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# AZOREDUCTASE ACTIVITY BY PURIFIED RABBIT LIVER ALDEHYDE OXIDASE

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**Abstract**—Our laboratory has investigated the azoreduction of dimethylaminoazobenzene (DAB) and its analogs by hepatic microsomal cytochrome P450. We have extended these studies to the cytosolic fraction of the mammalian liver using the molybdoflavoenzyme, aldehyde oxidase. Purified rabbit liver aldehyde oxidase readily reduced azo dyes which are mainly water soluble and contain charged groups. Lipophilic azo dyes, although readily reduced by microsomal cytochrome P450, were either poor substrates or not reduced at all. Kinetic measurements revealed no relationship between $V_{max}$ and $K_m$ for all dyes. More extensive studies were conducted on four azo dyes, *o*-methyl, red, 2′-pyridyl-DAB, sulfonazo III and Orange II, with characteristic functional groups. With each of these substrates, azoreductase activity was greatest when 2-hydropyrimidine (2-OHP) was the electron donor compared to $N^1$-methylnicotinamide (N-MN), propionaldehyde and butyraldehyde. With 2-OHP as the electron donor, *o*-methyl red and 2′-pyridyl DAB exhibited maximal activity at pH 5.0 while sulfonazo III and Orange II showed maximal activity at pH 9.5 and 7.0, respectively. $K_m$ values for *o*-methyl red and 2′-pyridyl DAB were lower at their pH optima whereas that for sulfonazo III was higher at its pH optimum. There was also no correlation between maximal activity and $K_m$; apparently $K_m$ is not a primary determinant for activity. The degree of ionization of function groups depends on pH. Since highest activity is seen at that pH in which maximal ionization of the substrate occurs, it can be concluded that rate of reduction is at least partially dependent on the charged state of the substrate. Azoreduction was inhibited by menadione and SKF 525-A. Sensitivity to inhibition by menadione was greatest at the pH where 2-OHP exhibited considerably higher activity than N-MN, but no differential was seen at the pH where activities with the two-electron donors were similar. On the other hand, sensitivity of azoreductase activity to inhibition by SKF 525-A was the same irrespective of electron donor, indicating that the mechanisms for these two inhibitors were different.

Azo dyes are used widely as colorants in food and drink, drugs and other pharmaceutical products and cosmetics [1–3]. The metabolism and toxicology of azo dyes, some of which are carcinogenic, have been the focus of numerous investigations for decades [4–10]. One such dye, *p*-dimethylaminoazobenzene (DAB†), is a known hepatocellular carcinogen [6, 7]. Its metabolism has been studied extensively in our laboratory [8–13]. It has been demonstrated that the N-demethylation, N-hydroxylation, and conjugation reactions result in the activation of some carcinogenic azo dyes [14, 15]. Detoxification is usually associated with reductive cleavage of the azo linkages; however, the reduced products of some azo compounds exhibit toxic and mutagenic effects [16–18].

The reduction of azo dyes to primary amines is catalyzed by intestinal bacteria [19, 20] and mammalian liver cytosolic [21] and microsomal enzymes [22–24]. Over 40 years ago, Mueller and Miller [5] demonstrated that DAB is reduced by rat liver microsomes. The reaction required NADPH, which suggested the involvement of NADPH–cytochrome P450 reductase. Fujita and Peisach [24] reported that microsomal reduction of amaranth is completely sensitive to carbon monoxide and attributed to all activity to cytochrome P450. Mason and his colleages [25, 26] found that sulfonazo III and arsenazo II are reduced anaerobically by hepatic microsomes and activity is blocked by oxygen. Sensitivity to oxygen was attributed to the one-electron reduced intermediates formed, which are reoxidized immediately under aerobic conditions to the parent azo compound with the formation of superoxide anion free radical. Azoreduction of *o*-methyl red has been shown to be catalyzed aerobically by liver cytosolic NAD(P)H:quinone reductase [21]. The enzyme contains two molecules of FAD and the oxygen-insensitivity is attributed to a two-electron reduction step, bypassing formation of a one-electron-reduced free radical intermediate which is reoxidized readily by oxygen. Alternatively, oxygen sensitivity may be due to slow reaction of the reduced enzyme with oxygen. The former explanation is favored. Although reduction of *o*-methyl red by quinone reductase is oxygen insensitive, reduction by microsomal cytochrome P450, presumably a one-electron step, is oxygen sensitive.

Our laboratory has established that polar electron-donating substituents, such as hydroxyl or primary, secondary and tertiary amines, are obligatory for the reduction of azo dyes by liver microsomal cytochrome P450 [13]. Azo dyes devoid of such substituents, will not bind to cytochrome P450 or be

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† Abbreviations: DAB, *p*-dimethylaminoazobenzene; 2-OHP, 2-hydroxypyrimidine; and N-MN, $N^1$-methylnicotinamide.

reduced. It was shown that microsomal reduction of azo dyes having only electron-donating substituents on either ring (e.g. DAB) is insensitive to oxygen and CO [8]. On the other hand, microsomal reduction of azo dyes having both electron-donating as well as electron-withdrawing substituents (e.g. *o*-methyl red) is sensitive to both oxygen and CO. These are referred to as I (insensitive) and S (sensitive) substrates, respectively. Intermediates in the reduction of both I- and S-substrates were studied by the use of cyclic voltammetry [27] which has lent considerable insight into the observed differential sensitivity to oxygen. S-Substrates exhibit one-electron-reduced radical intermediates which are quenched instantly upon exposure to air, whereas one-electron-reduced I-substrates are temporarily stable in air. This is consistent with the relative oxygen sensitivities of the microsomal reduction of I- and S-substrates. The two-electron-reduced intermediates are protonated to form hydrazo intermediates which spontaneously disporportionate to the parent azo dye and the fully reduced amine metabolites [8].

Reduction of several azo dyes has been shown to be catalyzed by liver aldehyde oxidase [28], a cytosolic molybdoflavoenzyme which catalyzes the oxidative metabolism of a variety of aldehydes and nitrogen-containing heterocyclic compounds using oxygen as electron acceptor [29–33]. In view of the importance of reduction in both the inactivation and toxic activation of azo dyes, we have extended those studies to provide insight into the mechanism of azoreduction by aldehyde oxidase. Structural requirements for substrates and the mechanisms of various inhibitors have been elucidated.

