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METABOLISM OF AZO DYES: IMPLICATION FOR DETOXICATION AND ACTIVATION*

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I. INTRODUCTION

The remarkable ability of the human eye to recognize hundreds of shades of color underlies the widespread use of dyes for aesthetic reasons as well as practical applications such as labeling, identification, etc. For example, food processing often results in natural colors fading and dyes are popular as a means of enhancing food attractiveness. Among the applications of dyes are textiles, food and drink, pharmaceuticals, cosmetics, plastics, paper and leather, printing inks, paint, varnish, lacquer, and wood stains. The history of dye usage, beginning several thousands of years ago, has been reviewed [1]. The color inherent in azo linkage derives from the conjugated system which facilitates pi-electron delocalization, giving rise to energy absorption at selective wavelengths in the visible spectrum. This permits the synthesis of a vast number of azo dyes of great intensity and variety of color. Thus, azo compounds represent a major proportion of total dye use. Although there are few comparable structures in nature, soil bacteria are able to convert certain aniline-based herbicides into various azo dyes [2]. Commercial dyes are prepared mainly by diazotizing aromatic primary amines and coupling the diazonium salts with phenols or aromatic amines with free ortho and/or para positions having high electron densities, or with other compounds having reactive positions. Dyes may contain one or more azo linkages. Electron-donating and -withdrawing groups on the aromatic ring markedly affect the coupling reaction through inductive and resonance effects. This fairly straightforward method of synthesis has facilitated preparation of hundreds of dyes with varying spectral properties. Unfortunately, some intermediates in azo dye manufacture, such as benzidine, 2-naphthylamine, and other aromatic amines, are carcinogenic or otherwise toxic. Efforts have been made to devise alternative routes of synthesis utilizing safer compounds and methods of production. Clarke discusses these problems in a recent review in this journal [3]. As seen for other chemicals that are so pervasive in the human environment, there is considerable concern about toxicity, and especially carcinogenicity, of these compounds among the consuming population. This area is dealt with briefly and the reader is referred to several reviews [1, 4, 5]. It appears that metabolic activation is required for all forms of toxicity by azo dyes and the extensive literature in this area is reviewed here. An excellent review on the metabolism and mutagenicity of select azo dyes appeared in this journal in 1985 [6]. Examples of representative azo dyes are found in Figures 1, 2, and 3. Many of these dyes contain benzidine in their structures (Figs. 2 and 3). This human bladder carcinogen may be released upon azoreduction of the dye, as discussed later.

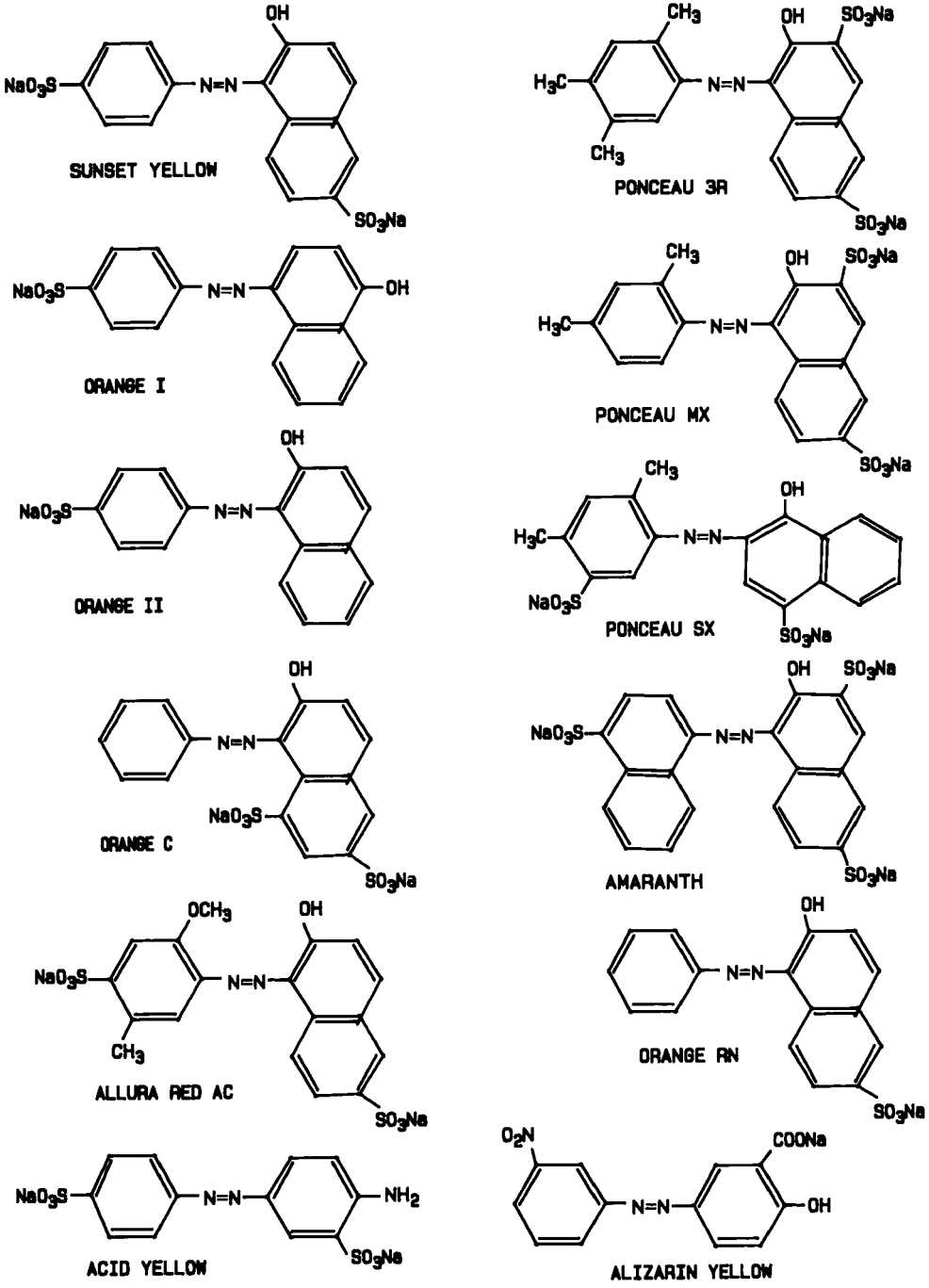

FIG. 1. Structures of representative azo dyes.

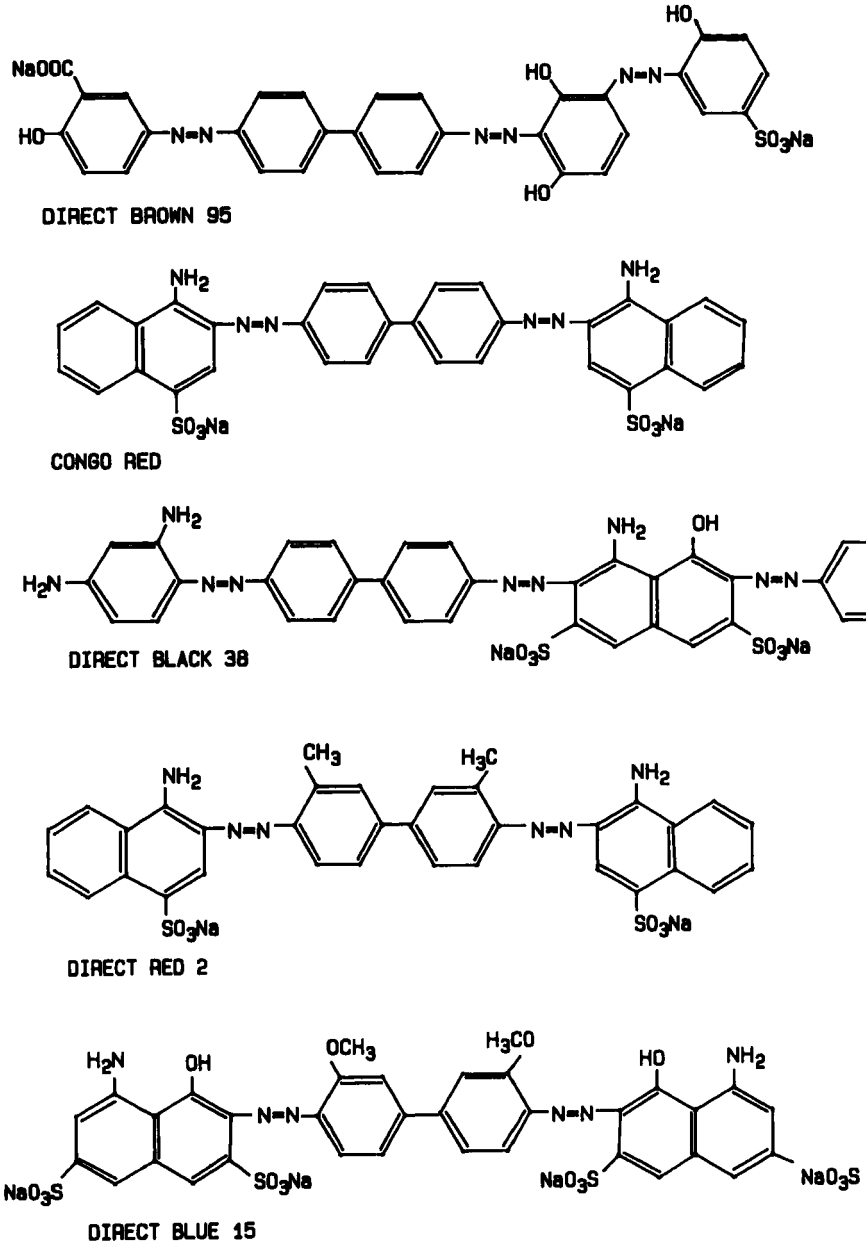

FIG. 2. Structures of representative benzidine-based azo dyes.

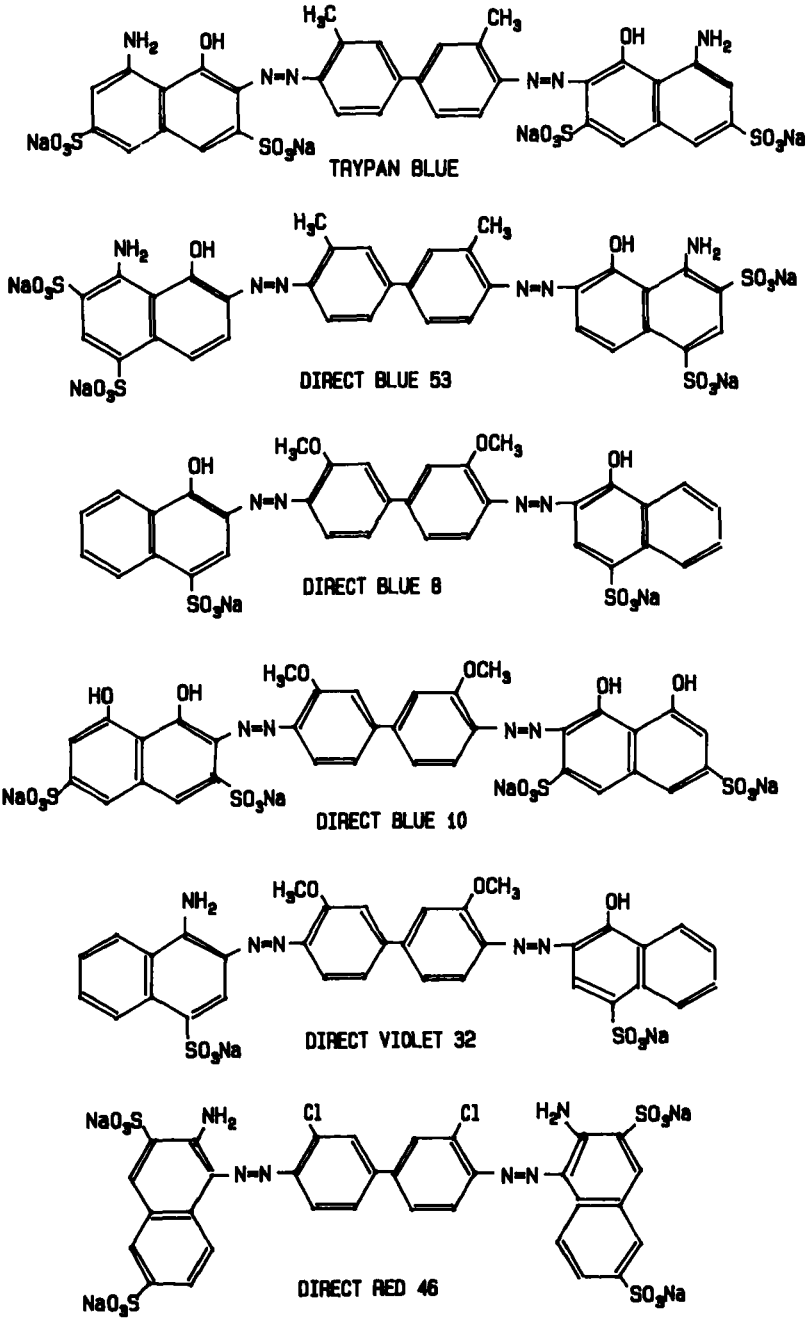

FIG. 3. Structures of representative benzidine-based azo dyes.

