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Autoxidation Reactions of Different Aromatic *o*-Aminohydroxynaphthalenes That Are Formed during the Anaerobic Reduction of Sulfonated Azo Dyes

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Mono- and disulfonated naphthalene derivatives with a hydroxy group in *ortho*-position to an amino group are commonly occurring products of the anaerobic reduction of azo dyes by microorganisms. These substituted *o*-aminohydroxybenzenes and *o*-aminohydoxynaphthalenes are oxygen-sensitive and decompose under aerobic conditions. To evaluate their behavior under aerobic conditions, 1-amino-2-hydroxynaphthalene-6-sulfonate [AHNS; a reduction product of Sunset Yellow FCF (FD&C Yellow No.6, C.I. No. 15985)], 1-amino-2-hydroxynaphthalene-3,6-disulfonate [AHNDS; a reduction product of Amaranth (Acid Red 27, C.I. No. 16185)], and 1,2,7-triamino-8-hydroxynaphthalene-3,6-disulfonate [TAHNDS; a reduction product of Naphthol Blue Black B (Acid Black 1, C.I. No. 20470)] were chemically prepared and exposed to air at neutral pH. The reactions were analyzed, and the autoxidation products were characterized mainly by LC−MS and UV/VIS spectroscopy. Autoxidation of AHNS led to the formation of a dimer. With AHNDS, 1,2-naphthoquinone-3,6-disulfonate was found as intermediate, which subsequently reacted to a disulfonated cinnamic acid derivative. From TAHNDS a stable autoxidation product with a deep blueish color was formed that was identified as a naphthoquinone imine derivative of the parent compound. The spontaneous autoxidation reactions were compared to the behavior of the *o*-aminohydroxynaphthalenes and their products in the presence of activated sludge under aerobic conditions over the period of 1 month. In the presence of activated sludge, a biological conversion of the stable autoxidation product of TAHNDS was observed.

Introduction

Sulfonated azo dyes are widely used as dyes for textiles, food, and cosmetics (*1*). Most sulfonated azo dyes are not degraded in conventional aerobic sewage treatment plants (*2, 3*). However, it is well-known that most azo dyes are reductively cleaved by various microorganisms under anaerobic conditions to the corresponding amines (*4*). The aromatic amines that are formed from the reduction of sulfonated azo dyes are in most cases sulfonated benzene or naphthalene derivatives. There are several reports on the mineralization of sulfonated aminobenzenes (*5*−*7*) and sulfonated aminonaphthalenes (*8*) by aerobic bacteria. Therefore, one approach for the bioremediation of azo dyes is a sequential anaerobic/aerobic treatment. In the anaerobic stage, the azo bond is reductively cleaved. The reduction products are further metabolized in the aerobic stage. The basic feasability of this strategy for the treatment of sulfonated azo dyes has been previously demonstrated with the model compound Mordant Yellow 3 (*9, 10*).

The products of the reductive cleavage of the various sulfonated azo dyes contain a hydroxy group in *ortho*-position to the amino group. These sulfonated *o*-aminohydroxynaphthalenes undergo autoxidation reactions in the presence of oxygen (*11*). Little is known about the constitution and stability of the autoxidation products formed at neutral pH in aerated solutions. However, the realization of an effective anaerobic/aerobic treatment of industrially important sulfonated azo dyes strongly depends on the behavior of the sulfonated aminohydroxynaphthalenes after reaeration. Therefore, the following study has been performed to characterize the chemical behavior of three representative reduction products of azo dyes in the presence of oxygen.

Materials and Methods

Chemical Reduction of Azo Dyes. Stock solutions of each dye (20 mM, in 50 mL of H_2O) were incubated under a hydrogen atmosphere in the presence of a hydrogenation catalyst (200 mg of palladium on barium sulfate, 5% Pd; Aldrich). After complete decolorization, the solutions were transferred anaerobically to rubber-stoppered serum bottles and stored in the dark under a nitrogen atmosphere. The reduction products were not stored longer than 6 weeks. HPLC analysis demonstrated that the compounds were stable under these conditions throughout this time period.

Kinetics of the Autoxidation Reactions. Samples were taken from the stock solutions with the reduction products of the dyes using a syringe and transferred to a test tube with buffer (0.5 or 1.0 mM initial concentration, 4 mL total volume). The solutions were slightly stirred at room temperature, and samples were taken at different time intervals and directly analyzed by HPLC.

Analytical HPLC. For analysis of the kinetics of the autoxidation reactions, a high-performance liquid chromatography (HPLC) system was used that consisted of two model 510 pumps, an automated gradient controller, and a 995 photodiode array (PDA) detector (Waters, Milford, MA). A reverse-phase column was used as the stationary phase (250 × 4 mm i.d. packed with 5-μm particles of C-18 Lichrosorb; Grom, Herrenberg, Germany). The flow rate was 1.0 mL min^{-1}. Separation was achieved by either isocratic or gradient elution using 5 mM tetrabutylammonium hydrogensulfate (Fluka, Neu-Ulm) as the ion-pair reagent and methanol as the organic modifier. The solvent had a pH of 3.0.

