# Complete Biodegradation of the Azo Dye Azodisalicylate under Anaerobic Conditions

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# Complete Biodegradation of the Azo Dye Azodisalicylate under Anaerobic Conditions

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Azo dyes are a widespread class of poorly biodegradable industrial pollutants. In anaerobic environments, azo bonds are reductively cleaved yielding carcinogenic aromatic amines, many of which are assumed to resist further metabolism by anaerobes. Here we report for the first time that an azo dye compound is completely biodegradable in the absence of oxygen. A pharmaceutical azo dye, azodisalicylate, constructed from two 5-aminosalicylic acid (5ASA) molecules was mineralized in an adapted methanogenic consortium to $CH_4$ and $NH_3$ with transient accumulation of 5ASA as a degradation intermediate in both batch assays and continuous bioreactors. The anaerobic metabolism of 5ASA was shown to provide the electrons required for the initial reductive cleavage of the azo group. Our results suggest that it is possible to design azo dyes that are fully mineralized in the environment; thereby, avoiding accumulation of notorious toxic intermediates.

## Introduction

Azo dyes are widespread environmental pollutants associated with the textile, cosmetic, food colorants, printing, and pharmaceutical industries (*1*−*3*). These synthetic compounds are resistant to aerobic degradation by bacteria. The strong electron-withdrawing character of the azo group stabilizes these aromatic pollutants against conversions by oxygenases (*4*−*6*). In studies with more than 100 azo dyes tested in aerobic activated sludge systems, only a few were actually biodegraded (*5*, *7*). Due to their recalcitrance in aerobic environments, the azo dyes eventually end up in anaerobic sediments, shallow aquifers, and groundwaters (*8*−*10*) or in the gastrointestinal tracts of higher animals (*11*). Under anaerobic conditions, azo dyes are readily cleaved via a four-electron reduction at the azo linkage generating aromatic amines (*12*, *13*). The aromatic amines are considered to be stable biotransformation products of azo dye metabolism by anaerobes (*6*, *13*, *14*). Unfortunately, as suspect mutagens and carcinogens, the aromatic amines cannot be regarded as environmentally safe end products (*11*, *15*). It is generally assumed that aromatic amines derived from azo dyes are not degraded under anaerobic conditions (*13*, *14*). This observation coupled with the fact that many aromatic amines are completely degraded under aerobic conditions has led to the proposal that anaerobic−aerobic systems might be effective in achieving the complete biodegradation of azo dyes (*6*, *14*). The azo dye Mordant Yellow 3 was degraded completely using this approach (*6*). However, in a separate study, we have demonstrated that some aromatic amine building blocks of azo dyes, such as 5ASA and 3-aminobenzoate, are mineralized by adapted methanogenic consortia (*16*). Therefore, azo dyes constructed from these aromatic amines could potentially be fully biodegradable in anaerobic environments. Results are presented in this study indicating the complete degradation under anaerobic conditions of a pharmaceutical azo dye, azodisalicylate (ADS), composed of two 5ASA units.

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## Materials and Methods

**Anaerobic Granular Sludge and Basal Medium.** A methanogenic consortium immobilized in sludge granules was obtained from a 160-mL laboratory upflow anaerobic sludge bed (UASB) bioreactor fed with an azo dye, Mordant Orange 1 (MO1; 5-(4-nitrophenylazo)salicylic acid), and glucose as cosubstrate for 7 months (17). The bioreactor was initially fed with a subtoxic concentration of MO1 (3 mg/L). The concentration of MO1 in the influent was increased periodically after at least 20 hydraulic retention times (HRT; 8 h) and when greater than 75% removal of MO1 and the cosubstrate chemical oxygen demand (COD) had been obtained. After 200 days of operation, the bioreactor was adapted up to a MO1 loading of 295 mg $L^{-1}$ $d^{-1}$ with >99% dye removal. MO1 was reductively cleaved to aromatic amines (5ASA and 1,4-phenylenediamine). At the end of the adaptation period, the granular sludge was able to mineralize 5ASA, whereas 1,4-phenylenediamine was not degraded.