## MATERIALS AND METHODS

### *Chemicals*

Sulfonazo III [3,6-bis(*o*-sulfophenylazo)-4,5-dihydroxy-2,7-naphthalenedisulfonic acid tetrasodium salt], DAB, metanil yellow, $N^1$-methylnicotinamide (N-MN), 2-hydroxypyrimidine (2-OHP), butyraldehyde, propionaldehyde and menadione were purchased from Sigma (St. Louis, MO). *p*-Methyl red [4′-carboxy-DAB,sodium salt], and Sudan III [1-(*p*-phenylazophenylazo)-2-naphthol] were purchased from the Eastman Kodak Co. (Rochester, NY). *o*-Methyl red [2′-carboxy-DAB], 2′-pyridyl-DAB [4-(2-pyridylazo)-*N*,*N*-dimethylaniline], AB [amino-azobenzene], 4′-OH-AB [4′-hydroxy-AB], methyl orange [4′-sulfonyl-DAB], DAB-arsonate, 3′-methyl-DAB, 2-methyl-DAB, Evans blue, and Sudan IV were purchased from Aldrich (Milwaukee, WI). Orange II [4-(2-hydroxy-1-naphthylazo)-benzenesulfonic acid, sodium salt], Trypan blue, and yellow AB [1-phenylazo-2-naphthalenamine] were purchased from Pfaltz & Bauer, Inc. (Waterbury, CT). MAB [4(methylamino)AB] was a gift from Dr. Fred Kadlubar, National Center for Toxicological Research (Jefferson, AR). The syntheses of 4′-OH-DAB [4′-hydroxy-DAB] [4] and methyl esters of *o*- and *p*-methyl red [13] were described previously. SKF 525-A was a gift of Smith Kline & French Laboratories (Philadelphia, PA). Other chemicals were obtained at the highest purity commercially available.

### *Enzyme purification*

Aldehyde oxidase was purified from the livers of young male rabbits purchased from Pel Freeze Biologists, Inc. (Rogers, AR). The procedure of Rajagopalan *et al.* [34] was modified as follows. The 50% ammonium sulfate precipitate was resuspended in 5 mM triethanolamine buffer, pH 7.8, containing 0.3 mM EDTA and 0.15 M potassium chloride, and dialyzed against the same buffer. The enzyme was then adsorbed to a DEAE Sephacel column (5 × 29 cm) equilibrated with the above buffer. The enzyme was eluted with a linear gradient of the above buffer, increasing the concentration of potassium chloride from 0.15 to 0.35 M. Activity appeared in a single peak. The appropriate fractions were combined, concentrated, and dialyzed against 10 mM potassium phosphate buffer, pH 7.8, containing 0.3 mM EDTA and 0.3 mM phenylmethanesulfonyl fluoride (PMSF). The enzyme was applied to a hydroxylapatite column (2.5 × 11 cm) equilibrated with the same buffer and eluted by increasing the ionic strength of the buffer to 30 mM. It was critical that the flow rate of the column in this step be no greater than 25 mL/hr. The final specific activities for the purified enzyme ranged from 2.5 to 3.4 μmol pyridone formed per min per mg protein, using N-MN as electron acceptor. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis produced only two bands of 141 and 133 kDa, corresponding to the known subunits of rabbit aldehyde oxidase.

### *Enzyme assay*

*Aldehyde oxidase activity.* This activity was assayed spectrophotometrically at 37° with oxygen as electron acceptor. With 2-OHP as electron donor, product (uracil) formation was measured by increased absorption at 259 nm ($E = 8.2\ cm^{-1}\ mM^{-1}$). With N-MN as electron donor, the 2-pyridone oxidation product was measured by the increased absorption at 300 nm ($E = 4.17\ cm^{-1}\ mM^{-1}$). The incubation mixture contained 0.1 mM 2-OHP or 1.0 mM N-MN, 3 mg bovine serum albumin (BSA) and the enzyme preparation in a final volume of 1 mL of 0.1 M potassium phosphate (pH 4.0 to 7.5). Since potassium ferricyanide is frequently used as an electron acceptor for studying aldehyde oxidase with various electron donors [35–40], its reduction was also measured by the increased absorption at 420 nm ($E = 1.0\ cm^{-1}\ mM^{-1}$) in the presence of either 2-OHP or N-MN as electron donor according to the methods described by Rajagopalan and Handler [41].

*Azoreductase activity.* This activity was assayed anaerobically at 37° by direct spectrophotometric monitoring of azo dye disappearance. Nitrogen, passed through a deoxygenating solution consisting of 0.5% sodium dithionite and 0.5% 2-anthraquinone sodium sulfonate in 0.4% NaOH [42], was bubbled for 5 min through a cuvette which was sealed with a rubber septum equipped with inlet and outlet ports. To ensure an oxygen-free environment, the system also contained 60 mM D-glucose and 14 units of glucose oxidase [43]. Samples were preincubated for

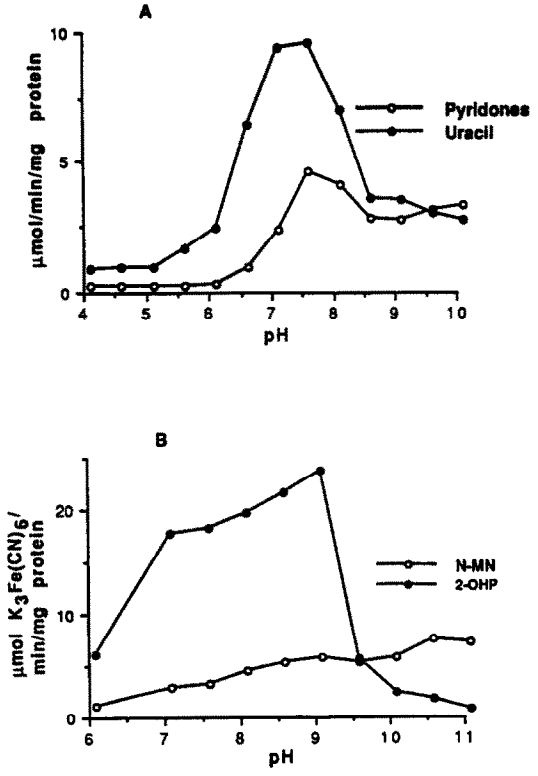


Fig. 1. Effect of pH on aldehyde oxidase activity. The reaction mixture consisted of 1.0 mM N-MN or 0.1 mM 2-OHP, 1.0 mM potassium ferricyanide in B, 3 mg BSA, 3–6 μg purified aldehyde oxidase, in a final volume of 1 mL with one of the following buffers (0.1 M): potassium phosphate (pH 4 to 7.5), HEPES [4-(2-hydroxyethyl)-piperazine-(cyclohexylamino)propane-sulfonic acid] (pH 7.5 to 8.5), glycine (pH 8.5 to 9.5) and CAPS [3-(cyclohexylamino)propane-sulfonic acid] (pH 9.5 to 11). (A) Oxidation of 2-OHP (uracil formation) and N-MN (pyridone formation), $O_2$ as electron acceptor. (B) Electron donors as indicated, potassium ferricyanide as electron acceptor.

