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AN ELECTRON SPIN RESONANCE STUDY OF A NOVEL RADICAL CATION PRODUCED DURING THE HORSERADISH PEROXIDASE-CATALYZED OXIDATION OF TETRAMETHYLHYDRAZINE

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Summary. The one-electron oxidation of tetramethylhydrazine by the horseradish peroxidase/hydrogen peroxide system forms a novel stable radical cation. The steady-state concentration of the tetramethylhydrazine radical cation increases with the hydrazine concentration, but depends on neither the enzyme concentration nor the hydrogen peroxide concentration. A mechanism involving the enzymatic one-electron oxidation of the tetramethylhydrazine radical cation to a dication is proposed to account for the enzyme independency. Incubations containing horseradish peroxidase, hydrogen peroxide and tetramethylhydrazine produced formaldehyde which was found to increase with either HRP or tetramethylhydrazine concentration. The cation radical, the dication, and the imine (the deprotonated dication) are proposed as intermediates in the mechanism of formaldehyde production.

Introduction.

Historically, radical cations were proposed as one-electron intermediates in the HRP-catalyzed peroxidations of aromatic nitrogenous hydrogen donors such as aromatic amines and benzidines on the basis of their color chemistry (1). ESR spectral evidence exists for the formation of radical cations during the HRP-catalyzed peroxidation of the nitrogenous substrates: aminopyrine (2), chlorpromazine (3), and N,N'-dimethyl-p-toluidine (4). The persistence of these N-substituted radical cations is apparently due to resonance stablization and the absence of α-

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Abbreviations: HRP, horseradish peroxidase; ESR, electron spin resonance; TMH, tetramethylhydrazine; TMH^{+}, tetramethylhydrazine radical cation; TMH^{++}, tetramethylhydrazine dication.

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hydrogens. The formation of dications or the related deprotonated iminium cations following one-electron oxidation of the substrate radical cation has been postulated in peroxidizing systems containing aminopyrine (2), N,N'-dimethylaniline (4), and chloropromazine (3) as substrates. Formaldehyde production during the HRP-catalyzed oxidation of aminopyrine (2) and N,N'-dimethylaniline (4) is thought to result from hydrolysis of the iminium cation. In this communication, we report the detection of a novel radical cation during the enzymatic one-electron oxidation of the carcinogen tetramethylhydrazine (5) by HRP and H_2O_2. The TMH radical cation is itself a substrate, and it is suggested that enzymatic one-electron oxidation of the TMH radical cation gives a diamagnetic TMH dication, which subsequently deprotonates to form an iminium cation. The production of formaldehyde during the peroxidation of TMH is attributed to the hydrolysis of iminium cation.

Methods. HRP (type VI) was purchased from Sigma Chemical Company. TMH was supplied by Isotope Chemicals. The ESR measurements were carried out on a Varian E-109 spectrometer operating at 100 kHz, equipped with an E-238 TM_{110} cavity (6). Hyperfine coupling constants were measured to ± 0.1G, so second-order corrections were neglected. The kinetic ESR measurements were made using a field-frequency locking device. Heat-denaturation of the HRP was accomplished by heating the HRP in boiling acetate-acetic acid buffer (pH 5.4) for 30-40 minutes. The heat-denatured HRP regained activity if left at room temperature for several hours, therefore the HRP was used soon after boiling. The formaldehyde formation was determined according to the method of Nash (7). A typical incubation for the Nash assay (7) consisted of TMH, HRP and hydrogen peroxide in a 2 ml acetic acid-acetate buffer (pH 5.4). The reaction was initiated by the addition of HRP and run in a Dubnoff incubator with shaking for 5 min at 37°C. Several experiments were performed where the concentration of TMH and HRP were varied. The appropriate blanks were run simultaneously. The reactions were terminated by the addition of 0.7 ml of zinc sulfate (15% solution), followed by 0.7 ml of saturated barium hydroxide.

Results.

