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MOLECULAR MECHANISMS FOR THE HYPOXIA-DEPENDENT ACTIVATION OF 3-AMINO-1,2,4-BENZOTRIAZINE-1,4-DIOXIDE (SR 4233)

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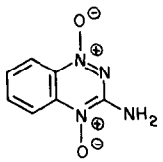
Abstract—The reduction of the hypoxic cell toxin 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233) was investigated using pulse radiolysis, radiation chemical reduction, and xanthine oxidase. Evidence was found that the one-electron reduction product of the parent compound is an oxidizing radical that caused single- and double-strand breaks in plasmid DNA and that produced a malondialdehyde-like thiobarbituric acid adduct from 2-deoxy-D-ribose. Possible forms of the reactive radical, either carbon- or nitrogen-centered, are suggested. The "natural" lifetime of the radical was sufficiently long that it could diffuse over significant distances within hypoxic cells and thus inflict oxidative damage on cellular targets. The radical reacted with O_2 at a rate comparable to those of the nitroimidazoles misonidazole and metronidazole. Thus, the selectivity for hypoxic cells is probably due to the elimination of "futile" reduction when the cellular oxygen concentration is sufficiently low.

The compound 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233; NSC 130181) **1** has been shown to have significant selective toxicity for hypoxic mammalian cells *in vitro* and *in vivo* [1]. Recently the reduction chemistry of **1** was investigated using radiation chemical, electrochemical and enzymatic methods [2]. Two major reduction products were found, 3-amino-1,2,4-benzotriazine-1-oxide **2** (SR 4317) and 3-amino-1,2,4-benzotriazine **3** (SR 4330). The reduction stoichiometry was determined using the one-electron reductants, $CO_2^{\cdot-}$ and e_{aq} at pH 7, and an apparent stoichiometry of approximately 0.5 one-electron donors per molecule of **1** was found. Two-electron stoichiometry is the expected result since **2** is a two-electron reduction product of **1**. Since **2** has been reported to be not measurably toxic to hypoxic cells *in vitro* at levels significantly greater than those which are toxic for **1** [1], it is likely that the damaging species exists at the one-electron reduction level. This result raised the possibility that the peculiar reduction stoichiometry observed earlier is relevant to the hypoxic cell toxicity. In the present work an attempt has been made to characterize the properties of the one-electron intermediate.



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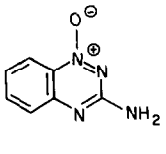

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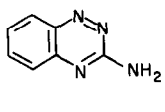

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MATERIALS AND METHODS

The compounds sodium cyanamide and malondialdehyde bis (dimethylacetal) were obtained from the Aldrich Chemical Co., Milwaukee, WI. Xanthine oxidase (grade III), xanthine, 2-deoxy-D-ribose and thiobarbituric acid (TBA) were purchased from the Sigma Chemical Co., St. Louis, MO. Barium [^{14}C]cyanamide (5 mg; 1 mCi/mg) was from Amersham, Oakville, Ontario, and desferrioxamine mesylate (Desferal) was from CIBA, Dorval, P.Q. Other materials, including Sepharose 4B (Pharmacia, Montreal, P.Q.) and pre-purified argon (Matheson Gas Products Canada, Toronto, Ontario), were commercial samples of high purity. Plasmid pDHd33 (3.3 kilobases) was provided by Dr. D. E. Pulleyblank of the University of Toronto, and plasmid pPM17 (5.5 kilobases) was obtained from Dr. G. F. Whitmore of the Ontario Cancer Institute. The latter plasmid was grown and purified by conventional microbiological techniques [3]. Both plasmids were >80% supercoiled.

Compound **1** was synthesized according to the procedure of Mason and Tennant [4]. Samples of **2** were obtained from this synthetic sequence. The labelled compound [3-^{14}C]-3-amino-1,2,4-benzotriazine-1,4-dioxide (**1**-^{14}C) was also produced in this way, but the required precursor, [^{14}C]cyanamide, was synthesized from barium [^{14}C]cyanamide by a modification of the procedure of Zbarsky and Fischer [5] in which 5 mg of labelled material was diluted with 2 g of sodium cyanamide. The yield of crude [^{14}C]cyanamide was 43%, and this was used directly in the synthesis of **1**-^{14}C. Purification of **1**-^{14}C was accomplished using a semi-preparative, Waters μBondapak C_{18} HPLC column and a mobile phase of 10% CH_3CN/20% CH_3OH/H_2O. The specific activity of the purified **1**-^{14}C was 2 μCi/mg.

The radioactivity detector used in the HPLC separation was a Radiomatic Instruments Flo-one/beta model IC flow detector. The same isocratic HPLC conditions and analytical C_{18} column were used with this detector.

The pulse radiolysis method has been reviewed [6]. The equipment used has been described previously [7], except that a Tektronix 7912D digitizer was used for transient analysis.

Reducing radicals were produced by gamma irradiation using an AECL ^{60}Co gamma-cell (approximately 0.5 Gy/sec). Solutions for irradiation were typically 0.5 mM in **1** and 100 mM in sodium formate or *t*-butanol. The *t*-butanol was distilled prior to use. The buffer used in the *t*-butanol scavenging experiments was 20 mM sodium phosphate, pH 6.8. The buffers used for the pH study of the radiation chemical reduction stoichiometry included 20 mM NaH_2PO_4 (pH 5.8), 20 mM NaH_2PO_4/Na_2HPO_4 (pH 6.8), 20 mM Na_2HPO_4 (pH 8.0), 40 mM $NaHCO_3$/4 mM NaOH (pH 9.1), 10 mM $NaHCO_3$/5 mM NaOH (pH 9.8) and 40 mM $NaHCO_3$/36 mM NaOH (pH 10.9). The ionic strength (μ) of each solution was adjusted where necessary to 0.2 with Na_2SO_4. Small volumes (1.5 ml) of the solutions were placed in glass vials and deoxygenated by bubbling with pre-purified nitrogen prior to sealing. The nitrogen was bubbled through a solution of 0.1 M $Na_2S_2O_4$/0.1 M Na_2CO_3 before entering the vials to remove any traces of oxygen.

