MOLECULAR PHARMACOLOGY, 23:766–770

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# An Electron Spin Resonance Study of the Activation of Benzidine by Peroxidases

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Received September 23, 1982; Accepted December 22, 1982

SUMMARY

The oxidation of benzidine, a carcinogenic aromatic amine, by $H_2O_2$ is catalyzed by horseradish peroxidase or lactoperoxidase. The resulting cation free radical is moderately stable at pH 5.0, and was identified by electron spin resonance spectroscopy. Two-electron oxidation yields the benzidine di-imine. This species reacts with phenol or catechol derivatives to give colored adducts. Monoacetylbenzidine is a relatively poor peroxidase substrate, and the biological implications of this difference are discussed.

## INTRODUCTION

Occupational exposure to benzidine resulted in greatly increased rates of bladder carcinoma among workers in the dyestuff industry (1). Benzidine-based azo dyes are reductively cleaved by intestinal anaerobic bacteria (2); this metabolism may constitute a previously unrecognized route of exposure to benzidine in the human population. The carcinogenicity of benzidine and other aromatic amines is probably a consequence of their metabolism to reactive species capable of binding to cellular macromolecules, particularly DNA. The possible role of peroxidases in the activation of chemical carcinogens is receiving increasing attention. Of the various proteins that possess peroxidative activity, horseradish peroxidase is the best-studied. Lactoperoxidase, an enzyme obtained from mammalian milk, is similar to horseradish peroxidase in physical properties and chemical activities. In addition, the microsomal enzyme prostaglandin synthase, which initiates the biosynthesis of prostaglandins from arachidonic acid, catalyzes the co-oxidation of xenobiotics via a peroxidase-like reaction (3). This enzyme catalyzes both the formation of a hydroperoxide (prostaglandin $G_2$) and the peroxidative reduction of prostaglandin $G_2$ to prostaglandin $H_2$.

Zenser and colleagues (4, 5) have demonstrated that benzidine is metabolized by rabbit renal medullary microsomal preparations. This metabolism is arachidonate-dependent and indomethacin-sensitive, suggesting the involvement of prostaglandin synthase. In the presence of exogenous nucleic acids, the microsomal system catalyzes the formation of benzidine metabolites that bind to macromolecules (5). Kadlubar *et al.* (6) have studied the prostaglandin synthase-catalyzed binding of radiolabeled aromatic amine carcinogens to DNA. Benzidine was the most reactive substrate examined, and gave more than 300 times as much bound product as did 2-aminofluorene, itself a potent carcinogen. However, the chemical nature of the metabolites formed by the prostaglandin synthase system has not been determined.

Benzidine is a well-known substrate for peroxidases. Indeed, the peroxidative activity of blood is the basis for the so-called "benzidine test": the presence of blood is revealed by the oxidation of benzidine to a blue product (7). The carcinogenic hazard presented by the use of benzidine in the blood test prompted its replacement by the noncarcinogenic derivative 3,5,3′5′-tetramethylbenzidine (7). We have studied the oxidation of 3,5,3′,5′-tetramethylbenzidine by horseradish peroxidase/$H_2O_2$ (8) and by prostaglandin synthase/arachidonic acid (9) and have demonstrated the formation of a cation free radical in both systems. In the present paper, we examine the oxidation of benzidine itself, using horseradish peroxidase and lactoperoxidase.

## MATERIALS AND METHODS

Benzidine dihydrochloride was obtained from Sigma Chemical Company (St. Louis, Mo.) in ISOPAC containers, the contents of which were dissolved in $H_2O$ or $^2H_2O$ as required. $H_2O_2$ (ACS grade) was obtained from Fisher Scientific Company (Pittsburgh, Pa.). Horseradish peroxidase (Type VI), lactoperoxidase (bovine milk), BHA,[4] guaiacol, epinephrine, serotonin, and 2,7-diaminofluorene were obtained from Sigma Chemical Company. Monoacetylbenzidine was obtained from ICN Pharmaceuticals, Inc. (Plainview, N. Y.) and recrystallized as recommended by Martin *et al.* (10).