II. CHEMISTRY

Basic information on the chemistry of azo dyes is contained in Refs. 7 and 8 and is reviewed briefly in this section. Simple aromatic azo compounds, such as azobenzene, exist preferentially in the trans configuration, which is planar and considerably more stable than the corresponding hydrazo form. The phenyl rings of the cis form of azobenzene, formed by photoisomerization, are twisted away from the plane and the isomer is relatively unstable. Interconversion of cis and trans forms of certain azo compounds is responsible for their phototropism [8]. As the complexity of the molecule increases, steric factors maintain the molecule exclusively in the trans form. With simple amino-substituted azobenzenes, the basicity of the azo nitrogen increases through delocalization of the unpaired electrons of the aromatic amine. Although the latter becomes less basic, it remains more basic than the azo nitrogen. In dimethylaminoazobenzene (DAB), the amino nitrogen is the first to be protonated in dilute acid and the first to form an *N*-oxide in the presence of a mild oxidizing agent such as perbenzoic acid [9]. Conversion to the tertiary amine *N*-oxide abolishes the intense absorption band of DAB at 410 nm while retaining the weak absorption at 440 nm seen with azobenzene itself. Azoxy derivatives on either azo nitrogen, on the other hand, retain spectra nearly identical to that of DAB.

Introduction of a hydroxy group on one ring ortho or para to the azo linkage presents the possibility of enol-keto (azophenol-quinone hydrazone) tautomeric forms which are detected spectrophotometrically. The degree of tautomerism varies, but addition of an electron-withdrawing substituent on the opposite ring favors the hydrazone form. The direction of the equilibrium also depends on the relative stability of each form. For example, several structural isomers of phenylazonaphthol exhibit tautomeric equilibria (Fig. 4) because the total bond energies of the two forms are comparable. However, the 2-phenylazo-3-naphthol isomer can exist only in the phenol form due to loss of aromaticity and stability of the quinone form. Changes in tautomeric equilibria upon addition of acid or base to aromatic amino- or phenol-substituted azo dyes, respectively, lead to color changes, enabling the use of such compounds as pH indicators. Hydroxyl or amino substituents ortho to the azo linkage can form hydrogen bonds with the azo nitrogens, resulting in a stable 5- or 6-member ring. This prevents protonation of the azo nitrogens, thereby blocking color changes in acidic or basic solution. On the other hand, such hydrogen bonding locks the azo linkage into coplanarity with the aromatic rings increasing the spectral influence of other ring substituents which easily alter color characteristics. In simple aminoazobenzenes devoid of hydrogen bonded ortho substituents, protonation usually alters spectral properties, although absolute assignment of the proton to a

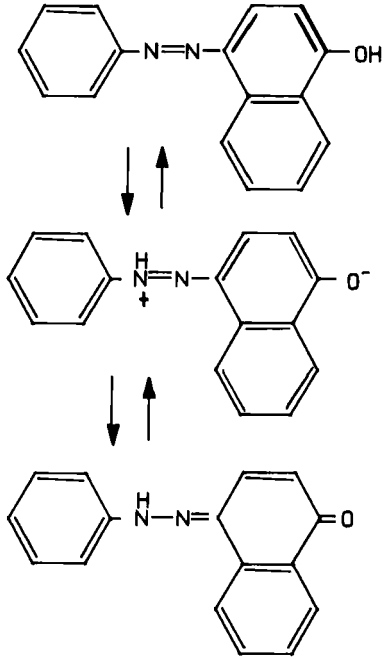

FIG. 4. Tautomeric equilibrium of 1-phenylazo-4-naphthol.

specific nitrogen is impossible and resonance between forms probably occurs. Formation of an azoxy group at the azo bond confers stability on the molecule due to different electronegativities of the two nitrogen atoms, leading to a somewhat ionic character of the linkage. Dual azoxy groups lead to repulsion of the two positively charged nitrogen atoms, although bond strength remains high. Reductive fission of the azo group, aside from the enzymic processes discussed below, can be effected by a number of reducing agents including sodium sulfide, stannous chloride, sodium hydrosulfite, titanous chloride, zinc dust, and Raney nickel. Loss of color follows reduction and these methods are used in dye detection and dye destruction when required in dyeing and printing.

Quantitative and qualitative characteristics of azo dye colors are dependent on (*a*) number and position of azo groups; (*b*) nature of aromatic nucleus, e.g., benzene, naphthalene, pyrazolone; and (*c*) nature and position of substituents, e.g., halo, alkyl, amino, hydroxyl, carboxyl, alkoxyl, nitro, and especially sulfonate. Increasing the number of azo linkages produces colorfast dyes although compounds with more than two linkages are considered to be less attractive. Color fastness also is increased by *N*-acylation and

halogenation of the acyl group. Specific examples of colors related to structural components are found in Ref. 7.

III. OXIDATIVE METABOLISM

Most commercially used azo dyes are substituted with large, highly charged groups, such as sulfonates, precluding significant oral absorption of intact dye and subsequent oxidative metabolism. Studies of Sudan I, Orange II, Tartrazine, Red 6B, Red 2G, Red 10B, Orange RN, Sunset Yellow, Ponceau MX, Ponceau 3R, Orange G [10], and Acid Yellow [11] indicate that only a few percent of an oral dose is excreted in the urine with the azo linkage (i.e., color) intact; apparently metabolism other than reduction does not occur. When intestinal bacteria, the major source of azoreductase for these dyes (see sec. IV.B), are bypassed through intravenous injection, substantial biliary excretion of intact dye is seen [12–17]. The lack of azoreduced products in the bile under these conditions may be attributable to the oxygen sensitivity of hepatic mechanisms for reduction of azo dyes. On the other hand, dyes with greater lipid solubility undergo significant oxidative metabolism. Early work on DAB by the Millers and others [18–23] revealed extensive oxidative and reductive pathways. Ring hydroxylation in the 4′-position and stepwise *N*-demethylation are major reactions. In the rat, oxidative metabolites with azo linkage intact are found mainly in the bile while products of azoreduction predominate in the urine [24–31]. After DAB administration, sulfate and glucuronide conjugates of aniline, *o*-aminophenol, *p*-aminophenol, and *p*-phenylenediamine appear in rat and dog urine, reflecting ring hydroxylation, *N*-demethylation, reduction, and conjugation, although not necessarily in that order [24]. After DAB or aminoazobenzene (AB) administration, small amounts of metabolites with azo linkage intact are also found in the urine including sulfates of 4′-hydroxyaminoazobenzene (4′-hydroxy-AB), 4′-hydroxymethylaminoazobenzene (4′-hydroxy-MAB), *N*-acetyl-4′-hydroxy-AB and 3-hydroxy-AB. However, it is unusual for xenobiotics of this size to be excreted in the urine of rats [29]. Biliary metabolites of DAB in the rat consist mainly of nonreduced structures including sulfates and glucuronides of 4′-hydroxy-DAB, 4′-hydroxy-MAB, and 4′-hydroxy-AB as well as AB itself. This indicates that the tertiary, secondary and primary amines can be hydroxylated in the 4′ position. Mono- and didemethylated DAB have also been identified in extrahepatic tissues and blood of rat after feeding, indicating either that extrahepatic tissue is capable of *N*-demethylating DAB or that these metabolites are derived from the liver prior to biliary or urinary excretion [18]. Biliary metabolites of orally

administered 3′-methyl-DAB include *N*-demethylated, ring-hydroxylated, and azo-reduced compounds [30] while urinary products are almost entirely derivatives of azo-reduced compounds.

More recently, identification of hydroxylated and *N*-demethylated metabolites in bile after DAB injection was confirmed and the role of hepatic cytochrome P-450 in their formation established through the use of selective inducing and inhibiting agents [28]. Treatment with either methylcholanthrene or phenobarbital, selective inducers of cytochrome P-450 activity, increases the rate of biliary excretion of DAB metabolites, whereas SKF 525-A and piperonyl butoxide, inhibitors of cytochrome P-450 activity, diminish their excretion. The response of each metabolic step to the inducing agents is highly selective. Using a 10,000-*g* supernatant fraction of rat liver, it was shown that the first demethylation of DAB is induced by methylcholanthrene but not phenobarbital; the latter, however, does induce the second demethylation [31]. Ring hydroxylation of the tertiary amine is induced by phenobarbital but not methylcholanthrene whereas both agents induce ring hydroxylation of the secondary and primary amines. Apparently the major pathways of oxidative metabolism of DAB, first *N*-demethylation, second *N*-demethylation, and 4′-hydroxylation, are catalyzed by different isozymes of cytochrome P-450. Partial confirmation of this selectivity is seen with a reconstituted mixed-function oxidase system using highly purified cytochrome P-450 prepared from methylcholanthrene-treated rat liver microsomes [32]. This form catalyzes the monodemethylation of DAB, but not the second demethylation or 4′-hydroxylation.

Glutathione (GSH) has a major involvement in the metabolism of DAB. Phorone, methyl iodide, and diethyl maleate are substrates for glutathione-*S*-transferase and their administration rapidly depletes hepatic GSH. Associated with depletion is depression of biliary excretion of DAB metabolites in rats [33, 34]. This is a specific effect on metabolism since biliary transport of preformed metabolites is unhindered by GSH depletion. Examination of individual biliary metabolites of DAB as well as *in vitro* experiments revealed a marked depression of metabolic formation and excretion of the didemethylated metabolite, AB, after GSH depletion while ring hydroxylation is unaffected [33, 34]. Inhibition of biliary excretion of DAB metabolites is reversed by administration of cysteine which permits rapid resynthesis of GSH. In a liver microsomal system, GSH stimulates both the *N*-demethylation and ring hydroxylation of DAB [35]. The effect is somewhat specific since nonphysiological thiols, 2-mercaptoethanol and dithiothreitol, depress *N*-demethylation; in contrast, both stimulate ring hydroxylation. The requirement that GSH be present at zero time to stimulate oxidative metabolism of DAB by rat liver microsomes suggests a protective effect associated with depression of lipid peroxidation [36] which is

detrimental to microsomal cytochrome P-450 activity [37–39]. The role of GSH in the fate of DAB was further elucidated by the identification in the bile of a major GSH metabolite, *N*-(glutathion-*S*-methylene)-4-aminoazobenzene [40, 41]. This labile conjugate easily decomposes to AB and has been proposed as the major source of the primary amine in the bile. This finding is consistent with the major route of metabolic activation of DAB which involves formation of a highly electrophilic center on the amino nitrogen capable of reacting with available cellular nucleophiles, including GSH (Sec. V). Thus, GSH regulates the fate of DAB through conjugate formation and through protection against lipid peroxidation. Also found in bile is the 4′-sulfonyloxy derivative of this labile GSH conjugate, suggesting that 4′-ring hydroxylation and sulfation precedes GSH conjugation. Interestingly, although the administration of DAB and related azo dye carcinogens might be expected to utilize a considerable amount of hepatic GSH, Neish and his colleagues reported that liver GSH levels are considerably increased 24 h after a single intraperitoneal dose of DAB and other azo dyes although levels rapidly diminished thereafter [42]. A rough correlation between carcinogenic potency and liver GSH levels was also seen [43]. These increases may be associated with initial cellular changes prior to transformation. Increased GSH parallels the increase in mitotic activity in regenerating liver and increased GSH is observed prior to division of cells in culture [44]. Therefore, GSH may be critical for cell proliferation in the early stages of azo dye tumorigenesis. A striking and rapid increase in liver GSH was also seen after injection of cobaltous chloride, an inhibitor of the mixed-function oxidase system [45]. Thus, the increase in GSH levels associated with administration of azo dyes may be unrelated to their metabolic fate. The response of GSH levels to azo dyes also exhibits seasonal variability, but this does not relate to the carcinogenicity of the dyes [42].