In LC−MS, tetrabutylammonium hydrogensulfate caused a very high background. Therefore, ammonium acetate was chosen as the ion-pair reagent for these analyses. To correlate the results with both ion-pair reagents, HPLC−photodiode array (HPLC−PDA) and HPLC−MS were used in parallel so that a sample was simultaneously analyzed on both systems to get a direct correlation of UV−visible and mass spectra.

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TABLE 1. Solvent Systems Used as Mobile Phases for HPLC for the Separation of the Reduction Products of Amaranth and Naphthol Blue Black and Their Autoxidation Products[a]

compound	solvent system	retention volume (mL)
AHNS	D	4.1
$AP1_{AHNS}$	D	7.3
$AP2_{AHNS}$	D	12.3
$AP3_{AHNS}$	D	14.4
AHNDS	A	5.4
4AN1S	A	5.6
AP2AHNDS	A	11.4
$AP2_{AHNDS}$	A	21.9
$AP3_{AHNDS}$	A	13.4
AHNDS	C	2.9
4AN1S	C	6.8
$AP2_{AHNDS}$	C	2.3
$AP3_{AHNDS}$	C	2.1
$AP1_{TAHNDS}$	B	9.3
$AP2_{TAHNDS}$	B	12.5
1,4-diaminobenzene	B	1.7
aniline	B	1.9
$AP2_{TAHNDS}$	C	3.0

[a] For analysis with solvent systems A and B, a Lichrosorb C-18 column was used (Fa. Grom). The injection volume was 10 μL, and the flow rate of the solvent was 1.0 mL/min. Individual compounds were detected at $\lambda = 210$ nm. The solvent systems were composed of the following solvents: A, 40% (v/v) methanol/60% (v/v) H_2O plus 5 mM tetrabutylammonium hydrogensulfate; solvent B, 30% methanol (v/v)/70 H_2O % (v/v) plus 5 mM tetrabutylammonium hydrogensulfate. For analysis with solvent C, a Hichrom C-18 column was used, and a flow rate of 0.7 mL/min was applied. The solvent contained 10 mM ammonium acetate in water. Solvent system D was a solvent gradient that is described under Materials and Methods.

The HPLC system consisted of a Hewlett-Packard 1090 gradient elution pump equipped with a 1100 autosampler and a PDA detector. A C-18 reverse-phase column (size 250 $\times$ 4 mm, 5 μm particles; Hichrom, Reading, U.K.) was used to separate individual compounds. Separation was achieved by isocratic elution using ammonium acetate (10 mM) as an ion-pair reagent at a flow rate of 0.7 mL min^{-1}.

The HPLC−MS system comprised a Waters 600-MS system controller/multisolvent delivery system (Waters, Milford, MA), an intelligent autosampler 851-AS, and an intelligent 875-UV/VIS detector that was operated at 210 nm (Jasco Corp., Hachioji, Tokyo, Japan). The analytical column and mobile phase were the same as for the HPLC−PDA system. Mass spectra were obtained using a Finnigan MAT TSQ-700 mass spectrometer (Finnigan MAT, San Jose, CA) fitted with an electrospray (ESI) source operated in negative ionization mode. The source was operated at a spray voltage of 4.5 kV, with nitrogen sheath and auxiliary gas at 60 and 10 psi, respectively, and a capillary temperature of 260 °C.

The autoxidation products of 1-amino-2-hydroxynaphthalene-3,6-disulfonate and 1,2,7-triamino-8-hydroxynaphthalene-3,6-disulfonate were analyzed by isocratic elution (Table 1).

For the analysis of the reaction of 1-amino-2-hydroxynaphthalene-6-sulfonate, the following solvent gradient was used (flow rate 0.7 mL min^{-1}): From 0 to 1 min, the mobile phase consisted of 40% methanol and 60% water. From 1 to 15 min, a linear solvent gradient was applied with a final concentration of 90% methanol and 10% water. From 20 to 30 min, a reverse solvent gradient was applied to re-equilibrate the column. All solvents contained 5 mM tetrabutylammonium hydrogensulfate as the ion-pair reagent.

Preparative HPLC. 1,2-Dihydroxynaphthalene-3,6-disulfonate ($AP1_{AHNDS}$) and 1,2-naphthoquinone-3,6-disulfonate ($AP2_{AHNDS}$) were purified by preparative HPLC [preparative version of HPLC pump model 510 (Waters) equipped with a variable wavelength monitor with spectrocontroller (Fa. Knauer, Bad Homburg) and an HP 3392A integrator (Fa. Hewlett-Packard, Palo Alto, CA). The preparative HPLC column was filled with Lichrosorb 100 RP 8 [column size 250 $\times$ 20 mm (Fa. Bischoff, Leonberg)]. The flow rate was 4.5 mL min^{-1}. The solvent system for the isolation of the autoxidation products consisted of 99.9% water and 0.1% (v/v) trifluoroacetate. The retention volumes (R_V) were $R_V = 69$ and 40 mL for $AP1_{AHNDS}$ and $AP2_{AHNDS}$, respectively.

