Alcohol Dehydrogenase-Dependent Reduction of 2-Nitrosofluorene and Rearrangement of *N*-Hydroxy-2-aminofluorene†

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ABSTRACT: Various *C*-nitroso compounds are intermediates of arylamine oxidation or nitroarene reduction. Reductive metabolism of *C*-nitroso compounds to their corresponding hydroxylamines is a necessary step in the activation of these compounds to mutagenic end points. In this study, 2-nitrosofluorene (2-NOF) has been investigated as an aldehyde substrate analogue for horse liver alcohol dehydrogenase (HLADH). The reaction products are assigned on the basis of their UV/visible spectra and coelution with authentic standards in reversed-phase HPLC. The direct product of 2-NOF reduction is *N*-hydroxy-2-aminofluorene (N-OH-2-AF), which undergoes further reduction to 2-aminofluorene (2-AF) and rearrangement to 1- and 3-hydroxy-2-aminofluorene (1- and 3-OH-2-AF). The formation of these products is potently inhibited by pyrazole indicating the involvement of active-site zinc ion in the role of a Lewis acid catalyst. It is suggested that the rearrangement reaction occurs *via* an inner-sphere N-OH 2AF-Zn^{2+}···E-coenzyme complex following the elimination of the hydroxyl group from the N-OH-2-AF intermediate and the hydrolysis of the fluorenyl nitrenium-derived carbocations to yield the hydroxy 2-AF products. Herein ADH is identified as a *C*-nitroso-reducing enzyme which must be considered in the mutagenic sequelae of nitro and nitrosoarenes.

Aromatic *C*-nitroso compounds are common intermediates in the hepatic metabolism of aromatic amines and nitroaromatics. Earlier reports have shown that the cytosolic fraction of rat liver contains at least two enzymes which in the presence of NADH or NADPH reduced certain *C*-nitroso compounds to the corresponding amines (Bernheim, 1973). Subsequent, more detailed characterization of the NADH-dependent C-nitrosoreductase activity of cytosolic fractions from liver and other animal tissues (Otsuka, 1961; Horie et al., 1980; Kuwada et al., 1980; Ogura & Horie, 1980; Horie & Ogura, 1980; Horie et al., 1982, Hajos & Winston, 1992) indicated, based on the sensitivity of the reaction to pyrazole, involvement of alcohol dehydrogenase (ADH, EC 1.1.1.1). ADH[1] catalyzed the four-electron reduction of *p*-nitrosophenol to *p*-aminophenol and showed a slightly acidic pH optimum. The results of kinetic studies indicated the transient formation of an enzyme-bound intermediate of *p*-nitrosophenol by two-electron transfer from one molecule of NADH (Ogura & Horie, 1980). Furthermore, Dunn and Bernhard (1971), Koerber et al. (1980), and recently. Trivic and Leskovac (1991) found that purified HLADH and yeast ADH catalyzed NADH-dependent reduction of *p*-nitroso-*N*,*N*-dimethylaniline to the corresponding amine.

Despite the fact that ADH has been known to catalyze the reduction of the *C*-nitroso functional group to its corresponding hydroxylamine for nearly 2 decades, the toxicological ramifications of this reaction have not been fully explored. The role of nitrosoarenes in nitroarene and arylamine mutagenesis and carcinogenesis is believed to be due to their facile interconversion with *N*-hydroxyarylamines by oxidation and reduction processes (Bernheim, 1972; Becker & Sternson, 1980; Leskovac & Trivic, 1988; Manson, 1974). It has been suggested that the protonation of *N*-hydroxyarylamines followed by the elimination of water can result in a highly electrophilic arylnitrenium ion which, in turn, forms adducts with nucleic acids (Kriek, 1965; Kadlubar & Beland, 1985). Reduction of 2-nitrosofluorene to the less toxic amine enables it to be removed by other biochemical pathways and decreases the probability that the N-OH-2-AF is metabolized to more toxic derivatives; the amine requires specific oxidizing enzymes e.g., cytochrome P450 to be converted back to the hydroxylamine. Thus, the mutagenic expression of nitroarenes and arylamines depends largely on the steady-state concentrations of the nitroso and hydroxylamino intermediates formed by oxidative and reductive metabolism. In the present study the potent mutagen, 2-nitrosofluorene, is used as an aldehyde substrate analogue for ADH. We report that ADH catalyzes the reduction of this substrate to the corresponding hydroxylamine and amine, and further, show that ADH catalyzes the rearrangement of the hydroxylamine to 1- and 3-hydroxy-2-aminofluorene.

