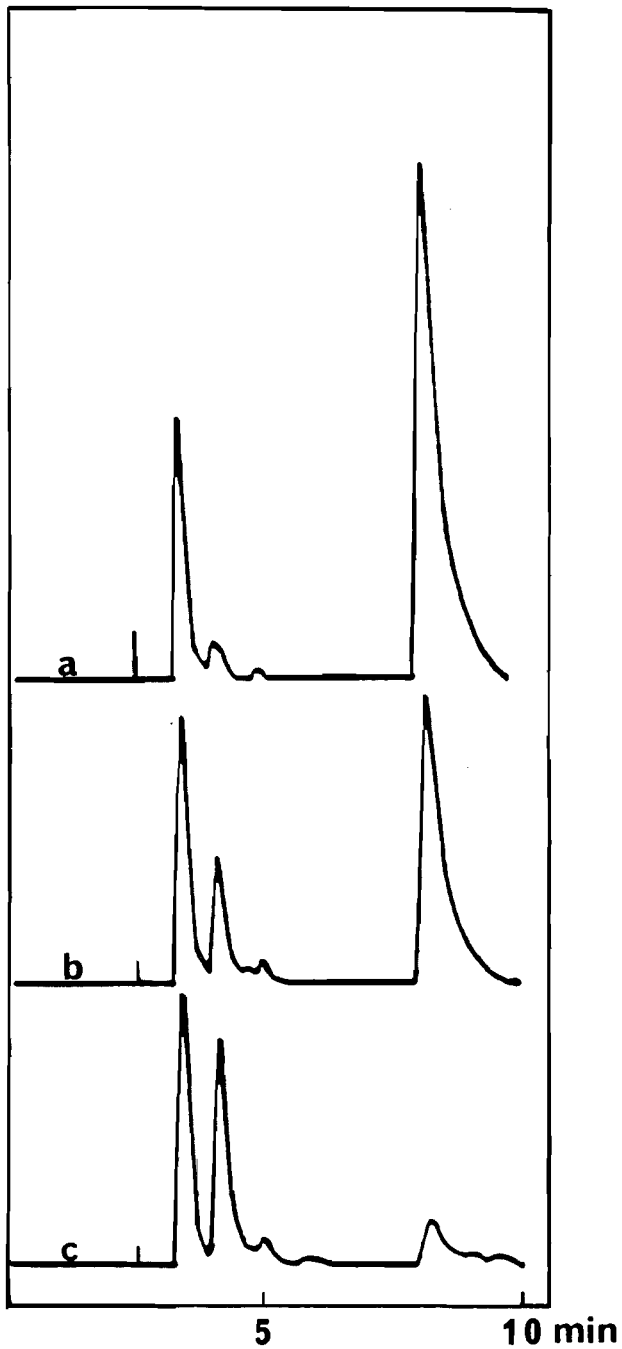



FIG. 3. HPLC chromatograms obtained during the electrochemical reduction at pH 7 of benznidazole; (a) 10% reduced, (b) 50% reduced, and (c) 90% reduced. The peaks at 8.7 min and 4.6 min correspond to benznidazole and **3***c* respectively. The peak at 3.4 min was present in all samples and is believed to be an impurity in the eluting solution. HPLC conditions: detector wavelength = 220 nm, Nova-pak $C_{18}$ column, with eluting buffer of 25% acetonitrile, 0.2 M glycine/hydrochloric acid, pH 2.5, plus 5 mM octanesulfonic acid, flow rate = 3 mL/min.