Table 1. $V_{max}$ and $K_m$ values of various azo dyes

| Azo dyes | $V_{max}$ (nmol/min/mg protein) | $K_m$ (nM) |
|---|---|---|
| Sulfonazo III | 2674 | 66 |
| DAB-arsonate | 740 | 339 |
| *o*-Methyl red ester | 660 | 110 |
| Amaranth | 482 | 63 |
| *o*-Methyl red | 474 | 129 |
| 2'-Pyridyl-DAB | 389 | 20 |
| Orange II | 217 | 61 |
| Methyl orange | 207 | 149 |
| Prontosil | 128 | 87 |
| 4'-OH-AB | 79 | 94 |
| AB | 65 | 194 |
| Azobenzene | 63 | 243 |
| 4'-OH-MAB | 31 | 213 |
| MAB | 19 | 131 |
| *p*-Methyl red | 0 | 0 |
| *p*-Methyl red ester | 0 | 0 |
| DAB | 0 | 0 |
| 4'-$NH_2$-DAB | 0 | 0 |
| 4'-OH-DAB | 0 | 0 |
| Yellow AB | 0 | 0 |
| Evans blue | 0 | 0 |
| Metanil yellow | 0 | 0 |
| Sudan III | 0 | 0 |
| Sudan IV | 0 | 0 |
| Trypan blue | 0 | 0 |
| Congo red | 0 | 0 |
| Benzoylamino-AB | 0 | 0 |
| Isopropyl-AB | 0 | 0 |
| 4-Nitro-AB | 0 | 0 |
| 2-Methyl-DAB | 0 | 0 |

The reaction mixture consisted of 1 mM N-MN, 20–150 μM azo dye, 3 mg BSA, 2500 units catalase, 50 mM D-glucose, 14 units glucose oxidase, purified aldehyde oxidase and 0.1 M potassium phosphate buffer, pH 7.4, in a final volume of 0.7 mL. The sample was equilibrated for 5 min with nitrogen passed through a deoxygenating solution, and preincubated for 3 min at 37°. The reaction was initiated with N-MN. Azoreductase activity was measured by monitoring dye disappearance spectrophotometrically. $K_m$ and $V_{max}$ were calculated from linear reduction rates using Lineweaver–Burk plots. Abbreviations: AB, aminoazobenzene; 4'-OH-AB, 4'-hydroxyaminoazobenzene; and 4'-OH-MAB, 4'-hydroxymethylaminoazobenzene.

3 min at 37° and the reaction was initiated by addition of an anaerobic solution of 2-OHP or N-MN (final concentrations 0.1 mM). Disappearance of each dye was monitored spectrophotometrically as its absorption maximum. The final incubation mixture contained enzyme, 10–100 μM azo dye, 3 mg BSA, 2500 units of catalase and 100 mM buffer in a total volume of 0.7 mL in a 1-mL spectrophotometric cell. $K_m$ and $V_{max}$ were calculated from initial linear rates using Lineweaver–Burk plots.

Protein was determined according to the method of Lowry *et al.* [44].

## RESULTS

Figure 1A shows the pH profile for purified aldehyde oxidase from rabbit liver. When oxygen was used as the electron acceptor, activity was considerably greater with 2-OHP (uracil formation) compared to N-MN (pyridone formation) as electron donor. With either electron donor, the optimum pH was 7.5. With ferricyanide as electron acceptor, optimum pH was 9.0 with 2-OHP as electron donor. However, with N-MN as electron donor, activity was much lower and gradually increased from pH 6 to 11 with no observable optimum (fig. 1B).

$V_{max}$ and $K_m$ values were measured with various azo dye substrates at pH 7.4 under anaerobic conditions, using N-MN as the electron donor (Table 1). Reducible dyes were mainly water soluble and in most cases contained highly charged groups. Such characteristics were not by themselves sufficient requirements since neither Trypan blue, Evans blue, nor Congo red was reduced. Based on $V_{max}$, sulfonazo III was by far the most reactive of all the hydrophilic azo dyes. This was true for several electron donors: 2-OHP, N-MN, propionaldehyde,

Table 2. Effects of electron donors on azoreduction

| Electron donors | Azoreductase activity (nmol/min/mg protein) | | | |
|---|---|---|---|---|
| | Sulfonazo III | *o*-Methyl red | 2′-Pyridyl-DAB | Orange II |
| 2-OHP | 8155 | 168 | 407 | 122 |
| N-MN | 3409 | 145 | 157 | 140 |
| Propionaldehyde | 3506 | 170 | 281 | 89 |
| Butyraldehyde | 3634 | 158 | 228 | 62 |

Conditions are described in the legend of Table 1. Each dye was present in a saturate concentration of 0.5 to 1.0 μM. A 1.0 mM concentration of each electron donor was used to initiate the reaction.

and butyraldehyde (Table 2). Some weakly soluble compounds (4′-OH-DAB, AB, azobenzene, 4′-OH-MAB, MAB) were reduced, but very slowly. $K_m$ value for reducible dyes were scattered and did not correlate in any way to $V_{max}$. In all cases, azoreduction was inhibited completely when the assays were carried out aerobically (data not shown). A number of lipophilic azo dyes such as DAB, 4′-OH-DAB, and others which are readily reduced by the microsomal cytochrome P450 [8–13], were either poor substrates or not reduced at all.