EXPERIMENTAL PROCEDURES

2-Aminofluorene, HLADH, NAD^+, NADH, NADPH, and deferoxamine mesylate were from Sigma (St. Louis, MO). Pyrazole and 2-nitrofluorene were from Aldrich Chemical Co., (Milwaukee, WI). N-Hydroxy-2-aminofluorene, 2-nitrosofluorene, 1- and 3-hydroxy-2-(acetylamino)fluorene were supplied by the National Cancer Institute Chemical Repository (Bethseda, MD). All other chemicals were of the highest purity grade commercially available.

The 1- and 3-OH-2-AF were synthesized by acidic deacetylation of the respective (acetylamino)fluorenes (2-AAF) according to the method given by Miller & McQueen (1986) by refluxing 1-OH-AAF and 3-OH-AAF in 6 N HCl for 2 h under argon. 1-OH-2-AF gave UV-visible absorption maxima at 269 nm and 3-OH-2-AF at 268 and 312 nm in 95% ethanol. 2,2′-Azoxybisfluorene was prepared by dis-

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[1] Abbreviations: HLADH or ADH, horse liver alcohol dehydrogenase; 2-NOF, 2-nitrosofluorene; 2-AF, 2-aminofluorene; N-OH-2-AF, *N*-hydroxy-2-aminofluorene; 1- and 3-OH-2-AF, 1- and 3-hydroxy-2-aminofluorene; 2-AAF, 2-acetamidofluorene; N-OH-2-AAF, *N*-hydroxy-2-acetamidofluorene.

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solving N-OH-2-AF in ethanol and bubbling with oxygen at room temperature for 20 min as described by Boyd et al. (1963). This product gave a UV-visible absorption maximum at 378 nm in 95% ethanol.

Reduction of 2-NOF and N-OH-2-AF was determined by a modification of the procedure described by Horie et al (1980); 0.2 mM 2-NOF or N-OH-2-AF was incubated in 1 or 2 mL for a specific period of time in the presence of 0.05–0.4 mM NADH, NAD^+, or NADPH and an appropriate amount of activating enzyme in 50 mM buffer. In some experiments pyrazole was added at concentrations of 0.01–5 mM. An argon atmosphere was employed and all solvents were throughly purged with argon. After a specific period of time the incubation was stopped by filtering 0.3–0.5 mL of the reaction mixture through a C_8 cartidge (Alltech) under argon pressure. The cartridge with adsorbed products was washed with 0.2 mL of distilled H_2O and the products eluted with 0.25–0.5 mL of ethanol. The cartridges were prewashed twice with 2 mL of methanol followed by 2 mL of 0.01% deferoxamine mesylate solution.

Analysis of products was performed with a Rainin HPX reversed-phase HPLC system, equipped with an Alltech econosphere C_8 5-μ column (150 × 4.6 mm) by a modification of the method of Smith & Thorgeisson (1981) and Miller & McQueen (1986). The initial solvent conditions were 60% 0.02 M acetic acid with 0.01% deferoxamine mesylate and 40% 2-propanol. The 2-propanol concentration was increased to 100% over 30 min at a flow rate of 1 mL/min and the eluant monitored at 290 nm. The results of analysis were confirmed by diode array detection on a Perkin-Elmer Model 410 HPLC equipped with a LC-235 Diode Array Detector with the column and conditions as described above. Mass spectra were acquired on a Hewlett-Packard 5971A quadrapole mass spectrometer linked to a Hewlett-Packard 5890 Series II gas chromatograph.

The spectrophotometric measurements were performed with a Hitachi U-3110 UV/vis spectrophotometer at room temperature (24 ± 1 °C).

