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Reaction of 4-substituted phenols with benzidine in a peroxidase system

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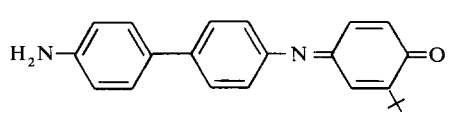
Benzidine and its derivatives are readily oxidized by peroxidases [1, 2]. The oxidation proceeds via a one-electron oxidized intermediate (free radical) which is further oxidized to a quinone-diimine. The oxidation of benzidine by peroxidases generates reactive electrophilic species, which bind to protein and DNA [3] and form glutathione adducts [4]. The peroxidase activity of prostaglandin synthase supports benzidine oxidation [5, 6] and may be involved in benzidine-induced human bladder carcinogenesis.

The reactive intermediate generated in the benzidine/peroxidase/H_2O_2 system may be chemically trapped by the antioxidant 2(3)-*tert*-butyl-*para*-hydroxyanisole (BHA). Thus, when BHA is included in such a system, the yellow color characteristic of the quinone-diimine is not seen; instead, a pink color forms [1]. No such reaction was observed with 2,6-di-*tert*-butyl-*para*-hydroxytoluene (BHT). Phenol reacts similarly to BHA and gives a pink benzidine/phenol adduct [7]. This adduct has been isolated and characterized by NMR and mass spectrometry. The structure of the product is analogous to that of the synthetic dye indoaniline, and results from addition of a benzidine N atom to the phenol ring at the position *para* to the hydroxyl group. Coupling occurred exclusively at the *para* position; no "ortho adduct" was detected. Therefore, the reaction with BHA (in which the *para* position is occupied) demands explanation. In this report, we show that the methoxy group is cleaved from the phenol ring during the trapping reaction.

The published procedure for synthesis of the benzidine/phenol adducts [7] was used. Benzidine, horseradish peroxidase (donor: H_2O_2 oxidoreductase EC 1.11.1.7), and the substituted phenol were mixed in 2 ml acetate buffer, pH 5. The reaction was initiated with H_2O_2. The concentrations of benzidine, phenol, and H_2O_2 were all 250 μM, and peroxidase was 50 μg/ml. After complete development of the color the product was extracted with 5 ml ethyl acetate. TLC was performed on silica gel plates in ether (100%) or $CHCl_3$/ethyl acetate (80 : 20).

The oxidation of benzidine by the horseradish peroxidase/H_2O_2 system yields the yellow quinone-diimine, which slowly decays to the poorly characterized polymeric precipitate "benzidine brown" [8]. In the presence of equimolar phenol, the pink-coloured benzidine/phenol adduct is formed [7]. The behaviour observed with *para*-methoxyphenol was similar to that reported for phenol itself, and the resulting adduct was identical. This was proven by co-chromatography of the *para*-methoxyphenol and phenol adducts in both TLC solvent systems, and identical optical and mass spectra. Thus, the methoxy group must be cleaved from the phenol ring during the reaction. To shed light on this unexpected mechanism, we studied a series of commercially available 4-substituted phenols. Organic-extractable colored adducts were obtained with (in order of decreasing yield) *para*-methoxyphenol, BHA, *para*-fluorophenol, phenol, *para*-chlorophenol, *para*-bromophenol, and *para*-hydroxybenzoic acid. In each case, the adduct was chromatographically identical to the benzidine/phenol adduct. In contrast, *para*-methylphenol (*para*-cresol) and *para*-nitrophenol gave no isolable products and did not prevent "benzidine brown" formation.

BHA (obtained from Sigma) gave one major pink product and numerous other bands (pink to purple colored). BHA is a mixture of 2- and 3-*tert*-butyl substituted isomers, and TLC examination of the commercial material revealed several minor components. Presumably, this accounts for the variety of adducts obtained. The major product was recovered from the TLC plate and submitted for mass spectrometry. The product gave a strong molecular ion (base peak, at m/e = 330, as expected for the structure:



Also present were 331 (^{13}C – parent; relative intensity = 24.8%) and 332 (18.3%). The M + 2 peak is commonly obtained with quinonoid compounds and corresponds to the reduced form of the quinone ring [9].