Experiments with Activated Sludge. The activated sludge was obtained from the aerobic part of the sewage treatment plant of the University of Stuttgart (Büsnau). Particulate material was collected by centrifugation (8500*g*, 30 min), resuspended in sodium/potassium phosphate buffer (50 mM, pH 7.4) and passed through a sieve with a mesh size of 1.0 mm to eliminate larger particles. A total of 19 mL of the resuspended sludge was transferred to 100-mL baffled Erlenmeyer flasks. One-half of the sample was sterilized. The experiments were started by adding a solution with the reduction products (stock solution 20 mM). All assays contained 26 $g_{dry\ weight}$/L and were shaken on a rotary shaker at 30 °C. The 100-μL samples were taken in intervals of 12 h, 1 day, or 4 days and centrifuged, and the supernatants were analyzed by HPLC.

Determination of Ammonia. The ammonia concentration was determined enzymatically using the glutamate dehydrogenase reaction (modified according to ref *12*). The reaction assay contained, in a total volume of 1 mL, 50 μmol of Bis−Tris buffer, pH 6.0; 2 μmol of ADP; 0.2 μmol of NADH; and 24 U of glutamate dehydrogenase. The reaction was started by the addition of 5 μmol of 2-oxoglutarate. The assay was measured after 10 min. The concentration of NADH was measured at $\lambda = 340$ nm.

Results

Autoxidation of 1-Amino-2-hydroxynaphthalene-6-sulfonate (AHNS). The reduction of Sunset Yellow (Figure 1A) yielded two products that were detected by HPLC. Sulfanilate was identified by comparison with an authentic standard. It was therefore concluded that the second signal corresponded to AHNS. The mixture of reduction products was transferred to buffers with different pH values, aerated, and analyzed at different time intervals by HPLC. As expected, sulfanilate was completely stable under these conditions. In contrast, a decrease of the concentration of AHNS was observed. The half-lifetime of AHNS became shorter with increasing alkalinity of the medium (Table 2). This effect was observed earlier for the autoxidation reactions of (substituted) 1,2-dihydroxynaphthalenes (*13*). At pH 7.0, three autoxidation products were formed from the original compound within the first 5 h of the reaction (Figure 2A). One autoxidation product, $AP1_{AHNS}$, was only detectable in trace amounts and had a much different retention behavior on the RP-phase than the second one ($AP2_{AHNS}$) (Table 1). The UV/VIS spectra of $AP1_{AHNS}$ and 1,2-naphthoquinone were very similar to each other exept for a shift of 14 nm toward higher wavelengths (Table 3). This is in accordance with the fact that the introduction of an additional sulfonate group leads to a bathochromic shift of the UV/VIS spectrum of the original unsulfonated compound (*14*). The UV/Vis spectrum of $AP2_{AHNS}$ had a characteristic absorption maximum at $\lambda = 475$ nm (Table 3). In the LC−MS analysis, a doubly charged ion (236.5 $[M - 2H]^{2-}$) and a single charged ion (474 $[M - H]^-$) were observed for $AP2_{AHNS}$ (Table 3). This suggested a molecule with two sulfonate groups and one nitrogen atom. Therefore, a coupling reaction seemed to be involved in the generation of $AP2_{AHNS}$. Another compound was observed to coelute with 1-amino-2-hydroxynaphthalene-6-sulfonate in the LC−MS chromatogram. This substance showed a single molecular ion at m/z 236, suggesting a molecular weight of

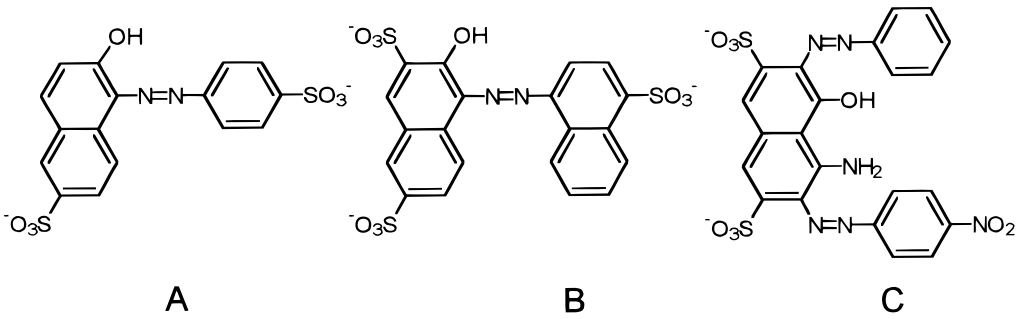

FIGURE 1. Structures of the azo dyes used in this study: (A) Sunset Yellow; (B) Amaranth; (C) Naphthol Blue Black B.