RESULTS

Identification of Reaction Products. A typical HPLC chromatogram of the products formed in the enzymatic reaction of 2-NOF with NAD(P)H is shown in Figure 1A. Figure 1B presents the separation of products obtained during the nonenzymatic reaction of 2-NOF with NAD(P)H. The product peaks coelute with standard solutions of N-OH-2-AF (0.38), 2-NOF (1.00), 1-OH-2-AF (1.68), 3-OH-2-AF (1.69), 2-AF (1.92) and 2,2′-azoxybisfluorene (3.38), respectively. The values in parentheses are the fractional retention times in minutes of these products relative to that of 2-NOF (11.7). HPLC eluants coincident with 1- and 3-OH-2-AF, 2-AF, and 2,2′-azoxybisfluorene were collected, and 2-AF and 2,2′-azoxybisfluorene were analyzed by mass spectrometry. The mass spectral analysis revealed peaks at the expected *m/e* values (181 and 374, respectively). The identification of 1- and 3-OH-2-AF were confirmed on the basis of the similarities of their spectra to those of corresponding products obtained from deacetylation of commercially available 1- and 3-OH-2-AAF.

Reduction of 2-Nitrosofluorene to N-Hydroxy-2-aminofluorene. The stability of 2-NOF under our experimental conditions was monitored spectrophotometrically by following the change in absorbance at 360 nm and confirmed by HPLC analysis. 2-NOF is stable in 50 mM buffer in the range of the pH values studied for at least 1 h. In the presence of

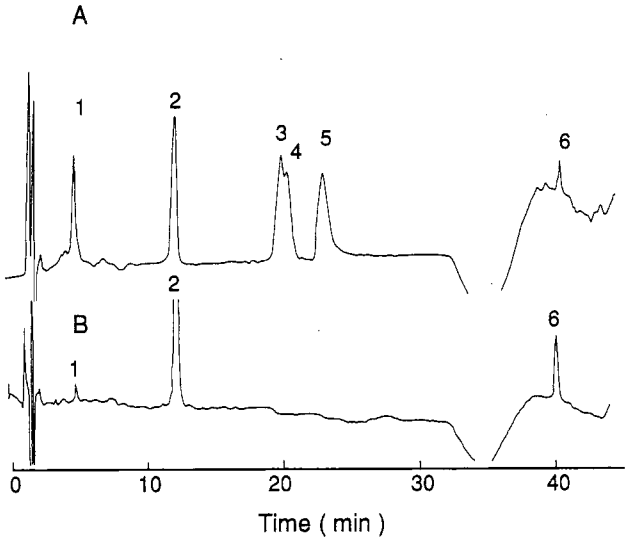

FIGURE 1: HPLC chromatogram of the products obtained during the enzymatic (A) and the nonenzymatic (B) reaction of 2-NOF with NADH. The products and the parent compound were eluted from a 150 × 4.6-mm Econosphere C8 5 μm reverse-phase column (Alltech) at a flow rate of 1 mL/min and detected by UV absorption at 290 nm. The initial solvent conditions were 60% 0.02 M acetic acid with 0.01% deferoxamine mesylate and 40% 2-propanol. Peak identification: (1) N-OH-2-AF; (2) 2-NOF; (3) 1-OH-2-AF; (4) 3-OH-2-AF; (5) 2-AF, (6) 2,2′-azoxybisfluorene.

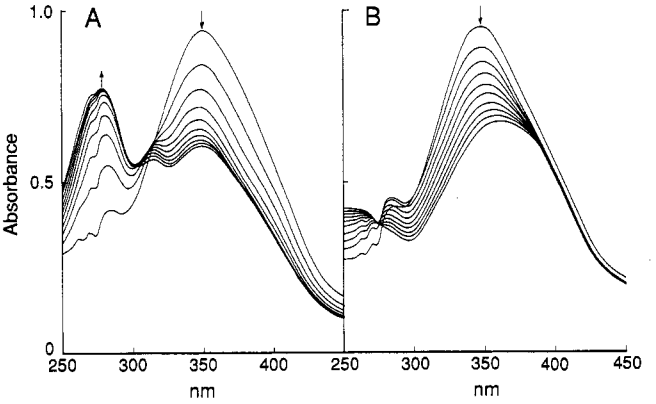

FIGURE 2: Nonenzymatic reduction of 2-NOF by NADH in phosphate buffer at pH 7.4 (A) and in pyrophosphate buffer at pH 8.8 (B). 2-NOF (0.1 mM) was reduced by NADH (0.05 mM) in an open cuvette and spectra were recorded at 1-min intervals.