Reduction stoichiometry, or the number of one-electron donors per molecule of **1** required for complete reduction of **1**, was calculated from the radiation dose which just eliminated the detectability of the parent compound by HPLC (less than a 6 μM solution of **1**). The hydroxyl radicals and hydrogen atoms H$^{\cdot}$ produced by the radiolysis of water are scavenged by formate to yield $CO_2^{\cdot-}$ reducing radicals, while e_{aq}^- reacts with **1** directly. The total yield of one-electron reductants was taken to be 0.61 μM Gy^{-1} in N_2-saturated, 0.1 M formate solution [8]. Nitrous oxide saturation converts e_{aq}^- to OH$^{\cdot}$ and hence to $CO_2^{\cdot-}$. Thus, for the 0.2 M formate/N_2O solutions used in pulse radiolysis, an increased yield of reductants (0.69 μM Gy^{-1}) was assumed because of this increased spur scavenging [8]. In *t*-butanol solutions, since the *t*-butoxyl radical is a much weaker reductant than $CO_2^{\cdot-}$ [9], it seemed likely that only e_{aq}^- and H$^{\cdot}$ would react with **1** and, thus, a reducing yield of 0.34 μM Gy^{-1} was assumed.

The reduction of **1** by xanthine oxidase was accomplished using vials containing 1.0- or 2.0-ml solutions that were deoxygenated by bubbling with pre-purified argon prior to sealing. Solutions contained 0.5 mM **1**, 0.4 mM xanthine, 0.6 mM of the Fe(III) chelator desferrioxamine mesylate, 10 mM sodium phosphate (pH 6.8) and 0.25 units of xanthine oxidase suspension. The DNA target for reduced **1** was 10 μg/ml of plasmid pDHd33 or 15–20 μg/ml of plasmid pPM17. To assay for strand breaks in plasmid pDHd33, reduction was allowed to proceed for either 10 or 40 min in the deoxygenated vials after addition of the enzyme and rapid sealing of the vials. Vials were opened under argon, and sodium arsenite solution was injected by capillary micropipette to a final concentration of 2 mM in order to poison the enzyme rapidly [10]. An aliquot was added to electrophoresis loading buffer, and the DNA molecules were separated on a 1% agarose gel.

Binding of reduced **1**-^{14}C to DNA was investigated using the plasmid pPM17. Oxygen-depleted reaction vials containing solutions of 0.5 mM **1**-^{14}C that were otherwise identical with those described above were kept sealed overnight at 20° along with an air-equilibrated control solution of **1** that did not contain the enzyme. The contents of a vial were loaded on a column containing a 200-ml slurry of the size separation medium Sepharose 4B in 10 mM Tris/1 mM EDTA, and fractions were eluted using the same buffer. These were assayed by UV-visible spectroscopy. The DNA and free reduced and unreduced **1**-^{14}C fractions were analyzed by liquid scintillation counting using an LKB Wallace 1217 Rackbeta counter.

In the enzymatic reduction experiments which involved 2-deoxy-D-ribose as a target, the solutions were either identical with those described above, but contained 5 mM sugar instead of DNA, or were large scale experiments as defined below. The latter experiments were performed in order to isolate the TBA-reactive material by HPLC for further analysis (semi-preparative Waters μBondapak C_{18} column; 10% CH_3CN). In the smaller scale experiments, after 3 hr, the vials were opened, and 0.1-ml aliquots were added to 0.4 ml of TBA solution (0.71 g TBA/0.7 ml 1.0 N NaOH in 100 ml H_2O) [11]. The solutions were heated with a boiling water bath for 20 min and cooled, and then the absorbance was measured at 532 nm. The larger scale solutions were 50 ml of **1**, xanthine and 2-deoxy-D-deoxyribose in the molar ratio 1:5:10 (0.3 mM **1**) to which 3.4 units of xanthine oxidase suspension were added. In the experiments which involved the use of sealed vials and shorter reaction times, an approximate 1:1 ratio of **1** to xanthine was used in order to limit the reaction to the two-electron reduction level at which **2** is formed. This was ascertained by HPLC. In fact, the production of **3** from **2** by xanthine oxidase/xanthine appeared to be a slow process. These solutions and controls lacking **1** were bubbled continually for several hours with pre-purified nitrogen which was initially passed through 0.1 M $Na_2S_2O_4$/0.1 M Na_2CO_3 for further deoxygenation. The authentic adduct of TBA and malondialdehyde was prepared according to the procedure of Sinnhuber *et al.* [11] using malondialdehyde bis (dimethylacetal).