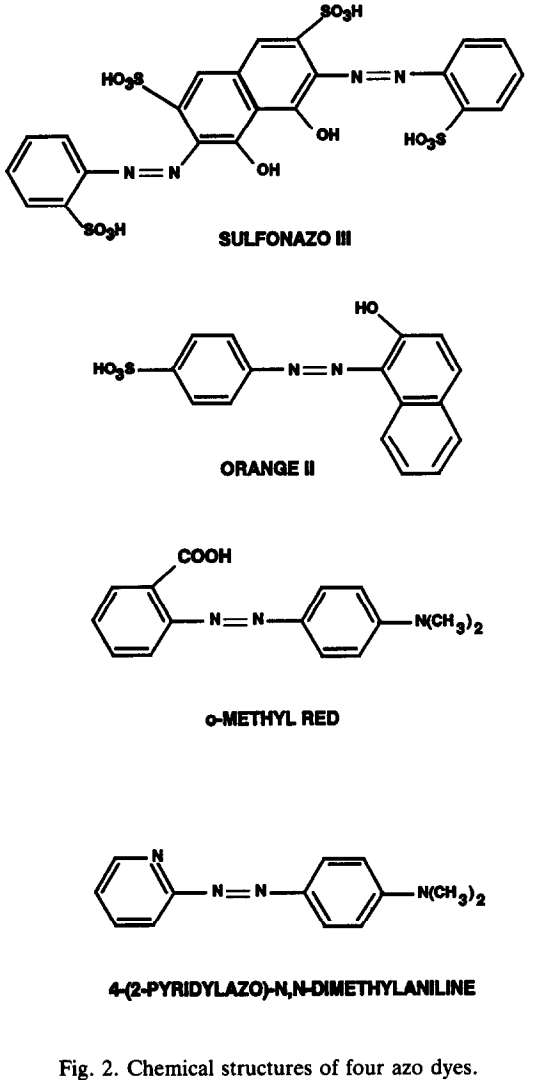


Fig. 2. Chemical structures of four azo dyes.

pH curves for azoreductase activity were measured with four azo dyes (sulfonazo III, Orange II, *o*-methyl red, 2′-pyridyl-DAB) with characteristic functional groups (Fig. 2). With 2-OHP as electron donor, *o*-methyl red and 2′-pyridyl-DAB exhibited maximal activity at pH 5.0 (Fig. 3). Sulfonazo III and Orange II showed maximal activity at pH 9.5 and 7.0, respectively. With N-MN as electron donor, lower activity, but similar pH optima were observed for sulfonazo III and Orange II, whereas there were no discernable pH optima for *o*-methyl red and 2′-pyridyl-DAB. Although $V_{max}$ rates with 2-OHP varied with pH, there was no obvious correlation with $K_m$. The $K_m$ values for *o*-methyl red and 2′-pyridyl DAB were lower at their pH optima, whereas that for sulfonazo III was higher (Table 3).

Menadione is a known inhibitor of aldehyde oxidase activity [34, 35, 45]. The effect of menadione on the reduction of ferricyanide by aldehyde oxidase at pH 7.5 is shown in Fig. 4. Activity was more sensitive to menadion inhibition when 2-OHP was the electron donor compared to N-MN. At this pH, the turnover rate of the non-inhibited enzyme was much greater with 2-OHP than with N-MN. Azoreductase activity was also inhibited by menadione. When inhibition was measured at a pH where both electron donors exhibited the same activity, sensitivity to menadione was nearly the same. Thus, rates of reduction of *o*-methyl red and 2′-pyridyl-DAB at pH 7.5 and 9.5, respectively, were similar (Fig. 3) and exhibited the same sensitivity to menadione (Fig. 5). However, at pH 5.0 where the turnover of both dyes is somewhat more rapid with 2-OHP compared to N-MN (Fig. 3), sensitivity to menadione was considerably greater with 2-OHP than with N-MN (Fig. 5). Similarly, sulfonazo III also exhibited sensitivity to menadione which reflected the different turnover rates with the two electron donors at pH 6.0 and 9.5 (Figs. 3 and 5).

SKF 525-A, an inhibitor of several forms of microsomal cytochrome P450 activity, also inhibited azoreductase activity by aldehyde oxidase (Fig. 6). In contrast to menadione, the sensitivity of SKF 525-A was the same with both electron donors, N-MN and 2-OHP, even at pH values where large differences in non-inhibited rates were seen for the two electron donors.