NADH or NADPH, 2-NOF is nonenzymatically reduced to N-OH-2-AF. The only product of this reaction, N-OH-2-AF (Figure 1B), is not stable under the conditions of the reaction and undergoes both acid- and base-catalyzed condensation (Pizzolatti & Yunes, 1990) with 2-NOF yielding 2,2′-azoxybisfluorene (Figure 1B). In solution under normal aerobic conditions, N-OH-AF is converted back to 2-NOF. The reaction of 2-NOF with NADH was investigated by spectral analysis in the range of 250–450 nm at pH 7.4 and 8.8 (Figure 2, parts A and B, respectively). At pH 7.4 a decrease in the NADH (340 nm) and 2-NOF concentration (360 nm) is observed concomitant with the generation of N-OH-2-AF. The latter is indicated by an absorption maximum at 280 nm and a shoulder at 315 nm. At pH 8.8, N-OH-2-AF is not seen due to its rapid oxidation in air to 2-NOF. The nonenzymatic reduction of nitroso compounds by NADH and NADPH in aqueous buffer has been reported previously (Bernheim, 1972; Becker & Sternson, 1980; Leskovac & Trivic, 1988); both cofactors quantitatively converted nitrosobenzene to phenylhydroxylamine (Bernheim,

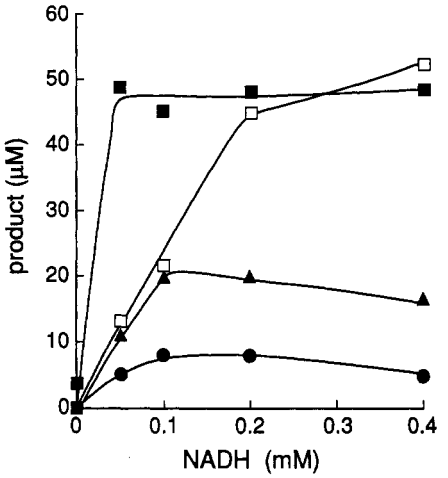

FIGURE 3: Nonenzymatic and enzymatic reduction of 2-NOF with NADH: effect of NADH concentration. The reactions of 0.2 mM 2-NOF with increasing concentrations of NADH were carried out for 10 min in the presence (closed symbols) or in the absence (open symbols) of HLADH in 50 mM phosphate buffer, pH 7.4. Symbols: (■) yield of *N*-hydroxy-2-AF; (▲) yield of 1- and 3-hydroxy-2-AF; (●) yield of 2-AF.

1972; Becker & Sternson, 1980) and 1-nitroso-2-naphthol to 1-amino-2-naphthol (Leskovac & Trivic, 1988).

To further probe the role of ADH in the reduction of 2-NOF the rate of reduction of 2-NOF was measured with NADH as the cofactor in the presence and absence of the horse liver enzyme (Figure 3). The nonenzymatic reaction rate is dependent on the concentration of NADH up to 0.2 mM. A similar reaction has been found to be second-order overall (Leskovac & Trivic, 1988). Under conditions of the present experiment (enzyme concentration = 7.5 μM), the enzymatic reduction of 2-NOF is completed within 10 min of incubation time. Additional experiments were conducted to determine the specific activity of HLADH in the reduction of 2-NOF. These experiments were determined under conditions (enzyme concentration ≪ 7.5 μM) in which the accumulation of the primary product, N-OH-2-AF, was linear for at least 10 min and the reduction was first order with respect to the concentration of enzyme. The extent of nonenzymatic reduction was determined under the same conditions and subtracted from the values obtained for the enzymatic reaction. The specific activities for the HLADH-dependent reduction of 2-NOF to N-OH-2-AF under these conditions were 0.8 and 0.2 μmol/min/mg with NADH and NADPH as cofactors, respectively.

Rearrangement of N-Hydroxy-2-aminofluorene: Inhibition by Pyrazole and Effect of pH. The primary product of the enzymatic and nonenzymatic reduction of 2-NOF, i.e., N-OH-2-AF, undergoes further enzymatic reactions yielding 2-AF and 1- and 3-OH-2-AF. These isomeric hydroxy derivatives of 2-AF were formed in equal amounts (±6% of each other) regardless of the reaction conditions.

