Oxidative Activation of Benzidine and Its Derivatives by Peroxidases

by P. David Josephy*

Benzidine (4,4'-diaminobiphenyl) is a known human carcinogen; exposure to this substance resulted in an epidemic of bladder cancer among workers in the dye industry in Europe and North America. The chemical or enzymatic oxidation of benzidine proceeds via a radical cation detectable by electron spin resonance. Peroxidase-catalyzed oxidation of benzidine generates reactive electrophiles which readily form adducts with phenol and thiol compounds. The structures of these novel metabolites are described. Peroxidases, including prostaglandin synthase, catalyze benzidine binding to protein and nucleic acid; the nature of the resulting adducts is unknown. The relevance of these processes to benzidine carcinogenesis in vivo is the subject of research and debate. A central question remains: is benzidine activated in extrahepatic target tissues such as bladder epithelium, or transported to these tissues following hepatic oxidative metabolism?

Introduction

The current interest in free-radical biology has been attributed to three major discoveries: superoxide dismutase, the involvement of lipid hydroperoxides in the arachidonate cascade, and the involvement of radical reactions in the metabolism of many environmental toxicants (1). Appropriately, most of the contributions to this volume touch on one or more of these themes, and several deal with the oxidation of xenobiocides catalyzed by the peroxidase activity of prostaglandin H synthase (PHS). This enzyme is responsible for the formation of prostaglandin H₂ from arachidonic acid in tissues which synthesize prostaglandins, and may be a major source of lipid hydroperoxides in mammalian systems. The initial report of cooxidation of polycyclic aromatic hydrocarbons by PHS (2) has stimulated a great deal of research in the past ten years, and a wide variety of xenobiocides has been shown to serve as substrates for PHS-dependent metabolism (3).

Aromatic amines comprise one of the most-studied classes of chemical carcinogens. Indeed, the pioneering studies by E. C. Miller and J. A. Miller and their colleagues on the metabolism of N,N-dimethyl-4-aminobenzene and 2-acylaminofluorene mark the beginning of the modern era of chemical carcinogenesis research (4). A recent issue of this journal is devoted to the proceedings of the Second International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds (5) held in 1982 and contains several useful reviews of aromatic amine metabolism and carcinogenesis.

Benzidine (4,4'-diaminobiphenyl) (Fig. 1) is one of the relatively few chemicals classified as a known human carcinogen, based on studies of workers in the dye industry who were exposed to very high levels of the substance (6,7). Improved industrial hygiene enforced by occupational safety legislation has largely removed this hazard, at least in the West. However, recent research has demonstrated a previously unsuspected route of exposure to benzidine. Azo dyes (synthesized by diazotization of arylamines and coupling of the resulting aryldiazonium cations) may be reduced by enzymes of the gut bacteria, releasing the parent arylamines. Reduction may be the fate of a large percentage of an oral dose of a benzidine-based azo dye (8), and thus, these dyes may pose a significant environmental hazard. Many of the benzidine-based dyes are mutagenic in the Ames test following preincubation under reductive conditions (9). The recognition of this potential hazard has prompted the establishment, by the National Toxicology Program, of a research initiative on benzidine dyes (10).

Benzidine is exclusively a bladder carcinogen in man and in some other species such as dogs (11). In contrast, benzidine induces liver, intestine, and ear duct tumors in the rat (12). The explanation of observed patterns of organ and species specificities of chemical carcinogenesis is a fundamental challenge to researchers (13). Two contrasting theories have been advanced to explain the activity of arylamines as bladder carcinogens. The studies of Kadlubar, Radomski, and others suggest that oxidation to the hydroxylamine and N-glucuronidation occur in the liver, and the resulting N-hydroxyarylamine N-glucuronides enter the circulation and are excreted in the urine. Acid-catalyzed hydrolysis of the glucuronide leads to generation of a reactive electrophile in the

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Benzidine: Chemical Oxidations

