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Original Contribution

OXIDATION OF TETRAHYDROBIOPTERIN BY BIOLOGICAL RADICALS AND SCAVENGING OF THE TRIHYDROBIOPTERIN RADICAL BY ASCORBATE

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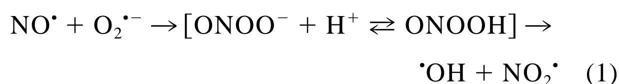
Abstract—One-electron oxidation of (6*R*)-5,6,7,8-tetrahydrobiopterin (H_4B) by the azide radical generates the radical cation ($H_4B^{\bullet +}$) which rapidly deprotonates at physiological pH to give the neutral trihydrobiopterin radical ($H_3B^{\bullet}$); pK_a ($H_4B^{\bullet +} \rightleftarrows H_3B^{\bullet} + H^+$) = (5.2 ± 0.1). In the absence of ascorbate both the $H_4B^{\bullet +}$ and $H_3B^{\bullet}$ radicals undergo disproportionation to form quinonoid dihydrobiopterin (qH_2B) and the parent H_4B with rate constants $k(H_4B^{\bullet +} + H_4B^{\bullet +}) = 6.5 \times 10^3\ M^{-1}\ s^{-1}$ and $k(H_3B^{\bullet} + H_3B^{\bullet}) = 9.3 \times 10^4\ M^{-1}\ s^{-1}$, respectively. The $H_3B^{\bullet}$ radical is scavenged by ascorbate ($AscH^-$) with an estimated rate constant of $k(H_3B^{\bullet} + AscH^-) \sim 1.7 \times 10^5\ M^{-1}\ s^{-1}$. At physiological pH the pterin rapidly scavenges a range of biological oxidants often associated with cellular oxidative stress and nitric oxide synthase (NOS) dysfunction including hydroxyl ($^{\bullet}OH$), nitrogen dioxide ($NO_2^{\bullet}$), glutathione thiyl ($GS^{\bullet}$), and carbonate ($CO_3^{\bullet -}$) radicals. Without exception these radicals react appreciably faster with H_4B than with $AscH^-$ with $k(^{\bullet}OH + H_4B) = 8.8 \times 10^9\ M^{-1}\ s^{-1}$, $k(NO_2^{\bullet} + H_4B) = 9.4 \times 10^8\ M^{-1}\ s^{-1}$, $k(CO_3^{\bullet -} + H_4B) = 4.6 \times 10^9\ M^{-1}\ s^{-1}$, and $k(GS^{\bullet} + H_4B) = 1.1 \times 10^9\ M^{-1}\ s^{-1}$, respectively. The glutathione disulfide radical anion ($GSSG^{\bullet -}$) rapidly reduces the pterin to the tetrahydrobiopterin radical anion ($H_4B^{\bullet -}$) with a rate constant of $k(GSSG^{\bullet -} + H_4B) \sim 4.5 \times 10^8\ M^{-1}\ s^{-1}$. The results are discussed in the context of the general antioxidant properties of the pterin and the redox role played by H_4B in NOS catalysis. © 2002 Elsevier Science Inc.

Keywords—Tetrahydrobiopterin, Tetrahydrobiopterin radical cation, Trihydrobiopterin radical, Free radicals, Radical scavenging, Antioxidant, Pulse radiolysis

INTRODUCTION

(6*R*)-5,6,7,8-Tetrahydrobiopterin (H_4B) is an essential cofactor for all nitric oxide synthases (NOS) and efficiently couples NADPH oxidation to nitric oxide ($NO^{\bullet}$) synthesis, thereby inhibiting superoxide ($O_2^{\bullet -}$) and hydrogen peroxide formation [1–3]. The pterin is believed to fulfill a range of functional roles in NOS but recent evidence suggests that H_4B is redox active in catalysis [4]. Involvement of a pterin radical in NOS catalysis was initially proposed on the basis of rapid-reaction kinetics with neuronal NOS [5]. This hypothesis was supported by rapid kinetic studies linking H_4B radical formation to heme-dioxy reduction and arginine hydroxylation in inducible iNOS [6]. Rapid freeze-quench EPR has provided useful information regarding pterin radical structure, which has been proposed to be either the trihydrobiopterin radical ($H_3B^{\bullet}$) [7], or the protonated tetrahydrobiopterin radical cation $(H_4B)^{\bullet +}$ [8]. Sub-optimal concentrations of H_4B during endothelial eNOS catalysis causes decoupling of the activating heme-dioxy complex to generate $O_2^{\bullet -}$ radicals [9]. The pterin may therefore modulate the ratio of $O_2^{\bullet -}$ to $NO^{\bullet}$, switching NOS to a 'peroxynitrite synthase' [2–10]. $NO^{\bullet}$ rapidly conjugates with the $O_2^{\bullet -}$ radical to generate peroxynitrite ($ONOO^-$), a potent oxidizing agent [11], which can lead to the formation of hydroxyl ($^{\bullet}OH$) and nitrogen dioxide ($NO_2^{\bullet}$) radicals following protonation [12].



$$NO^{\bullet} + O_2^{\bullet -} \rightarrow [ONOO^- + H^+ \rightleftarrows ONOOH] \rightarrow {}^{\bullet}OH + NO_2^{\bullet} \quad (1)$$

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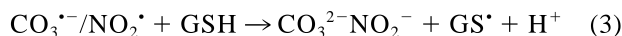
The interaction of $ONOO^-$ with carbon dioxide to generate the nitrosoperoxocarbonate anion ($ONOOCO_2^-$) can also result in the formation of two relatively strong one-electron oxidants, $NO_2^{\bullet}$ and the carbonate radical ($CO_3^{\bullet -}$) [13–18].