Earlier work suggested the possibility of DAB-*N*-oxide as an intermediate in the *N*-demethylation of DAB [46]. In the presence of iron porphyrin compounds, it rapidly forms demethylated derivatives. Tumorigenicity after treatment with the *N*-oxide has also been reported [47]. However, a definite role for such an intermediate has never been established. *N*-Hydroxylation, however, is an essential step in the activation of azo dye carcinogens and other toxic aromatic amines (Sec. V). *N*-Hydroxylation of aromatic amine carcinogens was first shown by Cramer et al. for 2-acetylaminofluorene through detection of the *N*-hydroxylated compound in the urine [48]. A similar reaction for MAB was reported by Kadlubar *et al.* [49] and it is probable that *N*-hydroxylation is critical for the carcinogenic activities of all aromatic amines, amides and nitro compounds [50]. The step is catalyzed by both microsomal cytochrome P-450 and flavin-containing monooxygenase [49, 50–53]. The species of cytochrome P-450 which is involved is in-

duced by methylcholanthrene but not by phenobarbital and is absent from uninduced microsomes [53]. This was demonstrated by selective effects of the inducing agents as well as selective inhibition of the reaction by antibodies to methylcholanthrene-induced cytochrome P-450 but not by antibodies to phenobarbital-induced cytochrome P-450 [53]. Formation of nitroxide radicals from *N*-hydroxy-MAB and *N*-hydroxy-AB has been reported [54, 55]. EPR signals consistent with such radicals have been detected during *in vitro* incubations of DAB or MAB with rat liver microsomes and in livers from rats fed 3′-methyl-DAB [54, 55]; signal formation *in vitro* is NADPH dependent. Although EPR signals formed from 3′-methyl-DAB are carbon monoxide sensitive, those from 3′-methyl-MAB are not. This is consistent with involvement of cytochrome P-450 in *N*-demethylation of the tertiary amine but not necessarily in *N*-hydroxylation of the secondary amine which is catalyzed to a significant extent by microsomal flavin-containing monooxygenase. The parallel between *N*-hydroxylation and carcinogenicity of DAB and MAB derivatives and the readily demonstrated radical formation from *N*-hydroxylated derivatives led to the proposal of direct involvement of nitroxide radical in the carcinogenic process [55]. In support of this is the finding that nitroxide radical binds covalently to DNA [55]. Strong binding to poly G implies that guanine is the site of attack. However, further work is required to unequivocally establish a critical role for such radicals.

IV. REDUCTIVE METABOLISM

A. Mammalian Systems

Biological reduction of azo linkage has been known for many years [10, 56–59]. The now classic work of Bovet and his colleagues [60] showed in rabbits that the antibacterial azo dye, prontosil, is active only after azoreduction yielding sulfanilamide. This was subsequently reported in humans [57]. It was the first demonstration of metabolic activation of a therapeutic agent. In contrast, benzeneazophenol and benzeneazoresorcinol are excreted in urine as their glucuronides with azo linkage intact, indicating resistance to reduction [61]. Nearly 50 years ago, Mueller and Miller [19, 63] showed that DAB was reduced by rat liver microsomes. The reaction required NADPH which suggested involvement of the flavin enzyme now known as NADPH-cytochrome P-450 reductase. (Note that these reports preceded the discovery of cytochrome P-450.) Fouts et al. [64] found that rabbit liver homogenate reduced water soluble dyes such as protonsil and Orange IV, as well as DAB. Kidney, lung, heart, and brain also exhibited activity which

was associated both with microsomal and supernatant fractions. FMN and FAD stimulated reduction while oxygen sensitivity of the reaction varied with substrate. In retrospect, these were important observations since they were predictive of later findings of multiple azoreductases with varying mechanisms and sensitivities to oxygen (see below). Purified NADPH–cytochrome P-450 reductase was shown to reduce neoprontosil; Hernandez et al. [65, 66] showed that reduction of this dye by rat liver microsomes was attributable both to the flavoprotein, using a partially purified enzyme, and to cytochrome P-450, based on inhibition by carbon monoxide and induction by treatment with phenobarbital and methylcholanthrene. Placental reduction of neoprontosil, on the other hand, is unaffected by oxygen and carbon monoxide and is not stimulated by flavins [67], distinguishing this activity from those previously described. In earlier work, it was not possible to determine whether full four-electron reduction occurred at once or whether there were separable steps. Evidence for a two-step reduction process came from the findings that administered azobenzene can be partially recovered in the urine as hydrazobenzene (two-electron-reduced form) and benzidine (formed by acid-mediated rearrangement of hydrazobenzene) [58, 68].

Fujita and Peisach [69] reported that the microsomal reduction of amaranth is almost totally inhibited by carbon monoxide and attribute all of the activity to cytochrome P-450 in contrast to that seen with prontosil and related dyes where both flavoprotein and cytochrome are involved. Treatment with either phenobarbital or methylcholanthrene induces activity, indicating lack of specificity among the various forms of cytochrome P-450. In microsomal systems, activity is proportional to the quantity of cytochrome P-450 present [69] and antibodies to both types of cytochrome inhibit activity [70]. Oxygen inhibits and added flavin stimulates reduction [71]. The flavin-stimulated activity is insensitive to carbon monoxide, implying that the flavin stimulates electron flow directly from flavoprotein (NADPH– cytochrome P-450 reductase) to dye. A highly purified form of cytochrome P-450 prepared from phenobarbital treated rats also reduces amaranth under anaerobic conditions [72]. Here too, added flavin [FMN] stimulates activity although flavoprotein alone does not reduce amaranth. More recent work [73] confirms that cytochrome P-450 is the sole source of oxygen- and carbon monoxide-sensitive amaranth azoreductase in hepatic microsomes.

Microsomal reduction of DAB exhibits unusual characteristics. It is not inhibited by oxygen [74, 75] or carbon monoxide [76, 77] although slight inhibition by the latter has been reported [78]. The unique character of this system is shown further by its selective induction after treatment with clofibrate, a hypolipidemic agent, but not other common inducers of cytochrome P-450 activity such as phenobarbital, methylcholanthrene, β-naphthoflavone, isosafrole, or pregnenolone-16α-carbonitrile [76, 79]. A form of

clofibrate-inducible cytochrome P-450 has been purified from rat liver microsomes [80, 81]. It is characterized principally as a laurate hydroxylase [82] and several pieces of evidence show that it is distinct from the form of cytochrome P-450 which catalyzes DAB azoreduction [76, 83]. First, undecynoic acid [84] selectively inhibits microsomal laurate hydroxylase activity but not DAB azoreductase activity (Fig. 5). Second, lauric acid inhibits microsomal *N*-demethylation of DAB, an oxidative pathway, probably as a competitive substrate, but has no effect on azoreduction of DAB (Table 1). Third, when rats were treated with a series of hypolipidemic compounds, mainly related to clofibrate, no correlation was found between effects on laurate hydroxylase and DAB azoreductase activities (Fig. 6) [79]. The lack of carbon monoxide inhibition initially cast some doubt on cytochrome P-450 as the DAB azoreductase since such inhibition is considered the hallmark of cytochrome P-450 activity. Nevertheless, suppression of azoreduction by known inhibitors of cytochrome P-450 activity and reconstitution of DAB azoreductase activity using purified enzymes established the fact unequivocally [84]. Although purified NADPH–cytochrome P-450 reductase exhibits weak DAB azoreductase activity, addition of cytochrome P-450 enhances activity manyfold, confirming the cytochrome as the major catalyst in this reaction [83, 85].

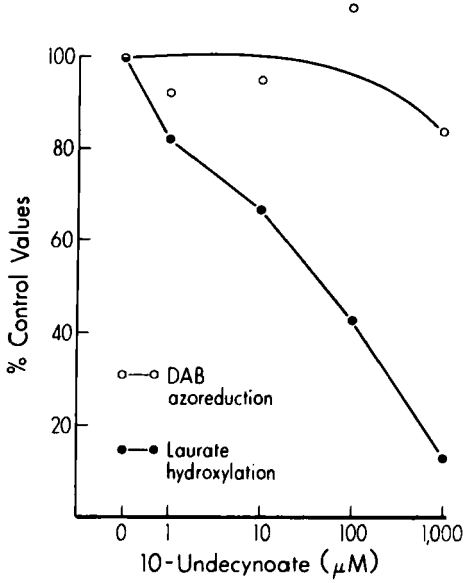

FIG. 5. Effect of 10-undecynoate on the microsomal reduction of DAB and hydroxylation of laurate. From Levine and Raza [83] with permission of the publisher.

TABLE 1
Effect of Lauric Acid on Oxidative and Reductive Pathways of DAB Metabolism

DAB metabolites[a]	Lauric acid concentration			
	0	0.01 mM	0.10 mM	1.0 mM
MAB	23.3 ± 1.5	23.4 ± 1.0	22.2 ± 1.3	17.0 ± 0.1
AB	11.4 ± 0.5	11.2 ± 0.6	7.7 ± 0.4	1.6 ± 0.1
4′-Hydroxy-DAB	5.5 ± 0.6	5.5 ± 0.6	5.6 ± 0.6	4.0 ± 0.3
Azoreductase products	10.4 ± 0.5	7.8 ± 0.7	10.0 ± 0.4	11.7 ± 0.7

[a] Measured as nmol of metabolite present after 10 min of incubation of DAB plus microsomes. Standard errors are indicated
From Raza and Levine [76] with permission of the publisher.

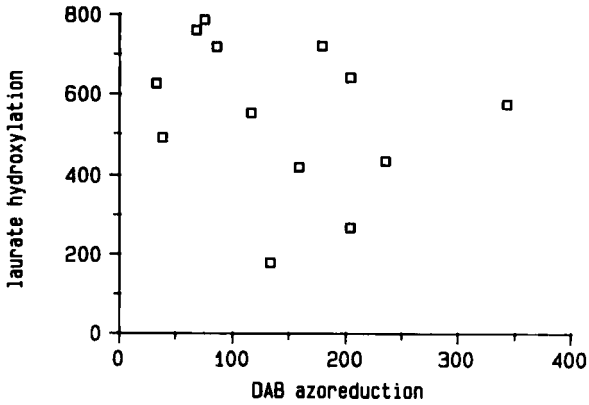

FIG. 6. Laurate hydroxylase and DAB azoreductase activities of hepatic microsomes prepared from rats treated with various hypolipidemic agents. The compounds used included clofibrate, clofibric acid, ciprofibrate, 2-(*p*-chlorophenoxy)-propionic acid, 2-(*o*-chlorophenoxy)-2-methylpropionic acid, 2-(*m*-chlorophenoxy)propionic acid, nafenopin, WY-14643, WY-14807, WY-15672, WY-14730, probucol, and di(ethylhexyl)phthalate. From Raza and Levine [79] with permission of the publisher.