Benzidine possesses two readily oxidized amino groups, and thus its oxidation chemistry is distinct from that of simple arylamines (such as 4-aminobiphenyl), just as the oxidation chemistry of hydroquinones differs from that of phenols. However, there have been relatively few studies of the benzidine system using chemical oxidation techniques. Willstätter and Kalb (17) reported the synthesis of azobenzidine (Fig. 1) by oxidation of benzidine with Pbo2, and suggested that the diimine was a likely intermediate. Hùnig and Richters (18) succeeded in isolating the dark yellow-colored diprotonated diimine dication, following oxidation of benzidine by sodium dichromate in perchloric acid solution. Recently, the oxidation of benzidine and derivatives by “chloramine-T” and by N-bromosuccinimide was reported (19). The products (λmax ~430 nm) were described as N,N-dichlorodiphenyquinonediimines on the basis of mass spectral data.

Several electrochemical studies of benzidine oxidation have been carried out. Oldfield and Bockris (20) performed potentiometric titration experiments using Br2 as oxidant, and concluded that the oxidation proceeded via a radical intermediate. Kuwana and Strojek (21) examined the oxidation of o-tolidine (3,3’-dimethylbenzidine), using optically transparent electrodes to measure absorption spectra during cyclic voltammetric experiments. The product of oxidation by cyclic voltammetry or controlled potential electrolysis was the yellow diimine (λmax = 437 nm). An intermediate blue species (λmax = 365, 630 nm) was produced, and then consumed, during both types of electrochemical oxidation. In the electrolysis procedure, the intermediate precipitated as dark blue crystals during the oxidation, and redissolved as the oxidation continued to the final product. The intermediate represents a one-electron oxidized form of o-tolidine and could be either a dimeric complex of the fully reduced and fully oxidized forms or a monomeric cation radical. In either case, the intermediate is in equilibrium with the fully reduced diamine and the fully oxidized diimine:

bladder lumen (14). An alternative theory (which is not exclusive of the previous view) holds that metabolic activation may occur in the bladder epithelial tissue. Langenbach and colleagues have shown that bovine bladder cells can activate aromatic amines, including benzidine, to mutagenic metabolites (15,16). Since prostaglandin H synthase activity is present in the kidney and bladder, the possible role of the enzyme in activation of bladder-specific carcinogens must be evaluated.

In this review, I discuss recent work on the peroxidase-mediated activation of benzidine and substituted benzidine derivatives.
for free-radical formation. The two cases can be distinguished, since only the former system is sensitive to dilution, which shifts the equilibrium towards the isolated diamine and diimine. On the basis of dilution experiments, Kuwana and Strojek identified the blue intermediate as a dimer charge-transfer complex. However, they noted that equilibrium analysis of this sort does not preclude the possibility that “the primary electron transfer step... is controlled by a one-electron transfer, and ... the free radical is formed first, followed by dimerization...” (21). Similar conclusions were reached in a later re-examination of potentiometric studies of o-tolidine oxidation (22).

ESR Studies of Benzidine Oxidation

\[ \begin{align*}
\text{H}_2\text{N-} & \text{C}_6\text{H}_4-\text{C}_6\text{H}_4-\text{NH}_2 + \text{H}_2\text{N-} & \text{C}_6\text{H}_4-\text{C}_6\text{H}_4-\text{NH}_2 \\
\downarrow & & \downarrow \\
\text{H}_2\text{N-} & \text{C}_6\text{H}_4-\text{C}_6\text{H}_4-\text{NH}_2 + \text{H}_2\text{N-} & \text{C}_6\text{H}_4-\text{C}_6\text{H}_4-\text{NH}_2
\end{align*} \]

for charge transfer complex formation, and

\[ 2 \text{H}_2\text{N-} \text{C}_6\text{H}_4-\text{C}_6\text{H}_4-\text{NH}_2 \]

Further reactions of these species might be regarded as proceeding via the radical as an intermediate or via the charge-transfer complex as an intermediate. This distinction is “certainly not clear-cut and possibly even meaningless” (30); as Colter has stressed (30), the most important chemical question is the structure of the transition state leading to product formation. On the other hand, reactions proceeding via the diimine itself should be distinguishable from reactions proceeding via one-electron-oxidized intermediates, on the basis of oxidation equivalents required for maximal reaction rate.