TABLE 2. Rates of Autoxidation of Substituted *o*-Aminohydroxynaphthalenes in Different Buffers[a]

	half-lifetime (min) at pH			
compound	5.5	6.0	6.5	7.0
AHNS from Sunset Yellow	75	70	60	55
AHNDS from Amaranth	130	110	50	20
$AP1_{TAHNDS}$ from Naphthol Blue Black	80	60	45	40

[a] Stock solutions of the reduction products (from 20 mM of the respective dye) were diluted (1:40) in the respective buffer (sodium acetate, pH 5.5; Bis-Tris, pH 6.0; sodium/potassium phosphate buffer, pH 6.5 or 7.0; 50 mM each).The reaction mixtures were aerobically incubated at room temperature and slowly stirred by a magnetic stirrer. Aliquots (10 μL) were taken every 30 min and immediately analyzed by HPLC. The remaining concentrations of the *o*-aminohydroxynaphthalenes were plotted half-logarithmically, and the half-lifetime was determined.

237, which would be consistent with the structure of the naphthoquinone imine corresponding to AHNS. A fourth major autoxidation product ($AP3_{AHNS}$) with an even higher retention on the RP-phase than $AP2_{AHNS}$ was generated when the concentration of $AP2_{AHNS}$ began to decrease. This compound had a characteristic absorbance maximum at λ = 520 nm.

These results suggested the following autoxidation mechanism (Figure 3-I): first, AHNS is oxidized rapidly to 1-amino-2-naphthoquinoneimine-6-sulfonate, followed by hydrolysis to the corresponding naphthoquinone (Figure 3-I, $AP1_{AHNS}$). 1,2-Naphthoquinone-6-sulfonate reacts immediately with AHNS by 1,4-addition to form *N*-(3,4-dihydroxy-6-sulfonyl-1-naphthyl)-1,2-naphthoquinone-1-imine-6-sulfonate (Figure 3-I, $AP2_{AHNS}$). The unsulfonated analogous compound was found when 1-amino-2-hydroxynaphthalene was electrochemically oxidized (*15*). Further oxidation of $AP2_{AHNS}$ possibly turned the two hydroxy groups into keto groups to form $AP3_{AHNS}$.

Autoxidation of 1-Amino-2-hydroxynaphthalene-3,6-disulfonate (AHNDS). The reduction of Amaranth (Figure 1B) yielded two products that were clearly separated by HPLC. One of these cleavage products was identified by comparison with an authentic standard as 4-aminonaphthalene-1-sulfonate (4AN1S), and the second signal was therefore tentatively identified as AHNDS. After the introduction of air, AHNDS decayed with approximately the same rate as AHNS (Table 2). The autoxidation of AHNDS at neutral pH yielded three compounds during the first 3 h of the reaction (Figure 2B). These compounds were labeled autoxidation products 1−3 ($AP1-3_{AHNDS}$). The disappearance of AHNDS was coupled with the formation of stoichiometric amounts of ammonia. To clarify the order of formation of the three APs, small quantities of $AP1_{AHNDS}$ were purified by preparative HPLC. When $AP1_{AHNDS}$ was incubated aerobically at neutral pH, it was converted to $AP2_{AHNDS}$; $AP3_{AHNDS}$ did not appear before $AP2_{AHNDS}$.

The LC−MS data and the UV/VIS spectrum of $AP2_{AHNDS}$ suggested that it was 1,2-naphthoquinone-3,6-disulfonate

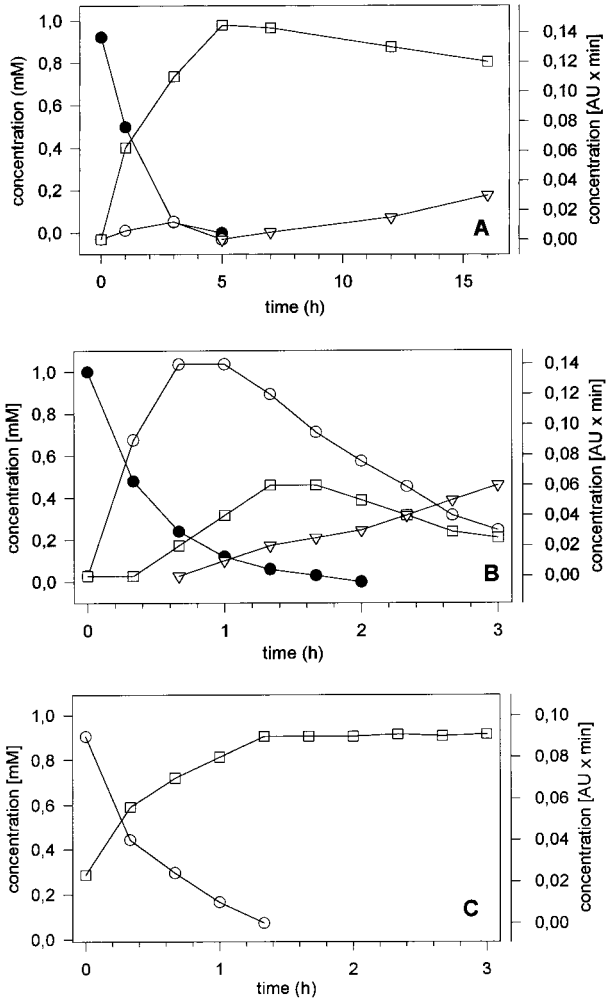

FIGURE 2. Autoxidation of 1-amino-2-hydroxynaphthalene-6-sulfonate (A), 1-amino-2-hydroxynaphthalene-3,6-disulfonate (B), and 1,2,7-triamino-8-hydroxynaphthalene-3,6-disulfonate (C). (●) in panel A, AHNS; in panel B, AHNDS. Autoxidation products 1 (○, 2 (□), 3 (▽). Filled symbols refer to the left *y*-axis, open symbols refer to the right *y*-axis. AU = relative absorbance units. Stock solutions of the reduction products (from 20 mM of the respective dye) were diluted (1:20) in 50 mM sodium/potassium phosphate buffer, pH 7.0. The reaction mixtures were aerobically incubated at room temperature and slowly stirred. Aliquots (10 μL) were taken every 30 min and immediately analyzed by HPLC.