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow NO_2^{\bullet} + CO_3^{\bullet -} \quad (2)$$

Potentially damaging reactions of $CO_3^{\bullet -}$ and $NO_2^{\bullet -}$ may be minimized by interception by low-molecular weight antioxidants; in the case of $NO_2^{\bullet}$, glutathione (GSH) and ascorbate ($AscH^-$) are most important in the cytosol [19].



$$CO_3^{\bullet -}/NO_2^{\bullet} + GSH \rightarrow CO_3^{2-}NO_2^- + GS^{\bullet} + H^+ \quad (3)$$

The glutathione thiyl radical ($GS^{\bullet}$) is rapidly scavenged by ascorbate, which provides a major radical sink via reaction 4 [20,21].

$$GS^{\bullet} + AscH^- \rightarrow GSH + Asc^{\bullet -} + H^+ \quad (4)$$

Recent work has shown that physiological concentrations of ascorbate increase the synthesis and biological activity of $NO^{\bullet}$ in cultured endothelial cells by increasing intracellular H_4B [22–24]. These observations may explain how supplementation with H_4B or ascorbate can reverse eNOS dysfunction in cardiovascular disease [25, 26]. H_4B appears to have a strong scavenging capacity for biological oxidants including peroxynitrite [27,28], and superoxide [29,30], implying that the pterin is not only an important regulator of NOS and other H_4B cofactor-dependent enzymes, but also may fulfill a role as an intracellular antioxidant [31]. However, the interaction of pterin with key biological radicals generated during NOS dysfunction and general cellular oxidative/nitrosative stress is relatively unexplored. Direct radical scavenging by H_4B and the interaction of the resultant H_4B radicals with other cellular antioxidants will define the antioxidant capability of the pterin.

In this investigation we have used the technique of pulse radiolysis to observe directly the reactions of $^{\bullet}OH$, $NO_2^{\bullet}$, $CO_3^{\bullet -}$ and $GS^{\bullet}$ radicals with H_4B, and the reaction of the resultant pterin radical with ascorbate.

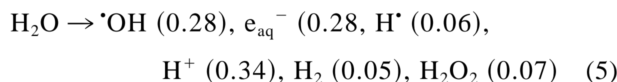
MATERIALS AND METHODS

Chemicals

(6*R*)-5,6,7,8-tetrahydrobiopterin dihydrochloride (H_4B), L-ascorbic acid, glutathione (GSH), sodium azide, sodium nitrite, sodium carbonate, and 2-propanol were obtained from the Sigma-Adrich Chemical Company Ltd, Poole, UK. Phosphate buffers were obtained from BDH Chemicals Ltd, Poole, UK. Prior to irradiation, all solutions were bubbled with zero-grade nitrous oxide (N_2O, oxygen content < 10 ppm) (British Oxygen Company, Kent, UK) from which trace oxygen was removed by an Oxisorb cartridge (Chromatography Services Ltd, Hoylake, UK).

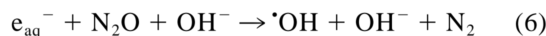
Radiation chemistry

The pulse radiolysis experiments were performed with a 6 MeV linear accelerator as described previously [32]. A pulse of 0.5 μs delivered doses of 2–35 Gy (typically 1–30 μM radicals), as determined by thiocyanate dosimetry [33]. Steady-state irradiations were performed with a ^{60}Co γ-source with a nominal activity of 2000 Ci. The radiolysis of water generates radicals, ions, and other molecular products (The numbers in parenthesis are the approximate radiation chemical yields in $\mu mol\ J^{-1}$.)



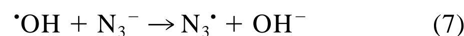
$$H_2O \rightarrow {}^{\bullet}OH\ (0.28),\ e_{aq}^-\ (0.28,\ H^{\bullet}\ (0.06),\ H^+\ (0.34),\ H_2\ (0.05),\ H_2O_2\ (0.07) \quad (5)$$

The azide radical ($N_3^{\bullet}$) was generated by radiolysis of N_2O-saturated solutions of NaN_3 (50 mM) in phosphate buffer (4 mM). The hydrated electron (e_{aq}^-) reacts with N_2O and is converted into the hydroxyl radical ($^{\bullet}OH$), which becomes the main primary product of water radiolysis:



$$e_{aq}^- + N_2O + OH^- \rightarrow {}^{\bullet}OH + OH^- + N_2 \quad (6)$$

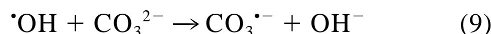
The resultant $^{\bullet}OH$ radical oxidizes the azide anion over the pH 4–9.5 to give the $N_3^{\bullet}$ radical [34]:



$$^{\bullet}OH + N_3^- \rightarrow N_3^{\bullet} + OH^- \quad (7)$$

The carbonate ($CO_3^{\bullet -}$) and nitrogen dioxide ($NO_2^{\bullet}$) radicals were generated by similar means by substituting the sodium azide with either sodium carbonate (20 mM) [35], and sodium nitrite (10 mM) [36].

$$^{\bullet}OH + NO_2^- \rightarrow NO_2^{\bullet} + OH^- \quad (8)$$



$$^{\bullet}OH + CO_3^{2-} \rightarrow CO_3^{\bullet -} + OH^- \quad (9)$$

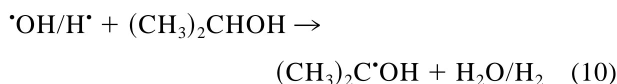
The acid-base equilibrium of the carbonate radical has been a matter of controversy in recent years but recent evidence suggests that $HCO_3^{\bullet}$ radical is a strong acid and is probably unprotonated at pH > 7.5 [37]. The glutathione thiyl radical ($GS^{\bullet}$) was generated by radiolysis of an N_2O-saturated aqueous solution containing 2-propanol (0.2 M) and glutathione (10 mM) following repair of