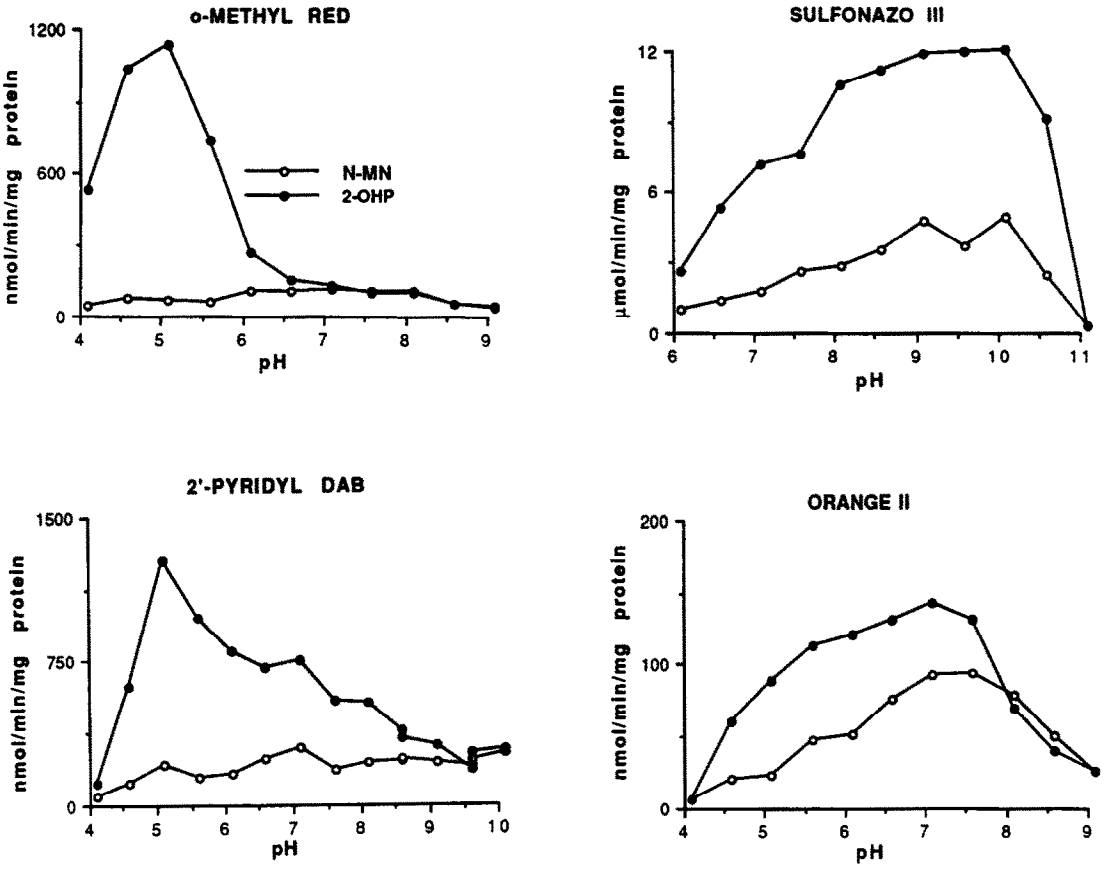


Fig. 3. Effects of pH on azoreduction by aldehyde oxidase. Reaction mixtures consisted of 50–100 µM azo dye, 3 mg BSA, 2500 units catalase, 60 mM D-glucose, 14 units glucose oxidase, and 0.1 M appropriate buffer as described in the legend of Fig. 1, in a final volume of 0.7 mL. The sample was bubbled for 5 min with nitrogen passed through a deoxygenating solution and preincubated at 37°. The reaction was initiated by addition of electron donor, final concentration 0.1 mM. Azoreductase activity was measured by monitoring dye disappearance spectrophotometrically.

## DISCUSSION

Previous studies have demonstrated that aldehyde oxidase can reduce *N*-oxides [46, 47], sulfoxides [35], nitrosamines [48], and several azo dyes [28]. Azo dyes can also be reduced by intestinal bacteria [19, 20] and mammalian liver microsomal and

Table 3. Effect of pH on $K_m$ values of typical azo dyes*

| Azo dyes | pH | $K_m$ (µM) |
|---|---|---|
| *o*-Methyl red | 5.0 | 8 |
| | 7.5 | 54 |
| 2'-Pyridyl-DAB | 5.0 | 37 |
| | 9.5 | 53 |
| Sulfonazo III | 9.5 | 96 |
| | 6.0 | 28 |

* Conditions are as described in the legend of Table 1. A 1.0 mM concentration of 2-OHP was used as the electron donor.

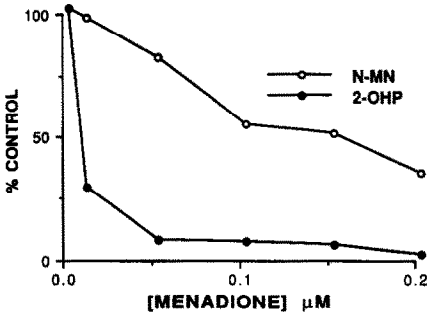


Fig. 4. Effects of menadione on aldehyde oxidase activity using potassium ferricyanide as electron acceptor. Various concentrations of menadione in 50% ethanol were added to a cuvette with 5 µg purified aldehyde oxidase, 3 mg BSA and 0.1 M potassium phosphate buffer (pH 7.5) in a final volume of 1 mL. Reaction was initiated by addition of 1.0 mM electron donor. The final concentration of ethanol was 0.5% in each case. Control rates using N-MN and 2-OH were 3.5 and 18 µmol/min/mg protein, respectively.