Mechanism of Benzidine Oxidation by Peroxidase Enzymes

The facile oxidation of benzidine derivatives to colored products has been of interest to biochemists and analytical chemists for many years. Some examples of the use of these compounds deserve mention. The “benzidine test” for blood has been routinely in clinical and forensic medicine. This procedure depends upon the peroxidase activity of blood proteins, especially methemoglobin; the test sample is added to a solution containing benzidine and \( \text{H}_2\text{O}_2 \), and the appearance of a blue color is a positive result (29). In coupled assays, such as that for glucose, one enzyme (glucose oxidase) oxidizes the test substance with production of \( \text{H}_2\text{O}_2 \), and the second enzyme (peroxidase) uses this \( \text{H}_2\text{O}_2 \) in the oxidation of the benzidine derivative to a colored product. Another application of benzidine derivatives in biochemistry is immunoperoxidase staining. This procedure relies on the spontaneous polymerization of the products of benzidine oxidation by peroxidase. The resulting polymer, known as “benzidine brown” does not diffuse, and so remains as a marker where it was formed. This immobilization is exploited in studies of the subcellular localization of antigens via the use of peroxidase-conjugated antibodies (31).

Van Duijn (32) discovered that the blue product of benzidine oxidation by peroxidase/\( \text{H}_2\text{O}_2 \) could be precipitated in the form of stable microcrystals, by the addition of \( \text{NH}_4\text{Cl} \). The product analyzed as \((\text{C}_8\text{H}_2\text{N}_2\text{O})^+\text{Cl}^{-}\text{2H}_2\text{O} \), interpreted as a “crystal in which
the positively charged odd ions [radicals] are separated by neutral benzidine molecules ... " (32).

Claiborne and Fridovich studied the oxidation of o-dianisidine by horseradish peroxidase (HRP)/H$_2$O$_2$ (33). Optical spectroscopy revealed a two-stage oxidation proceeding via a green intermediate ($\lambda_{\text{max}} = 704$ nm), identified as a dimer charge-transfer complex on the basis of dilution experiments. At neutral pH, the primary oxidation product rapidly transformed into a species ($\lambda_{\text{max}} = 476$ nm) suggested to be a bisazobiphenyl product analogous to azobenzidine. Several experiments were carried out with the intention of obtaining ESR evidence for a radical intermediate. However, no signal could be obtained, even with the use of a continuous-flow system, at low temperature, or with Ce (IV) oxidation. The authors concluded that “oxidation of dianisidine occurs in a rapid two-electron process." (33).

The report in 1979 that benzidine was an excellent substrate for cooxidation by PHS (discussed later in this review) stimulated renewed interest in the mechanism of benzidine oxidation. Holland et al. (34) described the synthesis of a novel derivative of benzidine, 3,5,3'5'-tetramethylbenzidine (TMB), which had little or no carcinogenic activity in rats (34) and was not mutagenic in the Ames test (35). Structurally, this compound is analogous to duroquinone (2,3,5,6-tetramethylbenzoquinone): all sites ortho to the redox-active substituents on the ring are protected by methylation. Thus, one might expect the TMB redox system to show enhanced stability relative to previously mentioned benzidine derivatives. This conclusion was borne out, and the use of TMB as a model substrate facilitated studies of benzidine oxidation by peroxidases.