The addition of HRP to a solution containing TMH and H_2O_2 forms a free radical characterized by an ESR spectrum of fifteen equally-spaced groups of lines (Fig. 1A). The ESR spectrum was recognized as that of the radical cation of TMH, based on previous reports on the chemical (8, 9) and the electrochemical (10) oxidation of TMH in water and organic solvents. The TMH radical cation possesses a "three-electron π bond" between the two nitrogens and is inherently stable due to favorable electronic interactions (11). The spectrum of the TMH radical cation shows electronic-nuclear hyperfine coupling constants of two equivalent nitrogens (a_N=13.5 G) and twelve equivalent protons (a_H=12.7 G) in agreement with the

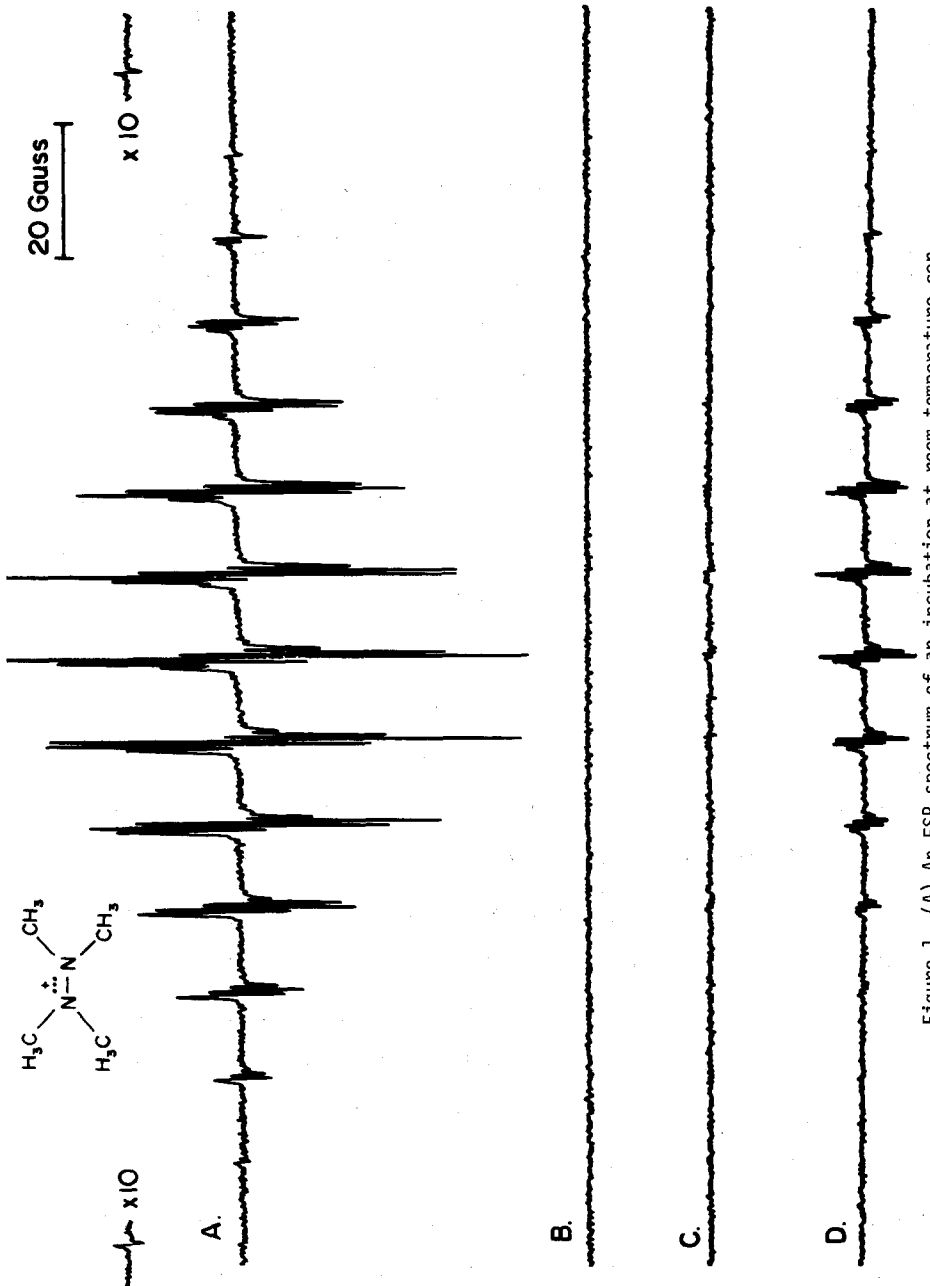


Figure 1. (A) An ESR spectrum of an incubation at room temperature containing 4.4 mM TMH, 42 μM H_2O_2 and 50 μg HRP in 2 ml of acetic acid-acetate buffer (pH 5.4). The reaction was initiated upon the addition of HRP. The ESR spectrometer conditions were as follows for all spectra.