RESULTS

Although it had been shown earlier [2] that the major products of the reduction of **1** in the absence of oxygen are **2** and **3**, it was not possible to account for up to ~50% of the starting material by the UV absorbances of the corresponding HPLC peaks. The ^{14}C-radioactivity chromatograms for a radiation chemical reduction presented in Fig. 1 confirmed that **2** was the major reduction product after 20 min of irradiation, and show that at least one other product besides **3** was formed at a low level. This latter product has not been identified. The peak for **3** was predominant at longer irradiation times (80–

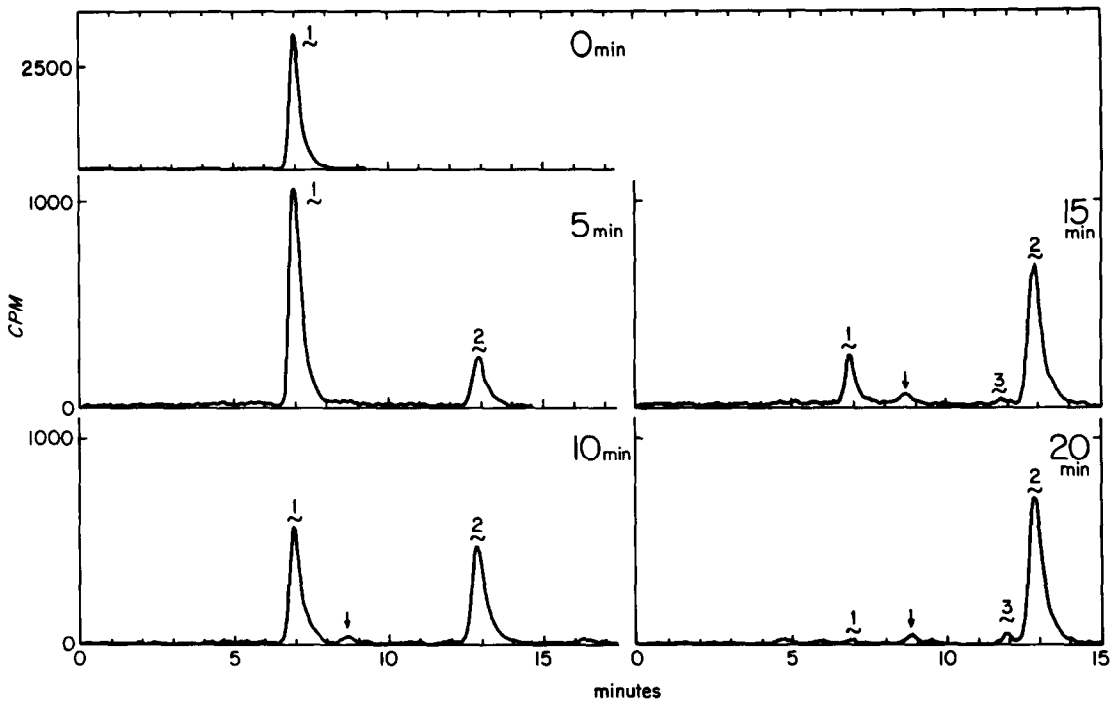

Fig. 1. HPLC chromatograms for the reduction of [3-^{14}C]-3-amino-1,2,4-benzotriazine-1,4-dioxide by ^{60}Co gamma radiation (approximately 0.5 Gy/sec) in 0.1 M $NaHCO_2$/10 mM sodium phosphate at pH 6.8. Each panel is designated by the time in minutes of irradiation of the sample. The arrow indicates the unidentified peak.

120 min). It was again found that not all of the starting material could be accounted for by HPLC since the number of counts per minute (cpm) eluted from the column was less than the total cpm injected for equal injections. These differences increased with irradiation time and reached maximum losses of ~20–40% for complete reduction of **1** (20–30 min irradiation times), depending on the experiment. This indicates that some material present in the original sample was either lost, eluted as background, or was tightly adsorbed to the C_{18} μBondapak HPLC column under the conditions employed. To determine whether or not ^{14}C had been lost in a volatile substance before chromatography, equal aliquots of samples corresponding to each time point of reduction were analyzed by liquid scintillation counting (20 μl/10 ml aqueous counting scintillant). However, no significant differences in total cpm were found among the samples.

Pulse radiolysis is a rapid reaction technique in which a time resolution of microseconds can be attained. Thus, a transient intermediate(s) which lies along the pathway from **1** to **2** can be observed spectroscopically in the absence of interference from **2**. The reaction between **1** and $CO_2^{\cdot-}$ to form the radical of **1** at pH 7.4 under N_2O was investigated by pulse radiolysis (0.2 μsec, 1.6 Gy) of a 52 μM solution of **1** in 0.2 M sodium formate/1 mM sodium phosphate. The spectral changes associated with the conversion of **1** to its one-electron reduction product are shown in Fig. 2; between 390 and 500 nm the goundstate **1** absorbed more than the radical and a net bleaching of the solution was observed. The true spectrum of the radical was obtained on adding to the observed changes the product of the groundstate extinction coefficient and the yield of conversion (0.68 μM/Gy). The latter value was determined from the one-electron reduction of $Fe(CN)_6^{3-}$ in a similar solution.

The buildup of absorption was exponential, as expected, since the initial concentration of **1** was much greater than the initial concentration of $CO_2^{\cdot-}$; the inset to Fig. 2 shows that the first-order rate constant obtained from the absorbance change at 550 nm was first order in [**1**] for three solutions of **1** (21–63 μM). The slope yielded a second-order rate constant for the reduction of **1** by $CO_2^{\cdot-}$ of $2.8 \times 10^9\ M^{-1}\ sec^{-1}$ at an ionic strength of 0.2. Thus, the formation of the radical in this system is essentially diffusion-controlled.