Oxidation of TMB by horseradish peroxidase/H$_2$O$_2$ was studied by optical spectroscopy (36). The reaction proceeds via a blue intermediate to a yellow final product; at pH 5, the oxidized products are indefinitely stable. The intermediate ($\lambda_{\text{max}} = 370, 652$ nm) is formed and then further oxidized as the reaction proceeds; this sequence was studied by titrating with H$_2$O$_2$ a solution containing TMB and peroxidase. The blue intermediate reaches maximum concentration at a molar ratio of H$_2$O$_2$ to TMB of 1 to 2, and thus corresponds to a one-electron oxidized product of TMB. Dilution experiments confirmed that this colored species was a dimer charge-transfer complex, as suggested by the studies described above. However, with this substrate we obtained strong, stable ESR spectra with partially resolved hyperfine structure (36), which were shown by computer simulation to correspond to the TMB$^+$ cation radical. The assignment was confirmed by deuteration substitution in D$_2$O buffer. Using TMB as a paradigm, we subsequently obtained satisfactory spectra of the cation radicals of o-dianisidine (36) and benzidine (37) in horseradish peroxidase/H$_2$O$_2$ systems, although these radicals are much shorter-lived than that of TMB. The formation of paramagnetic intermediates suggests that the enzymatic oxidation proceeds by a one-electron transfer mechanism. However, in this system, as in that of Kuwana and Strojek, the existence of comproportionation/disproportionation equilibria among the reduced, partially oxidized, and fully oxidized species precludes any definitive judgement of this issue, which is further discussed elsewhere (36).

### Benzidine Oxidation Catalyzed by the Prostaglandin H Synthase System

Prostaglandin H synthase is an enzyme found in the microsomal fraction of many mammalian tissues. PHS catalyzes the synthesis of prostaglandin H via incorporation of two molecules of O$_2$ into arachidonic acid. This reaction occurs in two enzymatic steps: cyclooxygenase-dependent conversion of arachidonic acid to the hydroperoxide PGG$_2$, and the hydroperoxidase-dependent reduction of PGG$_2$ to the corresponding alcohol, PGH$_2$. Both activities are contained on a single protein (38). While the peroxidase activity of PHS has many features in common with the plant peroxidases, such as horseradish peroxidase, there are important differences. PHS generates its own hydroperoxide substrate, PGG$_2$, whereas plant peroxidases require endogenous H$_2$O$_2$. PHS is a membrane-bound protein, whereas plant peroxidases are cytosolic. In general, PHS catalyzes cooxidation of a wide range of substrates including, but not limited to, the familiar substrates for plant peroxidases (e.g., guaiacol, phenylenediamine, pyrogallol). Recent investigations have demonstrated that PHS-dependent cooxidations may involve several different mechanisms, depending on the nature of the substrate. Marnett has reviewed the literature and describes examples of PHS-dependent oxygenation by O$_2$ (diphenylisobenzofuran), oxygenation by incorporation of the hydroperoxide oxygen (sulindac sulfide), and incorporation of oxygen from water (hydrolysis of diol epoxide derivatives of polycyclic hydrocarbons), as well as oxidation by the familiar electron-abstractation pathway elucidated by Chance and others for horseradish peroxidase (39).

Since benzidine is an excellent substrate for plant peroxidases, we anticipated that PHS-dependent oxidation of benzidine would proceed by a similar mechanism to that of horseradish peroxidase/H$_2$O$_2$-dependent oxidation. Indeed, incubation of TMB with a microsomal preparation from ram seminal vesicles (RSV) as a source of PHS activity and arachidonic acid, at pH 7.0, gave rise to the ESR signal of the TMB cation radical (40). The signal was not observed following pretreatment of the enzyme preparation with the cyclooxygenase inhibitor, indomethacin. On the other hand, radical formation could be initiated using 15-hydroperoxyarachidonic acid in place of arachidonic acid, thus bypassing the cyclooxygenase activity and driving the peroxidase reaction directly; in this case, indomethacin is without effect. Similarly, TMB oxidation by RSV microsomal preparation could be initiated with H$_2$O$_2$. Wise et al. (41) obtained an ESR spectrum of the benzidine cation radical using Tween 20-solubilized RSV preparation, and initiating
oxidation using H$_2$O$_2$ at pH 4.2. However, the authors did not report arachidonic acid-dependent metabolism studies, except to note that “results were essentially the same ... but with much reduced levels of metabolism at pH 5.0.” (41).