Scan range 200 G	Modulation amplitude 0.02 G
Time constant 0.25 s	spectrometer gain 6.3×10^3
Scan time 16 min	microwave power 20 mW

(B). As in (A) except the HRP was omitted. (C). As in (A) except that H_2O_2 was omitted. (D). As in (A) except that prior to its addition the HRP was denatured as described under Methods.

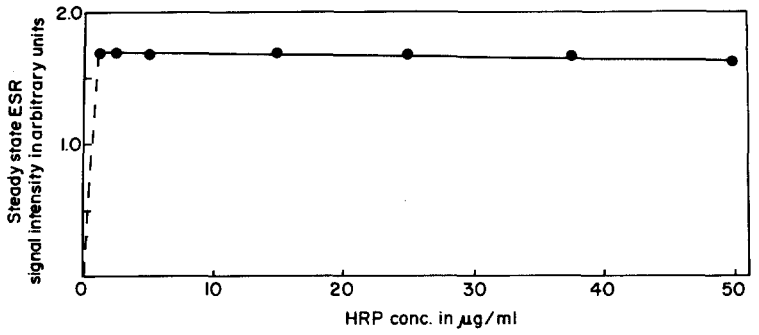


Figure 2. The steady-state concentration of the TMH radical cation versus the concentration of HRP. The incubation mixture consisted of 4 mM TMH, 42 μM H_2O_2 and HRP as indicated in acetate-acetic acid buffer (pH 5.4). The time-dependence of the radical's concentration was monitored by sitting on the center line of the ESR spectrum with the magnetic field scan off until the steady-state concentration was obtained, which required several minutes. The field-frequency lock was used to obtain the time-dependent spectra. ESR spectrometer conditions were as follows.

Time constant 0.25 s	Modulation amplitude 0.02 G
Scan time 16 min	Spectrometer gain 8.0×10^2

literature values reported in water (9). The appearance of the ESR spectrum is dependent on the enzyme as well as H_2O_2 (Figures 1B and 1C). Heat-denaturization of the enzyme significantly decreased the intensity of the TMH radical cation ESR spectrum (Fig. 1D).

The steady-state concentration of the TMH radical cation was found to be independent of the concentration of HRP (Fig. 2). This result is in contrast to the peroxidase-catalyzed substrate oxidations in which the radical intermediates decay by second-order kinetics due to dismutation, and the steady-state radical concentration is proportional to $[HRP]^{\frac{1}{2}}$ (12, 13). However, in the peroxidase-catalyzed oxidation of chlorpromazine, the dismutation reaction of the chlorpromazine cation radical was found to be slow (3), and under some conditions the free radical decayed mainly via the HRP-catalyzed one-electron oxidation of the chlorpromazine cation radical (3). It appears that a similar mechanism operates in the present case. Since the TMH radical cation is inherently very stable, the subsequent peroxidase-catalyzed oxidation of the radical cation to a dication is a reasonable decay mechanism (see scheme in the discussion). Moreover, cyclic voltametric studies have demonstrated the electrochemical one-electron oxidation of the TMH radical cation to an unstable dication (10). Hence, the steady-state concentration of the radical cation intermediate is dependent on