To investigate the role of pH in the one-electron reduction of **1**, absorbances of the groundstate and the one-electron species were determined as a function of pH from about 7 to 14. The colour of aqueous solutions of **1** changed reversibly from yellow to red-pink on raising the pH through this range. Absorbance values at 300, 455 and 540 nm from six solutions of pH of about 9 to 13.5 were fitted to the appropriate function using a non-linear least squares programme, and a pK_a of 12.5 was determined (isosbestic point at approximately 498 nm). This is presumably the ionization of the amino proton (cf. pK_a of trinitroaniline is 12.2) [12].

To ascertain whether the one-electron reduction product of **1** underwent a similar ionization, pulse radiolysis experiments were carried out like those

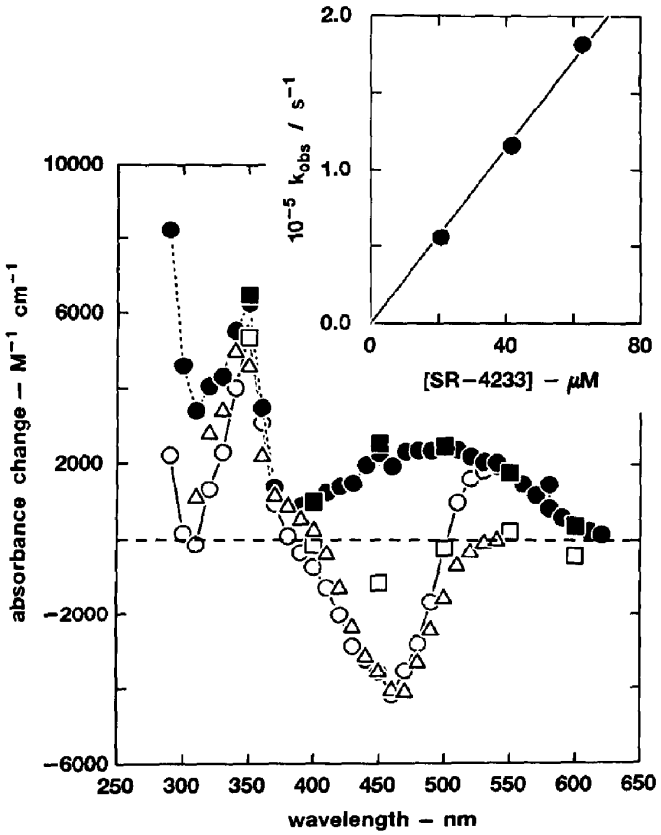

Fig. 2. Spectra changes (in extinction coefficient) observed on reduction of **1** by CO_2^- 50 μsec after the pulse (when <1% of the decay product **2** has been produced). Key: (○) absorbance changes at pH 7.4 (see text); (●) pH 7.4, corrected for groundstate conversion to yield true spectrum of radical; (□) absorbance changes at pH 12.3; (■) corrected radical spectrum at pH 12.3; and (△) absorbance changes at pH 2.7. Inset: First-order rate constants for formation of absorption at 550 nm in solutions containing various amounts of **1** at pH 7.4.

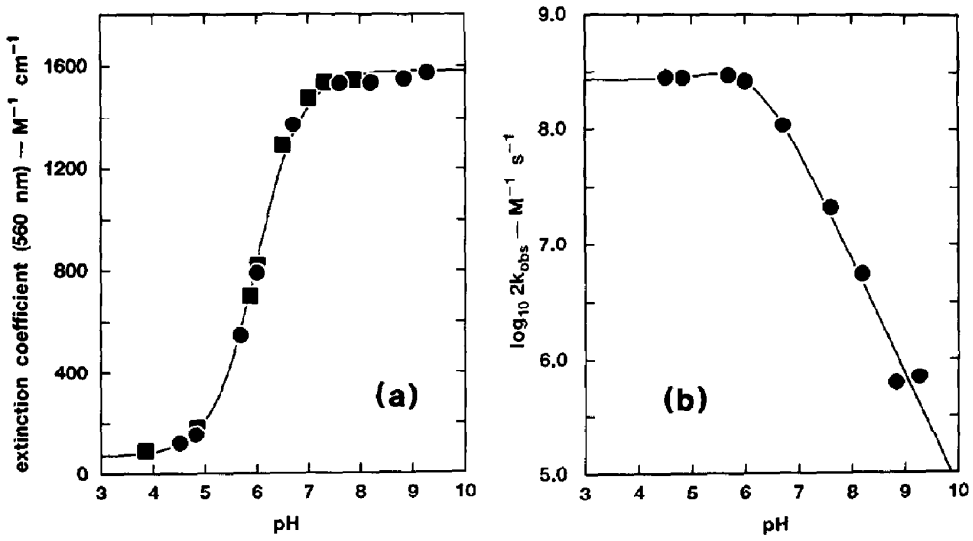

Fig. 3. (a) Absorbance of the radical from reduction of **1** by CO_2^- as a function of pH. Key: (●) 0.2 M and (■) 0.1 M formate. (b) Second-order rate constants $2k_{obs}$ for the reduction of **1** by CO_2^- as a function of pH.

described above but using solutions with a pH of 12.3. Representative data are plotted in Fig. 2 with points only every 50 nm for clarity. As expected, because the groundstate absorbances differ markedly at pH 7.4 and 12.3, the absorbance changes observed differed in the two experiments except at the isosbestic point. However, the corrected spectra show the true radical absorption to be very similar at both pH values. Thus, although the groundstate ionizes with a pK_a of 12.5, the radical does not appear to have a prototropic equilibrium with a pK_a in the range of 7 to 13. At pH 2.7 (Fig. 2), however, the absorption changes associated with the radical were shifted in wavelength compared to pH values above 7. In particular, there was little absorption above roughly 500 nm, whereas the groundstate absorption was unchanged. Analysis of absorbances at 560 nm from eighteen solutions of pH 3.9 to 9.3 (Fig. 3a) gave a pK_a of 5.99 ± 0.02 (the uncertainty ignores errors in the pH standards). Small differences in radical yield and ionic strength effects were not detectable in these sets of experiments.