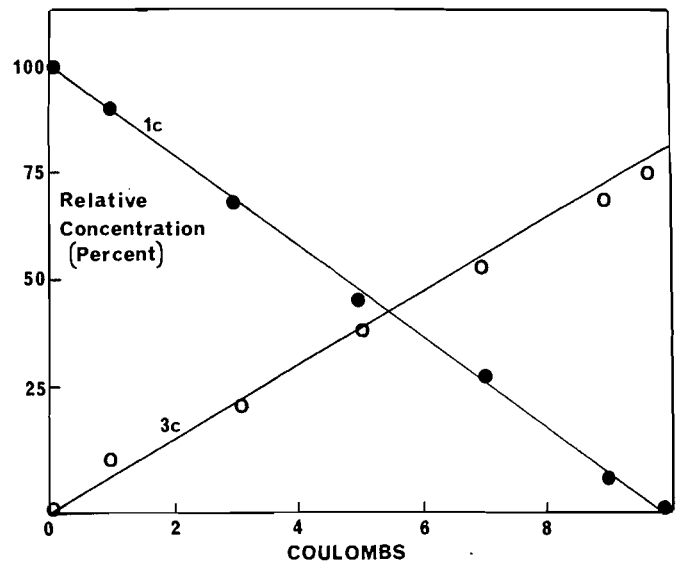



FIG. 4. Relative concentration of benznidazole **1***c* and the product **3***c* as a function of the number of coulombs passed through the solution in the electrochemical reduction of benznidazole. The initial concentration of benznidazole was 1.05 mM in a 25 mL volume (26.3 μmol). The relative concentrations of **1***c* and **3***c* were calculated from relative areas of the HPLC profiles, using standard curves constructed from injections of known amounts of authentic material.

reduce 26.2 μmol. This corresponds to a stoichiometry of 3.92 electrons per nitroimidazole, a result expected from previous studies (4, 6) where UV spectroscopy was employed to analyze for the nitro compound. As **1***c* disappears, **3***c* is formed. This production occurs in a linear fashion with the number of coulombs, indicative of the fact that **3***c* is a primary product of the reduction and that it is also stable under these experimental conditions. Using authentic samples of benznidazole and **3***c*, standard curves relating the HPLC integration units and concentration were constructed. After complete reduction, **3***c* accounts for 75% of the initial starting nitro compound.

Additional products are evident from the HPLC profiles but their identities are unknown. We have shown (10) that when reductions are carried out in phosphate buffers, a monophosphate of **3** is also formed, in yields of 10–20% even in 10 mM phosphate. Such a product is also possible in the present case, since phosphate was necessary to control the pH during the electrolysis. Another likely side product is an azoxy compound formed by coupling of the hydroxylamine product and the nitroso intermediate during reduction. This product is commonly encountered in the reduction of nitroaromatic compounds at neutral pH, and a yellow color observed in the reduced benznidazole solution was consistent with its presence. Whatever the nature of these other products, it is clear that their yields are small and they do not contribute significant signals to the NMR spectra. Moreover, the direct monitoring by HPLC has verified the conclusion of the NMR analysis, namely that **3***c* is the major product.

A product worth commenting upon, which was not present in significant yield with any of the three techniques, was the 2-aminoimidazole derivative, the result of a six-electron reduction. This substance, which was available in pure form as a standard, has in its $^1$H NMR spectrum a characteristic pattern of doublets at 6.7–6.8 ppm associated with H4 and H5 of the imidazole ring. These doublets were observed in the zinc reduction, but accounted for only 5–10% of the overall reduction mixture. With both the radiation chemical reduction and the electrochemical reduction, no signals remained at >6 ppm.

*Reaction of 3 with deoxyguanosine*

These experiments were conducted to demonstrate that the dihydroimidazoles **3** can transfer the elements of glyoxal to guanine, and also to quantitatively evaluate the position of the