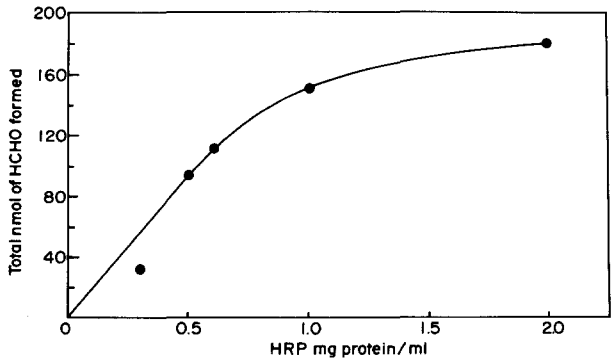


Figure 3. The total amount of formaldehyde formed as described in the Methods versus the amount of HRP added. The incubation mixture consisted of 4 mM TMH, 330 μM H_2O_2 and varying amounts of HRP in acetate-acetic acid buffer (pH 5.4).

the rate of peroxidase-catalyzed formation of the TMH radical cation, as well as the rate of peroxidase-catalyzed decay of the radical cation to a dication. At a concentration of 25 μg/ml HRP, the steady-state concentration of the TMH radical cation increased linearly with TMH concentration up to 10 mM. In identical incubations formaldehyde production was also found to increase with HRP concentration (Fig. 3) and TMH concentration (Fig. 4), as is expected for a product. Similar findings have been reported for the peroxidase-catalyzed oxidation of N,N'-dimethylaniline (4) and aminopyrine (2), where the formaldehyde formation was attributed to N-demethylation of the respective substrate.

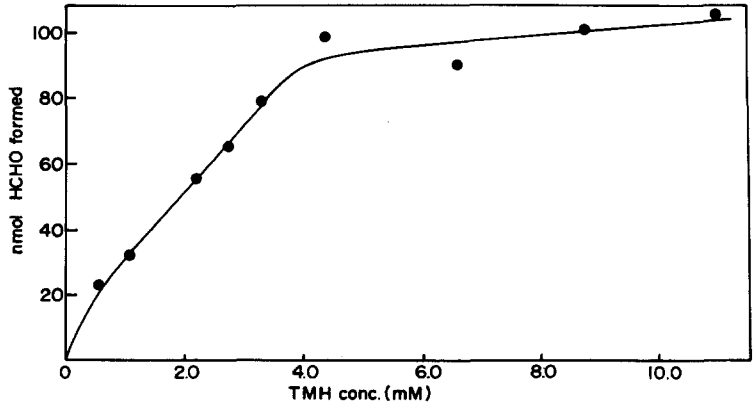
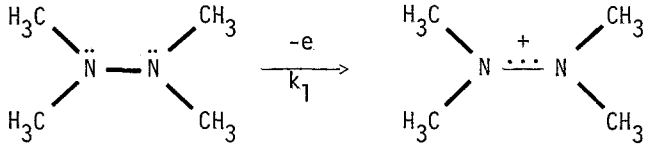


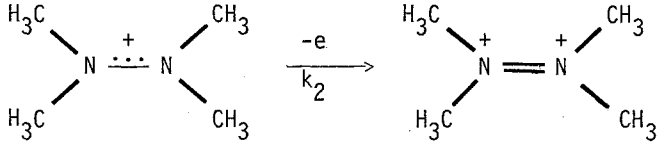
Figure 4. The total amount of formaldehyde formed as described in the Methods versus the amount of TMH added. The incubation mixtures consisted of 0.5 mg HRP, 330 μM H_2O_2 and varying amounts of TMH in acetate-acetic acid buffer (pH 5.4).

Discussion.

A scheme for the production and decay of the TMH radical cation can be formulated as follows, where, for an enzymatic reaction, the rate constants k_1 and k_2 may depend upon the substrate concentration. In Michaelis-Menten kinetics the rate constant k_1 would equal $V_{max}/(K_m + [TMH])$.