The stability of the product of the reduction of **1** by $CO_2^{\cdot-}$ was investigated by pulse radiolysis (2 μsec, 50 Gy) of 0.7 mM solutions of **1** in 0.2 M formate/N_2O. Up to 4 mM phosphate or 10 mM formic acid was used to vary the pH between 4.5 and 9.3. Observations of the decay at 560 nm showed the half-life to be inversely proportional to radiation dose (i.e. initial radical concentration). Plots of 1/absorbance versus time (i.e. second-order analysis) were linear over two half-lives, although minor contributions of another decay route, or possibly a weakly absorbing intermediate were evident at longer times. The extinction coefficients of the radical(s) were determined in each solution using doses of 6–12 Gy (Fig. 3a), and estimates of the second-order rate constants $2k_{obs}$ are plotted as a function of pH in Fig. 3b (the formalism $-d[\text{radical}]/dt = 2k[\text{radical}]^2$ is used).

At pH 12.3, the radicals decayed only ~5% in 0.1 sec. The decrease in $2k_{obs}$ by an order of magnitude for each pH unit increase above pH 7 demonstrated in Fig. 3b indicates that the unprotonated radicals disproportionate very slowly. Representing the radical anion of **1** by $R^{\cdot-}$, the second-order decay kinetics can be accounted for by the following scheme:

$$RH^{\cdot} \rightleftarrows R^{\cdot-} + H^+ \quad (1)$$

$$2RH^{\cdot} \rightarrow R + RH_2 \quad (2a)$$

$$RH^{\cdot} + R^{\cdot-} + H^+ \rightarrow R + RH_2 \quad (2b)$$

$$2R^{\cdot-} + 2H^+ \rightarrow R + RH_2 \quad (2c)$$

In fact, R is **1** and RH_2 becomes **2**. Evidently, like the analogous decay of $HO_2^{\cdot}/O_2^{\cdot-}$ [13] and many nitroaromatic radicals [14, 15], $k_{2c} \ll k_{2a}$ or k_{2b}. The pH-dependent decay can be approximated by the expression:

$$2k_{obs} = \frac{2k_{2a} + 2k_{2b}(10^{pH-pK_1})}{(1 + 10^{pH-pK_1})^2}$$

Non-linear least squares fit of the data in Fig. 3b to this function gave estimates of $2k_{2a}$ and $2k_{2b}$ of $2.7 \times 10^8\ M^{-1}\ sec^{-1}$ and $8.0 \times 10^8\ M^{-1}\ sec^{-1}$, respectively, and a value of pK_1 of 5.96 (cf. 5.99 estimated quite independently from absorbance changes described above).

At pH 7.4, $2k_{obs} \simeq 3 \times 10^7\ M^{-1}\ sec^{-1}$, and the first half-life of the radical at initial concentrations of ~4 μM (dose ~6 Gy) was about 8 msec ($T_{1/2} = 1/2k_{obs}[\text{radical}]$). In air and oxygen-saturated solutions of **1** (2 mM) in 0.2 M formate at pH 7.3, the radical absorption at 550 nm decayed exponentially with half-lives of 0.4 and 0.08 msec respectively. The reactions were first-order in $[O_2]$ and were readily separated from the disproportionation decay described by (2). For the reaction

$$R^{\cdot-} + O_2 \rightarrow \mathbf{1} + O_2^{\cdot-} \quad (3)$$

an estimate of k_3 of $6.2 \times 10^6\ M^{-1}\ sec^{-1}$ was obtained. Since traces of oxygen inhibit the reduction in **1** in cellular or chemical systems [2], it is assumed that (3) restores **1**.

The stoichiometry for the complete radiation chemical reduction of **1** at pH 6.8 in 0.1 M *t*-butanol was 1.8 ± 0.5 one-electron donors per molecule (average of four values). Product analysis by HPLC showed that the major product of reduction was **2**, although at least one new product was evident with a similar retention time to that of **1**. In contrast, the substitution of 0.1 M sodium formate for *t*-butanol under the same conditions resulted in a value for the stoichiometry of 0.5 ± 0.04 (Table 1). The major product was also **2**, as has been noted. Thus, the use of a radical scavenger which forms non-reducing radicals results in approximately the expected stoichiometry for the two-electron reduction of **1** to **2**.

The data in Table 1 show the effect of pH on the reduction stoichiometry. The values increased with increasing pH from ~0.5 at pH 5.8 to ~4 at pH 10.9, which is the expected stoichiometry for the four-electron reduction of **1** to **3**. Consistent with this result, HPLC analysis showed that the more abundant product of the reduction of **1** was **2** at pH values up to 8.0, and **3** at pH values between 8.0 and 10.9. At the latter pH, little **2** was present. Although the presence of a significant proportion of **2** with **1** as reduction proceeds to **3** leads to a competition between **1** and **2** for one-electron donors, and thus the stoichiometry calculation is not strictly applicable, the trend in the change in stoichiometry is clear, and suggests the operation of competitive mechanisms which are pH dependent. This idea is expanded in the Discussion.

Table 1. Reduction stoichiometry

pH*	Reducing equivalents/mol.†
5.8	0.5 ± 0.02
6.8	0.5 ± 0.04
8.0	0.9 ± 0.1
9.1	1.8 ± 0.2
9.8	3.1 ± 0.1
10.9	4.1 ± 0.2

* See Materials and Methods.