The basal medium used in all experiments (except when indicated otherwise) was as described by Donlon et al. (*18*) and contained the following (mg/L): $NaHCO_3$ (5000), $NH_4Cl$ (280), $CaCl_2 \cdot 2H_2O$ (10), $K_2HPO_4$ (250), $MgSO_4 \cdot 7H_2O$ (100), yeast extract (100), $H_3BO_3$ (0.05), $FeCl_2 \cdot 4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2 \cdot 4H_2O$ (0.05), $CuCl_2 \cdot 2H_2O$ (0.03), $(NH_4)SeO_3 \cdot 5H_2O$ (0.05), $AlCl_3 \cdot 6H_2O$ (2), $NiCl_2 \cdot 6H_2O$ (0.05), EDTA (1), resazurin (0.2), and 36% HCl 0.001 mL/L. The micronutrients were supplemented to the media for the continuous bioreactor experiments at a 10-fold lower concentration.

**Anaerobic Continuous Bioreactors.** Two identical UASB bioreactors were used during this study. The 160-mL glass bioreactors were operated in parallel using identical feed systems. Both bioreactors were placed in a temperature-controlled room at 30 °C. Each bioreactor was seeded with 20 g of volatile suspended solids (VSS) per liter of MO1-adapted granular sludge. The bioreactors were started up with either partially neutralized VFA mixture (acetate:propionate:butyrate, 23:34:41 on a COD basis; 1 g of COD/L) or glucose (1.5 g of COD/L) and ADS (25 mg/L). The concentration of ADS in the influent was increased periodically after at least 10 HRT. Glucose and the VFA mixture were used as a cosubstrate to provide the electrons for the ADS reductive cleavage. Methane production was measured with a 10-L Mariotte flask filled with a 3% (w/v) NaOH solution to scrub out the carbon dioxide from the biogas.

**Batch Experiments.** ADS-adapted sludge (0.7 g of VSS/L) withdrawn from the glucose reactor on day 130 was incubated at 30 °C under $N_2/CO_2$ (70%/30%) atmosphere in serum bottles (120 mL) containing 28 mL of basal medium. In this particular case, $NH_4^+$-N nutrient concentration was reduced to 22 mg/L, and the yeast extract was omitted from the basal medium. Serum bottles were incubated overnight, and only those bottles in which anaerobic conditions prevailed as indicated


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by the redox indicator resazurin were used for the assay. ADS was added as a sole source of substrate from a concentrated stock solution to give a final concentration of 200 of mg ADS/L. The serum bottles were incubated for a 50-day period on an orbital shaker (Gerhardt, Bonn, Germany) at 50 strokes/min. Sludge blanks, to correct for background $NH_4^+$-N and methane production from the sludge, were based on assays where no ADS was provided. Sludge that was autoclaved at 121 °C for 1 h received ADS and was used to measure abiotic reduction and/or adsoption of the azo dye. The ADS reduction, the temporal accumulation of 5ASA, and the $NH_4^+$-N released were measured periodically by sampling the liquid phase. Methane was sampled in the head space of the serum bottles using a pressure-lock (Dynatech Precision Sampling Corp., Baton Rouge, LA) gas-tight syringe (injection volume = 0.1 mL). The net values of $NH_4^+$-N released and methane produced were obtained by subtracting the values in the compound-amended sludge with those in the sludge blank controls. The corrected methane production was expressed as a percentage of the theoretical methane production (TMP) expected from the test azo dye mineralization based on the Buswell equation (*19*). The TMP expected for ADS was 2.76 mL. All the values reported are the means of triplicate or quadruplicate incubations. To elucidate intermediates during the metabolism of ADS, a parallel experiment was done. Incubations were made as described before, with the difference that 300 mg/L of the ADS breakdown intermediate, 5ASA, was added to the serum bottles instead of ADS. The methanogenesis was blocked using 50 mM of the methanogenic inhibitor 2-bromoethanesulfonate (BESA), allowing the accumulation of intermediates, which were determined by gas chromatography (GC) and high-performance liquid chromatography (HPLC). Sludge blank controls were also used in these determinations.