(1)



(2)

The rate of formation of the radical cation will then equal k_1 [HRP] [TMH], and the rate of decay of the radical cation will equal k_2 [HRP] [$TMH^{+\cdot}$] where [HRP], [TMH] and [$TMH^{+\cdot}$] denote the respective concentrations. At the steady state, the rate of radical formation is equal to the rate of radical decay.

$$k_1 [HRP] [TMH] = k_2 [HRP] [TMH^{+\cdot}]_{ss} \quad (3)$$

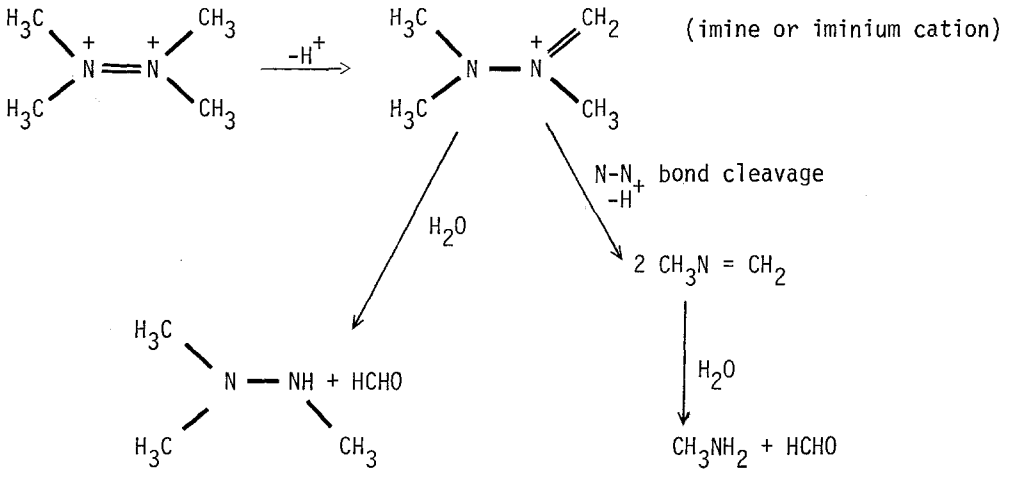
or

$$[TMH^{+\cdot}]_{ss} = \frac{k_1}{k_2} [TMH]$$

Equation (3) shows how the steady-state radical concentration can be dependent on the substrate concentration, but independent of the HRP concentration, as first indicated by Yamazaki and Piette (12). This derivation assumes that the disproportionation of the TMH radical cation (to the TMH dication and TMH) is slow, as has been observed in aprotic solvents (14), and that H_2O_2 is not limiting. In fact, the steady state [$TMH^{+\cdot}$] was invariant to [H_2O_2] over the range of 5-80 μM. These results are consistent with both TMH and $TMH^{+\cdot}$ being typical substrates for the HRP/H_2O_2 system (12,13).

In the earlier reports, it has been shown that the peroxidase-dependent N-demethylation reaction necessarily involves the intermediate radical cation and

the iminium cation (2, 4). Electrochemical studies on the oxidation of TMH have shown that the removal of the formally antibonding π electron in the TMH radical cation is fairly easy, and imply the formation of the TMH dication (10). The dication, with two adjacent positive charges, was found to be unstable and may decay via deprotonation to give a cationic imine intermediate with subsequent formation of N-methylene methylamine (10). In view of this work, two similar mechanistic schemes can be postulated to account for formaldehyde formation.



An imine intermediate in the formation of formaldehyde has also been proposed during the microsomal oxidation of 1,1-dimethylhydrazine by FAD-containing monooxygenase (15). As is indicated in the scheme, several other products are possible besides formaldehyde. In order to prove that the TMH radical cation is an obligatory intermediate in the production of formaldehyde, stoichiometric rate measurements of radical and formaldehyde formation will need to be carried out.

Both optical and ESR studies have provided evidence for the HRP-catalyzed one-electron (12, 13) and two-electron (16,17) oxidation of a wide variety of substrates. This investigation demonstrates for the first time that a hydrazine compound may be oxidized by the HRP/H_2O_2 system to a radical cation. It should be noted that hydrazine itself does not act as an electron donor for the HRP/H_2O_2 system (18). In fact, a reaction between hydrazine and the HRP apoprotein leads to the irreversible inhibition of HRP (19). Whether TMH reacts with HRP in this

manner is unknown, but tetramethyl-substitution of hydrazine clearly leads to an active substrate of the HRP/H_2O_2 system. Since the peroxidatic activity of the HRP/H_2O_2 system is often predictive of oxidation by mammalian peroxidases, it is possible that this free radical metabolism of TMH may be involved in its tumorigenicity, where methyl substitution also plays a role (5).

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