Table 1. Rate Constants for the Reaction of Biological Oxidants ($R^{\bullet}$) with Tetrahydrobiopterin (H_4B) Compared to Ascorbate ($AscH^-$), and Radiation Chemical Yields (G) for the Loss of the Pterin Relative to that of Oxidizing Radicals

Radical species ($R^{\bullet}$)	$k(R^{\bullet} + H_4B)/M^{-1}\ s^{-1}$	$k(R^{\bullet} + AscH^-)^c/\ M^{-1}\ s^{-1}$	$G(R^{\bullet})/\mu mol\ J^{-1}$	$G(-H_4B)/\mu mol\ J^{-1}$
$N_3^{\bullet}$	$(5.7 \pm 0.2) \times 10^9$	$2.9 - 4.8 \times 10^9$	0.55	0.40 ± 0.1
$^{\bullet}OH$	$^a(8.8 \pm 0.2) \times 10^9$ $^b(1.1 \pm 0.2) \times 10^{10}$	$3.3 - 5.6 \times 10^9$	0.55	0.39 ± 0.1
NO_2^-	$(9.4 \pm 0.1) \times 10^8$	$1.8 - 6.4 \times 10^7$	0.55	0.4 ± 0.1
$CO_3^{\bullet -}$	$(4.6 \pm 0.1) \times 10^9$	$1.1 - 1.4 \times 10^9$	0.61	0.37 ± 0.1
$GS^{\bullet}$	$(1.1 \pm 0.1) \times 10^9$	6×10^8	0.68	<0.01

[a] Absolute value measured directly from the rate of formation of the $H_3B^{\bullet}$ radical.
[b] Rate constant determined by kinetic competition with KSCN.
[c] Rate constants taken from the NRDL-NIST Solutions Kinetics Database ref. [49].

the 2-propanol radical by glutathione via reactions 5, 6, 10, and 11 [38].



$$^{\bullet}OH/H^{\bullet} + (CH_3)_2CHOH \rightarrow (CH_3)_2C^{\bullet}OH + H_2O/H_2 \quad (10)$$

$$(CH_3)_2C^{\bullet}OH + GSH \rightarrow (CH_3)_2CHOH + GS^{\bullet} \quad (11)$$

Under these experimental conditions the radiation chemical yields of radicals are $G(^{\bullet}OH) = G(N_3^{\bullet}) = G(NO_2^{\bullet}) = 0.55\ \mu mol\ J^{-1}$, $G(GS^{\bullet}) = 0.68\ \mu mol\ J^{-1}$, and $G(CO_3^{\bullet -}) = 0.61\ \mu mol\ J^{-1}$. Since H_4B rapidly auto-oxidizes in air [39], all stock solutions of the pterin were gassed separately with N_2O and added immediately prior to radiolysis.

HPLC quantification of H_4B oxidation

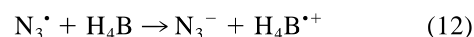
Following steady-state γ-radiolysis the oxidation of the pterin was monitored by HPLC. Separation was achieved on a Hichrom RPB 100 $\times$ 3.2 mm reverse-phase column (Hichrom, Reading, UK) at a flow rate of 1 ml min^{-1}. The solvents and gradients used were A: 5 mM heptane sulphonic acid, 5 mM KH_2PO_4, 5 mM H_3PO_4, and B: 75% acetonitrile, 25% water; gradient: 5–25% B, 5 min. Solvents were degassed with helium. In order to prevent H_4B auto-oxidation, samples were injected onto the HPLC directly from the irradiation syringe through a stainless steel luer needle via a Rheodyne 7120 valve fitted with a 20 μl loop. The solutions were irradiated in airtight syringes at a dose rate of 3.1 Gy min^{-1}, as determined by Fricke dosimetry [40]. Radiation chemical yields (G) in $\mu mol\ J^{-1}$ for the oxidation of H_4B were calculated from the slope of plots of change in concentration versus radiation dose.

RESULTS

Table 1 contains the absolute rate constants for the oxidation of H_4B at physiological pH 7.4 by the azide radical ($N_3^{\bullet}$) plus various biological one-electron oxidants determined by pulse radiolysis. Also included in Table 1 are the corresponding radiation chemical yields for loss of the pterin relative to that of the oxidizing radical determined by steady-state γ-radiolysis and HPLC.

Spectral characteristics and acid-base properties of pterin radicals

The azide radical is a weaker oxidant ($E^o(N_3^{\bullet}/N_3^-) = 1.33$ V) than $^{\bullet}OH$ ($E^o(^{\bullet}OH/OH^-) = 1.9$ V), and is capable of selectively oxidizing pterins by direct electron transfer [34]. The reaction of $N_3^{\bullet}$ radicals with H_4B was followed by observing the changes in absorption in the wavelength range 300–620 nm. Fig. 1A shows the typical absorption spectra of the $H_4B^{\bullet +}$ radical cation and the neutral $H_3B^{\bullet}$ radical generated by one-electron oxidation of H_4B by the $N_3^{\bullet}$ radical. Between pH 7–9.5 the spectrum of the $H_3B^{\bullet}$ radical is characterized by a strong peak ($\varepsilon \sim 6250 \pm 100\ M^{-1}\ cm^{-1}$) near 335 nm and a lower absorption above this, with a tail extending to 610 nm. Similar UV/visible spectra of trihydropterin radicals were previously reported by Armstrong and colleagues following oxidation of synthetic tetrahydropterins by the $N_3^{\bullet}$ radical [41]. For solutions of H_4B at a particular pH, the rate of increase in absorption of the pterin radical obeyed pseudo first-order kinetics (e.g., Fig. 1B) at all wavelengths with similar rates. Figure 1C shows a linear plot of the pseudo first-order rate constants versus H_4B concentration at pH 5, the slope of which gave the rate constant $k_{12} = (5.7 \pm 0.2) \times 10^9\ M^{-1}\ s^{-1}$ consistent with reaction 12.