The yields of benzidine/phenol adduct obtained with the most effective *para*-substituted phenols were calculated from the absorption maxima of the ethyl acetate extracts, using the reported extinction coefficient of the adduct [7]. (In the case of BHA, λ_{max} = 512 nm rather than 515 nm, presumably due to the effect of the *tert*-butyl substitution; the effect on ε_{max} was not determined but is undoubtedly small.) Calculated yields, expressed as percentages of the initial benzidine concentration, were: *para*-methoxyphenol, 80%; *para*-fluorophenol, 68%; phenol, 43%; and BHA, 80%.

BHA, but not BHT, reacts with the quinone-diimine formed from *o*-dianisidine in a peroxidase system. The proposed mechanism was "aromatic substitution upon the side of the aromatic ring away from the *tert*-butyl substitution, since the presence of a second *tert*-butyl group in BHT ... prevents reaction ... by a presumed steric effect" [1]. This explanation is no longer tenable, since the sterically non-hindered analogue, *para*-methylphenol, also forms no adduct. Apparently, the *ortho* position of the phenol ring is not sufficiently nucleophilic to allow adduct formation at that site. The nature of the electrophilic oxidation product of benzidine responsible for adduct formation is not certain, but a likely possibility is the benzidine nitrenium ion, which is a resonance hybrid form of the mono-protonated quinone-diimine [8]. Apparently, this species is a fairly weak electrophile, and reacts at only the most nucleophilic site on the phenol ring, *para* to the hydroxyl group. This behaviour is similar to that of the weakly electrophilic aryl diazonium cation. Benzene diazonium cation couples with phenoxide anion almost exclusively at the *para* position to give phenyl-azophenol [10].

The facile displacement of certain substituents from the *para* position, reported here, is consistent with the mechanism shown in Fig. 1. The attack of the nitrenium ion (1) on the phenol (2) yields a cationic intermediate (3) which deprotonates to yield (4). In the case R = H, (4) rearranges to the reduced (leuco) form of the adduct, which is oxidized to the benzidine/phenol adduct (5) by a second molecule of (1). An analogous mechanism has been suggested to explain the formation of indophenols [9]. Thus, the yield of adduct is less than 50%, and unchanged benzidine is recovered [7]. In the cases R = OCH_3 or R = halogen, (4) loses H^+ and R^- yielding (5) directly. This explains the nearly 2-fold higher yield of adduct obtained with *para*-methoxyphenol relative to phenol itself. BHA and BHT are widely used as antioxidant food additives. The ability of BHA to trap the electrophilic metabolite of benzidine *in vitro* suggests a chemical mechanism for the observation that BHA strongly inhibits the mutagenicity of certain aromatic amines in the Ames test [11].

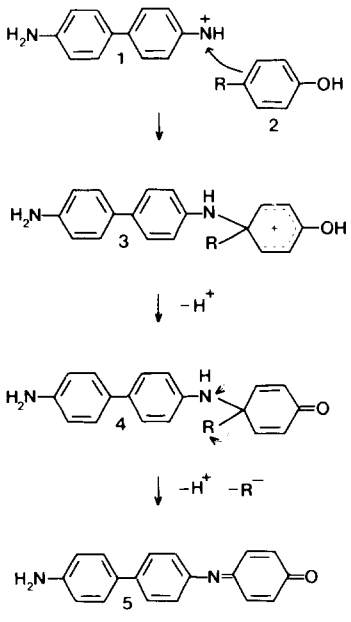


Fig. 1. Suggested mechanism for the reaction of 4-substituted phenols with benzidine in a peroxidase system.

The striking difference in the reactivities of BHA and BHT reported earlier [1] and explained by this study emphasizes that these two antioxidants may be metabolized very differently. For example, BHT becomes bound to microsomal protein via oxidation of the methyl group to BHT-alcohol and BHT-quinone methide [12], a pathway unavailable to BHA.

In this report, we have elucidated the mechanism of reaction of 4-substituted phenols with benzidine in a peroxidase system. Good leaving groups are displaced from the phenol ring to form a covalent benzidine/phenol adduct. The pharmacology of phenolic antioxidants will continue to be an active research frontier.

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Note added in proof. The BHA experiment was repeated using crystalline 3-tert-butyl-4-hydroxyanisole (98%) obtained from Fluka Specialities, Hauppauge, New York. As before, many colored products were apparent on TLC. Re-chromatography of the major band again showed the presence of the same pattern of products. Thus, the benzidine/BHA adduct appears to be unstable, and the additional components are decomposition products of the adduct.

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