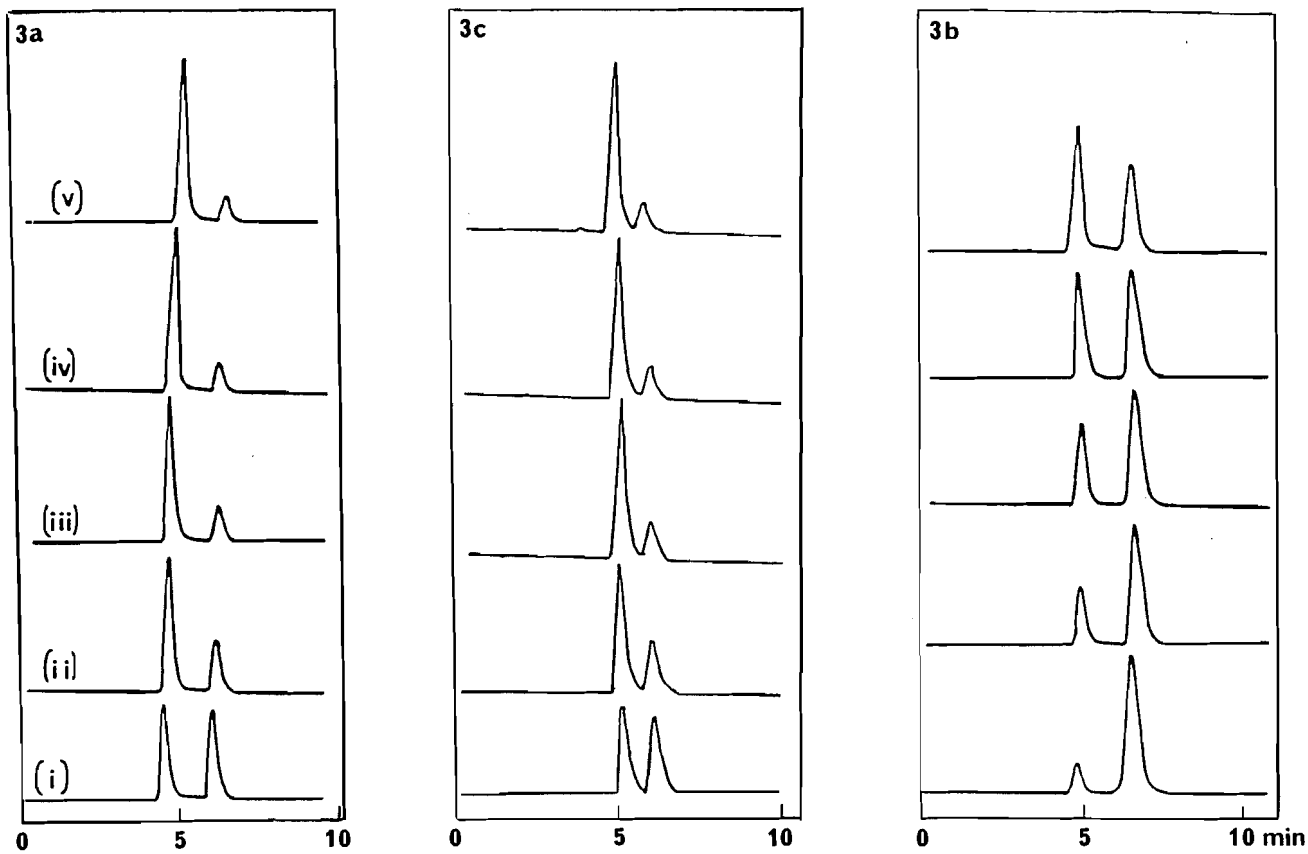
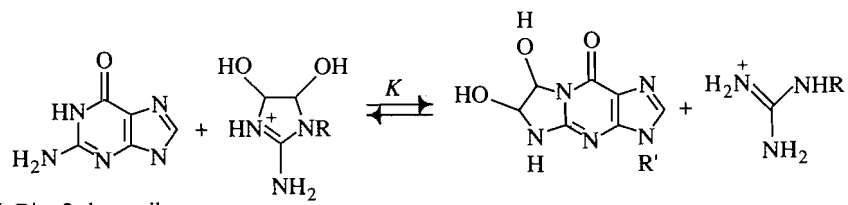



FIG. 5. HPLC chromatograms for the reaction of **3***a–c* with 2′-deoxyguanosine. Reaction conditions: 0.05 M phosphate buffer, pH 7.5, 25°C, 2′-deoxyguanosine = 0.1 mM, **3** = 1.0 mM (i), 2.0 mM (ii), 3.0 mM (iii), 4.0 mM (iv), and 5.0 mM (v). These chromatograms were obtained after 5–7 days and were unchanged on further standing. HPLC conditions: detector wavelength = 258 nm, C18–10μ–Bondapak column, with eluting buffer of 0.25 M ammonium acetate (pH ~ 7); methanol (95:5), flow rate = 3 mL/min.