$$N_3^{\bullet} + H_4B \rightarrow N_3^- + H_4B^{\bullet +} \quad (12)$$

Figure 2 shows the changes in absorbance at 300 nm and 420 nm fitted to the appropriate function giving a $pK_a = 5.2 \pm 0.1$ for the radical cation $H_4B^{\bullet +}$, which rapidly deprotonates at pH $> pK_a$ to give the $H_3B^{\bullet}$ radical:

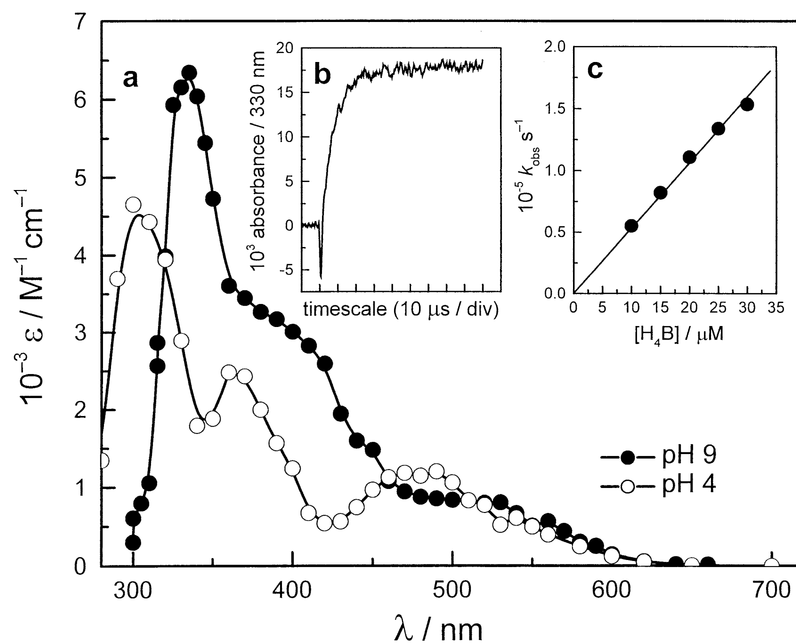


Fig. 1. (A) Spectra of the tetrahydrobiopterin radical cation (○) and the trihydrobipterin radical (●) obtained by pulse radiolysis (3 Gy) of an N_2O-saturated aqueous solution of H_4B (100 μM), NaN_3 (50 mM), sodium phosphate buffer (5 mM) at pH 4 and 9, respectively. The insert (B) is a typical kinetic trace showing the build-up of the $H_3B^{\bullet}$ radical following pulse radiolysis (2.3 Gy) of H_4B (25 μM) at pH 7.4. Insert (C) shows the linear correlation of the observed first-order rate constant versus the concentration of the pterin.

$$H_4B^{\bullet+} \rightleftarrows H_3B^{\bullet} + H^+ \quad (13)$$

In the absence of ascorbate the pterin radicals decayed via second-order kinetics with a half-life which increased with increasing radiation dose or initial concentration of oxidizing radicals ($[N_3^{\bullet}]$ ~ 3–30 μM), indicating that both the $H_4B^{\bullet+}$ and $H_3B^{\bullet}$ radicals undergo disproportionation to regenerate H_4B and probably quinonoid dihydrobiopterin (qH_2B) via reaction 14. The reciprocal of the first half-life of the pterin radicals varied linearly with the initial radical concentration, and from the slope of the fitted straight line, the rate constants $2k_{14} = (9.3 \pm 0.1) \times 10^4\ M^{-1}\ s^{-1}$ at pH 7.4 and $2k_{14} = (6.5 \pm 0.1) \times 10^3\ M^{-1}\ s^{-1}$ at pH 3.5 were obtained for the neutral and protonated pterin radicals, respectively.

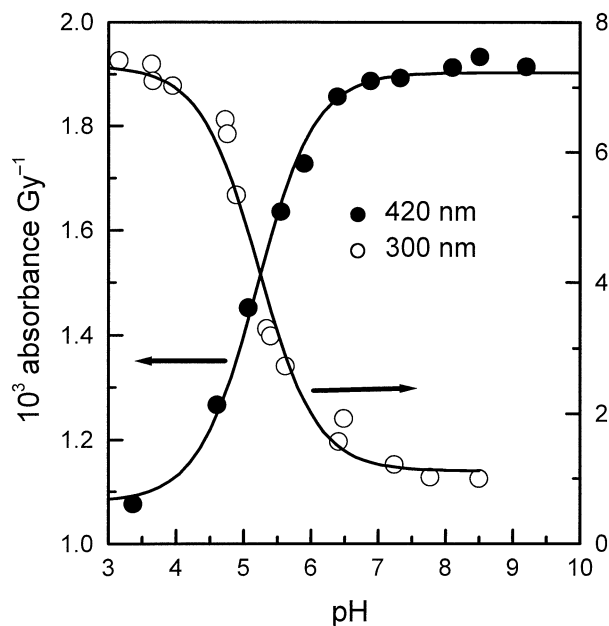
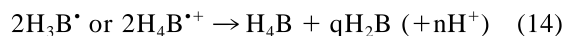


Fig. 2. The change in absorbance recorded at 300 nm (○) and 420 nm (●) between pH 3 and 9.3 following pulse radiolysis (3 Gy) of an N_2O-saturated aqueous solution of H_4B (50 μM), NaN_3 (50 mM), sodium phosphate buffer (5 mM).