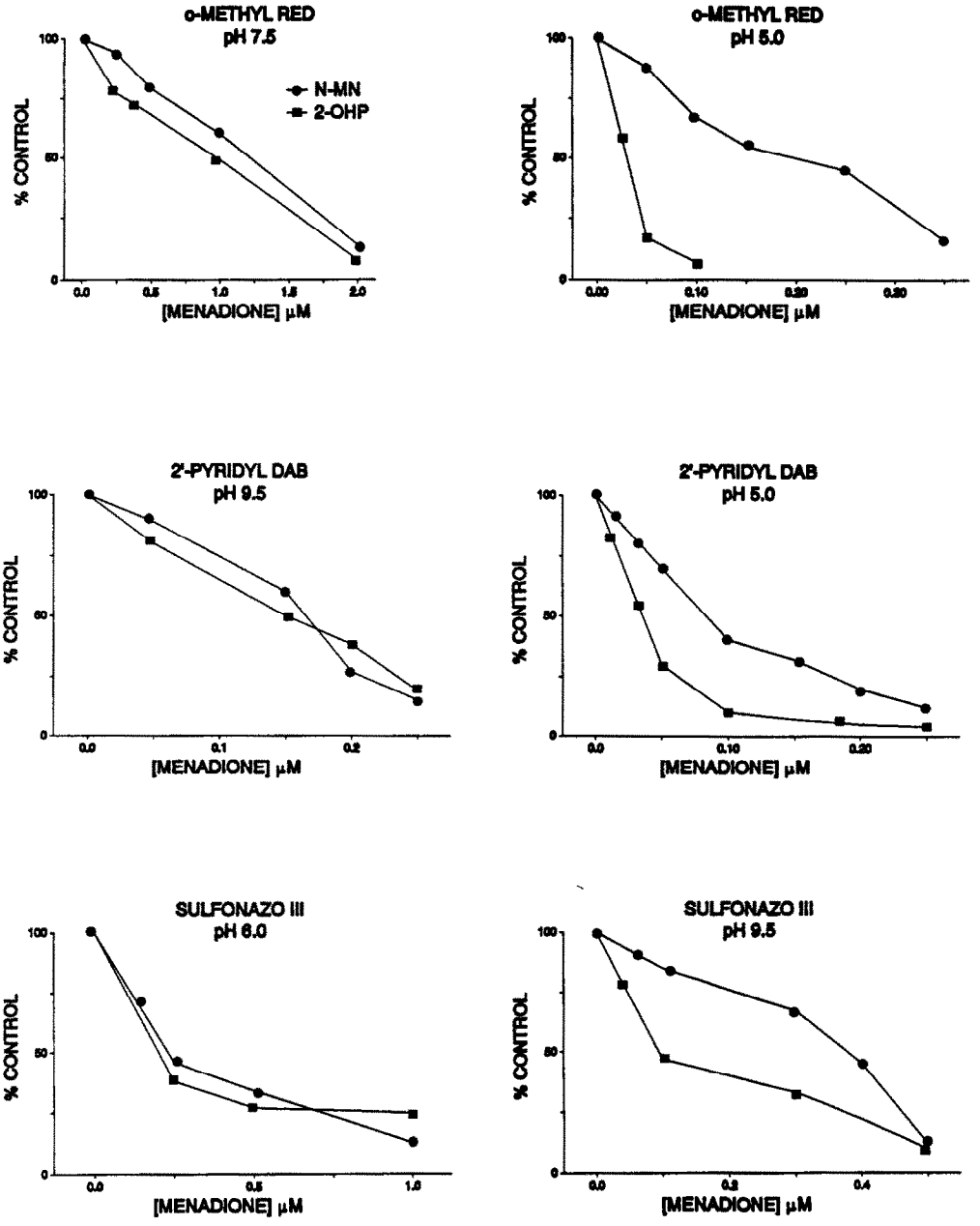


Fig. 5. Effects of menadione on azoreduction by aldehyde oxidase. Protocol is similar to that described in the legend of Fig. 3, except that the reaction mixture contained various concentrations of menadione, added as a 50% ethanolic solution, and was anaerobic. The final concentration of alcohol was 0.5% in all cases. Control rates (μmol/min/mg protein) were as follows: *o*-Methyl red, pH 7.5, 0.113 (N-MN), 0.113 (2-OHP); pH 5.0, 0.06 (N-MN), 1.13 (2-OHP). 2-Pyridyl DAB, pH 9.5, 0.20 (N-MN), 0.21 (2-OHP); pH 5.0, 0.21 (N-MN), 1.25 (2-OHP). Sulfonazo III, pH 6.0, 0.98 (N-MN), 2.64 (2-OHP); pH 9.5, 3.70 (N-MN), 11.8 (2-OHP).

cytosolic enzymes [22–24]. The present investigation provides insight into the mechanism of azoreduction by aldehyde oxidase. The results show that aldehyde oxidase can readily reduce a variety of hydrophilic azo dyes. Based on $V_{max}$ values, high reactivity was generally observed with dyes which were readily water soluble and contained highly charged groups, with a few exceptions. Sulfonazo III was the most reactive substrate. Lipophilic azo dyes such as DAB, 3′-methyl-DAB, and others which are substrates for microsomal cytochrome P450 [8–11], were not reduced by aldehyde oxidase (Table 1). Even at

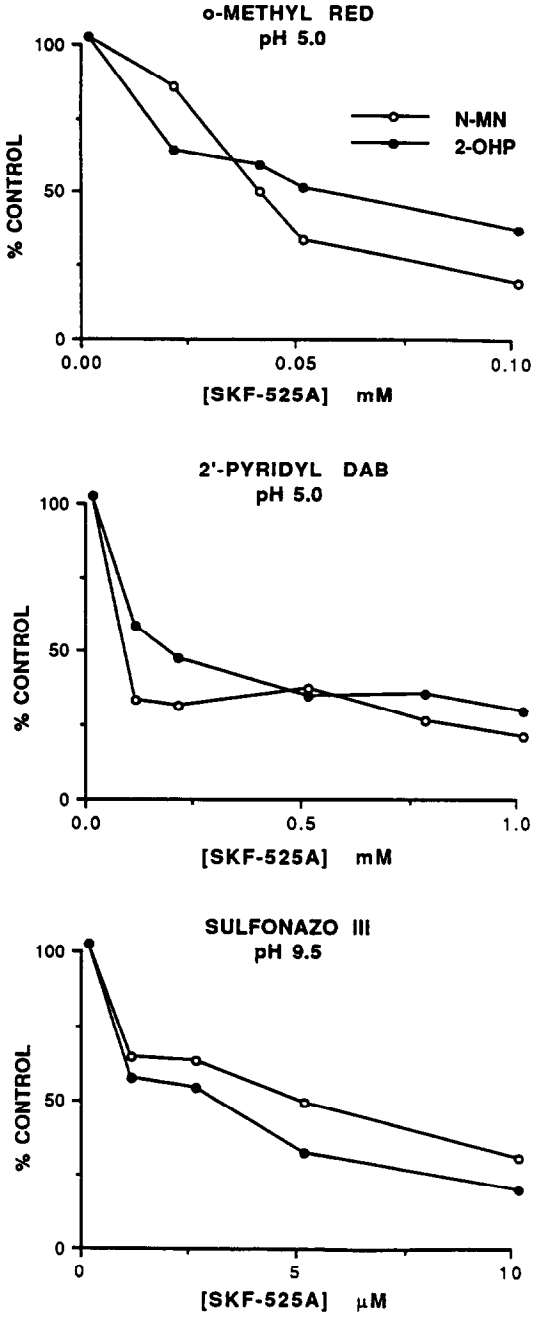


Fig. 6. Effect of SKF 525-A on azoreduction by aldehyde oxidase. Protocol is similar to that described in the legend of Fig. 5, except that the reaction mixture contained various concentration of SKF 525-A. Control rates (μmol/min/mg protein) were as follows: *o*-methyl red, 0.060 (N-MN), 1.13 (2-OHP); 2-pyridyl DAB, 0.21 (N-MN), 1.25 (2-OHP); and sulfonazo III, 3.70 (N-MN), 11.8, (2-OHP).

pH 5.0, where *o*-methyl red and 2'-pyridyl-DAB exhibited maximal activity, aldehyde oxidase did not reduce DAB. While polar electron-donating substituents are obligatory for azo dye reduction by liver microsomal cytochrome P450 [13], this may not be the only requirement for azo dye reduction by aldehyde oxidase.

Kinetic studies were conducted and values for $V_{max}$ and $K_m$ were obtained. However, no correlation between these two parameters was seen (Table 1). Furthermore, it is difficult to discern the significance of $K_m$ in an enzyme system requiring two substrates. Nevertheless, if, as in many cases, $K_m$ is a reflection of affinity between substrate and enzyme, then it could be concluded that affinity of the dye for enzyme is not a major determining factor in its rate of reduction.

Additional studies were conducted on four representative azo dyes (Fig. 3). The degree of ionization of their functional groups depends on pH. Sulfonazo III and Orange II, having sulfonate substituents, are ionized at basic pH [49]. 2'-Pyridyl-DAB with its basic functional group is expected to be protonated at acidic pH. *o*-Methyl red contains both acidic and basic residues and exhibits a color change between pH 4 and 6. This has been shown to be associated with protonation of the tertiary amine moiety but not of the 2'-carboxy moiety which is mainly protonated at pH 8.0*. Azoreduction of sulfonazo III and Orange II was maximal in the basic pH range (Fig. 3), whereas reduction of *o*-methyl red and 2'-pyridyl-DAB was maximal in the acidic pH range. Thus, optimal activity occurs at the pH at which maximal ionization of substrate occurs. $K_m$ values of the dyes tested also varied with pH (Table 3), but again there was no correlation between maximal activity and $K_m$. Consequently, it can be concluded that the rate of reduction is at least partially dependent on the charged state of the substrate but not on $K_m$. The fact that 2-OHP was the more effective electron donor in the reduction of these azo dyes is not unique to the present study. Other studies with aldehyde oxidase involving the reduction of sulfoxides [35], nitrosamines [48], and *N*-oxides [46, 47] have shown 2-OHP to be more effective than other electron donors. Furthermore, when N-MN was the electron donor, there was no apparent pH optimum for reduction of *o*-methyl red and 2'-pyridyl-DAB (Fig. 3) despite the obvious pH optimum at 7.5 seen in the aerobic system (Fig. 1A).