Concurrent studies by the NIEHS group revealed unexpected differences between the PHS-dependent and horseradish peroxidase-dependent reactions (42). Benzidine oxidation by the HRP/H$_2$O$_2$ system at pH 7.0 was studied by ESR; the resolved signal of the benzidine cation radical, previously observed at pH 5, is not seen. Instead, a short-lived, narrow, asymmetric spectrum is observed. The incubation mixture is initially blue, but quickly turns purple and finally brown. This ESR spectrum is probably indicative of rapid polymerization of the radical at neutral pH; benzidine brown soon precipitates from the solution. Indeed, the ESR spectrum is similar to those of synthetic melamins generated by oxidation of phenolic monomers. In contrast, arachidonic acid-initiated benzidine oxidation catalyzed by RSV microsomal preparations gives rise to a longer-lived, symmetric single-line ESR spectrum; the incubations remain deep blue-colored. The symmetric ESR spectrum indicates a more rapidly-tumbling paramagnetic species (shorter rotational correlation time) than for the HRP system. Thus, although the radical observed may be the same “benzidine melamin” polymer in both systems, the radical is observed in a different milieu or physical state.

We suspected this difference between the systems to be due to the presence of a large concentration of micellar lipid (e.g., 2.6 mM arachidonic acid) in the RSV incubations. This was confirmed by experiments in which arachidonic acid was added to the benzidine/horseradish peroxidase/H$_2$O$_2$ system. In the presence of arachidonic acid, the incubations were deep blue in color and the asymmetric spectrum was transformed into a longer lived, symmetric spectrum apparently identical with that obtained in the ram seminal vesicle microsomal system.

Both ESR results and optical experiments showed that the horseradish peroxidase system, supplemented with arachidonic acid, behaves very much as the prostataglandin synthase system does. The effect of arachidonic acid is apparently to stabilize the initial or early oxidation products. Perhaps the hydrophobic benzidine oxidation products are physically sequestered in fatty acid micelles and protected from base-catalyzed decomposition in the bulk aqueous medium.

We also studied the organic-extractable products of benzidine oxidation by these enzyme systems. In both the HRP/H$_2$O$_2$ and PHS/arachidonic acid incubations, the major products proved to be azobenzidine. This metabolite was identified by optical spectroscopy and mass spectrometry; at the time, we were unaware of previous chemical synthesis of this compound.

**Benzidine Adducts of Nucleophiles**

The free radical oxidation pathway of benzidine metabolism results in the formation of reactive electrophilic species. This is manifested by various subsequent reactions: spontaneous polymerization, formation of adducts with low molecular weight nucleophiles, and binding to macromolecules. In this section, I summarize recent work, in my laboratory and elsewhere, on the reactions of benzidine with thiols, phenols, and other small nucleophiles.

Rice and Kissinger (43) reported formation of thiol adducts of benzidine following oxidation with HRP/H$_2$O$_2$ or by the chemical procedures of Hüning and Richters. The reaction mixture for the enzymatic procedure contained 10 mM thiol, 0.1 mM benzidine, and 0.3 mM H$_2$O$_2$; the large molar excess of thiol is required to ensure that adduct formation competes effectively with polymerization of benzidine (self-reaction). The product mixture was separated by reversed-phase HPLC, with electrochemical detection of the eluted products. Glutathione, cysteine, and N-acetylcysteine each gave one major product peak, whose retention time was characteristic of the thiol; several smaller, more polar, product peaks were also noted. In addition, much of the oxidized benzidine was reduced back to the parent amine, presumably due to reduction by the thiol competing with adduct formation. The major product was suggested to be a ring-S-substituted thioether conjugate, but further structural assignment was not made.