$$2H_3B^{\bullet} \text{ or } 2H_4B^{\bullet+} \rightarrow H_4B + qH_2B\ (+nH^+) \quad (14)$$

The inclusion of 0.5 M $NaClO_4$ increased the rate of decay of the $H_4B^{\bullet+}$ radical 2.1-fold to $2k_{14} = (1.4 \pm 0.1) \times 10^4\ M^{-1}\ s^{-1}$. This is consistent with the acceleration of the rate of decay of a species with net charge ± 1 predicted from the Debye-Hückel-Brönsted-Davis equation [42]. In marked contrast, the decay of the $H_3B^{\bullet}$ radical at pH 7.4 was unaffected by increasing the ionic strength of the solution, consistent with an uncharged species. The radiation chemical yield for the loss of the pterin $G(-H_4B) = 0.40 \pm 0.1$ μmol J^{-1}. Of this, 0.07 μmol J^{-1} may arise from two-electron oxidation by H_2O_2 formed by reaction 5, leaving ~0.33 μmol J^{-1}, which is somewhat higher that half $G(N_3^{\bullet}) = 0.55 \pm 0.1$ μmol J^{-1}, the yield predicted from reaction 14.

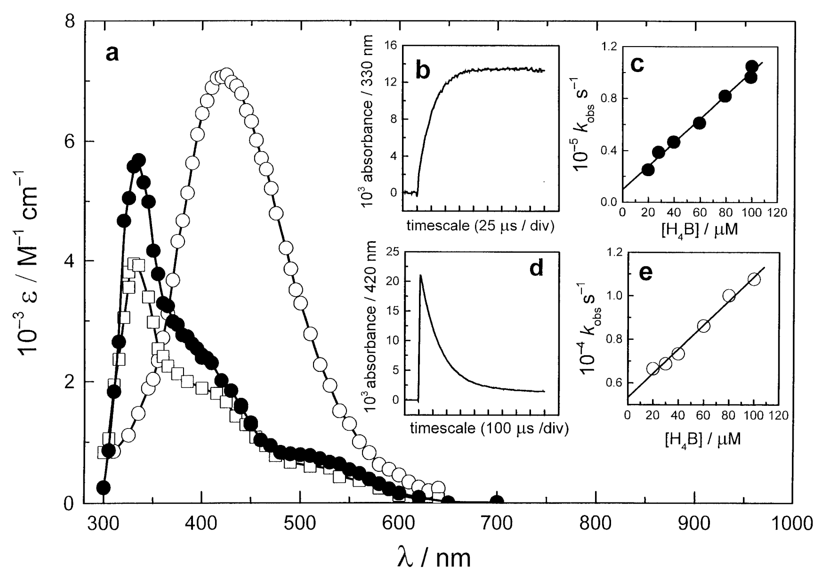


Fig. 3. (A) The spectrum of the trihydrobiopterin radical (●) generated by pulse radiolysis (2 Gy) of an N_2O-saturated aqueous solution of H_4B (100 μM), glutathione (10 mM), 2-propanol (0.2 M), sodium phosphate buffer (5 mM) at pH 6. The spectrum of the disulfide radical anion (○) and the tetrahydrobiopterin radical anion (□) generated by pulse radiolysis (2 Gy) of an N_2O-saturated aqueous solution of H_4B (100 μM), sodium formate (0.2 M), glutathione (10 mM) sodium phosphate buffer (5 mM) at pH 9.2 measured at 50 μs and 250 μs after pulse, respectively. Insert (B) is a typical kinetic trace showing the build-up of the $H_3B^•$ radical following oxidation of the H_4B (28 μM) by the $GS^•$ radical recorded at pH 7.4 and insert (C) shows the corresponding linear correlation of the observed first-order rate constant versus the concentration of the pterin (20–100 μM). Insert (D) shows a typical kinetic trace showing the decay of the disulfide radical anion in the presence of H_4B (30 μM) under similar experimental conditions described in (A) and insert (E) shows the corresponding linear correlation of the observed first-order rate constant versus the concentration of the pterin (20–100 μM).

Oxidation of H_4B by the glutathione thiyl radical and reduction by the glutathione disulfide radical anion

Figure 3A (●) shows the spectrum obtained when H_4B is oxidized by the $GS^•$ radical and Fig. 3B a typical kinetic trace recorded at 330 nm. The spectral similarities with Fig. 1A for oxidation of H_4B by the $N_3^•$ radical indicates that scavenging of the $GS^•$ radical by H_4B generates the $H_3B^•$ radical according to reaction 15.

$$GS^• + H_4B \rightarrow GSH + H_3B^• \qquad (15)$$

At pH 4 the $GS^•$ radical gives the $H_4B^{•+}$ radical cation suggesting that at physiological pH, interaction with the pterin involves electron transfer followed by deprotonation rather than hydrogen atom transfer to form the $H_3B^•$ radical directly. Figure 3C shows a linear plot of the pseudo first-order rate constants versus H_4B concentration at pH 6, the slope of which gave the rate constant $k_{15} = (1.1 \pm 0.1) \times 10^9\ M^{-1}\ s^{-1}$ for reaction 15. However, the yield of $GS^•$ and therefore reaction 15 decreases above pH 6 due to the competing equilibrium reaction 16 coupled to the prototropic equilibrium reaction 17 [43].