Sensitivity to oxygen has been observed selectively in azoreduction by microsomal cytochrome P450, depending on the structure of the dye [8, 13]. Reduction of substrates containing only electron-donating substituents is insensitive to oxygen. On the other hand, microsomal reduction of azo dyes containing both electron-donating and electron-withdrawing substituents is sensitive to oxygen [13]. In the case of aldehyde oxidase, reduction of all dyes is sensitive to oxygen. This may be due to competition for electrons by oxygen, the physiological electron acceptor or to reoxidation of the one-electron reduced form of the dye. It should be pointed out that most of the substrates showing substantial activity with aldehyde oxidase (Table 1) are also reduced by microsomal cytochrome P450 in an oxygen-sensitive manner [13]. Such substrates have been shown by cyclic voltammetry to have highly labile one-electron reduced derivatives [13].

* Zbaida S, personal communication, cited with permission.

Menadione has been established as a potent inhibitor of aldehyde oxidase [34, 35, 45]. This has been observed when oxidation of electron donor is directly measured with oxygen as electron acceptor. It is also seen when reduction of electron acceptors, ferricyanide and azo dyes (this paper) is measured. In this case a contributory factor may be menadione, a quinone, acting as a competing electron acceptor since a resonance-stabilized semiquinone may form through one-electron reduction [50]. Sensitivity to inhibition by menadione was greater when 2-OHP was the electron donor compared to N-MN, but only at the pH optimum for each dye (Fig. 5). Aldehyde oxidase contains three redox centers, molybdenum, flavin and iron-sulfur, and is capable of taking up more reducing equivalents in the steady state when 2-OHP rather than N-MN is the electron donor. Under such circumstances, electron transfer to menadione may be facilitated (the site of electron transfer is unknown). This could lead to menadione being a more effective electron acceptor (the site of electron transfer is unknown) and greater inhibition seen. SKF 525-A has also been shown to be an inhibitor of aldehyde oxidase activity [33]. However, sensitivity of azoreductase activity to SKF-525A inhibition was the same irrespective of electron donor, suggesting that the mechanism of inhibition by SKF 525-A differs from that of menadione.

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## REFERENCES

1. Wolff AW and Oehme FW, Carcinogenic chemicals in food as an environmental issue. *J Am Vet Med Assoc* **164**: 623–629, 1974.
2. Collins TFX and McLaughlin J, Teratology studies on food colourings I. Embryotoxicity of amaranth [FD and C red No. 2] in rats. *Food Cosmet Toxicol* **10**: 619–624, 1972.
3. Euris TE (Ed.), *Handbook for Food Additives*. The Chemical Rubber Co., Cleveland, OH, 1968.
4. Miller JA and Miller EC, The carcinogenicity of certain derivatives of *p*-dimethylaminobenzene in the rat. *J Exp Med* **87**: 139–156, 1948.
5. Mueller GC and Miller JA, The reductive cleavage of 4-dimethylaminoazobenzene by rat liver: Reactivation of carbon dioxide-treated homogenates by riboflavin-adenine dinucleotide. *J Biol Chem* **185**: 145–154, 1950.
6. Miller JA and Miller CE, The carcinogenic aminoazo dyes. *Adv Cancer Res* **1**: 339–396, 1953.
7. Kinosita R, Research on the carcinogenesis of various chemical substances. *Gann* **30**: 423–426, 1936.
8. Zbaida S, Stoddart AM and Levine WG, Studies on the mechanism of azo dye carcinogen reduction by rat liver microsomes. *Chem Biol Interact* **69**: 61–71, 1989.
9. Levine WG, Metabolism of azodyes: Implication for detoxication and activation. *Drug Metab Rev* **23**: 253–309, 1991.
10. Levine WG, Azoreduction of drugs and other xenobiotics. *Prog Drug Metab* **13**: 179–216, 1992.
11. Stoddart AM and Levine WG, Azoreduction of dimethylaminoazobenzene (DAB) in primary cultures of rat hepatocytes. Effect of hypolipidemic agents. *Drug Metab Dispos* **18**: 36–41, 1990.
12. Levine WG and Lu AYH, Role of isozymes of cytochrome P-450 in the metabolism of N,N-dimethyl-4-aminoazobenzene in the rat. *Drug Metab Dispos* **10**: 102–109, 1982.
13. Zbaida S and Levine WG, Characteristics of two classes of azo dye reductase activity associated with rat liver microsomal cytochrome P450. *Biochem Pharmacol* **40**: 2415–2423, 1990.
14. Kadlubar FF, Miller JA and Miller EC, Microsomal *N*-oxidation of the hepatocarcinogen, *N*-methyl-4-aminoazobenzene and the reactivity of *N*-hydoxy-*N*-methyl-4-aminoazobenzene. *Cancer Res* **36**: 1196–1206, 1976.
15. Delclos KB, Miller EC Miller JA and Liem A, Sulfuric acid esters as major ultimate electrophilic and hepatocarcinogenic metabolites of 4-aminoazobenzene and its N-methyl-derivatives in infant male C57BL/6J × C3H/HeJ $F_1$ (B6C3$F_1$) mice. *Carcinogenesis* **7**: 277–287, 1986.
16. Chung K-T, The significance of azo-reduction in the mutagenesis and carcinogenesis of azo dyes. *Mutat Res* **114**: 269–281, 1983.
17. Søderlund EJ, Dybing E, Nordenson S, and Tjelta E, The role of ethyl and fluorine substitution in the 4'-position of N,N-diethyl-4-aminoazobenzene mutagenicity and azo reduction. *Acta Pharmacol Toxicol* **47**: 175–182, 1980.
18. Ashby J Lefevre PA and Callander RD, The possible role of azoreduction in the bacterial mutagenicity of 4-dimethylaminoazobenzene (DAB) and 2 of its analogs (6BT and 5I). *Mutat Res* **116**: 271–279, 1973.
19. Walker R, The metabolism of azo compounds. A review of the literature. *Food Cosmet Toxicol* **8**: 656–676, 1970.
20. Scheline RR, Drug metabolism by the gastrointestinal microflora. *Monogr Pharmacol Physiol* **5**: 551–580, 1980.
21. Huang M-T, Miwa GT, Cronheim N and Lu AYH, Rat liver cytosolic azoreductase. Electron transport properties and the mechanism of dicumarol inhibition of the purified enzyme. *J Biol Chem* **254**: 11223–11227, 1979.
22. Levine WG and Raza H, Mechanism of azoreduction of dimethylaminoazobenzene by rat liver NADPH–cytochrome P-450 reductase and partially purified cytochrome P-450. Oxygen and carbon monoxide sensitivity and stimulation by FAD and FMN. *Drug Metab Dispos* **16**: 441–448, 1988.
23. Hernandez PH, Gillette JR and Mazel P, Studies on the mechanism of action of mammalian hepatic azoreductase—I. Azoreductase activity of reduced nicotinamide adenine dinucleotide phosphate-cytochrome *c* reductase. *Biochem Pharmacol* **16**: 1859–1875, 1967.
24. Fujita S and Peisach J, Liver microsomal cytochrome P-450 and azoreductase activity. *J Biol Chem* **253**: 4512–4513, 1978.
25. Mason RP and Peterson FJ, Inhibition of azoreductase by oxygen. The role of the azo anion free radical metabolite in the reduction of oxygen to superoxide. *Mol Pharmacol* **4**: 665–676, 1978.
26. Docampo R, Moreno SNJ and Mason RP, Generation of free radical metabolites and superoxide anion by the calcium indicators arsenazo III, antipyrylazo III and murexide in the rat liver microsome. *J Biol Chem* **258**: 14920–14925, 1983.
27. Zbaida S and Levine WG, A novel application of cyclic voltammetry in microsomal azoreduction. *Chem Res Toxicol* **4**: 82–88, 1991.
28. Kitamura S and Tatsumi K, Azoreductase activity of liver aldehyde oxidase. *Chem Pharm Bull (Tokyo)* **31**: 3334–3337, 1983.
29. Hille R and Massey V, Molybdenum-containing