**Analytical Methods.** Methane and VFA were determined by GC methods described elsewhere (*20*). The ADS and 5ASA concentrations were determined with an HPLC. The HPLC determinations were conducted on an Spectra-Physics SP8810 HPLC (Thermo Separation Products, Breda, The Netherlands). A $C_{18}$ (250 × 3 mm, particle size 5 $\mu$m) reverse-phase column (Chrompack, Bergen op Zoom, The Netherlands) was used to separate individual compounds that were detected using a Kratos Superflow 773 UV−VIS detector (Separations, H. I. Ambacht, The Netherlands). The solvent phases were 98% methanol and 0.5% acetic acid in demineralized water (adjusted to pH 4.5 with NaOH). The column was run with methanol/acetic acid solution in a ratio 2/98 for 5 min, and then the ratio was changed to 80/20 over the next 2.5 min and sustained for 7.5 min. The solvent flow rate was 0.3 mL/min, and the column temperature was 20 °C. The retention times of 5ASA and ADS were 5 and 15.5 min and were detected at 330 and 300 nm, respectively.

In some cases, ADS was also measured spectrophotometrically by UV−VIS absorbance with a Spectronic 60 spectrophotometer (Milton Roy/Analitycal Products Division, Ostende, Belgium) and a Model 100-QS (Hellma Benelux, The Hague, The Netherlands) 1-cm quartz cuvette. Absorption is reported as the absorption of the medium containing N-aromatic compounds minus the absorption of the control medium (which contained no test compound). All samples were diluted to less than 1 absorbance unit in 0.2 M phosphate buffer (pH 7.0). Azo dye reduction was monitored at an absorbance maxima of 380 nm, which is a value that does not overlap with the products of azo reduction; 5ASA has no absorbance at this wave length. In some cases, 5ASA was monitored colorimetrically at 440 nm after reacting with 4-dimethylaminobenzaldehyde hydrochloride (Ehrlich Reagent) according to the method for measuring aromatic amines described by Oren et al. (*21*). $NH_4^+$-N was measured spectrophotometrically based on the modified Berthelot reaction with an Skalar autoanalyzer (Skalar Analytical B.V., Breda, The Netherlands), according to Dutch standards (*22*).

The pH was determined immediately after sampling to avoid any change due to the $CO_2$ evolution, using a pHmeter 511 (Knick, Berlin, Germany) and a double electrode N61 (Scot Gerade, Hofheim, Germany). All the other analytical determinations were performed as described in *Standard Methods for Examination of Water and Wastewater* (*23*).

**Chemicals.** ADS (under the commercial name Olsalazine) was kindly supplied by Pharmacia AB (Uppsala, Sweden). ADS was 99.5% pure. The other chemicals were purchased from Acros Chimica (Geel, Belgium). All chemicals were of the highest purity commercially available and were not purified further.

## Results

**Anaerobic Treatment of ADS in Continuous UASB Bioreactors.** Two 160-mL UASB bioreactors were operated in parallel under several operational conditions to study the anaerobic transformation and mineralization of ADS. The bioreactors were fed with ADS and either a VFA mixture (R1) or glucose (R2). VFA mixture and the glucose were used as cosubstrates to provide the reducing equivalents for the reduction and the cleavage of the azo chromophore. The applied influent concentrationss of ADS ($ADS_{in}$), cosubstrate ($CoS_{in}$), organic loading rate (CoS-LR), ADS loading rate (ADS-LR), and HRT as well as the assessed treatment efficiencies in the various periods of the experiment are listed in Tables 1 and 2 for R1 and R2, respectively. The daily azo dye treatment performance is also shown in Figures 1 and 2 for R1 and R2, respectively.