$$GS^• + GS^- \rightleftarrows GSSG^{•-} \qquad (16/-16)$$

$$GSH \rightleftarrows GS^- + H^+ \qquad (pK_{17} = 9.2) \qquad (17)$$

Whereas thiyl radicals are moderately strong oxidants [$E^o(RS^• + H^+/RSH) = 1.3$ V (vs. NHE)], the conjugated $RSSR^{•-}$ intermediate is an extremely powerful reductant [$E^o(RSSR/RSSR^{•-}) = -1.6$ V] [44]. Previous work demonstrated that pterin and pterin-6-carboxylate ions are rapidly reduced to pterin radicals by the model one-electron reductant the formate radical ($CO_2^{•-}$) [45], which has similar reducing properties to $RSSR^{•-}$. Following pulse radiolysis of an N_2O-saturated solution containing 0.2 M HCOONa, 10mM GSH, 4 mM phosphate buffer at pH 9.2 the majority of $GS^•$ radicals produced are rapidly converted to the $GSSG^{•-}$ radical anion ($\varepsilon_{420nm} \sim 8000\ M^{-1}\ cm^{-1}$) which is produced in high yield $G(GS^•) \sim G(GSSG^{•-}) = 0.6\ \mu mol\ J^{-1}$ (see Fig. 3A (○)) [46]. Under these experimental conditions the half-life of the $GS^•$ radical in the presence of the glutathione anion is extremely short $t_{1/2} = 0.7/(k_{16}[GS^-] + k_{-16}) \sim 0.1$ μs and even in the presence of 50 μM H_4B easily out-competes reaction 14 with a half-life of $t_{1/2} = 0.7/k_{15}[H_4B] \sim 14$ μs. Figure 3A (□) shows a typical spectrum of the radical obtained on the reduction of H_4B by the $GSSG^{•-}$, which is ascribed to the tetrahydrobiopterin radical anion ($H_4B^{•-}$) formed via reaction 18.

$$GSSG^{•-} + H_4B \rightarrow GSSG + H_4B^{•-} \qquad (18)$$

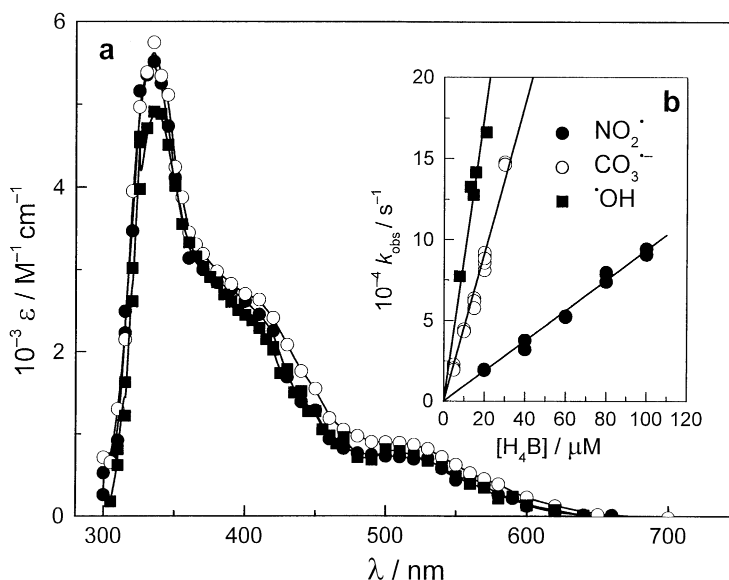
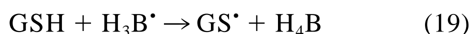


Fig. 4. Spectra of the trihydrobiopterin radical obtained on the oxidation of H_4B by the $^{\bullet}OH$ radical (■), $NO_2^{\bullet}$ radical (●) and $CO_3^{\bullet -}$ radical (○). All spectra were determined by pulse radiolysis (2.5 Gy) of N_2O-saturated solutions containing the pterin (100 μM), potassium phosphate buffer (5 mM) at pH 7.4. The $NO_2^{\bullet}$ and $CO_3^{\bullet -}$ radicals were generated by introducing either $NaNO_2$ (10 mM) or Na_2CO_3 (10 mM) as described in the experimental. The insert (B) shows the corresponding linear correlation of the observed first-order rate constant for the build-up of the trihydrobiopterin radical at 340 nm versus the concentration of the pterin (20–100 μM) for each of the radical oxidants.

Figure 3E shows a linear plot of the pseudo first-order rate constant for the decay of the $GSSG^{\bullet -}$ radical anion (Fig. 3D) versus H_4B concentration at pH 9.2, the slope of which gave the rate constant $k_{18} = (4.5 \pm 0.1) \times 10^8$ M^{-1} s^{-1} for reaction 18.

Despite the fact that the $GS^{\bullet}$ radical rapidly oxidizes H_4B via reaction 15, steady-state γ-radiolysis experiments revealed that GSH protected H_4B from oxidation (see Table 1). However, pulse radiolysis did indicate that the decay of the $H_3B^{\bullet}$ radical was unaffected by the concentration of GSH (5–20 mM) ruling out fast 'repair' of the pterin radical by the thiol.

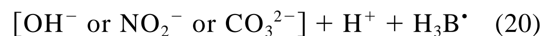


$$GSH + H_3B^{\bullet} \rightarrow GS^{\bullet} + H_4B \qquad (19)$$

It is more likely that GSH protects H_4B from oxidation by reducing an intermediate product(s), for example qH_2B formed in reaction 14, back to pterin. The ability of thiols to convert qH_2B to H_4B is a commonly used strategy for preventing H_4B auto-oxidation [47].