We have isolated the benzidine/N-acetylcysteine adduct by HPLC, and obtained optical and $^1$H-NMR spectra of the compound (P. D. Josephy and D. C. Iwaniw; unpublished data). Examination of the aromatic region of the $^1$H-NMR spectrum established the position of S-substitution as the 3 position on the ring (i.e., ortho to the NH$_2$ group, Fig. 2). Optical spectra of the minor products obtained in the enzymatic incubation were consistent with multiple substitution by N-acetylcysteine: the initially formed adduct may itself be oxidized and react with N-acetylcysteine at the free ortho position, to give substituted and even polysubstituted adducts.

Claiborne and Fridovich (33) observed that the product of o-dianisidine peroxidation reacted rapidly with butylated hydroxyanisole (BHA) to give a stable colored product; the reaction appeared analogous to the reaction between Gibbs reagent (2,6-dichlorobenzoquinone-4-chloroimine) and BHA. The NIEHS group explored this reaction in more detail (44). 2,6-Dimethylphenol was found to react in a manner similar to BHA, and the resulting adduct was isolated and characterized by $^1$H-NMR and mass spectrometry. The product is intensely-colored ($\lambda_{max} = 515$ nm), consistent with a structure
analogous to the indoaniline dyes. $^1$H-NMR confirmed the structure shown in Figure 3, resulting from addition of the benzidine N to the para position of the phenol ring. The adduct is formed in high yield; in contrast to the thiol adducts, addition of equimolar nucleophile to the benzidine peroxidase incubation will convert most of the product formation from "benzidine brown" to adduct. Thus dimethylphenol can be used effectively as a "trap" to quantitate benzidine metabolism in, for example, RSV preparations (42).

The nature of the reaction of benzidine with BHA remained puzzling, since BHA is substituted (methoxy group) at the para position. We found that the substituent is lost during the reaction, and so the product formed in the benzidine/H$_2$O$_2$/BHA/peroxidase system is the same as is formed with 2-tert-butylphenol in place of BHA (45). Presumably, the methoxy group leaves as H$_2$CO$^-$. An analogous reaction was noted between Gibbs reagent and para-substituted phenols, in those cases where the substituent gives a good anionic leaving group such as X$^-$ (X = halogen), RO$^-$, and ArO$^-$ (46, 47).

The facile trapping of the reactive product of benzidine oxidation by phenolic antioxidants suggests a possible route for inhibition of benzidine carcinogenesis. BHA was recently shown to inhibit arylamine mutagenesis in the Ames test (48).

Many nucleophiles are also reducing agents. We have seen that phenols react with benzidine in peroxidase systems principally by adduct formation, but thiols act both as nucleophiles and as reducing agents. Ascorbic acid, in contrast, acts purely as a reducing agent, and converts benzidine diimine stoichiometrically back to benzidine itself (40, 43, 49).

**Macromolecular Binding and Mutagenesis**

Carcinogen binding to biological macromolecules, particularly in DNA, is a decisive event in the metabolic activation of diverse chemicals. Several groups have investigated the ability of peroxidases to generate nucleic acid-reactive species from benzidine. Zenzer and colleagues demonstrated arachidonic acid-dependent and indomethacin-inhibitable metabolism of $^{14}$C-benzidine by microsomal preparations from rabbit renal medulla (50). Radioactivity was recovered as aqueous, trichloroacetic acid (TCA)-precipitable material and as aqueous, non-TCA-precipitable material. Metabolism was completely inhibited by the antioxidant ethoxyquin. Glutathione or cysteine caused a shift from TCA-precipitable to non-TCA-precipitable products; presumably, this reflects adduct formation competing with protein binding.