(Table 3). $AP1_{AHNDS}$ was isolated by preparative HPLC and analyzed by ^{1}H NMR spectroscopy. The ^{1}H NMR data gave evidence for hydrogen atoms at positions 4, 5, 7, and 8 on the naphthalene ring (data not shown). Since AHNDS was deaminated yielding both $AP1_{AHNDS}$ and $AP2_{AHNDS}$ and the oxidation of $AP1_{AHNDS}$ led to $AP2_{AHNDS}$, the only liable structure for $AP1_{AHNDS}$ seemed to be 1,2-dihydroxynaphthalene-3,6-

TABLE 3. Physical Characteristics of the Autoxidation Products from 1-Amino-2-hydroxynaphthalene-6-sulfonate, 1-Amino-2-hydroxynaphthalene-3,6-disulfonate, and 1,2,7-Triamino-8-hydroxynaphthalene-3,6-disulfonate[a]

parent compound	autoxidation product	in-situ UV spectrum λ_{max} (nm)	LC−MS (*m/z*)
AHNS from Sunset Yellow	$AP1_{AHNS}$	254/339/455	
	$AP2_{AHNS}$	235/280/475	236.5 $[M - 2H]^{2-}$, 474 $[M - H]^{-}$
	$AP3_{AHNS}$	223/253/277/283/392/520	
1,2-naphthoquinone		240/325/442	
AHNDS from Amaranth	$AP1_{AHNDS}$	244/301/348	158 $[M - 2H]^{2-}$, 317 $[M - H]^{-}$
	$AP2_{AHNDS}$	269/345/478	
	$AP3_{AHNDS}$	208/260/297	175 $[M - 2H]^{2-}$, 351 $[M - H]^{-}$
TAHNDS from Naphthol Blue Black B	$AP1_{TAHNDS}$	267/305/350/510/667	
	$AP2_{TAHNDS}$	250/320/595	173 $[M - 2H]^{2-}$, 347 $[M - H]^{-}$

[a] The autoxidation reactions were performed as described under Materials and Methods. The UV/Vis spectra were recorded in-situ using a photodiode array (PDA) detector. Mass spectra were obtained using a Finnigan MAT TSQ-700 mass spectrometer fitted with an electrospray (ESI) source operated in negative ionization mode. The source was operated at a spray voltage of 4.5 kV, with nitrogen sheath and auxiliary gas at 60 and 10 psi respectively, and a capillary temperature of 260 °C.