A structure-activity study of various azobenzenes revealed that a polar electron-donating group (amino or hydroxy) para to the azo linkage is required for substrate activity with microsomes (Fig. 7) [86]. Azobenzene itself, lacking any ring substituents, is unreactive, although its reduction

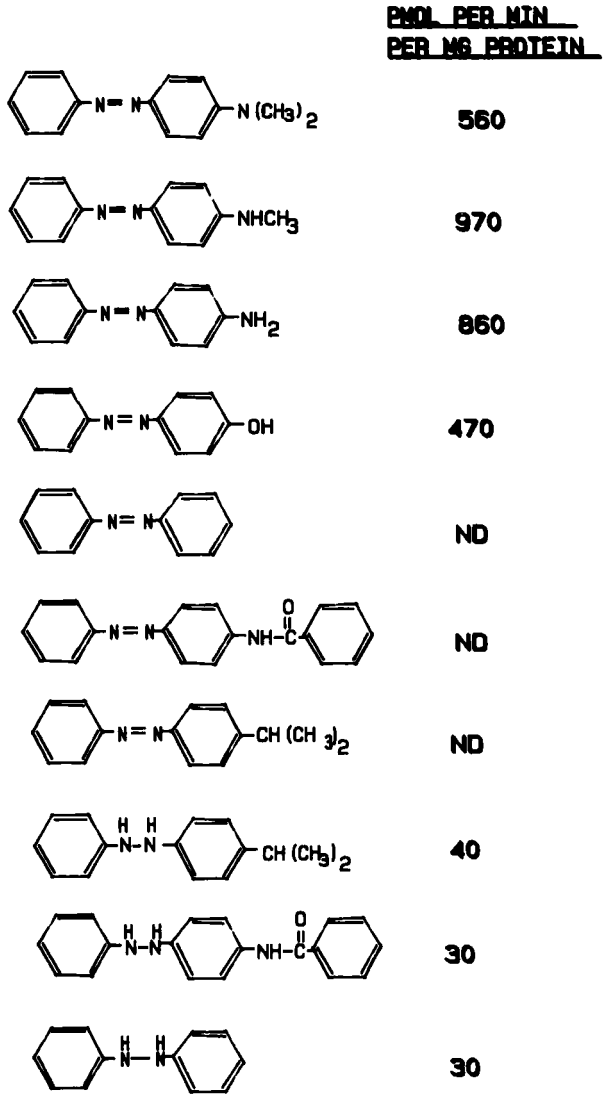

FIG. 7. Structure-activity relationship of azo dyes and microsomal azoreductase activity. Activity was determined from the fluorescence of primary amine reduction products formed during incubation of substrate and hepatic microsomes. From Zbaida et al. [85] with permission of the publisher.

products have been isolated from rat urine after feeding [58]. If the tertiary amine group of DAB is replaced by an isopropyl structure which has approximately the same bulk but is nonpolar, no activity is seen. Furthermore, if the primary amine, AB, a readily reducible substrate, is benzoylated at the 4-amino position, the electron-withdrawing carbonyl group on the benzoyl moiety prevents electron donation to the azo ring system and the resulting product is inactive. Similarly, substituting a nitro group (electron-withdrawing) for the tertiary amine of DAB destroys activity. The polar electron-donating substituents may be responsible for binding to microsomal cytochrome P-450 since known substrates, DAB and so forth, exhibit typical binding spectra when added to microsomes whereas no spectra are apparent upon addition of enzymically nonreducible dyes [85]. Chemically reducing these enzymically inactive compounds to their two-electron-reduced derivatives with metallic zinc does not confer ability to induce binding spectra and azoreductase activity is minimal (Fig. 7). On the other hand, similar attempts at two-electron reduction of azo substrates yielded only fully reduced amines. It appeared that the two-electron-reduced intermediates spontaneously degrade to primary amines. The one exception is DAB, where a spectrally definable two-electron-reduced intermediate is seen upon reduction by zinc. It is exquisitely sensitive to reoxidation by molecular oxygen and, even under rigorous anaerobic conditions, disproportionates upon addition of water to the parent azo form and free amines (Fig. 8) [85]. It has been

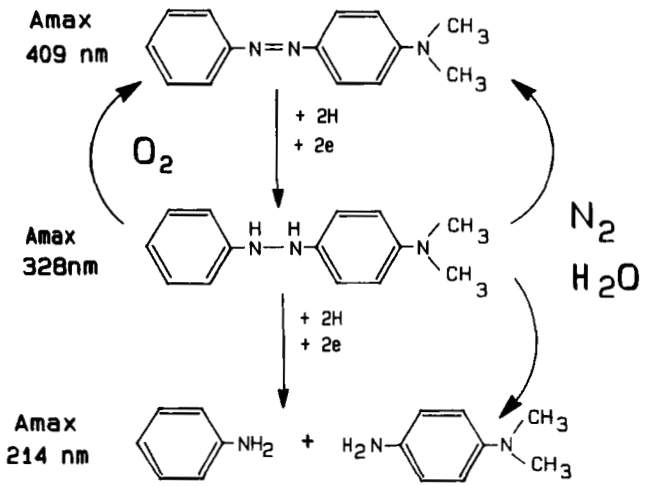

FIG. 8. Two-electron reduction of DAB by metallic zinc and subsequent aerobic and anaerobic pathways.

postulated that the proton on the electron-donating group (amino, phenolic) of most substrates migrates to an azo nitrogen, destabilizing the azo linkage and preventing the reaction from stopping at the two-electron-reduced stage. DAB, a tertiary amine lacking this proton, can form a two-electron-reduced intermediate in the zinc system which is stable under appropriate anaerobic conditions. Even for this intermediate, addition of water facilitates protonation of the azo linkage and further reduction. It appears that enzymic reduction of DAB, and possibly related azo structures, involves a two-electron transfer with formation of an intermediate which spontaneously disproportionates, yielding primary amines. One must then reconcile these chemical results with the oxygen-insensitive microsomal reduction of DAB. It would be expected that, whether cytochrome P-450 transfers one or two electrons to the dye, the resultant intermediate would be highly sensitive to oxygen. Therefore one could postulate a hydrophobic binding site within the cytochrome where DAB would be protected from both oxygen and water, permitting oxygen-insensitive, two-electron reduction. Primary amine formation may involve disproportionation of this intermediate upon release from the hydrophobic environment. Additionally, since DAB disproportionates in an aqueous environment [85], the rate of primary amine formation actually measured in azoreductase experiments may reflect this mechanism rather than an uninterrupted 4-electron reduction.

Further insight into microsomal reduction mechanism was gained through a structure-activity study of DAB-related dyes using compounds containing electron-donating (Fig. 9) or electron-withdrawing (Fig. 10) ring substituents. Microsomal reduction of the former group exhibits characteristics of DAB reduction, i.e., insensitivity to oxygen and carbon monoxide, whereas reduction of the latter is sensitive to both [86, 87]. The former group is designated as I (insensitive) and the latter as S (sensitive). Azoreduction of the I substrates responds selectively to induction by clofibrate, whereas azoreduction of the S substrates is induced by phenobarbital, β-naphthoflavone, isosafrole, pregnenolone-16α-carbonitrile, as well as clofibrate (Fig. 11). In view of sensitivity to carbon monoxide, it can be concluded that the S substrates are reduced in a microsomal system mainly by cytochrome P-450. Furthermore, substrates such as amaranth, neoprontosil and other highly polar azo dyes are also reduced by cytochrome P-450 in an oxygen- and carbon monoxide-sensitive manner. These may also be considered S substrates. Mason and his colleagues have demonstrated that oxygen-sensitive reductions proceed through a one-electron-reduced free radical which is detectable by EPR and readily reoxidized by molecular oxygen [88]. Recent studies have been carried out in our laboratory on reduction potentials of azo dyes using cyclic voltametry [89]. Both I and S substrates, as well as nonsubstrate dyes such as azobenzene, exhibited two

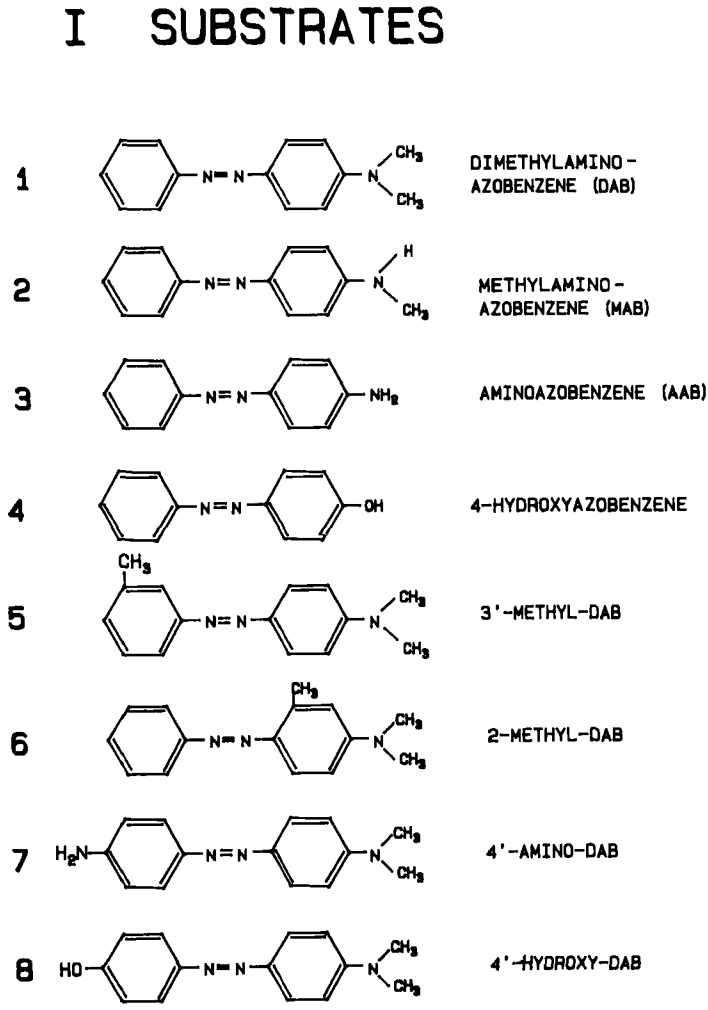

FIG. 9. Azo dyes reduced by rat liver microsomes by oxygen- and carbon monoxide-*insensitive* mechanism (I = insensitive substrate). The structures contain only electron-donating substituents.

negative potentials (Table 2) consistent with previous observations on electrochemical reduction of azo dyes [90–92], reflecting, respectively, one- and two-electron-reduced forms. In the case of I substrates, the signal for the first (one-electron-reduced) potential is temporarily stable in air whereas that of the S substrates is immediately quenched in air. Therefore, another possible explanation for the differential oxygen sensitivities of the I and S

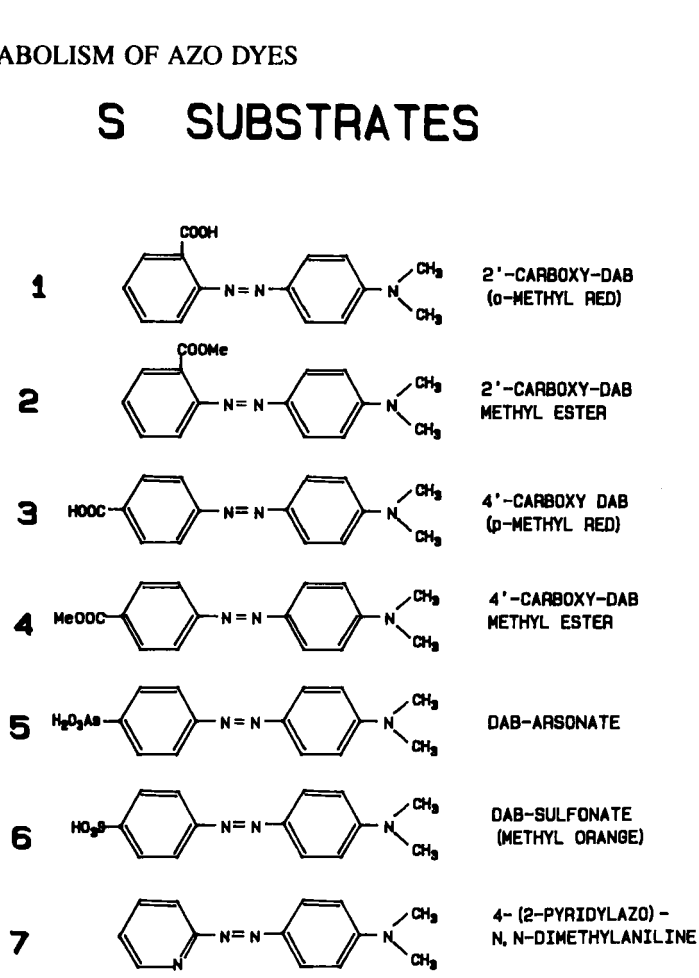

FIG. 10. Azo dyes reduced by rat liver microsomes by oxygen- and carbon monoxide-*sensitive* mechanisms (S = sensitive substrate). The structures contain both electron-donating and electron-withdrawing substituents.

substrates may be found in the stability of their one-electron-reduced intermediates. If water is admitted to the anaerobic system, allowing protonation of reduced forms, the second electron signal is rapidly quenched while the first is quenched more slowly; this is seen for both I and S substrates. Subsequently, fully reduced amines are formed [89]. These results support the probable mechanism of reduction illustrated in Fig. 12. Addition of the first electron yields a free radical, although its location in the molecule cannot be accurately indicated. Since addition of water, which allows protonation of the azo linkage, immediately quenches the second electron signal, it is apparent that addition of the second electron occurs prior to protonation.