R1 was operated under four different operational conditions for a 205-day period at 30 °C (Table 1 and Figure 1). During the four periods, ADS was reduced on the average by 70% (Figure 1). Variations in the OLR and/or the ADS-LR did not cause any significant improvement in the ADS reduction. The ADS breakdown product (5ASA) was recovered in the effluent at a relatively high molar yield of the ADS removed (88%) during period 2. In period 4, 5ASA was detected at very low concentrations (molar yield <0.5% of ADS removed), indicating that further metabolism of this aromatic amine had occurred (Figure 1).

R2 was operated under seven different operational conditions for a 340-day period (Table 2 and Figure 2). In periods 1 and 2, the ADS was partially removed to an extent of more than 60%; whereas, the ADS breakdown product 5ASA was recovered at low concentrations in the effluent (Figure 2). During periods 3 and 4, the ADS was greatly removed (at least by 95%), and the corresponding aromatic amine was recovered by less than 2% (molar yield of ADS removed). This indicates that the 5ASA released from azo dye cleavage was being metabolized. The cosubstrate removal was also quite high during all these periods, accounting for more than 86% of the influent COD (Table 2).

In the subsequent periods (5, 6, and 7), the cosubstrate was no longer included in the influent in order to determine if ADS could be fully degraded as a sole carbon and energy source. In this case, the reducing equivalents required for the azo bond reduction would need to be generated by 5ASA metabolism. After the cosubstrate was excluded (period 5), ADS continued to be reduced by more than 60%. On day 236, the upflow velocity was increased in the reactor from 0.02 to 0.2 m/h by recirculating the effluent. Nonetheless, the increase in upflow velocity did not produce any improvement in the ADS reduction as can be observed in Figure 2. 5ASA was still detected at very low concentration levels in the effluent (<1% molar yield of the ADS removed). In period 6, the HRT was doubled from 8 to 16 h, and there was a slight increase in the ADS reduction to 70%. In this period, the sulfates and nitrogen nutrient sources were removed from the basal medium to avoid any possible competition for electron acceptors and forcing the consortia to utilize ADS

**TABLE 1. Operational Conditions and Treatment Efficiency of Continuous UASB Bioreactor (R1) Treating ADS with VFA Mixture as a Cosubstrate at 30 °C**

| parameter[a] | experimental periods[b] 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| | **Operational** | | | |
| $ADS_{in}$ (mg/L) | 25 | 50 | 50 | 50 |
| $CoS_{in}$ (g of COD/L) | 1 | 1 | 2 | 2.5 |
| CoS-LR (g of COD $L^{-1}$ $d^{-1}$) | 3 | 3 | 6 | 7.5 |
| ADS-LR (mg $L^{-1}$ $d^{-1}$) | 75 | 150 | 150 | 150 |
| HRT (h) | 8 | 8 | 8 | 8 |
| | **Efficiency (%)** | | | |
| ADS removal[c] | 73.2 ± 7.9 | 74.7 ± 2.7 | 72.8 ± 4.7 | 74.4 ± 9.3 |
| 5ASA recovered[c] | 24.1 ± 10.1 | 88.2 ± 8.1 | 2.5 ± 4.9 | 0.5 ± 0.04 |
| CoS removal (% COD) | 88.2 ± 2.5 | 84.1 ± 1.7 | 93.1 ± 2.7 | 96.7 ± 1.3 |
| $CH_4$ (% $CoS_{in}$-COD)[d] | 73.1 ± 14.4 | 65.5 ± 9.5 | 111.2 ± 10.9 | 105.8 ± 9.9 |

[a] Azodisalicylate concentration influent ($ADS_{in}$), cosubstrate influent concentration ($CoS_{in}$), cosubstrate volumetric loading rate (Co-LR), ADS volumetric loading rate (ADS-LR), and hydraulic retention time (HRT). [b] Periods: 1 (day 0−25), 2 (day 26−40), 3 (day 41−136), and 4 (day 137−205). [c] ADS removal and 5ASA recovered were determined by HPLC, except for periods 1 and 2 when the determinations were done by spectrophotometry and colorimetry; respectively (see Materials and Methods). 5ASA recovered is expresed as a molar yield of ADS removed (see Figure 1 caption for definition). [f] Yield of methane in COD as a % of $CoS_{in}$-COD.