† Number of one-electron donors or reducing equivalents per molecule of **1** for complete reduction of **1**. Averages of three experiments (0.1 M $NaHCO_2$).

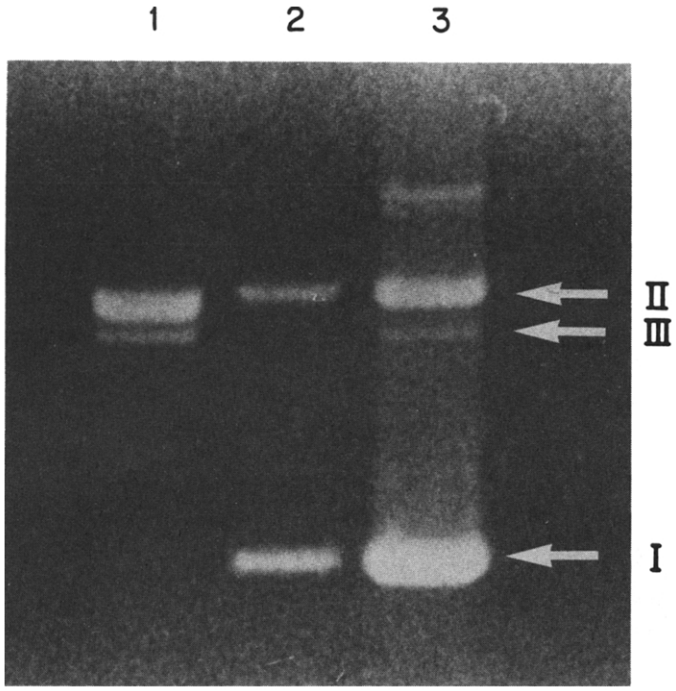

Fig. 4. Gel electrophoretic analysis of the reaction(s) in the absence of oxygen between the one-electron reduction product of **1** and plasmid pDHd33. Lane 1, plasmid + **1** + xanthine oxidase/xanthine; lane 2, plasmid + xanthine oxidase/xanthine; lane 3, plasmid stock solution. DNA was visualized by ethidium bromide fluoresence.

The absorption spectra of the TBA-adduct material from the reaction mixture of 2-deoxy-D-ribose and **1** reduced by xanthine oxidase/xanthine and the authentic TBA-malondialdehyde adduct were virtually identical. However, although the former adduct appeared as a single peak in an HPLC chromatogram, it had a different retention time from the standard (TBA-adduct: 12 min; TBA-malondialdehyde adduct: 5 min; 10% CH_3CN/30 × 3.9 cm Waters μBondapak C_{18} column; 1 ml/min). It has not been isolated as a pure substance. If oxygen were excluded completely, no detectable TBA-reactive material was present in control samples.

The ability of **1** reduced by xanthine oxidase in the presence of supercoiled plasmid DNA to cause single- and double-strand breaks is exemplified by the photograph of the agarose gel shown in Fig. 4. In these experiments the major stable reduction product was **2**. Lane 1 in the gel contained an aliquot of supercoiled plasmid pDHd33 which had been exposed to **1** under argon in the presence of xanthine oxidase and xanthine. The electrophoretic mobility of the supercoiled covalently closed circular DNA (form I) was greater than that of the linear form (form III), which was in turn, greater than that of the nicked or relaxed covalently closed circular DNA (form II). These three forms of the plasmid are evident in the figure in lane 3 which contained stock solution DNA. It can be seen that lane 1 did not have any detectable supercoiled plasmid, but had significantly more nicked plasmid than was present in the control sample loaded in lane 2. In addition, lane 1 had linear DNA. Since nicked plasmid arises from one single-strand break or nick per DNA molecule, and linear plasmid DNA results from a double-strand break, the one-electron reduction of **1** can produce a species which causes nicking of DNA. With a sufficient density of such events, double-strand cleavage can occur. Unreduced **1** and authentic **2** did not show any activity in this assay.

In an attempt to find evidence for the binding of reduced **1** to plasmid DNA, essentially identical experiments were undertaken using **1**-^{14}C and plasmid pPM17. Liquid scintillation counting of the separated fractions containing reduced **1** and DNA revealed that a low level of binding of the label had occurred representing ~0.05 to 0.1% of the total cpm recovered from the column. Consistently, this value was above the background level of ~0.01% which was determined using air-equilibrated solutions that did not receive any enzyme.

DISCUSSION

The unusually low stoichiometry for the radiation chemical reduction of **1** at pH values less than ~8 indicates that the reduction of **1** occurs at enhanced rates within this pH range. The change in stoichiometry from ~0.5 at pH 6 and 7 to ~4 at pH 11 is consistent with a pH-dependent change in reduction mechanism. This was reflected in the alteration in product distribution when **1** was consumed from a predominance of the two-electron product **2**, to that of the four-electron product **3** at higher pH values. These results, taken with the observation made following pulse radiolysis that the second-order half-

life of the radical derived from **1** increased with increasing pH, provide the following molecular rationale for the reduction chemistry of **1**.