An activity peak attributed to the benzidine-glutathione conjugated was detected by TLC analysis of the lyophilized aqueous material. Metabolism was also demonstrated by using renal inner medullary slices (51).

The rabbit renal inner medullary microsomal preparation also catalyzes $^{14}$C-benzidine binding to exogenous yeast tRNA or calf thymus DNA (52). The PHS inhibitor, aspirin, effectively inhibits $^{14}$C-benzidine binding to TCA-precipitable material, whether added to microsomal preparations from "control" rabbits, or used to inhibit the enzyme in vivo (15 mg/kg IV bolus 30 min before sacrifice) (53).

Kadlubar and colleagues (54) compared a variety of radionuclide aromatic amines as substrates for arachidonic acid-dependent binding to DNA catalyzed by PHS activity of solubilized rat seminal vesicle microsomal preparation. Their results showed that benzidine was by far the most active compound studied; DNA binding was more than 300 times greater for benzidine than for 2-aminofluorene, 2-naphthylamine, or 4-aminobiphenyl. For the latter compounds, binding to microsomal protein was about 1000 times higher than binding to DNA, under the experimental conditions. But, for benzidine, this ratio was only about 10; this suggests that there may be a specific DNA binding mechanism for benzidine.

Morton et al. performed similar studies using tRNA rather than DNA as target (55). Again, benzidine was far more effective than other aromatic amines, including the N-acetyl and N,N'-diacetyl derivatives of benzidine. The authors noted that "the unusually high reactivity of benzidine ... could be due to the potential for extended conjugation between 4,4'-aminostyrenes. In contrast to these results, N-acetylbenzidine is much more mutagenic than benzidine in liver microsome-mediated mutagenicity assays ... This difference may be a reflection of the fact that the liver is relatively low in P450 synthase activity and high in N-hydroxylase activity." (55). Morton and colleagues studied the base specificity of benzidine binding using synthetic homopolypyrinucleotides as macromolecular targets. Binding was essentially specific for guanosine (about 100 times higher binding for poly G than for poly A, poly C, or poly U).

The HRP/H$_2$O$_2$ system also catalyzes benzidine binding to DNA (56). Binding can be quantitated by spectral changes as well as by radiolabeling; in this way, o-tolidine, o-dianisidine, and o-dichlorobenzidine were shown to bind, whereas TMB does not do so.

Apparently, the DNA-binding mechanism requires the participation of the ortho position. This is in contrast to the reaction with phenols, which occurs at the N atom and proceeds with TMB as well as with benzidine, but is consistent with the thiol adduct formation, which occurs at the 3 position on the ring. The nature of the benzidine DNA adduct formed in the peroxidase system is of obvious significance. Preliminary studies suggest
that a single adduct is formed (57), but complete characterization has not yet been accomplished.

Benzidine is weakly mutagenic in the standard Ames test, using Salmonella typhimurium strain TA 98 and rat liver S-9 supernatant for metabolic activation; for unknown reasons, hamster liver S-9 is much more effective than rat liver S-9 (58). NIEHS scientists have developed a modified Ames test using PHS/arachidonic acid activation in place of the S-9/NADPH system (59). Robertson et al. (60) reported that the PHS system (using ram seminal vesicle microsomal preparation) is about equally effective compared to rat liver S-9 as an activation system for benzidine.

Future Directions

The studies summarized in this review establish the potential role of peroxidase mechanisms in the activation of benzidine. The nature of the peroxidase-mediated interaction of benzidine with DNA is probably distinct from the nitrenium ion mechanism elucidated for arylamines such as 2-acetylaminofluorene. Characterization of the DNA adducts formed in vivo in the bladder and other target organs may help distinguish mechanisms of bioactivation. On the other hand, the interaction of benzidine with endogenous or synthetic nucleophiles may provide an opening for study of chemical carcinogenesis in vivo.

NOTE ADDED IN PROOF: The identification of the benzidine–thiol adduct is described in two recent publications (61,62).

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