hydroxylases: Xanthine oxidase, aldehyde oxidase, and sulfite oxidase. In: *Molybdenum Enzymes* (Ed. Spiro TG), Chap. 9, pp. 443–518. John Wiley, New York, 1985.

30. Beedham C, Molybdenum hydroxylases. Biological distribution and substrate-inhibitor specificity. In: *Progress in Medical Chemistry* (Eds. Ellis GP and West GB), pp. 85–127. Elsevier Science Publisher, U.K., 1987.
31. Rajagopalan KV and Handler P, hepatic aldehyde oxidase III. The substrate binding site. *J Biol Chem* **239**: 2227–2035, 1964.
32. Krenitsky TA, Neil SM Elion GB and Hitchings GH A comparison of the specificities of xanthine oxidase and aldehyde oxidase. *Arch Biochem Biophys* **150**: 585–599, 1972.
33. Beedham C, Molybdenum hydroxylases as drug-metabolizing enzymes. *Drug Metab Rev* **16**: 119–156, 1985.
34. Rajagopalan KV, Fridovich I and Handler P, Hepatic aldehyde oxidase. I. Purification and properties. *J Biol Chem* **237**: 922–928, 1962.
35. Yoshihara S and Tatsumi K, Guinea pig liver aldehyde oxidase as a sulfoxide reductase. Its purification and characterization. *Arch Biochem Biophys* **242**: 213–224, 1985.
36. Hall WW and Krenitsky TA, Aldehyde oxidase from rabbit liver: Specificity toward purines and their analogs. *Arch Biochem Biophys* **251**: 36–44, 1986.
37. Banks RB and Barnett SD, Tropylium tetrafluoroborate, a novel substrate for aldehyde oxidase. *Biochem Biophys Res Commun* **140**: 609–615, 1986.
38. Johnson C, Beedham C and Stell JGP, Reaction of 1-amino- and 1-chlorophthalazine with mammalian molybdenum hydroxylases *in vitro*. *Xenobiotica* **17**: 17–24, 1987.
39. Gristwood W and Wilson K, Kinetics of some benzothiazoles, benzoxazoles, and quinolines as substrates and inhibitors of rabbit liver aldehyde oxidase. *Xenobiotica* **18**: 949–954, 1988.
40. Beedham C, Bruce SE, Critchley DJ and Rance DJ, 1-Substituted phthalazines as probes of the substrate-binding site of mammalian molybdenum hydroxylases. *Biochem Pharmacol* **39**: 1213–1221, 1990.
41. Rajagopalan KV and Handler P, The absorption spectra of iron-flavoproteins. *J Biol Chem* **239**: 1509–1515, 1964.
42. Tatsumi K and Kitamura S and Yamada H, Sulfoxide reductase activity of liver aldehyde oxidase. *Biochim Biophys Acta* **747**: 86–92, 1983.
43. Mallett AK, King LJ and Walker R, A continuous spectrophotometric determination of hepatic microsomal azo reductase activity and its dependence on cytochrome P450. *Biochem J* **201**: 589–595, 1982.
44. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
45. Ohkubo M, Sakiyama S and Fujimura S, Purification and characterization of $N^1$-methylnicotinamide oxidases I and II separated from rat liver. *Arch Biochem Biophys* **221**: 534–542, 1983.
46. Kitamura S and Tatsumi K, Reduction of tertiary amine N-oxides by liver preparations: Function of aldehyde oxidase as a major N-oxide reductase. *Biochem Biophys Res Commun* **121**: 749–754, 1984.
47. Kitamura S and Tatsumi K, Involvement of liver aldehyde oxidase in the reduction of nicotinamide N-oxide. *Biochem Biophys Res Commun* **120**: 602–606, 1984.
48. Tatsumi K, Yamara H and Kitamura S, Evidence of involvement of liver aldehyde oxidase in the reduction of nitrosamines to the corresponding hyrazines. *Chem Pharm Bull (Tokyo)* **31**: 764–767, 1983.
49. Solomon S, *Introduction to General, Organic and Biological Chemistry*, pp. 305–340. McGraw-Hill, New York, 1983.
50. Prince RC, Lloyd-Williams P, Bruce JM and Dutton PL, Voltammetric measurement of quinones. *Methods Enzymol* **125**: 109–119, 1986.