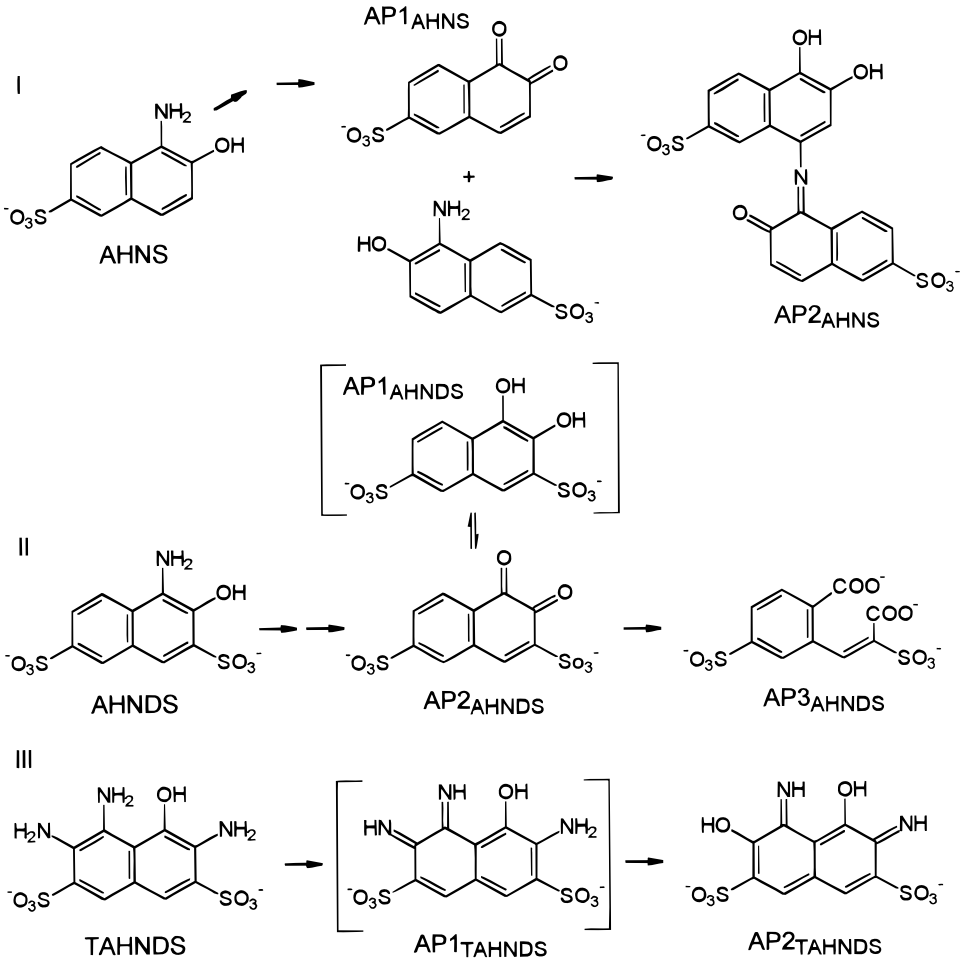

FIGURE 3. Proposed initial intermediates in the autoxidation processes of AHNS (I), AHNDS (II), and TAHNDS (III). AHNS, 1-amino-2-hydroxynaphthalene-6-sulfonate; $AP1_{AHNS}$, 1,2-naphthoquinone-6-sulfonate; $AP2_{AHNS}$, *N*-(3,4-dihydroxy-6-sulfonyl-1-naphthyl)-1,2-naphthoquinone-1-imine-6-sulfonate; AHNDS, 1-amino-2-hydroxynaphthalene-3,6-disulfonate; $AP1_{AHNDS}$, 1,2-dihydroxynaphthalene-3,6-disulfonate; $AP2_{AHNDS}$, 1,2-naphthoquinone-3,6-disulfonate; $AP3_{AHNDS}$, 4-carboxy-3-(2-carboxy-2-sulfonylvinyl)benzene-1-sulfonate; TAHNDS, 1,2,7-triamino-8-hydroxynaphthalene-3,6-disulfonate; $AP1_{TAHNDS}$, 7-amino-8-hydroxy-1,2-naphthoquinone-3,6-disulfonate-1,2-diimine; $AP2_{TAHNDS}$, dihydroxynaphthoquinone-3,6-disulfonatediimine. (The compounds shown in brackets and the positions of the hydrolyzed amino groups in $AP1_{TAHNDS}$ and $AP2_{TAHNDS}$ are hypothetical.)

disulfonate (Figure 3-II, $AP1_{AHNDS}$). AHNDS, a reductive agent also used for the reduction of silver salt in photography (*16*), apparently reduced 1,2-naphthoquinone-3,6-disulfonate (Figure 3, $AP2_{AHNDS}$) to the corresponding dihydroxynaphthalene derivate.

$AP3_{AHNDS}$ was formed from $AP2_{AHNDS}$. In the presence of catalase (50 U) however no $AP3_{AHNDS}$ was formed. This suggested that H_2O_2 was involved in the formation of $AP3_{AHNDS}$. Moreover, the addition of H_2O_2 greatly enhanced the formation of $AP3_{AHNDS}$ from a mixture of $AP1_{AHNDS}$ and $AP2_{AHNDS}$. No formation of sulfite or sulfate was found during the conversion of $AP2_{AHNDS}$ to $AP3_{AHNDS}$. The LC−MS data and the loss of the VIS maximum at about 300−400 nm (Table 3) led to the conclusion that 1,2-naphthoquinone-3,6-disulfonate was converted to 4-carboxy-3-(2-carboxy-2-sulfonylvinyl)benzenesulfonate (Figure 3-II, $AP3_{AHNDS}$). An

analogous reaction had been previously described that resulted in the conversion of 1,2-naphthoquinone to 4-carboxy-3-(2-carboxyvinyl)benzene in the presence of H_2O_2 (*17*).

Autoxidation of 1,2,7-Triamino-8-hydroxynaphthalene-3,6-disulfonate (TAHNDS). The reduction of Naphthol Blue Black B (Figure 1C) theoretically yielded three aromatic amines: aniline, 4-aminonitrobenzene, and 1,2,7-triamino-8-hydroxynaphthalene-3,6-disulfonate. As expected, the hydrogenation reaction lead also to the reduction of 4-aminonitrobenzene to 1,4-diaminobenzene. Aniline and 1,4-diaminobenzene were identified by comparison with authentic standards. TAHNDS was rapidly oxidized even in the presence of trace amounts of oxygen and could not be detected by HPLC. The initially clear solution of the reduction products then turned quickly dark blue. Two major peaks were observed in the autoxidation process during the first 3 h (Figure 2C) with intense absorption maxima at wavelengths above 590 nm (Table 3) and were designated autoxidation products $AP1_{TAHNDS}$ and $AP2_{TAHNDS}$. As shown by HPLC, these two compounds were different from the parent azo dye Naphthol Blue Black B. With decreasing concentration of $AP1_{TAHNDS}$, the concentration of $AP2_{TAHNDS}$ increased. In the LC−MS analysis, a doubly charged molecule with a molecular weight of 348 was found for $AP2_{TAHNDS}$. Therefore, it was concluded that $AP2_{TAHNDS}$ is one of the possible monomeric dihydroxynaphthoquinonediimine derivatives with an even number of nitrogen atoms (Figure 3-III, $AP2_{TAHNDS}$). $AP1_{TAHNDS}$ is consequently likely to be the corresponding aminohydroxynaphthoquinonediimine (Figure 3-III). The long-term stability of $AP2_{TAHNDS}$ is amazing since naphthoquinone imines are usually rapidly hydrolyzed in aqueous solutions (*18*). However, the structurally similar *p*-quinoneimine 5-amino-8-hydroxy-1,4-naphthoquinone-1-imine proved to be comparably stable (*19*).