The time course for the HLADH-dependent formation of 2-AF and 1- and 3-OH-2-AF with NADH and NADPH as cofactors is presented in Figure 4A. It is seen that the HLADH-catalyzed rearrangement of N-OH-2-AF to 1- and 3-OH-2-AF and the reduction of N-OH-2-AF to 2-AF proceeds with NADH or NADPH as cofactor, albeit much more slowly with the latter. With NADH as the cofactor, formation of both the rearrangement and reduction products display biphasic kinetics. The first step of this reaction is more rapid and is completed within 5-15 min, while the slower phase continues up to 60 min. This slower rate appears to parallel that of the NADPH-dependent reaction. At the plateau points NADH-dependent activity is approximately 1 order of magnitude greater than that with NADPH. The direct formation of these products from the reaction of N-OH-2-AF with NADH (Figure 4B) or with NAD^+ (data not shown) supports the idea that N-OH-2-AF is an intermediate in this process (Figure 4B). Very early work (Kaplan and Ciotii, 1954, and van Eys et al., 1958) have suggested the formation of the strongly inhibitory ternary complex; hydroxylamine–NAD^+–ADH. This possibility can be excluded for N-OH-2-AF since the HLADH-dependent reduction of 2-NOF to N-OH-2-AF is continuous (Figure 3).

To confirm a role for HLADH in each step of the overall process, pyrazole was utilized as a rapid and specific inhibitor of the enzyme active site; pyrazole forms a "dead-end" HLADH–NAD^+–pyrazole complex (McFarland and Bernhard, 1972). Figure 5 shows the effect of varying the concentration of pyrazole on the activity of HLADH in the primary reaction which yields N-OH-2-AF, and the two consecutive reactions that yield 1- and 3-OH-2-AF and 2-AF. At 2 mM pyrazole, the inhibition of 2-AF formation and the rearrangement is greater than 90% and remains unaffected by higher concentrations of pyrazole. The formation of N-OH-2-AF decreases continuously with increasing concentration of inhibitor and reaches about 25% of the control at 5 mM pyrazole. We noted above that N-OH-2-AF formation can proceed *via* a nonenzymatic reaction, which can explain the relatively lower inhibition of this product by pyrazole.

Figure 6 shows the dependence of the formation of the secondary products as a function of pH during the enzymatic and nonenzymatic reaction of 2-NOF with NADH. The rearrangement of N-OH-2-AF to 1- and 3-OH-2-AF increases with increasing pH and reaches a maximum at approximately pH 7.4. The reduction of N-OH-2-AF to 2-AF proceeds more rapidly below pH 6 at which values HLADH is partially denatured (Vallee & Hoch, 1957). The oxidation of N-OH-2-AF to 2-NOF and the formation of 2,2′-azoxybisfluorene occur at pH values above pH 7.4, and the rates are noted to increase rapidly with increasing pH. The observed maximum at pH 7.4 may result from spontaneous oxidation of the N-OH-2-AF intermediate at pH values above 7.4 and from a decrease in the HLADH activity due to partial enzyme denaturation at values below pH 6. The nonenzymatic formation of secondary products is negligible over the range of pH studied. Traces of 2-AF and the rearrangement products were formed at pH 4.

DISCUSSION

Herein, evidence is presented to show that horse liver alcohol dehydrogenase catalyzes the reduction of 2-NOF by NAD(P)H. The overall mechanism for this process, which occurs *via* both nonenzymatic and enzymatic pathways, may be described in accord with the scheme shown in Figure 7. 2-NOF is reduced to N-OH-2-AF, which undergoes further reactions: (1) the acid- and base-catalyzed condensation with 2-NOF yielding 2,2′-azoxybisfluorene and (2) spontaneous oxidation to 2-NOF. The presence of HLADH affects both the formation of 2-AF and the rearrangement of N-OH-2-AF to 1- and 3-OH-2-AF. The relative proportions of the 1- and 3-hydroxy products are essentially 1:1.

A similar rearrangement of aromatic hydroxylamines *in vivo* has been reported by Miller and Miller (1960) who observed that *N*-hydroxy-2-acetamidofluorene (N-OH-2-AAF) gives rise to 1-hydroxy-2-AAF among the urinary metabolites of rats. 3-(Methylthio)-2-AF and 1- and 3-(meth-