**TABLE 2. Operational Conditions and Treatment Efficiency of Continuous UASB Bioreactor (R2) Treating ADS with Glucose or No Cosubstrate at 30 °C**

| parameter[a] | experimental periods[b] 1 | 2 | 3 | 4 | 5[c] | 6 | 7 |
|---|---|---|---|---|---|---|---|
| | **Operational** | | | | | | |
| $ADS_{in}$ (mg/L) | 25 | 50 | 50 | 75 | 75 | 75 | 75 |
| $CoS_{in}$ (g of COD/L) | 1.5 | 1.5 | 3 | 3 | 0 | 0 | 0 |
| CoS-LR (g of COD $L^{-1}$ $d^{-1}$) | 4.5 | 4.5 | 9 | 9 | NA[d] | NA | NA |
| ADS-LR (mg $L^{-1}$ $d^{-1}$) | 75 | 150 | 150 | 225 | 225 | 114 | 69 |
| HRT (h) | 8 | 8 | 8 | 8 | 8 | 16 | 26 |
| | **Efficiency (%)** | | | | | | |
| ADS removal[e] | 69.4 ± 6.6 | 63.5 ± 4.5 | 96.7 ± 1.5 | 98.8 ± 0.8 | 63.4 ± 9.1 | 69.1 ± 2.8 | 88.9 ± 1.8 |
| 5ASA recovered[e] | 13.4 ± 3.6 | 11.5 ± 2.0 | 1.0 ± 1.2 | 0.8 ± 1.1 | 0.12 ± 0.6 | <0.06 | <0.06 |
| CoS removal (% COD) | 89.0 ± 2.9 | 88.7 ± 0.2 | 93.3 ± 1.7 | 94.8 ± 0.9 | NA | NA | NA |
| $CH_4$ (% $CoS_{in}$-COD)[f] | 74.6 ± 15.8 | 70.5 ± 3.6 | 96.7 ± 12.6 | 100.9 ± 8.0 | NA | NA | NA |

[a] Azodisalicylate concentration influent ($ADS_{in}$), cosubstrate influent concentration ($CoS_{in}$), cosubstrate volumetric loading rate (Co-LR), ADS volumetric loading rate (ADS-LR), and hydraulic retention time (HRT). [b] Periods: 1 (day 0−25), 2 (day 26−40), 3 (day 41−136), 4 (day 137−205), 5 (day 206−257), 6 (day 258−307), and 7 (day 308−340). [c] On day 236, the upflow velocity in the bioreactor was increased from 0.02 to 0.2 m/h through recirculation of the effluent. This upflow velocity was applied until the end of the experiment. [d] NA, not applicable. [e] ADS removal and 5ASA recovered were determined by HPLC, except for periods 1 and 2 when the determinations were done by spectrophotometry and colorimetry; respectively (see Materials and Methods). 5ASA recovered is expresed as molar yield of ADS removed. [f] Yield of methane in COD as a % of $CoS_{in}$-COD.

as an N source. An HRT increase to 26 h in period 7 resulted in a great increase in the ADS reduction to 89%.