Oxidation of H_4B by hydroxyl, nitrogen dioxide, and carbonate radicals

Oxidation of H_4B at pH 7.4 by $^{\bullet}OH$, $NO_2^{\bullet}$, or $CO_3^{\bullet -}$ radicals gave spectra indicative of $H_3B^{\bullet}$ radical formation (see Fig. 4A). Even the $^{\bullet}OH$ radical, which reacts by addition with most biomolecules, gave the same spectrum. In all cases the absolute rate constants for oxidation of H_4B via reaction 20 were determined directly by monitoring the exponential build-up in absorbance ~ 350 nm ascribed to the $H_3B^{\bullet}$ radical with H_4B concentration (5–100 μM) shown in Fig. 4B. As before the absolute rate constants for radical scavenging were determined from the slopes of the linear plots of the observed rate constant versus concentration of H_4B (see Fig. 4B) and are displayed in Table 1.



$$[^{\bullet}OH \text{ or } NO_2^{\bullet} \text{ or } CO_3^{\bullet -}] + H_4B \rightarrow [OH^- \text{ or } NO_2^- \text{ or } CO_3^{2-}] + H^+ + H_3B^{\bullet} \qquad (20)$$

The absolute rate constant for $^{\bullet}OH$ attack on H_4B was also determined indirectly by kinetic-competition with thiocyanate ion using the rate constant $k(^{\bullet}OH + SCN^-) = 1.1 \times 10^{10}$ M^{-1} s^{-1} from the literature [48]. However, the resultant thiocyanate radical anion ($SCN_2^{\bullet -}$) also reacted with H_4B and the absolute rate constant determined directly $k_{20}(^{\bullet}OH + H_4B) = (8.8 \pm 0.2) \times 10^9$ M^{-1} s^{-1} is therefore favored over the higher value determined by competition kinetics. As shown in Table 1 all the radicals reacted significantly faster with the pterin than with ascorbate. The radiation chemical yields in Table 1 for the oxidation of the pterin by $^{\bullet}OH$, $NO_2^{\bullet}$ and $CO_3^{\bullet -}$ radicals were approximately equal to that of the $N_3^{\bullet}$ radical reflecting similar yields of the $H_3B^{\bullet}$ radical which undergo disproportionation via reaction 14.

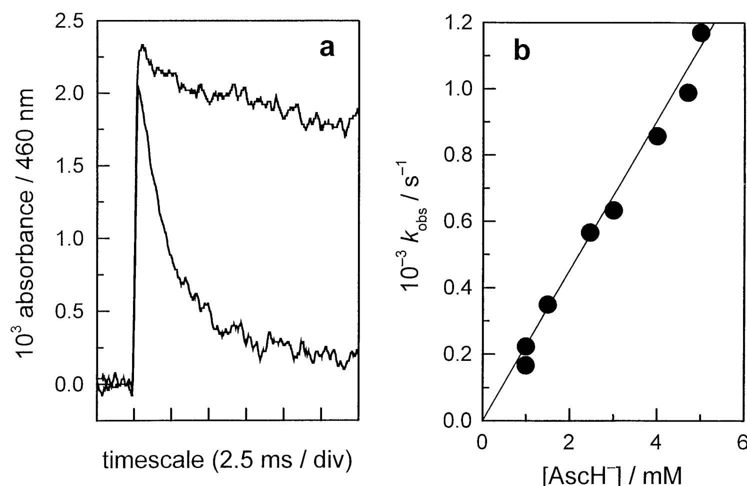


Fig. 5. (A) shows two kinetic traces showing the decay of the trihydrobiopterin radical in the absence and presence of the ascorbate. The upper trace was recorded by pulse radiolysis (~ 1 Gy) of an N_2O-saturated solution containing H_4B (10 mM), $NaNO_2$ (50 mM) and potassium phosphate buffer (5 mM) at pH 9.3. The lower trace shows the decay of the tetrahydrobiopterin radical in the presence of ascorbate (1 mM). The right panel (B) shows the increase in the observed rate of decay of the tetrahydrobiopterin radical with increasing concentration of ascorbate.

Scavenging of the trihydrobiopterin radical by ascorbate

Ascorbate is capable of stabilizing H_4B in biological systems. One possible mechanism by which intracellular ascorbate stimulates NOS activity is regeneration of H_4B from the trihydrobiopterin radical via reaction 21.

$$H_3B^{\bullet} + AscH^{-} \rightarrow H_4B + Asc^{\bullet -} \qquad (21)$$

In order to determine the rate constant for reaction 21 the decay of the $H_3B^{\bullet}$ radical was studied in the absence and presence of ascorbate. The $NO_2^{\bullet}$ radical was the chosen oxidant to generate the $H_3B^{\bullet}$ radical since $NO_2^{\bullet}$ reacts with H_4B over an order of magnitude faster than ascorbate (see Table 1). Pulse radiolysis was performed with N_2O-saturated solutions containing H_4B (10 mM), $NaNO_2$ (20 mM), potassium phosphate buffer (4 mM), and ascorbate (0–5 mM). Under these experimental conditions $<$ 5% of the $NO_2^{\bullet}$ radicals generated react with ascorbate and the $H_3B^{\bullet}$ radical is produced with equivalent yield to the $NO_2^{\bullet}$ radical even in the presence of 5 mM $AscH^{-}$. At pH 9.2 the $H_3B^{\bullet}$ radical decays by second-order kinetics reflecting the disproportionation reaction 14. Figure 5A shows typical kinetic traces showing the decay of the $H_3B^{\bullet}$ radical in the absence and presence of ascorbate. A low dose per pulse (2 Gy producing ~ 1.1 μM $H_3B^{\bullet}$ radicals) was used to minimize the decay of the $H_3B^{\bullet}$ radicals via reaction 14. In the absence of ascorbate the half-life of the pterin radical was $t_{1/2} = 1/2k_{14}[H_3B^{\bullet}] \sim 9.8$ s. In the presence of ascorbate (1 mM) the decay of the $H_3B^{\bullet}$ changes to first-order (lower kinetic trace in Fig. 5A), reflecting scavenging of the pterin radical by ascorbate via reaction 21. Figure 5B shows the linear plot of the observed rate constant versus the concentration of ascorbate (0.5–5 mM), the slope of which yields $k_{21}(H_3B^{\bullet} + AscH^{-}) \sim 1.7 \times 10^5$ M^{-1} s^{-1} at pH 9.2. The decay of the $H_3B^{\bullet}$ radical at 360 nm resulted in an increase in absorbance at the same wavelength ascribed to the formation of the $Asc^{\bullet -}$ radical via reaction 21. Since both the $H_3B^{\bullet}$ and $Asc^{\bullet -}$ radicals absorb at 360 nm a longer wavelength was favored for kinetic measurements.