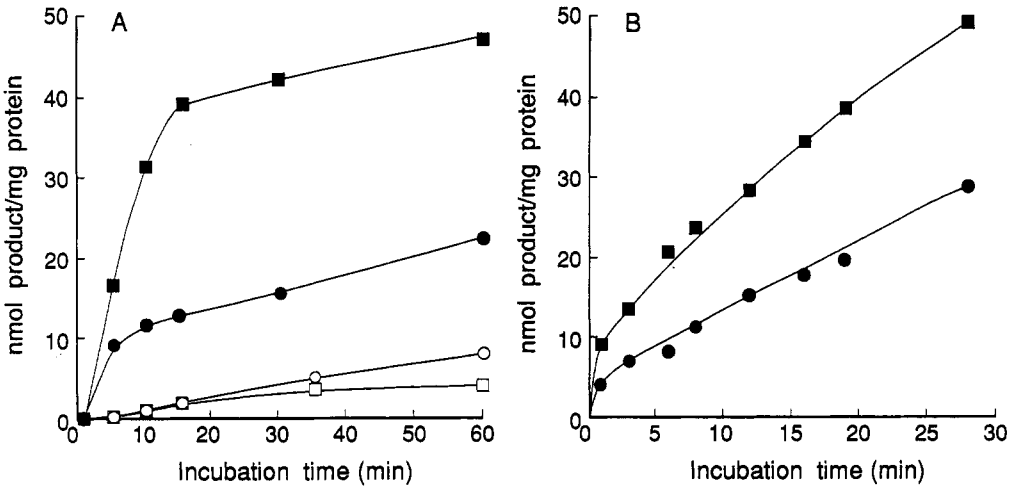

FIGURE 4: Time courses of the formation of 1-OH- and 3-OH-2-AF (■,□) and 2-AF (●, ○) during the HLADH-dependent (A) reaction of 0.2 mM 2-NOF with 0.2 mM NADH (closed symbols) or 0.2 mM NADPH (open symbols); (B) reaction of 0.2 mM N-OH-2-AF with 0.2 mM NADH in 50 mM phosphate buffer at pH 7.4. HLADH was present at 8.6 μM.

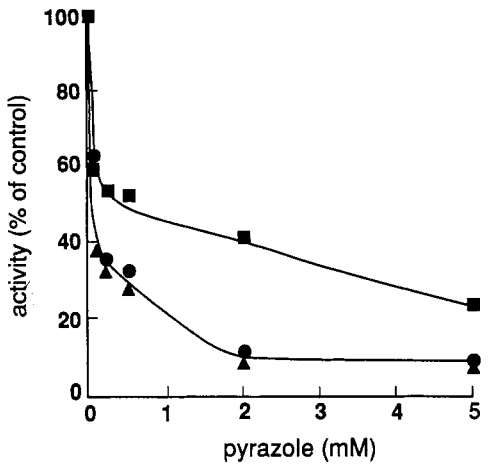

FIGURE 5: Inhibition by pyrazole of N-OH-2-AF (■), 1- and 3-OH-2-AF (▲), and 2-AF (●) formation during the reaction of 0.2 mM 2-NOF with 0.2 mM NADH in the presence of 8.6 μM HLADH. The reaction was carried out for 10 min in 50 mM phosphate buffer, pH 7.4.

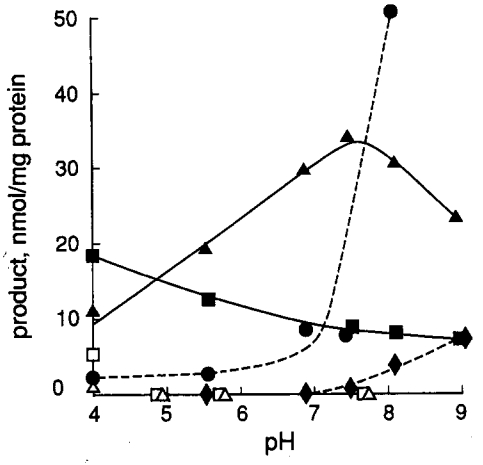

FIGURE 6: Dependence of product formation on pH during the reaction of 0.2 mM 2-NOF with 0.2 mM NADH in the presence (closed symbols) and the absence (open symbols) of 8.6 μM HLADH. The reaction was carried out for 10 min in 50 mM pyrophosphate, phosphate or acetate buffer. Symbols: (▲,△) 1- and 3-OH-2-AF; (■,□) 2-AF; (●) 2-NOF; (◆) 2,2′-azoxybisfluorene.