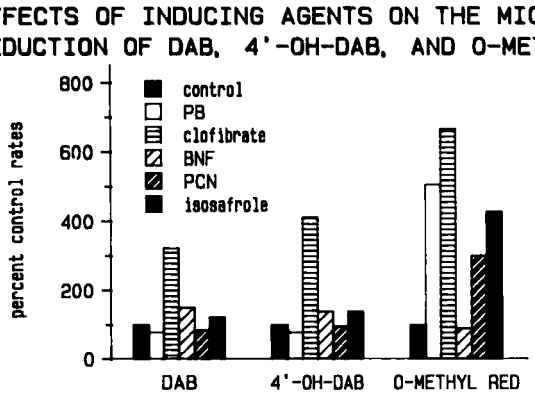

FIG. 11. Microsomal reduction of I and S substrates. Response to pretreatment with various inducers of cytochrome P-450 activity. From Zbaida and Levine [86] with permission of the publisher.

Further addition of water also quenches the first electron signal suggesting that slower protonation of the one-electron-reduced intermediate may also occur. The mechanism of further reduction of the hydrazo intermediate is not known but it must involve the addition of two electrons and two protons to form primary amines. Based on observations with zinc-reduced DAB [85], final reduction may proceed through disproportionation.

Transfer of electrons to dyes from cytochrome P-450 or NADPH–cytochrome P-450 reductase is not explainable based on the negative potentials of the substrates (Table 2) since the enzyme potentials have been reported to be -0.3 V to -0.4 V [93, 94] and electron transfer can only proceed in the direction of more positive potential. However, each substrate also exhibits a positive potential ($+0.98$ V to $+1.64$ V), which is absent or low in nonsubstrates, as does each of the primary amine reduction products (Table 3). It is likely that the positive potential relates to the polar electron-donating groups present in both substrates and reduced products. These groups are therefore responsible not only for binding to cytochrome P-450 [87] but for providing the appropriate potential for electron transfer to the dye.

A priori, one might not expect that cytochrome P-450-catalyzed azoreduction would be carbon monoxide sensitive since simple displacement of oxygen on the sixth ligand of the iron heme should not affect a reaction which is not oxygen dependent. Yet the microsomal reduction of amaranth [69], neoprontosil [66] and S substrates [87] is completely or partially inhibited by carbon monoxide. The change in redox potential of cytochrome P-450 upon binding with carbon monoxide [95] might alter azoreductase

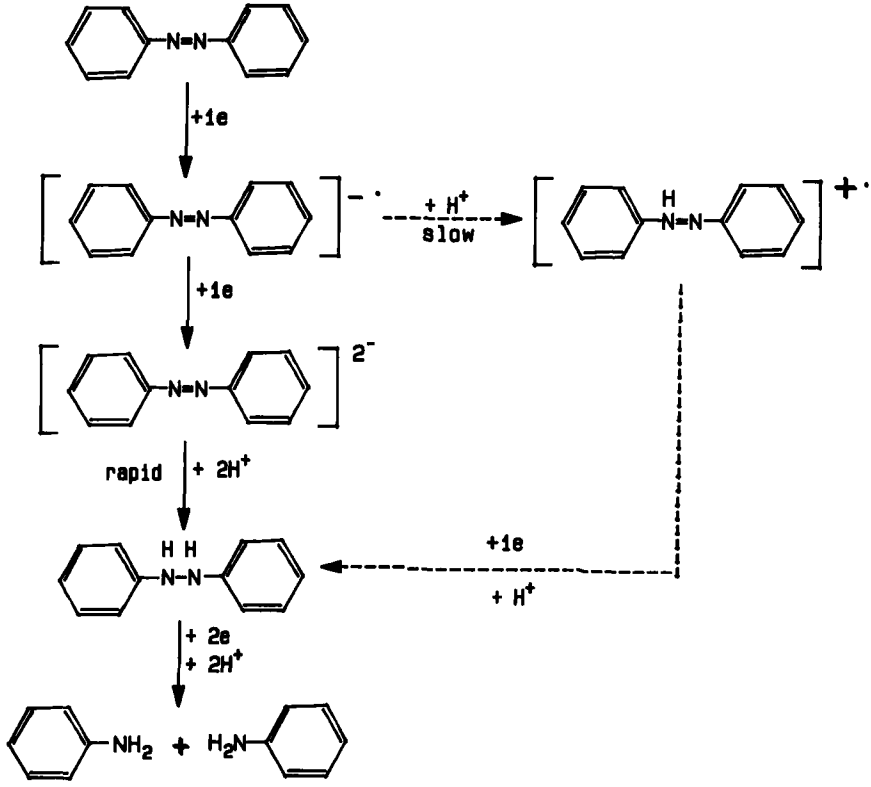

FIG. 12. Proposed mechanism for the microsomal azoreduction of I and S dyes. The first electron is received forming a free radical. Following transfer of the second electron, protonation is rapid forming the true hydrazo intermediate which spontaneously reduces to primary amines. Protonation of the one-electron-reduced free radical also occurs but less readily than protonation of the two-elctron-reduced form.

activity since the difference in redox potential between enzyme and azo dye substrate would be a significant factor in the reaction. Changes in the redox potential of cytochrome P-450 also occur upon addition of selective substrates [96]. In addition, slow electron transfer between reduced P-450 and amaranth can occur in the presence of carbon monoxide [71]. Since carbon monoxide binding is reversible, involving an on-rate and off-rate, the question of azoreduction in the presence of carbon monoxide may simply be a competition between the rate of electron flow to dye and on-rate of carbon monoxide. As indicated above, the measurable rate of dye reduction may be somewhat slower than the actual rate of electron transfer from cytochrome

TABLE 2
Reduction Potentials for Various Azo Dyes Structurally Related to DAB

Compound	Negative potentials	Positive potentials
Nonsubstrates		
Azobenzene (AB)	−1.34, −2.26	—
4-Isopropyl-AB	−1.41, −2.22	—
4-Benzoylamino-AB	−1.25, −1.64	—
Hydrazobenzene[a]	—	+0.48
4-Isopropylhydrazobenzene[a]	—	+0.44
4-Benzoylhydrazobenzene[a]	—	+0.41
I substrates		
DAB	−1.55, −2.28	+1.03
MAB[b]	−1.66, —	+0.98
AAB[b]	−1.70, —	+1.04
p-Hydroxy-*AB*	−1.38, −2.04	+1.54
4′-Hydroxy-MAB	— , −2.22	+0.82
4′-Hydroxy-DAB[c]	— , −1.50	+0.63
4′-Amino-DAB[c]	— , −1.88	+0.68
2′-methyl-DAB	−1.56, −2.30	+0.99
3′-methyl-DAB	−1.56, −2.34	+1.04
S substrates		
2′-carboxy-DAB (*o*-methyl red)	−0.90, −1.72	+1.09
2′-Carboxy-DAB methyl ester[c]	— , −1.45	+1.06
4′-Carboxy-DAB (*p*-methyl red)	−1.17, −1.53	+1.05
4′-Carboxy-DAB methyl ester	−1.27, −1.84	+1.09
DAB arsonate[c]	— , −1.88	+1.05
DAB sulfonate[c] (methyl orange)	— , −1.55	
4-(2-Pyridylazo-*N*,*N*-dimethylaniline)	−0.80, −1.38	+1.14

[a]No negative potentials were visible.
[b]Only the first negative potential was visible.
[c]Only the second negative potential was visible.
From Zbaida and Levine [89] with permission of the publisher.

TABLE 3
Reduction Potentials for Aromatic Amino and Phenolic Reduction Products of Azo Dyes

Compound	Potential
Aniline	+1.07
N,*N*-Dimethyl-1,4-phenylenediamine	+0.86, +1.35
N-Methyl-1,4-phenylenediamine	+0.60, +1.41
o-Aminobenzoic acid	+1.31
p-Aminophenol	+1.39
Methyl-4-aminobenzoate	+1.25

From Zbaida and Levine [89] with permission of the publisher.

P-450 to dye due to subsequent disproportionation of the reduced intermediate. Therefore, direct comparisons between CO on-rate and electron flow to dye is not possible at the present time. Other CO-insensitive, cytochrome P-450-catalyzed reactions are known. Aromatase cytochrome P-450 from human placenta catalyzes conversion of androstenedione to estrone by a CO-insensitive mechanism [97–101]. Evidence suggests that substrate is bound to the heme site in such a manner that the C-19 methyl group interferes with CO binding. Aromatization of substrates lacking this substituent is inhibited by CO [100, 101].

Azoreduction is also catalyzed by cytosolic enzymes. NAD(P)H:quinone reductase (DT-diaphorase) reduces methyl red (2′-carboxy-DAB) [102]. Unlike the microsomal system, substrate specificity for this enzyme is highly restrictive. Removing or methylating the carboxyl group or shifting it to the opposite ring destroys all activity as does substituting a hydroxyl for the carboxyl, eliminating the possibility that hydrogen bonding from carboxyl to azo nitrogen is critical. Except for methyl red, the azo dyes in Figs. 5 and 6 exhibit little or no activity with the enzyme. The enzyme contains two molecules of FAD and catalyzes a two-electron reduction which is oxygen insensitive [102]. The oxygen sensitivity of cytochrome P-450-catalyzed reduction of methyl red and the oxygen insensitivity of its reduction by NAD(P)H:quinone reductase is consistent with the theory that oxygen-sensitive azo dye reduction is in part attributable to a readily reoxidizable one-electron-reduced intermediate as demonstrated by free radical formation [88] and oxygen-sensitive redox potentials [89]. NAD(P)H:quinone reductase activity is inhibited by dicumarol and induced *in vivo* by treatment with methylcholanthrene, *trans*-stilbene oxide, 2-acetylaminofluorene, 2(3)-*tert*-butyl-4-hydroxyanisole, ethoxyquin, disulfiram, Sudan III (an azo dye), and a large series of phenolic antioxidants, azo dyes, polycyclic aromatic hydro-

carbons, coumarins and other lactones, flavonoids, and selective sulfur compounds such as dithiocarbamate [103, 104]. The enzyme has been proposed as a protective mechanism against the toxicity of various quinones [104, 105]. Chronic treatment of hamsters with estradiol results in a temporary (1 month) but marked decrease in enzyme activity in the kidney but not the liver [106]. Thereafter activity is considerably induced in liver but not kidney. The loss of kidney activity at 1 month was offered as an explanation for the induction of kidney tumors by estrogen, possibly through decreased ability to reduce estrogen free radicals derived from microsomes. Another cytosolic enzyme, aldehyde oxidase, was reported to reduce methyl red, amaranth, methyl orange, and DAB anaerobically [107]. Electron donors include acetaldehyde, 2-hydroxypyrimidine and N^1-methylnicotinamide, but not NADPH or NADH. Dicumarol, an inhibitor of NAD(P)H:quinone reductase, and SKF 525-A, an inhibitor of cytochrome P-450 activity, blocked azoreduction by aldehyde oxidase. Preliminary work in our laboratory indicates that this enzyme will utilize as electron acceptors selective azo compounds that are reduced by the microsomal system [108] and contain polar electron-donating substituents. It is not known which of its three electron transfer centers—molybdate, iron–sulfur, and FAD—donates electrons to azo dye substrates. However, this molybdoflavin does reduce a number of hydroxyamic acids [109], sulfoxides [110], nitrosamines [111], and aromatic nitro compounds [112]. Aldehyde oxidase and xanthine oxidase are members of a group of cytosolic (and possibly mitochondrial) molybdenum hydroxylases which catalyze oxidative and reductive metabolism of a number of xenobiotics, especially nitrogen-containing heterocyclic compounds [113, 114]. Recently a diaphorase-like activity has been identified in rat brain microsomes [115]. It contains one molecule each of FAD and FMN, thus bearing a similarity to liver microsomal NADPH–cytochrome P-450 reductase. With NADPH as electron source, the enzyme reduces nitrobluetetrazolium anaerobically, although it is not known whether reduction proceeds to the hydrazo or fully reduced primary amine. The reaction is not inhibited by dicumarol or SKF 525–A.