[4]




**6**, R′ = 2-deoxyribose

**3***a–c*

**4**

**7**

equilibrium. HPLC was used to monitor the course of the reaction, as previously described by Varghese and Whitmore (23). This involved analyzing for the relative concentrations of starting 2′-deoxyguanosine **6** and its adduct **4** by monitoring near the $\lambda_{max}$ of the guanine chromophore. The HPLC elution profiles showed two peaks (Fig. 5), that for the deoxyguanosine coming at 6.0 min with the glyoxal–guanosine adduct at 4.5 min. An authentic sample of the latter was prepared as previously described, from the reaction of glyoxal and 2′-deoxyguanosine (27, 28). Standard curves were constructed for the two samples. Under the HPLC conditions both gave a single peak, indicative that interconversion between the two was not occurring on the column. As shown in Fig. 5, the dihydroimidazoles **3** did transfer glyoxal. Under the reaction conditions, aqueous solution at 25°C and pH 7.5, the reaction was slow, 2–4 days being required to reach an equilibrium condition. Moreover, the reaction did not proceed to completion. Even with a 50-fold excess of **3** some unreacted deoxyguanosine remained. Equilibrium constants ([**4**][**7**]/[**6**][**3**]) were calculated, the equilibrium concentrations of **4** and **6** being determined from the peak areas in the HPLC chromatograms, the concentration of **7** being set equal to that of **6** and the concentration of **3** being calculated by difference. (We have previously demonstrated that the equilibrium involving **3** and glyoxal plus guanidinium ion lies well to the adduct side in aqueous solution (10), so that the reaction can be represented as written in eq. [4].) Values of the equilibrium constants are summarized in Table 1. This table also shows that there is good agreement between measurements with different initial concentrations of **3**.

## Discussion

We have used an HPLC method to demonstrate that the electrochemical reduction of benznidazole under neutral aqueous conditions results in a 75% yield of the corresponding dihydroimidazole **3***c*. This arises from a further nonreductive reaction of the four-electron reduction product, the 2-hydroxylaminoimidazole. The result, which is based on a direct analy-

TABLE 1. Equilibrium constants for the reaction of **3***a–c* with 2′-deoxyguanosine (0.1 mM) in 0.05 M phosphate buffer, pH 7.5 at 25°C

| Substituent R in **3** | [**3**], M | Equilibrium constant |
|---|---|---|
| $CH_3$ | 0.005 | 0.030 |
| | 0.004 | 0.024 |
| | 0.003 | 0.027 |
| | 0.002 | 0.024 |
| | 0.001 | 0.025 |
| $CH_2CONHCH_2Ph$ | 0.005 | 0.090 |
| | 0.004 | 0.083 |
| | 0.003 | 0.093 |
| | 0.002 | 0.089 |
| | 0.001 | 0.090 |
| $CH_2CHOHCH_2OCH_3$ | 0.005 | 0.11 |
| | 0.004 | 0.11 |
| | 0.003 | 0.10 |
| | 0.002 | 0.10 |
| | 0.001 | 0.11 |

sis while reduction is proceeding, verifies previous conclusions reached on the basis of $^1$H NMR spectroscopy that simple model reductions of 2-nitroimidazoles produce high yields of this type of product. 2-Nitroimidazoles are reductively activated to compounds that generate covalent adducts in protein and DNA (34–36) and breaks in DNA (37), and that result in depletion of the intracellular thiol glutathione (38). We have suggested that the unstable hydroxylamine may in part be responsible for these effects, since mechanistically a reactive electrophile capable of binding cellular nucleophiles can be identified for the conversion of the hydroxylamine to products such as **3** (10, 21, 22). In general, in these model reductions the 2-aminoimidazole, which is the product of a six-electron reduction, is not observed or is found only in small quantities. This derivative, however, has been identified in biological systems, in studies both with misonidazole (39, 40) and with benznidazole (17). The origin of this species would appear to be the further reduction of the 2-hydroxylaminoimidazole and what can be proposed is that there is a competition at the hydroxylamine stage between this further reduction and the nonreductive reaction producing products such as **3**. There is evidence in fact for this competition in some of the model systems. In the present study small amounts of the amine were observed in the zinc reduction. Previous workers employing radiation chemical reduction have demonstrated with misonidazole that the amine can be produced with very high radiation doses but not with low doses (4). Moreover, catalytic hydrogenation of 2-nitroimidazoles produces only the amine derivative (41, 42).[1] Our suggestion is that in cells, there are enzymes capable of reducing the hydroxylamine further in competition with the decomposition reaction.