DISCUSSION

This study has demonstrated that H_4B is a potent scavenger of radical species generated during oxidative stress and NOS dysfunction. The oxidants $N_3^{\bullet}$, $^{\bullet}OH$, $GS^{\bullet}$, $NO_2^{\bullet}$ and $CO_3^{\bullet -}$ radicals all generate the $H_3B^{\bullet}$ radical at physiological pH. To emphasize the potent radical scavenging ability of H_4B one needs to compare the measured rate constants from this study with the corresponding rate constants for scavenging of these radicals by ascorbate displayed in Table 1. The latter are taken from compilations of rate constants available from the NDRL Radiation Chemistry Data Center [49]. A recent electrochemical study has indicated that the pterin may well be more easily oxidized [$E^{o}(H_4B/H_4B^{\bullet +}) = +0.27$ V vs. NHE] [50] than ascorbate [$E^{o}(AscH^{-}/H^{+}, Asc^{\bullet}) = +0.33$ V vs. NHE] [51]. For all radical oxidants the rate of scavenging by H_4B is faster than with ascorbate. The most dramatic difference is for the $NO_2^{\bullet}$ radical, which oxidizes the pterin over an order of magnitude faster than ascorbate. Using EPR-kinetic analysis Vásquez-Vivar and colleagues determined the rate constant for the re-

action of superoxide with H_4B to be $k = (3.9 \pm 0.2) \times 10^5\ M^{-1}\ s^{-1}$ at pH 7.4, a value similar to that of superoxide with ascorbate [52].

However, the ability of H_4B to act as an antioxidant will depend on the relative concentrations of the pterin to other low molecular weight antioxidants such as ascorbate and GSH. For example, the typical concentration of H_4B in endothelial cells is $\sim 7\ \mu M$ [53], the half-life for the reaction of the $NO_2^{\bullet}$ radical at this concentration is $t_{1/2} = 0.7/k_{20}[H_4B] \sim 106\ \mu s$. The corresponding half-life of the $NO_2^{\bullet}$ radical in the presence of a physiologically relevant concentration of ascorbate ($\sim 100\ \mu M$) is estimated to be is $t_{1/2} = 0.7/k[AscH^-] \sim 5\ \mu s$, suggesting that ascorbate is capable of protecting against the oxidation of H_4B by directly competing for oxidizing radical species. We have also shown that ascorbate may also protect H_4B indirectly by 'repairing' the $H_3B^{\bullet}$ radical. These conclusions are supported by recent work showing that physiological concentrations of ascorbate increase the synthesis and biological activity of nitric oxide in cultured endothelial cells by increasing intracellular H_4B [22–24]. Other cell types including MCF-7 breast tumor cells and MOLT-4 human T-cell leukemia contain similar quantities of H_4B as HUVECS, typically 5–10 μM [54]. However, it should be noted that higher levels of H_4B will be present in cells stimulated by cytokines where a higher concentration of the pterin cofactor is required to support NOS activity [55]. It is argued that in cells devoid of ascorbate the pterin substitutes as the sink for radicals that would otherwise be intercepted by ascorbate. In the absence of ascorbate, glutathione (~ 2 mM) will probably play a central role in scavenging radicals, e.g., the half-life of the $NO_2^{\bullet}$ radical is likely to be $t_{1/2} = 0.7/k[GSH] \sim 25\ \mu s$. The resultant $GS^{\bullet}$ radicals rapidly oxidize H_4B with a rate constant of $k_{15} = (1.1 \pm 0.1) \times 10^9\ M^{-1}\ s^{-1}$. Interestingly, recent work has demonstrated that unlike ascorbate, GSH does not appear to be an important determinant of endothelial cell H_4B content [23]. This is in contrast to work by Hofmann and Schmidt, which showed that GSH can influence NOS kinetics and therefore $NO^{\bullet}$ production by recycling or preventing the auto-oxidation of the pterin cofactor [56]. The conjugation of $GS^{\bullet}$ with the GS^- to form the $GSSG^{\bullet -}$ radical anion is recognized as an important reaction in biology since the reaction with oxygen sources $O_2^{\bullet -}$radicals.

$$GSSG^{\bullet -} + O_2 \rightarrow GSSG + O_2^{\bullet -} \qquad (22)$$

The pterin is also rapidly reduced by the $GSSG^{\bullet -}$ radical anion to produce the $H_4B^{\bullet -}$ radical anion, $k_{18} = (4.5 \pm 0.1) \times 10^8\ M^{-1}\ s^{-1}$. This rate is similar to $k_{22} \sim 5.1 \times 10^8\ M^{-1}\ s^{-1}$ previously reported by Prütz and colleagues [46]. The biological significance of reaction 18 and the fate of the $H_4B^{\bullet -}$ radical anion in cells remains to be elucidated but may prove an important reaction in hypoxic environments.

There is increasing evidence to support a role of H_4B as a one-electron donor in NOS catalysis [1–3]. The actual protonation state of the resultant pterin radical is a matter for debate but this study has demonstrated that the $H_4B^{\bullet +}$ radical cation rapidly deprotonates to the neutral $H_3B^{\bullet}$ radical at physiological pH.

In conclusion, this work contains the rate constants for the scavenging of key biological radicals with the enzyme cofactor H_4B. The high scavenging efficiency of H_4B compared to ascorbate supports the role of H_4B as a significant biological antioxidant.

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