B. Bacterial Systems

Early work on the metabolism of azo dyes by microorganisms was reviewed in 1970 [116] and a discussion of the factors which affect their metabolic activity has recently appeared in this journal [117]. The ability of microorganisms to reduce azo dyes was established long before intestinal bacteria were implicated in the reduction of orally ingested dyes [116]. *In vitro* bacterial and yeast systems reduce many azo dyes quite nonspecifi-

cally. It appears that reduction of the majority of azo dyes is sensitive to oxygen diminishing the likelihood of hepatic reduction of these compounds, although the pO_2 in most parts of the liver is considerably below that of air. Early observations that reduction products of azo dyes such as FD&C Red no. 2, FD&C Red no. 4, FD&C Red no. 6, and Acid Yellow appear in the urine [10, 11] has focused a great deal of attention on the intestinal bacteria since the lumen of the lower bowel is essentially anaerobic and is populated by hundreds of facultative and obligate anaerobes. Many species of bacteria reduce numerous azo dyes and the reaction appears to lack specificity [117–122]. Although much of the work has been done with crude preparation from intestinal contents and feces, a number of specific species were shown to be active including *Streptococcus faecalis, Proteus vulgaris, Klebsiella aerogenes, Escherichia coli,* and others. Chung et al. [122] reported that 10 distinct intestinal anaerobes reduce amaranth, Ponceau SX, Allura Red, Sunset Yellow, Orange II, methyl orange, and tartrazine. The enzymes were assumed to be flavorproteins but unambiguous proof was not offered. There is no inhibition by carbon monoxide or cyanide, ruling out most cytochromes, although lack of carbon monoxide inhibition does not necessarily eliminate cytochrome P-450 as the catalyst (see Sec. IV.A). Reduction of Red 2G by a crude cell-free extract of *Streptococcus faecalis* is markedly stimulated by exogenous FMN or FAD [119]. These flavins are directly reduced by the bacterial enzyme and, in turn, rapidly and independently reduce the dye. It has been suggested that flavin acts as an electron shuttle between the bacterial enzyme and the dye [119], a concept supported by several groups [120, 122]. In view of their charged nature, it is unlikely that FAD and FMN could readily shuttle across bacterial cell walls and membranes under physiological conditions. However, riboflavin may fit such a role. Bacterial reduction of tartrazine is also stimulated by the synthetic electron carriers, methyl viologen, benzyl viologen, phenosafronin, neutral red, crystal violet, menadione, and Janus Green B [122]. Zero-order kinetics of the reduction of amaranth, Orange II, Ponceau 3R, Ponceau SX, Sunset Yellow, and tartrazine by *Proteus vulgaris* suggest nonenzymic reduction and is consistent with the idea of an electron shuttle concept [120]. This concept is helpful in rationalizing reduction of the many highly charged sulfonated dyes by intact bacterial cells since it is unlikely that such dyes could pass through the cell wall and plasma membrane. An electron shuttle could effect the reduction with the dyes remaining extracellular. It also may serve as a partial explanation for the oxygen sensitivity of these reductions if one assumes that reduced flavin has greater affinity for oxygen than for dye.

One of the few studies on purified bacterial azoreductase is that of Zimmerman [123, 124]. By growing Pseudomonas KF46 on media containing either Orange I or Orange II as the sole source of carbon and nitrogen, he

was able to induce and purify two enzymes which selectively reduced one, but not the other, dye. In contrast to most of the reports with crude bacterial preparations, these enzymes have specific substrate structural requirements. Slight deviations in the position or nature of the hydroxy or sulfonate substituents on the dyes result in total loss of activity in most cases. Molecular weights of the enzymes (21,000 and 30,000) differ considerably; both use NAD(P)H as electron source and function aerobically, which is unusual for bacterial systems. There is no enzyme-bound FAD and the catalytic mechanisms are unknown. Substrate reactivity correlates directly with the electronegativity of ring substituents.

Although bacterial azoreduction generally requires an anaerobic atmosphere, there are reports on the reduction of 4,4′-dicarboxyazobenzene [125], 2,4- and 4,4-diaminoazobenzene, DAB, *p*-hydroxyazobenzene, phenylazoresorcinol, *p*-nitrophenylazorescorcinal, *p*-aminoazobenzene, and phenylazobenzene [126, 127] in the presence of air. *p*-Aminoazobenzene is also reduced aerobically by liver microsomal cytochrome P-450, as are the corresponding secondary (MAB) and tertiary (DAB) amines [75]. No simple mechanistic conclusion can be drawn from this since DAB, which can be reduced aerobically by liver microsomes, requires an anaerobic environment for bacterial reduction [74].

A concern in the spectrophotometric measurement of dye reduction by bacteria is nonspecific adsorption to cell walls. This results in an immediate decrease in dye absorbance and varies greatly from one dye to another, complicating assays based on dye disappearance [126]. The presence of nonspecifically adsorbed dyes may or may not affect bacterial reduction of other dyes by blocking access to intracellular enzymes, a complication which must be addressed in such assays.

Among the azo dyes used in the textile, paper printing, and leather industries are benzidine and benzidine congener based compounds such as Direct Blue 6, Direct Blue 15, Direct Black 15, Direct Black 38, Direct Black 39, Direct Red 2, Direct Brown 95, and Congo Red (representative dyes are seen in Figs. 2 and 3). Upon reduction of both azo linkages by rat and human gut bacteria, these diazo compounds yield potential bladder carcinogens, benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine (Fig. 13), which are detected in the urine [6, 129–135]. The metabolic activation of benzidine and related aromatic amines and their carcinogenic potential is well documented [6, 136]. The carcinogenicity of the benzidine-based dye, Direct Blue 6, may not necessarily depend on azoreduction since a DNA adduct identified after oral feeding contains an intact azo linkage but no benzidine moiety [137].

Large quantities of azo dyes find their way into the ground through the wastewaters from chemical and textile industries. Therefore, considerable

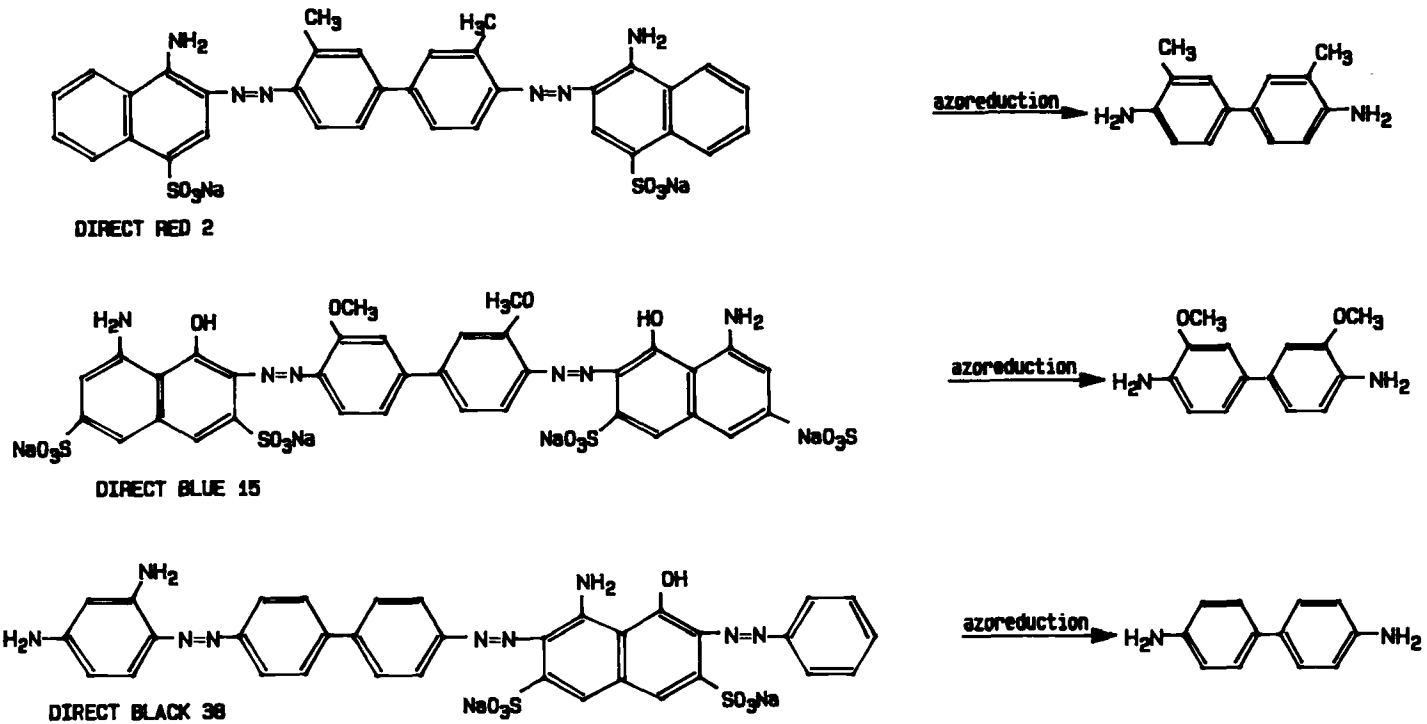

FIG. 13. Azoreduction of three dyes to yield the bladder carcinogen benzidine and its derivatives.

interest has been generated in dye reduction by soil bacteria. Wuhrmann et al. [128] found that the facultative anaerobe, *Bacillus cerus*, and the aerobes *Sphaerotilus natans* and *Arthrobacter* (strain Co), from soil, sewage or sludge, reduce 1-phenylazo-2-naphthol derivatives of Orange II under anaerobic conditions. First-order kinetics were observed, but incomplete dye disappearance suggested product inhibition. Some compounds not reduced by whole cells were readily reduced by cell extracts indicating that, for some dyes, transport through cell wall and plasma membrane is a limiting factor. This is especially true for sulfonated dyes which are highly charged under all but extremely acid conditions. Nitrite, an inevitable contaminent in sewage, is inhibitory. A large series of azo dyes was tested for reduction by cultures prepared from sewage sludge [138]. Predictably, many of the sulfonated compounds resisted reduction whereas the more hydrophobic compounds were almost completely reduced. Even so, sulfonated compounds with hydroxyl and amino substituents were reduced although those with methyl, methoxy, and chloro substituents were not, suggesting membrane transport of the former but not the latter. *Aeromonas hydrophilia* from dye factory wastewater has shown to reduce a number of lipophilic azo dyes: *p*-aminoazobenzene, *p*-acetamidoazobenzene, 2,4-diaminoazobenzene, DAB, *p*-hydroxyazobenzene, phenylazoresorcinol, *p*-nitrophenylazoresorcinol [127]. Reduction is apparently oxygen insensitive since there was no indication that anaerobic conditions were established. However, aerated sludge is generally unable to reduce a number of azo dyes which are readily reduced anaerobically [139]. This may be of considerable environmental concern since wastewater processing is done under aerobic conditions.

V. CARCINOGENESIS

A review of early work on azo dye carcinogenesis can be found in Refs. 140 and 141. The relationship between aromatic amines and human cancer was shown nearly 100 years ago by Rehn [142], who reported the development of bladder cancer among workers in the aniline dye industry. High incidence of bladder cancer was also observed in Japanese oriental script writers and kimono painters [143]. Yoshita [144] in 1933 reported hepatic tumors in rats and mice fed the azo dyes scarlet red, *o*-aminoazotoluene, and 2′,3-dimethyl-4-aminoazobenzene; and Kinosita [145] later reported the strong carcinogenicity of DAB. Since these early reports, numerous azo dyes have been tested for carcinogenicity. Hair dyes represent a major application of azo compounds [146]), several of which are carcinogenic in lower animals. Some are absorbed through the skin, creating a potential hazard.

Benzidine-based dyes are of great concern since, upon reduction, many yield benzidine, a known human urinary bladder carcinogen [6, 129–136, 147, 148]. In 1978 American products and imports of these dyes exceeded 4 million pounds, implying widespread exposure by industrial workers and the general public. Among the benzidine-based structures in hair dyes are Direct Black 6 and Direct Black 38.

Liver is the major site of tumorigenesis for most azo dyes. However, *o*-aminoazotoluene also produces pulmonary adenomas, hemangioendotheliomas, and bladder tumors. It is carcinogenic in rats, mice, hamsters, dogs, and possibly rabbits. 4-Aminoazobenzene (AB) and 4-phenylazoacetanilide (AAB) were originally thought to be noncarcinogenic; however, high, but nontoxic, doses of AB were later shown to induce liver tumors in rats [4]. A tabulated summary of results with nearly 100 dyes can be found in Ref 4. A more detailed description of testing of 32 dyes can be found in Ref. 5.