Incubation of the *o*-Aminohydroxynaphthalenes with Activated Sludge. An important point for the feasibility of an anaerobic/aerobic treatment of sulfonated azo dyes is the question if the anaerobic reduction of the azo bond really enhances the aerobic biodegradability. To test the effect of an unadapted microflora on the autoxidation behavior, the reduction products from Sunset Yellow, Amaranth, or Naphthol Blue Black B (0.5 mM each) were incubated for 30 days in sodium/potassium phosphate buffer (pH 7.4, total volume 20 mL) either alone or together with activated sludge or a sterilized control of the sludge.

In all the assays with AHNS, $AP2_{AHNS}$ (*N*-(3,4-dihydroxy-6-sulfonyl-1-naphthyl)-1,2-naphthoquinone-1-imine-6-sulfonate) was generated in the first few hours. After 3 days, however, $AP2_{AHNS}$ had disappeared in all the assays. Over the observed period of time, up to 10 peaks of unidentified compounds were detected in all of the assays.

In the assays with AHNDS, within the first 24 h $AP1_{AHNDS}$ (1,2-dihydroxynaphthalene-3,6-disulfonate) and $AP2_{AHNDS}$ (1,2-naphthoquinone-3,6-disulfonate) were stabilized in the assay with activated sludge, probably due to the catalase activity of the cells. In the assays with inactivated sludge or buffered solution, $AP3_{AHNDS}$ (4-carboxy-3-(2-carboxy-2-sulfonylvinyl)benzene-1-sulfonate) was generated faster, and $AP1_{AHNDS}$ and $AP2_{AHNDS}$ could be detected only in trace amounts. After 3 days, compounds $AP1-3_{AHNDS}$ disappeared in all the assays, and new, unknown compounds were formed. However, the patterns of product formation in the different assays were different. Whereas six different compounds were detected in the assay with activated sludge after 3 days, 18 peaks were observed in the inactivated control. In all assays, however, after 30 days of incubation several unknown compounds remained in solution; five major peaks beside the one for 4AN1S were found in the assay with activated or inactivated sludge, respectively, whereas some 30 peaks were observed in the assay with the buffered solution.

With TAHNDS, the autoxidation behavior was completely different. $AP2_{TAHNDS}$ proved to be stable when incubated in buffered aqueous solution over the period of 27 days. In the same period of time, $AP2_{TAHNDS}$ was converted to two compounds with no absorption in the visible range in the presence of activated sludge. One of these compounds was also generated from $AP2_{TAHNDS}$ in the assay with inactivated sludge, but there, enough $AP2_{TAHNDS}$ was left to give the supernatant a deep blue color.

Discussion

Mono- and disulfonated naphthalene derivatives with a hydroxy group in *ortho*-position to an amino group are reduction products of a wide range of azo dyes. The comparison of the autoxidation kinetics of these compounds and the analysis of the products formed demonstrated the importance of the substitution pattern of the labile aromatic amines for the autoxidation behavior.

The autoxidation of AHNS led to the rapid generation of a dimer, probably by 1,4-addition of 1,2-naphthoquinone-6-sulfonate and AHNS. The intermediate 1,2-naphthoquinone-6-sulfonate seemed to react quickly and was detected only in traces. It seemed therefore possible that this compound also reacts with the nucleophilic groups in the matrix of activated sludge. It is known that xenobiotic compounds, particularly with quinoide structures, react with humic substances (*22*). Nucleophilic addition reactions are also known for aromatic amines (*23*). These reactions may lead to precipitation and reduced toxicity. However, the formation of the dimer from 1,2-naphthoquinone-6-sulfonate and AHNS was not influenced by the presence of activated sludge. Furthermore, no indications for the formation of water-insoluble polymers were found during the autoxidation of AHNS.