Oxidation of *t*-butanol by $OH^{\cdot}$ yields principally a non-reducing radical, the *t*-butoxyl radical [9], whereas oxidation of formate by $OH^{\cdot}$ yields the reductant $CO_2^{\cdot-}$. Hence, a higher yield of reducing equivalents (approximately 2-fold) in N_2-saturated solutions of formate compared to *t*-butanol is expected. However, since the reduction of **1** to primarily **2** at pH 6.8 was about 4-fold more efficient with formate compared to *t*-butanol, after allowing for the increased yield of reducing species, it follows reasonably that the reduction of **1** produces a radical which is capable of oxidizing formate but not *t*-butanol, to initiate a short chain reaction. Further, protonation of radical anions is not uncommon, and this can result in enhanced rates of disproportionation. This effect has been observed in the disproportionation of $O_2^{\cdot-}/HO_2^{\cdot}$ [13] and with the radical anions of a number of nitroaromatics [14, 15]. It is interesting that the changes in absorption accompanying protonation of the radical anions of **1** were qualitatively similar to those observed on protonation of nitroimidazole radical ions [15].

The present work does not reveal whether or not the site of protonation on the radical anion is at the nitrogen at position 3. The pK_a for the latter group is presumably ≤ 3 in the gound state because of the electron-withdrawing N-oxide groups (cf. pK_a values of 2.47 and 1.0 for 3- and 4-nitroaniline respectively [16]), but it will be increased on adding the electron to form the radical. Protonation of the nitrogen at position 2 seems unlikely because of the electropositive nitrogen at position 1.

The radical from **1** disproportionates about three orders of magnitude faster than most nitroaromatic radicals at similar pH values, and about an order of magnitude faster than $HO_2^{\cdot}/O_2^{\cdot-}$ [13, 15]. The values for $2k_{obs}$ shown in Fig. 3b imply "natural" lifetimes of the radical of **1** of many milliseconds in the absence of oxygen and at the micromolar or lower, steady-state concentrations likely to occur in cells. This would permit significant diffusion from the site of formation.

The pH-dependent stoichiometry of the reduction of **1** in formate suggests a plausible mechanism for the observed hypoxic cell toxicity. First, facile oxidation of the radical by O_2 according to (3) is entirely analogous to the "futile metabolism" cycle of formation and oxidation of nitroaromatic radical anions responsible for the hypoxic specific toxicity of these species [17]. In fact, the rate constant k_3 is very similar to that for the oxidation of the radical anions of metronidazole and misonidazole by O_2 [18]. Second, the unusually low reduction stoichiometry for the conversion of **1** to **2** must mean that a reduction intermediate is formed which is capable of oxidizing formate to $CO_2^{\cdot-}$. This suggests that analogous hydrogen abstractions from sugar residues in DNA, leading to strand breaks, could be the basis for the toxicity of **1** in hypoxic cells.

The present study has not identified the nature of the oxidizing intermediate. Perhaps the simplest possibility is that the radical itself oxidizes formate, but only when protonated since the reduction stoichiometry is pH dependent.

$$RH^{\cdot} + HCO_2^{-} \rightarrow RH_2 + CO_2^{\cdot-} \qquad (4)$$

This can be compared to the greatly enhanced reactivity of $HO_2^{\cdot}$ relative to the conjugate base $O_2^{\cdot-}$ towards compounds of biological interest [19]. The enhanced oxidizing ability of $RH^{\cdot}$ compared to $R^{\cdot-}$ is in competition with decay by disproportionation according to (2) as the pH is decreased. Thus, a dose-rate effect upon the observed stoichiometry would be expected, and this has been observed in preliminary experiments.

The proposed structure of $RH^{\cdot}$ is shown in Fig. 5 (**4**). This is given on theoretical grounds, with no spectroscopic evidence. The protonation site for the pK_a of 6.0 is assumed to be on the oxygen at position 4, in order to postulate OH^- (or H_2O) as a reasonable leaving group for cleavage of the N–O bond to form **2**. This is discussed below. The preferred site for the unpaired spin is assumed to be on the carbon at position 3, rather than the nitrogen at position 2, since the former site is surrounded by two-electron donor groups and one-electron acceptor group. The canonical forms shown in Fig. 5 illustrate the con-

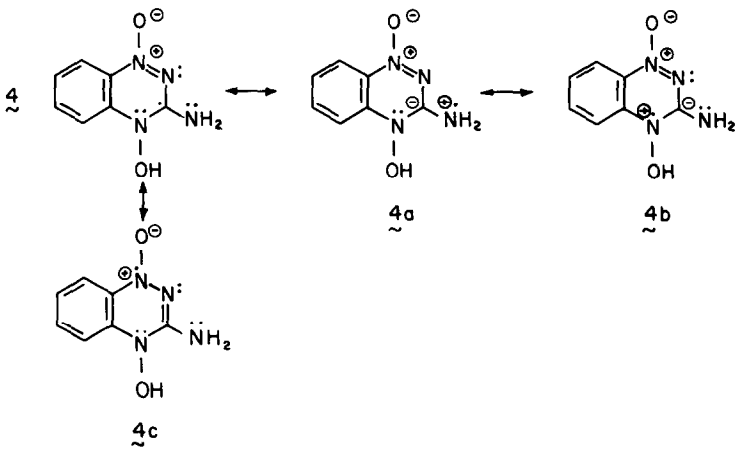

Fig. 5. Hypothetical structure (4) of the one-electron product of **1**. The structures 4a–c are canonical forms.