The carcinogenicity and metabolic activation of *N*-aryl amines, including azo dyes, have been the subject of intensive study for many years and numerous reviews have been published [1, 4, 22, 23, 50, 55, 149–152]. Early reports on skin hyperplasia [153], liver adenomas and tumors [144, 145] induced by azo dyes gave rise to a great interest in these compounds as carcinogenic agents. These suspicions have been born out by epidemiological studies indicating that workers in the dye industry have an abnormally high incidence of bladder tumors [142, 148, 154]. Due to its potent hepatocarcinogenic response, DAB and many of its derivatives have been widely tested and were among the first chemical carcinogens to be studied mechanistically. An intact azo linkage (–N=N–) is required for carcinogenic activity and DAB analogs with –N=CH–, –CH=N–, –C–NH, or –CH=CH– linkage lack tumorigenic capacity, as do primary reduction products or DAB, *N*,*N*-dimethyl-*p*-phenylenediamine, and aniline [20, 148]. High dietary riboflavin facilitates azoreduction and partially protects against DAB-induced carcinogenesis, whereas riboflavin deficiency enhances the response [155, 156]. Simple modification of DAB substituents results in considerable change in carcinogenic potency. For example, 3′-methyl-DAB has a much greater potency than DAB, whereas 2-, 3-, and 4′-methyl-DAB are, at most, weakly tumorigenic [140]. MAB is as potent a carcinogen as DAB. Its 3′-methyl derivative is very potent although its reduction products are noncarcinogenic. *N*-Ethyl-*N*-methylaminoazobenzene, 3′-nitro and 3′-chloro-DAB are as effective as DAB. In early experiments, AB was only weakly carcinogenic after oral feeding but skin painting readily produced tumors. Later the dye was shown to exhibit a pronounced carcinogenic effect in weanling mice although it was noncarcinogenic in weanling rats, indicating marked species selectively [158, 159]. This has yet to be explained on a metabolic basis. Carboxy substitution in the 2′-position, as in methyl red, destroys carcino-

genic activity. Several fluorene-substituted forms of DAB vary in tumorigenicity from moderate to weak [21]. Methoxy-substituted derivatives of DAB, *N*-methyl- and *N*,*N*-dimethyl-4-(phenylazo)-*o*-anisidine, and 2-methoxy-aminoazobenzene are unusual in that they induce extrahepatic tumors, e.g., ear duct, intestine, and skin [4]. The 4′-hydroxy derivatives of DAB and MAB are inactive. These are also major metabolites formed after feeding the carcinogens and represent a critical pathway for inactivation.

In the broad area of testing of chemically induced carcinogenesis, some of the negative effects reported may be attributable to inadequate dosage. Inaccuracies may also derive from lack of sufficient purity of azo dye preparations, many of which are suitable for commercial dying applications but are inappropriate for scientific investigation. Reports of long-term feeding experiments rarely mention repurification of dye preparations. A recent study [160] with several food dyes, FD&C Yellow No. 5, FD&C Yellow No. 6, FD&C Red No. 40, and FD&C Red No. 2 (amaranth) showed little or no mutagenesis with or without FMN-supplemented S9 activation. However, ether extracts of aqueous solutions of FD&C Red No. 40 were mutagenic after chemical reduction, as were very high concentrations of the dye itself, suggesting that activity was due to lipophilic contaminents. Furthermore, each parent dye was inhibitory in mutagenesis tests with chemically reduced metabolites and with 2-acetylaminofluorene. Similar discrepancies in mutagenic results with crude versus purified dyes have been reported [161]. These factors are rarely considered and bring into question the validity of much of the carcinogenic and mutagenic testing. On the one hand, some purified dyes may be less biologically active than previous testing suggests. On the other hand, if dyes are utilized mainly in impure form, the activity of the entire preparation must be considered.

Early observations on the tight binding of dye to rat liver after DAB administration as well as its lack of spontaneous activity suggested a relationship between binding and carcinogenesis and the necessity for metabolic intervention [50, 162]. Subsequent recognition of the genetic role of DNA established covalent binding to nucleic acid as a primary event in chemical carcinogenesis. The *N*-hydroxy metabolite of 2-acetylaminofluorene was identified in the urine of rats fed this carcinogen and shown to be more potent than the parent compound [48]. This key observation suggested that DAB may also be activated via *N*-hydroxylation. A correlation was found between the carcinogenic activity of a series of DAB derivatives and rate of *N*-hydroxylation of the corresponding MAB derivatives [51]. The fact that MAB, the secondary amine, exhibited carcinogenic potency equal to that of DAB pointed toward *N*-demethylation as an initial activation step. The lack of direct reaction of *N*-hydroxy acetylaminofluorene with tissue *in vitro* suggested that a further reaction was required and that this would also hold for

N-hydroxy-MAB. The latter compound being exceedingly unstable, *N*-benzoyloxy-MAB was synthesized and shown to react spontaneously with protein and nucleic acids and to possess greater carcinogenic potency than the parent dye [163]. This suggested that a conjugate was the ultimate carcinogenic metabolite. It was subsequently revealed that sulfation of the *N*-hydroxy derivative by PAPS and sulfotransferase created a derivative which spontaneously deesterified to a highly reactive electrophilic intermediate which bound covalently to protein and DNA [49, 164 ,165]. Degradation of dye-bound DNA yielded adducts with guanosine through the amino nitrogen (*N*-guanosin-8-yl-MAB) suggesting the formation of a nitrenium ion intermediate. Degradation of dye-bound protein yielded 3-methylmercapto-MAB, indicating that a carbonium ion formed ortho to the amine which reacted with methionyl residues in the protein. Consistent with the proposal that carbonium and nitrenium ions are active intermediates is the identification of their respective glutathione adducts, 3-(glutathion-*S*-yl)-*N*-methyl-4-aminoazobenzene and *N*-(glutathion-S-methylene)-4-aminoazobenzene, in the bile following administration of DAB [40, 41] and *in vitro* using *N*-hydroxy-MAB and a sulfotransferase system [165]. Furthermore, covalent binding of azo dye metabolites to DNA in the *in vitro* system was partially blocked by glutathione, a strong nucleophile which readily binds to highly electrophilic sites, successfully competing with macromolecular nucleophilic sites. Further support for the role of a reactive sulfate ester was seen in the inhibition of DNA adduct formation and tumorigenesis after DAB or AB administration by pentachlorophenol, an inhibitor of sulfotransferase [166]. In addition, high dietary sulfate facilitates carcinogenesis by 3′-DAB [167] although this has not been confirmed. Sulfate esters may not be the only activated forms of azo dye carcinogens since several acyl esters of *N*-hydroxylated dyes have been shown to react directly with proteins and nucleic acid as well as to be directly mutagenic [168]. Acyl and other esters are also implicated in the metabolic activation of acetylaminofluorene [169] and 3,2′-dimethyl-4-aminobiphenyl [170], and binding of *N*-hydroxy-azobenzene to nucleic acids has been associated with serine acylation of the amino nitrogen [171].

Several dye–DNA adducts have been identified after administration of MAB [136]. The significance of each in the carcinogenic process is not readily determined with assurance, particularly in view of DNA repair mechanism [172]. After a single dose of MAB, major adducts include *N*-(deoxyguanosin-8-yl)-MAB, accounting for 70% of bound dye [173], and 3-(deoxyguanosin-N^2-yl)-MAB [174]. The first adduct quickly disappears while the second persists for at least 2 weeks. Both adducts plus *3*-(deoxyadenosine-N^6-yl)-MAB are present after chronic administration of MAB [175]. It has been speculated that, despite its rapid disappearance

after a single injection of MAB, *N*-(deoxyguanosin-8-yl)-MAB may be critical in tumorigenesis since it persists during chronic tumorigenic dye treatment [176] and is associated with mutagenesis in bacterial systems [177]. Furthermore, its concentration in the liver, a target organ for MAB, is many times that in nontarget organs, kidney and spleen [176]. It is also the major product formed spontaneously by reacting *N*-benzoyloxy-DAB with deoxyguanosine or DNA *in vitro* [178]. Although AB is not carcinogenic in adult rats, its administration to preweanling mice, in which it is carcinogenic, leads to formation of a single adduct, *N*-(deoxyguanosin-8-yl)-AB.

Other routes of activation of azo dyes have been considered. Nitroxide radicals are reported to form *in vitro* from *N*-hydroxy-MAB and *N*-hydroxy-AB as well as *in vivo* after feeding 3′-methyl-DAB [55]. Incubation of DAB or MAB with rat liver microsomes generates NADPH-dependent EPR-detectable free radicals [55]. MAB is somewhat more active in this respect and the signal is inhibited 40% by carbon monoxide, suggesting the involvement of cytochrome P-450. Selective induction of radical formation by methylcholanthrene and inhibition by α-naphthoflavone, but not SKF 525-A or metyrapone, implies activation by selective forms of the cytochrome. It was postulated that nitroxide radicals bind to DNA and a correlation was demonstrated between radical formation and carcinogenic activity of a series of substituted MAB derivatives. To what extent such a radical contributes to azo dye carcinogenesis relative to activation via *N*-hydroxylation and sulfate conjugation is unknown. The role of free radicals in chemical carcinogenesis has long been debated [179]. These include oxygen radicals possibly generated through reactive metabolic intermediates described above. For example, it has been proposed that both superoxide and hydrogen peroxide are generated from the nitroxide formed from *N*-hydroxy-MAB [180]. In addition, nitroxide radicals of several aromatic amines bind to microsomal membranes and may subsequently induce formation of activated forms of oxygen [181]. Most of the carcinogenic amines tested by Stier et al. [181] including MAB and AB, formed nitroxide radicals while most of the noncarcinogens did not. Activation and binding of MAB through prostaglandin synthetase has also been reported [182].

3′-Methyl-DAB is a somewhat more potent carcinogen than DAB. Labuc and Blunck [183] noted that rat liver preparations catalyzed the covalent binding of its metabolites to yeast RNA and to tissue protein by apparently different mechanisms. RNA binding required both microsomes and cytosol with PAPS. Binding to tissue protein did not require the sulfotransferase system. Thus a second electrophilic metabolite may be involved in the activation of 3′-methyl-DAB which does not arise from the *N*-demethylation, *N*-hydroxylation, sulfation pathway. Mori and co-workers have shown that

oxidation of the 3′-methyl group makes a major contribution to metabolic activation [184–187]. 3′-Hydroxymethyl-DAB is a more potent carcinogen than 3′-methyl-DAB or 3′-carboxy-DAB in feeding experiments [184]. 3′-Hydroxymethyl derivatives of DAB, MAB, and AB are mutagenic in the Salmonella typhimurium system [185, 188]. Activation with S9 rat liver fraction is required and preparations from polychlorinated biphenyl- and 3-methylcholanthrene-induced animals are more active than those from phenobarbital-induced animals. 7,8-Benzoflavone, but not metyrapone or SKF 525-A, inhibits activation, implying selective cytochrome P-450 involvement. Products of side chain oxidation and azoreduction, 3–aminobenzoid acid and 3-acetaminobenzoic acid, have been detected *in vivo* [30]. The high carcinogenic potency of 3′-hydroxymethyl-DAB suggested that the activation pathway may be similar to that described by Watabe and his colleagues [189, 190] for 7-hydroxymethyl-12-methylbenz[a]anthracene in which sulfation of the hydroxymethyl group gives rise to covalent binding and mutagenecity. These effects are blocked by glutathione [187] and adduct formation *in vivo* is blocked by prior administration of dehydroepiandrosterone, a sulfotransferase inhibitor [191]. Enhancement of microsomal mediated mutagenicity of 3′-hydroxymethyl-DAB but not 3′-methyl-DAB is seen with a cytosolic fraction in the molecular weight range of 43,000–47,500 [192]. However, this is not related to sulfotransferase which has a higher molecular weight. Furthermore, sulfotransferase inhibitors and sulfatase do not inhibit mutagenicity. The cytosolic fraction does not act through nonspecific protection of microsomal enzymes. One or more cytosolic macromolecules also stimulate *N*-demethylation and ring hydroxylation of DAB [193]. Here too the mechanism is unknown although suppression of lipid peroxidation has been ruled out. Therefore, the ultimate carcinogenic form of 3′-DAB has not yet been defined although it undoubtedly involves an activated form of 3′-hydroxymethyl-DAB in addition to the well-established activation sequence centered in the aryl amine moiety. In keeping with the critical position of ring substituents, Mori et al. [186, 187] found that 2′-hydroxymethyl-DAB and 4′-hydroxymethyl DAB induced far fewer hepatic tumors in rats than did 3′-hydroxymethyl-DAB. In addition, Dipple [194], using the model compounds 3′- and 4′-chloromethyl-DAB, found that, although both bound spontaneously to DNA *in vitro*, binding for each was spectrally distinct and the 4′-product diminished with time.