There is evidence that products derived from the hydroxylamine are produced in biological systems, in the findings that the guanosine–glyoxal adduct can be formed in vitro (29) and in vivo (30), and the observation in vitro of a glutathione adduct derived from the hydroxylamine (43). As demonstrated in this study, the dihydroimidazoles can transfer glyoxal to guanine, and we suggest it is these species, not glyoxal itself, that are responsible for the formation of **4** both in the model systems and in the biological systems. Our results, however, show that the reaction is incomplete, the equilibrium in fact being quite unfavorable. On this basis we suggest that guanine (or one of its derivatives) cannot be used to quantitatively analyze for glyoxal in the reduction mixtures of 2-nitroimidazoles. The unfavorable equilibrium also accounts for the low yields of the glyoxal derivative obtained in those cases where this method was employed. It is in fact interesting, considering the position of the equilibrium, that the glyoxal–guanidine adduct was observed in the biological systems. Since the equilibrium for the formation of this derivative is so unfavorable, the implication is that the amount of the precursor must have been considerably higher.

## Experimental

*General methods*

$^1$H NMR and $^{13}$C NMR spectra were recorded with a Varian 400 MHz spectrophotometer at the Department of Chemistry. Electrochemical reductions were carried out with an ECO Instruments model 550 potienstat equipped with a model 721 integrator. HPLC experiments were performed with a Varian LC 5500 system equipped with a Varian 601 data system integrator. Radiation chemical reductions were performed using a $^{60}$Co Gamma cell (Atomic Energy of Canada) located at the Princess Margaret Hospital.

*Materials*

Benznidazole and the amine derivative of benznidazole were kindly provided by Paul Workman. 2′-Deoxyguanosine and aqueous glyoxal were from the Aldrich Chemical Co. and were used directly. The glyoxal-2′-deoxyguanosine adduct **4** was prepared as described in the literature (27, 28), and had an $^1$H NMR spectrum identical to that reported previously. HPLC analysis of this material showed that there was no unreacted 2′-deoxyguanosine. The 4,5-dihydro-4,5-dihydroxyimidazolium salts **3***a* and **3***b* were prepared through the combination of aqueous glyoxal and the appropriate guanidinium ion, as previously described (10). The sample of **3***b*, which was obtained as the >98% *trans* isomer, was isolated in this way by dissolving the *cis*:*trans* mixture in methanol, followed by the slow addition of diethyl ether until a precipitate just formed.

*N*-Benzyl-2-bromoacetamide was prepared from bromoacetyl bromide (Aldrich) and benzylamine (44). *N*-Benzyl-2-phthalylamidoacetamide was prepared from *N*-benzyl-2-bromoacetamide by reacting with potassium phthalimide (45). *N*-Benzyl-2-aminoacetamide was prepared by the reaction of *N*-benzyl-2-phthalylamidoacetamide with hydrazine (46). *N*-Benzyl-2-guanidinoacetamide was obtained as its sulfate salt ($2RNHC(NH_2)_2 \cdot SO_4$) by the reaction of *N*-benzyl-2-aminoacetamide with 2-methyl-2-thiopseudourea sulfate (47). *N*-Benzyl-(2′-amino-4′,5′-dihydro-4′,5′-dihydroxy-1′-imidazoyl)acetamide was prepared as the sulfate salt ($(\mathbf{3}c)_2 \cdot SO_4$) from the reaction of *N*-benzyl-2-guanidinoacetamide sulfate with aqueous glyoxal (10).

*Reduction procedures. NMR analysis*

Details of these procedures have been given previously (10).