ylthio)-2-AAF have been observed as degradation products of fluorene derivatives bound to liver protein after administration of N-OH-2-AAF to rats (Bartsch et al., 1972, 1973). Booth and Boyland (1964) have suggested that this rearrangement is a more general reaction for these types of compounds because *N*-hydroxyacetanilide, *N*-hydroxy-2-acetamidonaphthalene and N-hydroxy-4-acetamidobiphenyl are also converted to the corresponding *o*-hydroxy derivatives. Sternson and Gammans (1975) suggested that this rearrangement is catalyzed by a hydroxylamine isomerase enzyme system present in rat or rabbit liver cytosol. Such hepatic isomerase-catalyzed rearrangements of hydroxylamines can be envisaged to proceed *via* pathways analogous to those described for certain chemical model systems. In acidic solution aromatic hydroxylamines undergo a Bamberger-type rearrangement to aminophenols. This reaction is intermolecular and occurs by a monoprotonation mechanism involving the rate-limiting elimination of water from $ArNHOH_2^+$ to form the arylnitrenium ion (Heller et al., 1951; Sone et al., 1980; Kadlubar et al., 1978).

The presence of 1- and 3-OH-2-AF among the reaction products can indicate that they are formed during the hydrolysis of the corresponding 1- and 3-fluorenyl carbocations, which in accord with a Bamberger-type mechanism, exist in equilibrium with the fluorenyl nitrenium ion. (Figure 7, scheme B). The relative yield of isomeric 1- and 3-fluorenyl carbocations is likely to depend on the distribution of the electron density in the N-OH-2-AF molecule. Molecular orbital calculations performed by the semiempirical AM1 method (Dr. Andrzej Sygula, personal communication) indicate that the charges at the carbon 1 and 3 positions are similar. These calculations are in agreement with our experimental data which show that the 1- and 3-hydroxy products are produced in essentially equal amounts.

We have previously reported on the ADH-catalyzed reduction of *p*-nitrosophenol to *p*-aminophenol (Hajos & Winston, 1992). Presumably this occurred via subsequent two-electron steps; the reduction of the nitroso group to a hydroxylamine species precedes the further reduction to the amine. The hydroxylamine species could conceivably exist as a transient intermediate either bound to or free from the ADH active site, but no direct evidence for the formation of the hydroxylamine compound has been obtained (Ogura & Horie, 1980). The direct product of 2-NOF reduction is N-OH-2-AF, which is not transient but has a finite steady-state concentration in solution and undergoes the further reactions of rearrangement to 1- and 3-OH-2-AF, and slow reduction