A novel route of activation was suggested by Stiborova et al. [195], who proposed the formation of benzenediazonium ion from the carcinogen, 1-phenylazo-2-hydroxy-naphthalene [Sudan I), through an oxidative reaction catalyzed by rat liver microsomes. This reactive intermediate could then bind covalently to DNA [191]. Evidence for this mechanism is based on the use of a trapping agent, 2-phenyl-3-methyl-5-pyrazolone, although final

proof for the structure of the adduct has yet to be published. In connection with this mechanism, it is of interest that a diazonium cation was proposed as an intermediate in the conversion of 3,4-dichloroaniline into azodye catalyzed by bacterial nitrate reductase in the presence of inorganic nitrate [197]. Thus diazonium intermediates may function in both the biological synthesis and degradation of azo dyes.

VI. MUTAGENESIS

The widespread acceptance of mutagenesis as a major mechanism underlying conversion of a normal cell to a tumor cell has led to mutagenicity testing as a short-term, inexpensive means of screening potential chemical carcinogens. Azo dye carcinogenicity stemming from chronic feeding appears to require an intact azo linkage. In contast, most mutagenicity studies imply that azoreduction is a prerequisite for activity. In two studies there was no correlation between reductive cleavage and carcinogenicity in a series of compounds related to DAB [198, 199]. It is difficult to reconcile these differences if one assumes that most carcinogens act through mutagenic mechanisms. The Salmonella typhimurium test developed by Ames [200] is the most widely applied assay for mutagenicity but others include the hepatocyte primary culture unscheduled DNA synthesis (DNA repair) test [201]. In a large series of azo compounds, relatively good correlation was shown between the two systems [202]. Hamster hepatocytes have been shown to be more effective than rat hepatocytes in demonstrating mutagenicity of a series of azo dyes and their reduction products, including *o*-aminoazotoluene, Ponceau SX, Evans Blue, Trypan Blue, Congo Red and 4′-dimethylaminobenzeneazo-1-naphthoflavone [203]. 6-Dimethylaminophenylazobenzothiazole and 5-dimethylaminophenylazoindazole, carcinogenic and noncarcinogenic derivatives of DAB, respectively, are potent bacterial mutagens although only the carcinogen induces unscheduled DNA synthesis in rat liver *in vivo* [204–206]. After *in vivo* administration, the noncarcinogen binds to liver DNA less effectively than the carcinogen, probably due to differential metabolism [207, 208]. The *N*-demethylated form of 5-dimethylaminophenylazoindazole is a considerably more potent mutagen than is the parent compound. This would be expected if activation were analogous to DAB where formation of the secondary amine is a required step. Whether the derived compound is carcinogenic is not yet known and is difficult to predict. For some dyes, mutagenicity is more readily demonstrated using the S9 fraction from uninduced hamster liver than from Aroclor 1254-induced rat liver S9 [209] since the latter may inactivate reduced products. Consistent with the assumption that mutagenicity is associ-

ated with reduced products, some groups have included FMN in their mutagenicity protocol to facilitate azoreduction [209]. Mutagenicity of chemically reduced azo dyes is consistent with biological findings. On the other hand, many aromatic amines are too unstable in air to be tested in conventional mutagenicity test sytems. This is true of many dyes permitted for human consumption which have hydroxyl substituents ortho to the amino function. For most reduced products, S9 activation is required, confirming that the reduced primary amine is not the final activated form [210]. On the other hand, a relatively lipophilic dye, Solvent Yellow 3 (4′-amino-2,3′-dimethylazobenzene) appears to be mutagenic in the unreduced form since chemically reduced products are nonmutagenic [210]. Furthermore the mutagenicity of rat urine after its administration is probably due to the parent compound which is excreted unchanged to a significant extent. A good correlation between carcinogenicity and mutagenicity, based on the Salmonella assay, has been shown for a series of dyes related to 4-aminoazobenzene, including nitro-, nitroso-, and methoxy-substituted compounds [211]. Several *N*-hydroxy derivatives are mutagenic in the absence of S9 although the parent azo dyes require S9, confirming *N*-hydroxylation as the major activation pathway. However, several nitroso derivatives are mutagenic in the absence of S9, indicating activation through an alternate pathway. The appearance of mutagenic metabolites in the urine after administration of azo dyes has been reported frequently and is a major source of concern for human exposure to such chemicals. This has been shown for a number of benzidine-based dyes [129, 132–134, 212–216]. For example, oral administration of Direct Red 39, Direct Red 14, Direct Blue 53, Direct Orange 6, Direct Blue 8, Direct Blue 10, Direct Violet 32, and Direct Red 46 yields measurable urinary levels of the corresponding dimethyl, dimethoxy, and dichloro benzidines and their *N*-acetyl derivatives [215]. These derivatives are more mutagenic than benzidine [213, 215]. Administration of Direct Black 39 and Direct Brown 95 also produces mutagenic urinary products [133]. Tartrazine, which is widely used in human consumption, also yields mutagenic urinary and fecal products after oral feeding [216, 217]. Since each of these dyes is highly sulfonated, and therefore charged within a broad range of physiological pH, it can be assumed that absorption is minimal and that reduction is entirely due to gut bacteria. Direct evidence that intestinal microflora are responsible for mutagenic activation has come from many sources (see sec. IV.B). The benzidine-based dyes Direct Black 39 and Direct Black 39 are weakly mutagenic in conventional Salmonella systems [213, 218, 219], even in the presence of rat liver S9. However, after anaerobic incubation with cultures from human feces, the resulting metabolites are directly mutagenic in the Salmonella system [219]. Individual metabolites of the reaction, 4-aminobiphenyl, 4-acetylaminobiphenyl, benzidine, and monoacetylbenzidine

are mutagenic but require S9 fraction activation. Addition of FMN to accelerate azoreduction increases the mutagenicity only of Direct Black 39 itself, supporting azoreduction as the activating pathway for this compound.

In structure-activity studies, compounds expected to yield *p*-phenylenediamine in the presence of rat liver microsomes have been shown to be mutagenic although an oxidation product of the amine is proposed as the active intermediate [224]. Sulfonation and carboxylation reduce mutagenicity of the amine, probably through resistance to metabolic activation. Similarly, 4,4′-diaminoazobenzene, a potent mutagen, loses activity upon addition of 2-hydroxyethyl substituents on either amino function, the site of metabolic activation [221]. Joachim and Decad [222]) reported that several benzidine-based dyes, Direct Blue 1, Direct Blue 14, Direct Brown 95, and Direct Red 46, are not directly mutagenic in primary hepatocyte cultures, using unscheduled DNA synthesis as criterion. However, hepatocytes isolated from rats treated with these dyes exhibit mutagenicity with this test, implying *in vivo* activation of the dye, probably through bacterial action. Nevertheless, there still is a potential for hepatic reduction since some dyes also form mutagenic products aerobically in the presence of rat liver S9 fraction or hepatocytes. In view of the insensitivity to oxygen, the reductase activity may be is associated with NAD(P)H:quinone reductase in the cytosolic fraction or selective forms of cytochrome P-450. Hepatic reduction may also be a partial source of urinary mutagens after intraperitoneal administration of azo dyes. Another reduction product of Direct Black 39, 1,2,4-triaminobenzene, is also mutagenic, but is somewhat weaker than benzidine and its derivatives [134]. A contrasting series is that of DAB, MAB, and their respective 3′-methyl derivatives which are activated to mutagenic metabolites by oxidative reactions catalyzed by the mixed-function oxidase system [223]. Antibodies to NADPH–cytochrome P-450 reductase and to methylocholanthrene-induced cytochrome P-450 almost totally block mutagenesis. Antibodies to phenobarbital-induced cytochrome P-450 are ineffective, demonstrating the enzyme specificity of the system. A relation between mutagenicity and azo reduction of DAB-like compounds has been demonstrated [224]. The *N-N*-diethyl congener of DAB is slightly mutagenic after S9 activation whereas a 4′-ethyl substituent prevents azoreduction but has little effect on mutagenicity. A 4′-fluoro substituent, on the other hand, increases both azo reduction and mutagenicity, implying again that activity resides in the reduced metabolites. In contrast, another study found that mutagenic activation of DAB is not due to the reduced amine produces [205]. Two azo dyes, Alizarin Yellow GG and acid Alizarin Yellow R, also exhibit direct mutagenicity apparently without intervening metabolism [225]. These dyes, however, contain nitro substituents which tend to confer mutagenicity on aromatic compounds [226].

Although evidence of mutagenicity is often used to predict carcinogenicity, 2′- and 4′-methyl DAB, which are very weak carcinogens, were shown by Mori and co-workers to exhibit pronounced S9-dependent mutagenicity in the Ames test [185]. They also compared rat liver S9, which is used almost exclusively in the Ames test, with S9 from other organs and species. 3′-Methyl-DAB and 3′-hydroxymethyl-DAB were not mutagenic in the presence of S9 from rat kidney, lung, intestine, brain, or heart and from human liver. Rat liver S9 was more effective than S9 from mice or hamsters. Kennelly et al. [137] provided evidence that a substantial proportion of direct blue 6, administered intraperitoneally to rats to avoid intestinal metabolism, bound to liver DNA with the azo linkage intact. This reflected the poor ability of liver to reduce this dye as well as its greater mutagenicity compared to Congo Red and other dyes believed to act solely through azo reduction products.

Vitamin A and other retinoids have been investigated in the prevention and treatment of various types of cancer [227, 228]. They have been shown to modify DNA binding and mutagenesis of 2-acetylaminofluorene and 2-aminofluorene [229, 230], aflatoxin B_1 [231], benzo(a)pyrene [232], 7,12-dimethylbenzanthracene [233], and other polycyclic hydrocarbons [234]. Inhibition of mutagenicity of *o*-aminoazotoluene [235] has been reported using S9 from mouse, gerbil, hamster, and rat. The mechanism is unknown but is probably related to metabolic activation of the carcinogen, since S9 is required for mutagenesis by *o*-aminoazotoluene whereas retinol does not inhibit direct acting mutagens such as adriamycin [229], diepoxybutane [220], 4-nitro-*o*-phenylenediamine [236], and others [237].

VII. SUMMARY

Azo dyes are consumed and otherwise utilized in varying quantities in many parts of the world. Such widely used chemicals are of great concern with regard to their potential toxicity and carcinogenic properties. Their metabolism has been studied extensively and is significant for detoxication and metabolic activation. Both oxidative and reductive pathways are involved in these processes. The majority of azo dyes undergo reduction catalyzed by enzymes of the intestinal microorganisms and/or hepatic enzymes including microsomal and soluble enzymes. The selectivity of substrate and enzyme may to a large extent be determined by the oxygen sensitivity of reduction since a normal liver is mainly aerobic in all areas, whereas the microorganisms of the lower bowel exist in an anaerobic environment. However, it should be pointed out that the pO_2 of centrilobular cells within the liver is

only a fraction that of air, where pO_2 = 150 torr. Therefore, an azo dye reduction experiment performed aerobically may not be an accurate predictor of reductive metabolism in all areas of the liver. Many of the azo dyes in common use today have highly charged substituents such as sulfonate. These resist enzymic attack and for the most part are poorly absorbed from the intestinal tract, providing poor access to the liver, the major site of the mixed-function oxidase system. Lipophilic dyes, such as DAB, which are often carcinogenic, readily access oxidative enzymes and are activated by both mixed-function oxidase and conjugating systems. Reduction of the carcinogenic dyes usually leads to loss of carcinogenic activity. By contrast, most of the highly charged water-soluble dyes become mutagenic only after reduction. Even then, most of the fully reduced amines required oxidative metabolic activation. An outstanding example is the potent human bladder carcinogen benzidine, which derives from the reduction of several azo dyes. Many problems regarding mutagenic and carcinogenic activation remain to be solved. At the present time, it is apparent that both oxidative and reductive pathways yield toxic products. Toxicologic assessment of azo dyes must consider all pathways and particularly the oxygen sensitivity of azoreduction. This is critical in the treatment of waste from chemical plants where there is a great need for soil bacteria which catalyze reduction aerobically. Consideration of secondary pathways are also of great concern. For example, azoreduction of carcinogenic dyes such as DAB removes carcinogenic activity although oxidative metabolism of the primary amines yield mutagenic products. Such apparent dilemmas must be dealt with when considering metabolism/toxicity relationships for azo dyes.

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