*Electrochemical reduction of benznidazole with HPLC analysis*

This reduction was carried out with a standard H-cell with a fritted disc separating the anode and cathode compartments. The anode was a 50 cm$^2$ sheet of platinum foil; this compartment contained 0.05 M NaCl. A mercury cathode (12 cm$^2$ surface area) was used; this was set at a constant potential of −800 mV with reference to a calomel electrode. A phosphate buffer was used to maintain pH on the cathode compartment near neutrality (pH 6.5–7.5). Both compartments were continually bubbled with nitrogen during the electrolysis. In a typical experiment 10 mg of benznidazole was dissolved in 25 mL of phosphate buffer. The reduction was monitored by periodically withdrawing 0.1 mL aliquots of the cathode solution, diluting to 1.0 mL, and determining the relative concentrations of unreduced benznidazole and **3***c* by HPLC. The number of coulombs passed at each point was read directly from the integrator. HPLC analyses were carried out on a Nova-pak $C_{18}$ column (3.9 mm × 15 mm) with a mobile phase con-

sisting of 25% acetonitrile (HPLC grade, Aldrich) in 0.2 M glycine buffer, pH 2.5, containing 5 mM octanesulphonic acid. The column was eluted isocratically at a flow rate of 1 mL/min (column pressure 1000 psi; 1 psi = 6.9 kPa) and the absorbance was monitored at 220 nm.

*Reactions of 3 with 2'-deoxyguanosine*

2'-Deoxyguanosine (0.1 mM) and various concentrations of **3***a–c* were mixed in a phosphate buffer (0.05 M) and the pH adjusted to 7.5. These solutions were stored at 25°C, with aliquots being periodically withdrawn for analysis by HPLC. Generally 5–7 days were required before equilibrium was reached as judged from a constant integration of the two peaks in question. HPLC analyses were carried out using a $C_{18}$-10μ-Bondapak column (3 mm × 15 mm) with the mobile phase consisting of 0.25 M ammonium acetate:methanol (95:5). The column was eluted at a flow of 1 mL/min (column pressure 1000 psi) with the absorbance monitored at 258 nm.