Optical experiments were performed using an Aminco DW-2A spectrophotometer. ESR experiments were performed using a Varian E-104 spectrometer equipped with a $TM_{110}$ cavity and aqueous flat cell; the cell was filled using a modified Gilford rapid sampler.

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[4] The abbreviations used are: BHA, butylated hydroxyanisole; HPLC, high-pressure liquid chromatography.

0026-895X/83/030766-05$02.00/0
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The rates of oxidation of benzidine and monoacetylbenzidine by horseradish peroxidase were determined by HPLC. Aliquots of reaction mixtures were stopped by addition of an equal volume of 1 N HCl, and a sample was injected into the chromatograph. A Waters Radial-Pak C-18 column was eluted with methanol/10 mM phosphate buffer, pH 6.0 (50:50) at a flow rate of 5.5 ml/min. Substrates were detected by UV absorption at 280 nm; peak height was linear in concentration, over the range studied.

## RESULTS

We observed a two-stage color change upon oxidation of 3,5,3′5′-tetramethylbenzidine by horseradish peroxidase/$H_2O_2$ (8). A similar sequence of color changes was seen with benzidine; however, its oxidized products were less stable, and decomposed to a polymeric precipitate in a few minutes. Thus, it was not possible to follow the oxidation by repetitive scanning of a single incubation mixture. Instead, a series of incubations was prepared. Each mixture contained benzidine and sufficient enzyme so that, upon addition of $H_2O_2$, the oxidation was complete in a few seconds. The product was then scanned rapidly and the optical spectrum was recorded before the products decayed. (The rapid scan procedure introduces slight distortion of peak shapes.) The amount of $H_2O_2$ was varied in a series of incubations (Fig. 1). With increasing $H_2O_2$, the resulting solutions were blue, green, and (finally) yellow. We identified the blue product as a charge-transfer complex of benzidine and its two-electron oxidation product, the di-imine derivative, by analogy with the results for 3,5,3′5′-tetramethylbenzidine (8). This species has the same oxidation state as the cation free radical of benzidine and exists in equilibrium with the radical via comproportionation/disproportionation (Scheme 1). The yellow product is the di-imine itself; the green stage is an intermediate mixture of the blue and yellow products. Incubations containing low concentrations of enzyme do not proceed past the blue stage, even in the presence of excess peroxide, since the decay to precipitate occurs more rapidly than the enzymatic oxidation to the di-imine.