Hypothetical Reaction Schemes

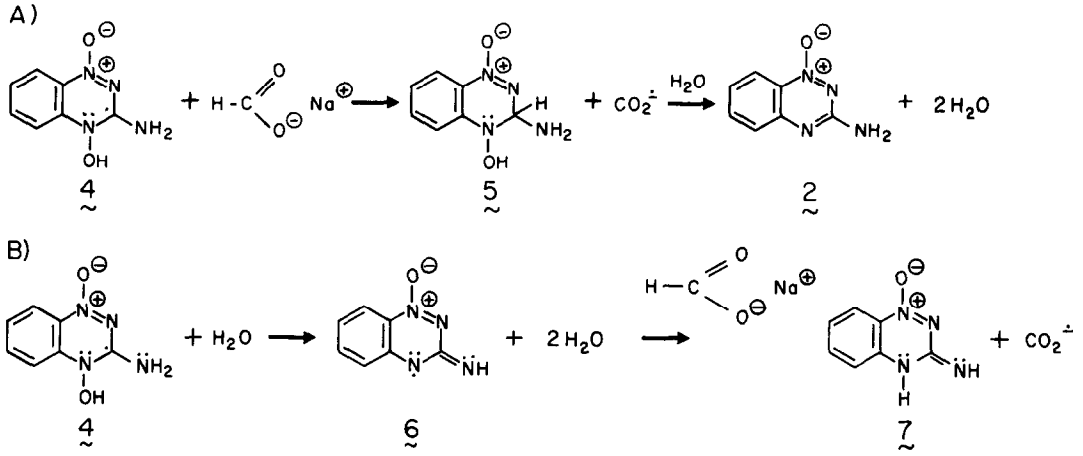

Fig. 6. Hypothetical reaction schemes for the radiation chemical reduction of **1** by CO_2^- at pH 7.

tributions of electron donation (**4a**, **4b**) and electron withdrawal (**4c**) to the stability of the carbon-centered radical.

In this proposal, the radical anion is a member of the captodative or merostabilized class of radicals [20] which derive their stability from the presence of such donor and acceptor groups through the delocalization of the unpaired spin. Since a nitrogen-centered radical at position 2 lacks the amino group donor, it would be less stable than structure **4**.

The hypothetical reaction schemes in Fig. 6 are based on structure **4**. In Fig. 6A, **5** is the unstable species formed by the abstraction of a hydrogen atom from formate. This reaction replaces the original CO_2^- reducing radical which was consumed by the formation of **4**, and thus sets up a chain-reaction mechanism of reduction. Subsequent pseudounimolecular dehydration of **5** is driven thermodynamically by rearomatization to form **2**. The participation of H_2O as a base in the dehydration step is conjectural and a specific or general base (or acid) catalysis could also be invoked at this step. The main point in mechanism A is that **4** is a carbon-centered oxidizing radical which undergoes a rate-determining decomposition following hydrogen abstraction. In Fig. 6B, a hypothetical mechanism is shown in which **4** is converted to a nitrogen-centered oxidizing radical, and the product of hydrogen-abstraction **7** forms **2** following tautomerization. Some earlier pulse radiolysis studies offer support for this sequence. The protonated, nitrogen-centered radical derived from tryptophan is able to oxidize thiocyanate at high thiocyanate concentrations [21]. In addition, since the amino group is isoelectronic with the hydroxyl group, **4** can be compared with the radicals derived from the addition of OH$^\cdot$ to either phenol [22] or 1,4-hydroquinone [23], which eliminate water in a unimolecular, acid or base-catalyzed step. The mechanism suggested earlier [2], namely production of a hydroxyl radical from **4**, seems less likely than these proposals, and no direct evidence for it has been obtained thus far. Certainly, OH$^\cdot$ can oxidize *t*-butanol. In all of the postulated mechanisms, the damaging or toxic species which is formed by the reduction of **1** under hypoxia is considered to be an oxidizing radical which is manifested by the initiation of a chain mechanism of radiation chemical reduction. The chemical nature of this radical, however, cannot be determined in the absence of ESR spectra. Based on limited ESR results, it was suggested that the toxic form of the structurally related compound quinoxaline-1,4-dioxide is the protonated radical anion [24].

The ability of the intermediate which arises from the one-electron reduction of **1** to damage nucleic acids was assessed with the TBA assay, and with supercoiled plasmid DNA. The formation of TBA-reactive material within air-equilibrated, aqueous solutions of DNA that has been degraded by ionizing radiation [25], ferrous iron [26], and Fe(II) bleomycin [27] has been attributed, at least in part, to hydroxyl radical attack on the deoxyribose residues of the sugar-phosphate backbone, but of course any oxidizing radical with H-abstraction properties will suffice. The decomposition of 2-deoxy-D-ribose in the presence of ferrous iron and O_2 to produce malondialdehyde which can be detected using the TBA assay has been described [28]. The occurrence of both single- and double-strand breaks in supercoiled plasmid DNA in the presence of the one-electron reduction product of **1** also provides evidence for the presence of an oxidizing radical. For comparison, the ability of the hydroxyl radical to cause damage leading to strand cleavage under anoxic conditions has been amply demonstrated [29]. Again, since the major stable product was **2**, it is likely that the reactive species is produced by one-electron reduction. The occurrence of some DNA binding as shown by the association of ^{14}C with plasmid DNA following enzymatic reduction of **1**-^{14}C cannot be assessed presently, but it is conceivable that some of the reduced parent molecules may have

undergone ring fragmentation to release products that can bind to DNA. Thus far, no evidence has been obtained which shows that **1**, **2** or **3** can associate noncovalently with DNA.

In summary, the present studies indicate that the selective toxicity of **1** (SR 4233) towards hypoxic mammalian cells is mediated by a reactive oxidizing radical which is formed directly from the parent compound. This mode of action appears to be different from those of other classes of bioreductively activated drugs such as the nitroimidazoles [30] or the mitomycins [31]. Ultimately, it may be possible to develop the benzotriazine dioxides as alternative chemotherapeutic agents in the targeting of hypoxic cells in solid tumors.

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