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1. S. DISCHE. Radiother. Oncol. **3**, 97 (1985).
2. G. E. ADAMS and I. J. STRATFORD. Biochem. Pharmacol. **35**, 71 (1986).
3. A. M. RAUTH. Int. J. Radiat. Oncol. Biol. Phys. **10**, 1293 (1984).
4. D. W. WHILLANS and G. F. WHITMORE. Radiat. Res. **86**, 311 (1981).
5. T. KAGIYA, H. IDE, S. NISHIMOTO, and T. WADA. Int. J. Radiat. Biol. **44**, 505 (1983).
6. R. A. MCCLELLAND, J. R. FULLER, N. E. SEAMAN, A. M. RAUTH, and R. BATTISTELLA. Biochem. Pharmacol. **33**, 303 (1984).
7. A. R. J. SILVER, S. S. MCNEILL, P. O'NEILL, T. C. JENKINS, and I. AHMED. Biochem. Pharmacol. **35**, 3923 (1986).
8. M. V. MIDDLESTADT and A. M. RAUTH. Int. J. Radiat. Oncol. Biol. Phys. **8**, 709 (1982).
9. R. J. KNOX, R. C. KNIGHT, and D. I. EDWARDS. Biochem. Pharmacol. **32**, 2149 (1983).
10. R. A. MCCLELLAND, R. PANICUCCI, and A. M. RAUTH. J. Am. Chem. Soc. **109**, 4308 (1987).
11. A. J. VARGHESE and G. F. WHITMORE. Chem.-Biol. Interact. **36**, 141 (1981).
12. A. J. VARGHESE and G. F. WHITMORE. Chem.-Biol. Interact. **56**, 269 (1985).
13. E. D. CLARKE, P. WARDMAN, and K. H. GOULDING. Biochem. Pharmacol. **29**, 2684 (1980).
14. P. D. JOSEPHY, B. PALCIC, and L. D. SKARSGARD. Biochem. Pharmacol. **30**, 849 (1981).
15. J. A. RALEIGH, S. F. LIU, and F. Y. SHUN. Int. J. Radiat. Oncol. Biol. Phys. **8**, 701 (1982).
16. E. D. CLARKE, K. H. GOULDING, and P. WARDMAN. Biochem. Pharmacol. **31**, 3237 (1982).
17. D. C. HEIMBROOK and A. C. SARTORELLI. Mol. Pharmacol. **29**, 168 (1986).
18. M. I. WALTON and P. WORKMAN. Biochem. Pharmacol. **36**, 887 (1987).
19. K. R. LADEROUTE, E. ERYVAIC, R. A. MCCLELLAND, and A. M. RAUTH. Int. J. Radiat. Oncol. Biol. Phys. **12**, 1215 (1986).
20. R. PANICUCCI, R. A. MCCLELLAND, and A. M. RAUTH. Int. J. Radiat. Oncol. Biol. Phys. **12**, 1227 (1986).
21. R. A. MCCLELLAND, R. PANICUCCI, and A. M. RAUTH. J. Am. Chem. Soc. **107**, 1762 (1985).
22. J. L. BOLTON and R. A. MCCLELLAND. J. Am. Chem. Soc. In press.
23. A. J. VARGHESE and G. F. WHITMORE. Cancer Res. **43**, 78 (1983).
24. J. A. RALEIGH and S. F. LIU. Biochem. Pharmacol. **32**, 1444 (1983).
25. J. A. RALEIGH and S. F. LIU. Int. J. Radiat. Oncol. Biol. Phys. **10**, 1337 (1984).
26. A. J. R. SILVER and S. S. MCNEILL. Biochem. Pharmacol. **19**, 3537 (1987).
27. R. SHAPIRO and J. HACHMANN. Biochemistry, **5**, 2799 (1966).
28. N. E. BROUDE and E. O. BOROWSKY. Biochem. Biophys. Acta, **54**, 340 (1971).
29. A. J. VARGHESE and G. F. WHITMORE. Radiat. Res. **97**, 262 (1984).
30. A. J. VARGHESE and G. F. WHITMORE. Int. J. Radiat. Oncol. Biol. Phys. **10**, 1361 (1984).
31. C. A. BARCLAY, J. A. CERISOLA, H. LUGONES, and O. LEDESMA. *In* Current chemotherapy. Vol. 1. *Edited by* W. Siegenthaler and R. Luthy. American Society for Microbiology, Washington, DC. 1978. p. 158.
32. P. R. TWENTYMAN and P. WORKMAN. Br. J. Cancer **48**, 17 (1983).
33. J. T. ROBERTS, N. M. BLEEHEN, F. Y. F. LEE, P. WORKMAN, and M. I. WALTON. Int. J. Radiat. Oncol. Biol. Phys. **10**, 1745 (1984).
34. A. J. VARGHESE and G. F. WHITMORE. Cancer Res. **40**, 2165 (1980).
35. G. G. MILLER, J. NGAN-LEE, and D. J. CHAPMAN. Int. J. Radiat. Oncol. Biol. Phys. **8**, 741 (1982).
36. A. J. FRANKO. Int. J. Radiat. Oncol. Biol. Phys. **12**, 1195 (1986).
37. D. I. EDWARDS. Br. J. Vener. Dis. **56**, 285 (1980).
38. E. A. BUMP, Y. C. TAYLOR, and J. M. BROWN. Cancer Res. **44**, 997 (1983).
39. A. J. VARGHESE. Anal. Biochem. **110**, 197 (1981).
40. J. L. BORN and V. M. HADLEY. Int. J. Radiat. Oncol. Biol. Phys. **11**, 1157 (1985).
41. I. R. FLOCKHART, P. LARGE, D. TROUP, S. L. MALCOLM, and D. R. MARTEN. Xenobiotica, **8**, 97 (1978).
42. R. FULLER. M.Sc. Thesis, University of Toronto (1983).
43. A. J. VARGHESE and G. F. WHITMORE. Int. J. Radiat. Oncol. Biol. Phys. **10**, 1341 (1984).
44. R. HUISGEN and R. REIMLINGER. Ann. **599**, 161 (1956).
45. R. F. BROWN and N. M. VAN GULICK. J. Am. Chem. Soc. **77**, 1090 (1956).
46. F. E. KING, J. W. CLARK-LEWIS, D. A. KID, and G. R. SMITH. J. Chem. Soc. 1041 (1956).
47. B. T. STOREY, W. W. SULLIVAN, and C. L. MOYER. J. Org. Chem. **29**, 3118 (1964).