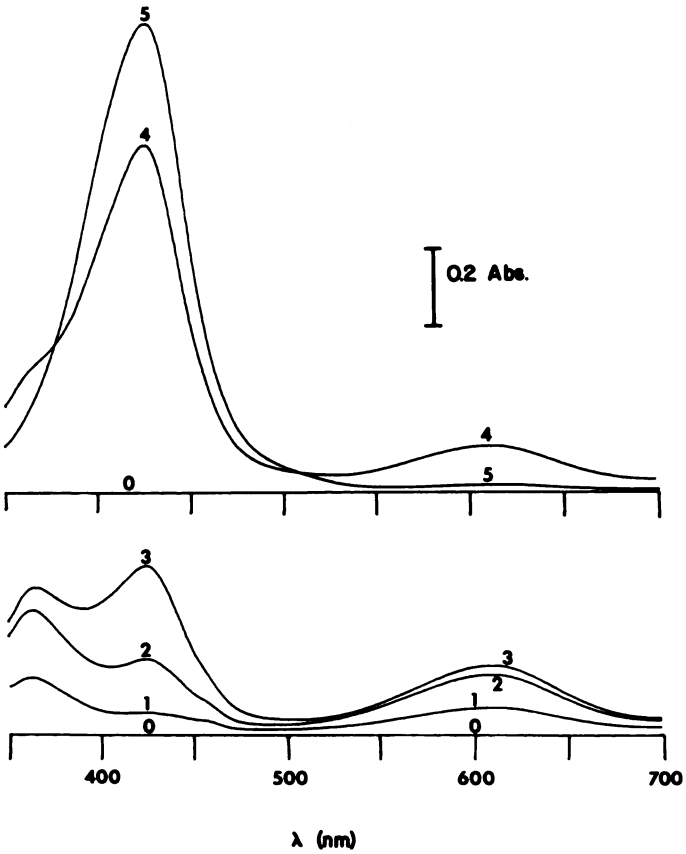


FIG. 1. *Oxidation of benzidine: optical spectrum*

The sample cuvette contained benzidine (25 μM) and horseradish peroxidase (25 μg/ml) in 2 ml of acetate buffer, pH 5.0. The reaction was initiated by the addition of hydrogen peroxide, and the spectrum was recorded immediately. The concentrations of hydrogen peroxide were 0, 5, 10, 15, 20, and 25 μM for spectra labeled *0* through *5*. The reference cuvette contained all components except hydrogen peroxide. Instrumental conditions were: 20 nm/sec scan rate, fast response.

We used ESR to identify the presence of a free radical intermediate. Both lactoperoxidase and horseradish peroxidase gave identical results in all studies, except for the greater specific activity of the latter enzyme. Incubations prepared as described in Fig. 2 were blue-colored, and yielded a well-resolved multiline ESR signal that lasted for about 30 min. This gradual decay resulted in asymmetry of peak heights between the low- and high-field regions of the spectra. Note that ESR spectra are symmetrical, and one-half of an ESR spectrum suffices to determine all hyperfine splitting constants. The ESR spectrum of the benzidine cation radical has been observed in organic solvents using chemical oxidation (11) and a "slow-flow" system (12). Our analysis of the spectrum in Fig. 2A began with the hyperfine splitting constants reported in the earlier work; slight adjustment was needed to obtain an adequate computer simulation of the spectrum (Fig. 2B). The assignment of the hyperfine splitting constant for the amino protons ($a^{H}_{NH_2}$) was confirmed from the deuterium-exchanged species, using the ratio of the gyromagnetic ratios of H and D nuclei. The predicted value of $a^{D}_{ND_2}$ (0.56 G) agreed well with the smallest hyperfine splitting observed in $^2H_2O$ buffer. A summary of the reported hyperfine splitting constants for this radical is given in Table 1. In all experiments, the appearance of color changes and ESR signals was dependent upon the presence of benzidine, $H_2O_2$, and enzyme. Denatured enzyme (boiled for 10 min) was ineffective.

We also examined the compound 2,7-diaminofluorene, which is structurally similar to benzidine and is a powerful carcinogen in rats (13). This substrate was also oxidized to a blue product by $H_2O_2$ and peroxidase. A partially resolved ESR spectrum was recorded; however, the limited resolution of the hyperfine pattern precluded a complete analysis of the spectrum, which should possess 2,025 lines.

The oxidation of benzidine in the presence of phenol or 2,6-dimethylphenol yields violet-colored products which have been characterized chemically (14). These products result from coupling of the phenol to the N atom of benzidine di-imine, yielding an adduct analogous to indoaniline, which is formed by the oxidation of *p*-phenylenediamine in the presence of phenol (15). A similar reaction occurred with other phenol derivatives, such as BHA, guaiacol (2-methoxyphenol), epinephrine, and serotonin (Fig. 3).