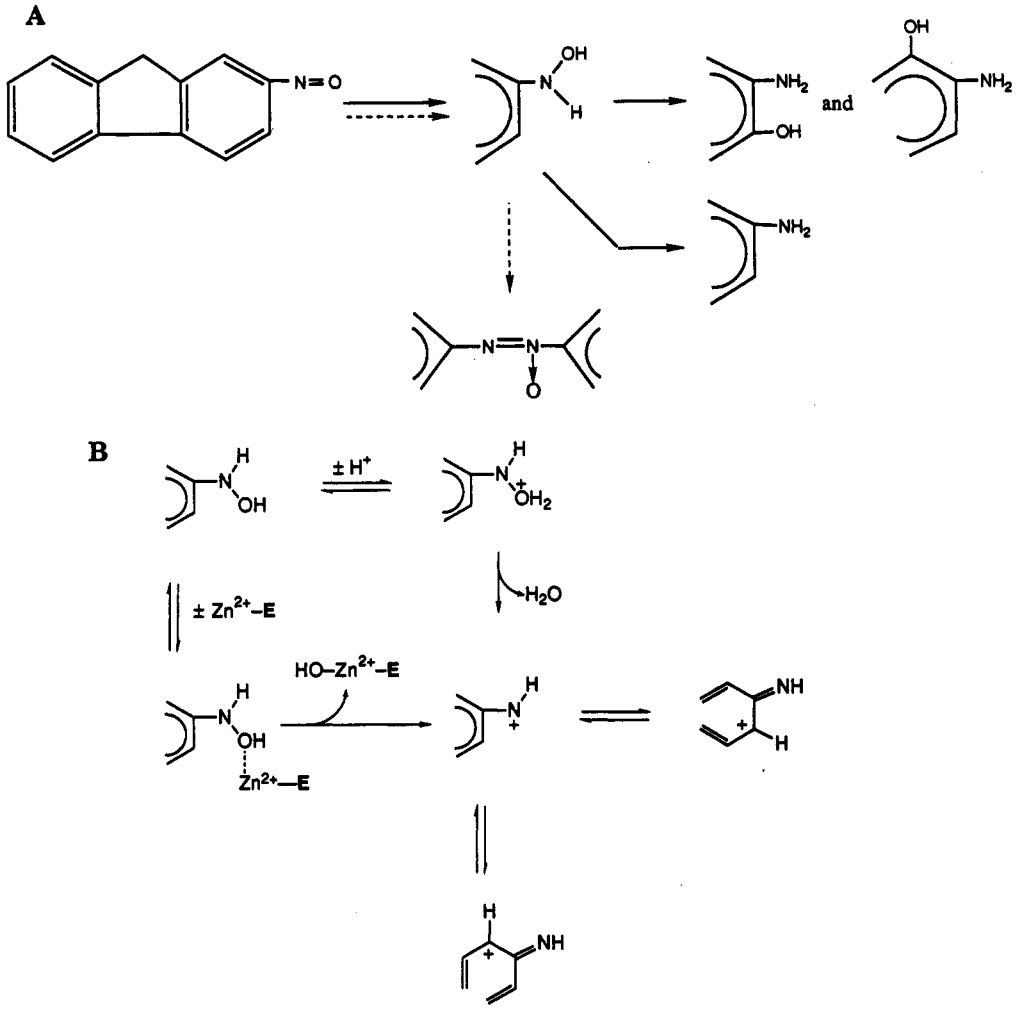

FIGURE 7: (A) Reaction scheme for the formation of products during the enzymatic (solid arrows) and nonenzymatic (dashed arrows) reduction of 2-NOF by NAD(P)H. (B) Proposed mechanism for rearrangement of N-OH-2-AF involved by the active site zinc of HLADH acting as a Lewis acid (bold arrow) in comparison with a Bamberger-type reaction.

to 2-AF. The reduction of 2-NOF or N-OH-2-AF to 2-AF previously has been observed to be catalyzed by rat, rabbit, or hamster cytosolic fractions (Booth & Boyland, 1964; Lotlikar et al., 1965), but the nature of this reduction is still unknown as various enzymes are capable of this catalysis. Kadlubar et al. (1976) have proposed that the arylamine arises from the reaction of arylnitrenium ion with *N*-hydroxyarylamine; however, Koerber et al. (1980) have found evidence that it can be formed in the reaction of free nitrenium ions with free NADH.

Our studies demonstrate that during ADH-dependent metabolism of 2-NOF with NADH as a cofactor, 2-NOF is reduced to the more mutagenic *N*-hydroxy-2-AF (Shane and Winston, 1993) which can be subsequently converted to the electrophilic fluorenylnitrenium ion and carbocation species. The role of the flourenylnitrenium ions and carbocations in the formation of promutagenic lesions with DNA is well documented (Frederick et al., 1982; Kadlubar et al., 1980; Beland & Kadlubar, 1985; Krauss et al., 1989). The covalent binding of N-OH-2-AF and N-OH-2-AAF, as well as their analogs *N*-hydroxy-1-naphthylamine and *N*-hydroxy-2-naphthylamine to DNA and RNA at slightly acidic pH have been reported and both *ortho*- and N-substituted arylmine–nucleotide adducts have been obtained.

In accord with a Bamberger-type rearrangement reaction, the formation of both fluorenylnitrenium ions and carbocations would require protonation of N-OH-2-AF. The pK_a for protonation of the *N*-hydroxy group has been established to be between 5 and 6 (Kriek, 1965; Kadlubar & Beland, 1985); thus, some amount of the *N*-hydroxy derivative should exist in the protonated form at neutral pH. We did not observe the rearrangement reaction in the absence of enzyme nor coenzyme; thus, both ADH and cofactor are necessary for this rearrangement. It implicates ADH at least in part as a hydroxylamine isomerase. The formation of the delocalized fluorenylnitrenium ion can occur *via* transfer of a proton from a water molecule bound to zinc or by a transfer of the hydroxyl group from N-OH-2-AF to the active-site zinc ion (Figure 7, scheme B). The results obtained by Dunn and Hutchison (1973) and Koerber et al. (1980) have suggested that the active-site zinc ion can act as a Lewis acid catalyst in this process. Therefore, the elimination of the hydroxyl group from the *N*-hydroxy substrate occurs *via* an inner sphere complex in which the oxygen atom of the *N*-hydroxy group is bound directly to the zinc of the enzyme–coenzyme complex. The possible role of horse liver alcohol dehydrogenase in the formation of both fluorenylnitrenium ion and carbocations at neutral pH is presently under investigation in our laboratory.

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