In contrast to 1,2-naphthoquinone-6-sulfonate, 1,2-naphthoquinone-3,6-disulfonate was found in comparatively high concentrations during the autoxidation process of AHNDS in buffered aqueous solution. In contrast to 1,2-naphthoquinone-6-sulfonate, 1,2-naphthoquinone-3,6-disulfonate showed no tendency to form adducts and was slowly transformed to a benzene derivative in the presence of hydrogen peroxide. Apparently, 1,4-addition at the 4-position of substituted naphthoquinones is sterically hindered by the bulky sulfonate group in the 3-position. This view was further confirmed by the finding that there were no addition reactions observed in the autoxidation process of TAHNDS. A stabilizing effect of catalase on 1,2-naphthoquinone-3,6-disulfonate and 1,2-dihydroxynaphthalene-3,6-disulfonate was observed. Catalase activity of microorganisms is therefore likely to be responsible for the stabilizing effect on the APs of AHNDS in the presence of aerobic sludge. In contrast, the strong tendency for addition reactions apparently prevented 1,2-naphthoquinone-6-sulfonate from reacting with H_2O_2 in a similar manner.

In contrast to the characterized autoxidation product of AHNS and AHNDS, $AP2_{TAHNDS}$ remained stable in buffered aqueous solution. $AP2_{TAHNDS}$ showed an intense absorption maximum above $\lambda = 500$ nm. This indicates that azo dye reduction does not necessarily lead to decolorization of effluents. TAHNDS, the reduction product of many economically important azo dyes and similar naphthalene derivatives, may prove to form rather stable and colored autoxidation products. The stability of this compound can be explained by intramolecular hydrogen bonding among the imino, amino, and hydroxy groups (*11*). However, a biological conversion of $AP2_{TAHNDS}$ was shown that led to the loss of color in the supernatant.

This study shows that the chemical reactivity of the oxygen-sensitive *o*-hydroxyaminonaphthalenes formed by the reductive cleavage of azo dyes heavily depends on their substitution patterns. With the results of this study, it should be easier to predict the autoxidation behavior of a range of different oxygen-sensitive *o*-hydroxyaminonaphthalenes with comparable structures.

Literature Cited

(1) Zollinger, H. *Color chemistry*, 2nd ed.; VCH: Weinheim, 1991.
(2) Pagga, U.; Brown, D. *Chemosphere* **1986**, *15*, 479−491.
(3) Shaul, G. M.; Holdsworth, T. J.; Dempsey, C. R.; Dostal, K. A. *Chemosphere* **1991**, *22*, 107−119.
(4) Walker, R. *Food. Cosmet. Toxicol.* **1970**, *8*, 659−676.
(5) Feigel, B. J.; Knackmuss, H.-J. *Arch. Microbiol.* **1993**, *159*, 124−130.
(6) Locher, H. H.; Thurnheer, T.; Leisinger, T.; Cook, A. M. *Appl. Environ. Microbiol.* **1989**, *55*, 492−494.
(7) Thurnheer, T.; Köhler, T.; Cook, A. M.; Leisinger, T. *J. Gen. Microbiol.* **1986,** *132*, 1215−1220.
(8) Nörtemann, B.; Kuhm, A. E.; Knackmuss, H.-J.; Stolz, A. *Arch. Microbiol.* **1994**, *161*, 320−327.
(9) Haug, W.; Schmidt, A.; Nörtemann, B.; Hempel, D. C.; Stolz, A.; Knackmuss, H.-J. *Appl. Environ. Microbiol.* **1991**, *57*, 3144−3149.
(10) Kudlich, M.; Bishop, P. L.; Knackmuss, H.-J.; Stolz, A. *Appl. Microbiol. Biotechnol.* **1996**, *46*, 597−603.
(11) Grünanger, P. *Methoden der Organischen Chemie* 4th ed.; Georg Thieme Verlag: Stuttgart, 1978; Vol. 7, pp 1−195.
(12) Da Fonseca-Wollheim, F.; Bergmeyer, H. U.; Gutmann, I. In *Methoden der enzymatischen Analyse*, 3rd ed.; Bergmeyer, H. U., Ed.; VCH: Weinheim, 1974; pp 1850−1853.
(13) Kuhm, A. E.; Stolz, A.; Ngai, K.-L.; Knackmuss, H.-J. *J. Bacteriol.* **1991**, *173*, 3795−3802.
(14) Koepernik, H.; Borsdorf, R. *J. Prakt. Chem.* **1983**, *325*, 1002−1010.
(15) Bonastre, J.; Castetbon, A.; Mericam, P. *Bull. Soc. Chim. Fr.* **1977**, 1099−1106.
(16) Junge, K. W.; Hübner, G. *Fotographische Chemie-aus Theorie und Praxis*, 2nd ed.; VEB Fotokinoverlag: Leipzig, 1972.
(17) Böeseken, M. J. *Recl. Trav. Chim. Pays-Bas.* **1911,** *30*, 142−144.
(18) Irving, Ch. C. *J. Org. Chem.* **1960**, *25*, 464−465.
(19) Merian, E. *Chimia* **1959**, *13*, 181−212.
(20) Mason, H. S.; Peterson, E. W. *J. Biol. Chem.* **1955**, *212*, 485−493.
(21) Rees, J. R.; Pirie A. *Biochem. J.* **1967**, *102*, 853−863.
(22) Bollag, J.-M.; Loll, M. J. *Experientia* **1983**, *39*, 1221−1231.
(23) Weber, E. J.; Thorn, K. A.; Pettigrew, P. J.; Goldenberg, W. S. *Environ. Sci. Technol.* **1996,** *30*, 2764−2775.

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