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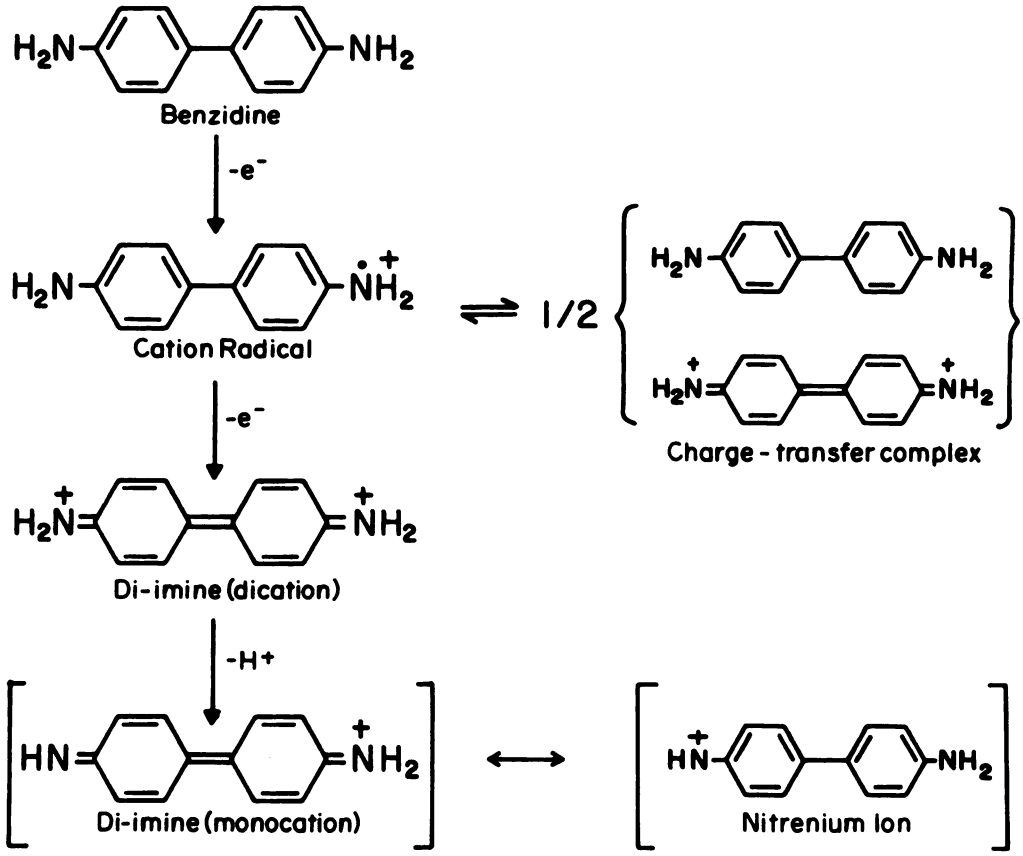
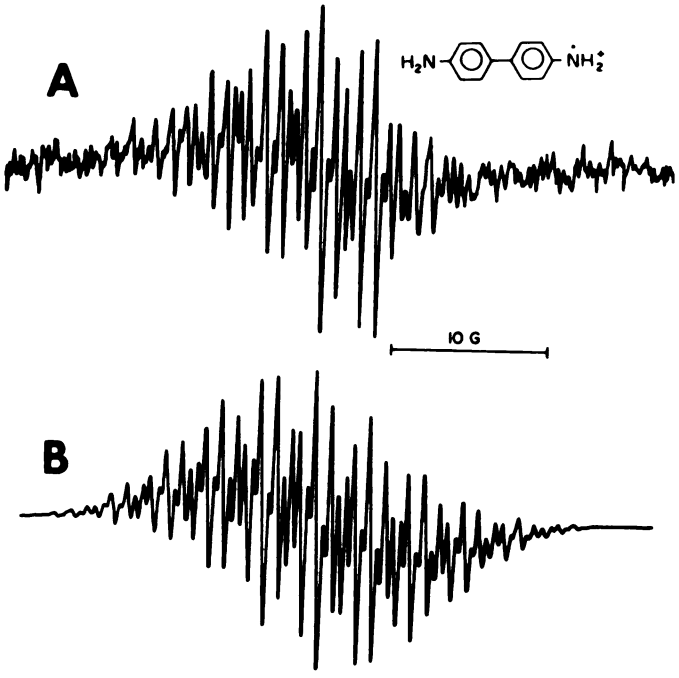



SCHEME 1

*The oxidation products of benzidine discussed in the text*

There are two one-electron oxidation products, the benzidine cation radical and the charge-transfer complex of benzidine and its di-imine derivative. The radical is detected by ESR and the charge-transfer complex by its blue color. Two-electron oxidation of benzidine yields the di-imine (drawn here as a dication, which is the likely form at pH 5). Deprotonation of this species yields a monocation of the di-imine, which is a resonance structure of the benzidine nitrenium ion.




Monoacetylbenzidine is oxidized by the horseradish peroxidase/$H_2O_2$ system, but much more slowly than either benzidine itself (Fig. 4) or 2,7-diaminofluorene (which was oxidized at a rate similar to that of benzidine itself; data not shown). No strongly colored products were observed, and no free radical was detected by ESR. The greater ease of oxidation of the nonacetylated compound is shown clearly in Fig. 5. Here, an equimolar mixture of benzidine and monoacetylbenzidine was oxidized enzymatically; the former compound is consumed much faster than the latter.

## DISCUSSION

Both benzidine and noncarcinogenic 3,5,3′5′-tetramethylbenzidine are oxidized to di-imine species by the

FIG. 2. *Oxidation of benzidine: ESR spectra*

A. The sample contained benzidine (1.7 mM), hydrogen peroxide (0.8 mM), and lactoperoxidase (8 μg/ml) in acetate buffer (pH 4.6). Instrumental conditions were: scan time 30 min; time constant, 2 sec; modulation amplitude, 0.14 G; gain 125,000; nominal power, 20 mW.

B. Computer simulation of spectrum in A. Hyperfine splitting constants were as follows: $a^{N}_{NH_2} = 3.52$ G; $a^{H}_{NH_2} = 3.62$ G; $a^{H}_{2,6,2',6'} = 1.03$ G; $a^{H}_{3,5,3',5'} = 1.53$ G. Lorentzian peak-to-peak linewidth = 0.37 G.

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TABLE 1

*Hyperfine splitting constants reported for the benzidine cation radical*

| Solvent | $a^{N}_{NH_2}$ | $a^{H}_{NH_2}$ | $a^{H}_{2,6,2',6'}$ | $a^{H}_{3,5,3',5'}$ | Ref. |
|---|---|---|---|---|---|
| | *G* | *G* | *G* | *G* | |
| $CH_3CN$ | 3.60 | 3.97 | 1.08 | 1.62 | 14 |
| MeOH | 3.39 | 3.52 | 1.10 | 1.44 | 15 |
| $H_2O$, pH 4.6 | 3.52 | 3.62 | 1.03 | 1.53 | This work |

peroxidase/$H_2O_2$ system. The di-imine metabolite of benzidine is a highly reactive species. This is indicated by its tendency to polymerize, its reactivity with phenols (14), and its reactivity with glutathione and other sulfhydryls (16). We anticipate that this species will also be capable of reacting with nucleic acids.

The effect of BHA on benzidine carcinogenicity is unknown. However, the ability of BHA to "trap" the benzidine di-imine suggests that this antioxidant might diminish DNA binding and carcinogenicity. The reaction of benzidine di-imine with endogenous phenols and catechols may represent a new pathway of xenobiotic metabolism.

Both peroxidative oxidation by prostaglandin synthase and oxygen insertion by the monooxygenase system could generate reactive metabolites from benzidine. In liver there is little prostaglandin synthase activity, and the monooxygenase should predominate. Furthermore, the structure of the benzidine-DNA adducts from rat liver implies that benzidine is rapidly acetylated in that organ, and the acetylated derivative is much less easily oxidized by peroxidases. However, benzidine is a bladder carcinogen in man and dogs, and benzidine binding has been observed in dog bladder epithelium (17). The structure of the benzidine-DNA adduct in this tissue is unknown. Since prostaglandin synthase activates benzidine to a reactive species *in vitro* (4–6) and catalyzes the formation of mutagenic products from benzidine in a modified Ames test (18), the possible involvement of peroxidases in benzidine bladder carcinogenesis merits further consideration.

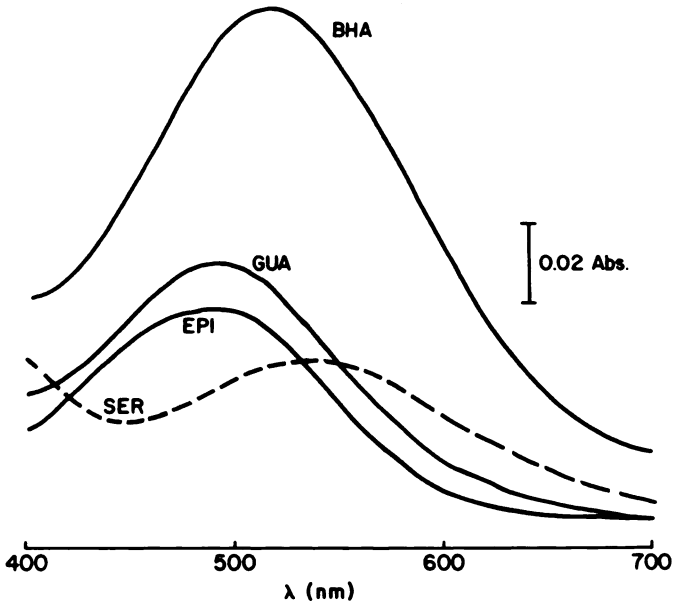


FIG. 3. *Benzidine/phenol adducts: optical spectra*

Benzidine (25 μM) and hydrogen peroxide (25 μM) were added to 2 ml of acetate buffer (pH 5.0). Horseradish peroxidase (5 μg) was added, and the solution became yellow in a few seconds. The indicated phenolic compound (25 μM) was then added to the solution, which turned light violet. The optical spectrum was recorded. Instrumental conditions were: 5 nm/sec scan rate, medium response. *GUA*, Guaiacol; *EPI*, epinephrine; *SER*, serotonin.

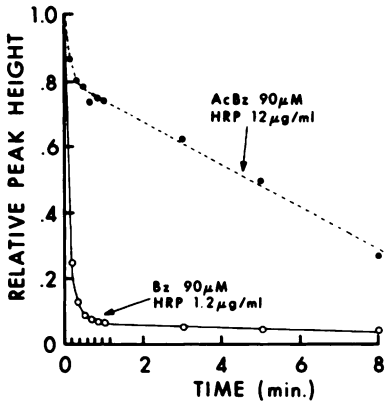


FIG. 4. *Oxidation of benzidine (Bz) and monoacetylbenzidine (AcBz) by horseradish peroxidase (HRP)*

Both incubation mixtures contained substrate (90 μM), $H_2O_2$ (230 μM), and enzyme in phosphate buffer (pH 7.0) containing 10% ethanol. Incubations were initiated by addition of $H_2O_2$ at room temperature. At intervals, aliquots were removed and stopped with an equal volume of 1 N HCl. Remaining substrate concentration was determined by HPLC, as described in the text. Enzyme concentrations were 1.2 μg/ml for benzidine and 12 μg/ml for monoacetylbenzidine (the higher enzyme concentration was required to obtain significant oxidation of the latter compound).

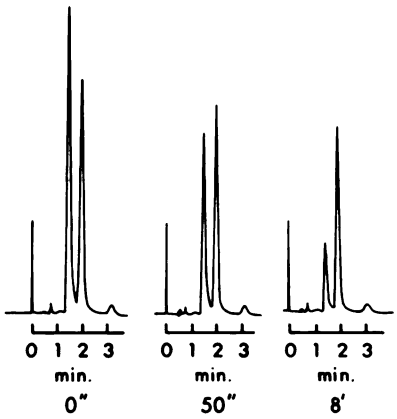


FIG. 5. *Oxidation of a mixture of benzidine and monoacetylbenzidine by horseradish peroxidase: HPLC chromatograms*

A single incubation contained both substrates, each 90 μM, and enzyme (1.2 μg/ml) in phosphate buffer (pH 7.0) containing 10% ethanol. The reaction was initiated with $H_2O_2$ (final concentration, 450 μM) at room temperature. HPLC traces of aliquots stopped with equal volumes of 1 N HCl are at 0 sec, 50 sec, and 8 min. Benzidine was eluted at 1.5 min, and monoacetylbenzidine at 2.0 min.

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## ACKNOWLEDGMENTS

We wish to thank Mr. Peter Jordan for technical assistance, and Dr. F. Kadlubar for providing an advance copy of ref. 6.

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