**Batch Experiments.** In order to confirm that ADS could be mineralized into methane and $NH_3$, ADS-adapted granular sludge withdrawn from R2 was incubated in anaerobic serum bottles with and without 200 mg/L of ADS as a sole source of substrate. During the course of the 50-day incubation period, the HPLC-determined ADS concentration steadily decreased at an average rate of 8.3 mg (g of VSS)$^{-1}$ $d^{-1}$ (Figure 3a). No significant decrease in the ADS concentration was observed in the autoclaved sludge after more than 50 days, indicating that the dye removal in the living sludge was biologically mediated. The elimination of ADS was associated with a net increase in methane production and ammonium concentration beyond that observed in the sludge blanks (Figure 3b). The net methane production and ammonium released at the end of the 50-day incubation period accounted for 78% COD and 75% of the nitrogen contained in the ADS, respectively. These results indicate that ADS was extensively mineralized by the adapted methanogenic consortium. During ADS mineralization, 5ASA was detected as a transient degradation intermediate (Figure 3a), demonstrating that reductive cleavage of the azo dye was the first step in the degradation. By day 20, this intermediate was no longer detectable suggesting that it was metabolized as was confirmed in a separate experiment by the conversion of exogenously added 5ASA to methane in the same sludge (net methane production accounted for 76% of 5ASA-COD).

During the degradation of 5ASA, it is possible that electrons are used to reduce the azo dye. This hypothesis was confirmed by adding exogenous 5ASA (300 mg/L) to the sludge incubated with ADS (200 mg/L). The rate of azo dye reduction was enhanced by 65% with the addition of exogenous 5ASA (results not shown).

To elucidate intermediates during the metabolism of 5ASA, a specific methanogenic inhibitor (BESA) was added to sludge incubated with 5ASA. BESA effectively blocked the methane production from 5ASA. Several VFA were identified in the BESA-amended cultures after 50 days of incubation accounting for up to 60% of the COD contained in 5ASA. Acetate was the major VFA formed, responsible for 79% of the VFA pool.

## Discussion

**Azo Dye Reduction.** It is well known that azo dyes are easily reduced under anaerobic conditions. The stoichiometric reduction of an azo bond yielding aromatic amines requires four reducing equivalents, which are typically supplied by an

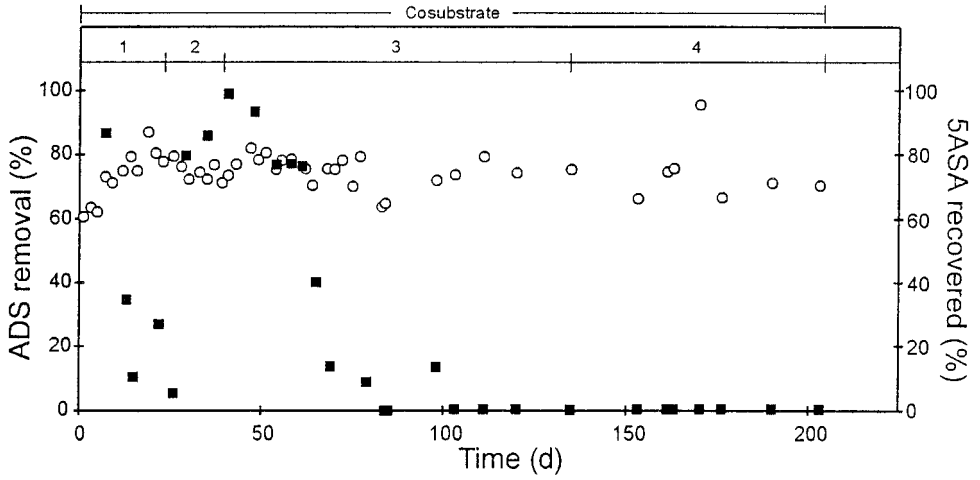


**FIGURE 1. Treatment efficiency of ADS degradation with the VFA mixture as a cosubstrate in UASB bioreactor (R1); %ADS removal (○), %5ASA recovered in effluent (as a molar yield of ADS removed = 100 × [5ASA effluent/2 × ADS removed]) (■). Numbers in the top of the figure indicate the experimental periods (see Table 1).**

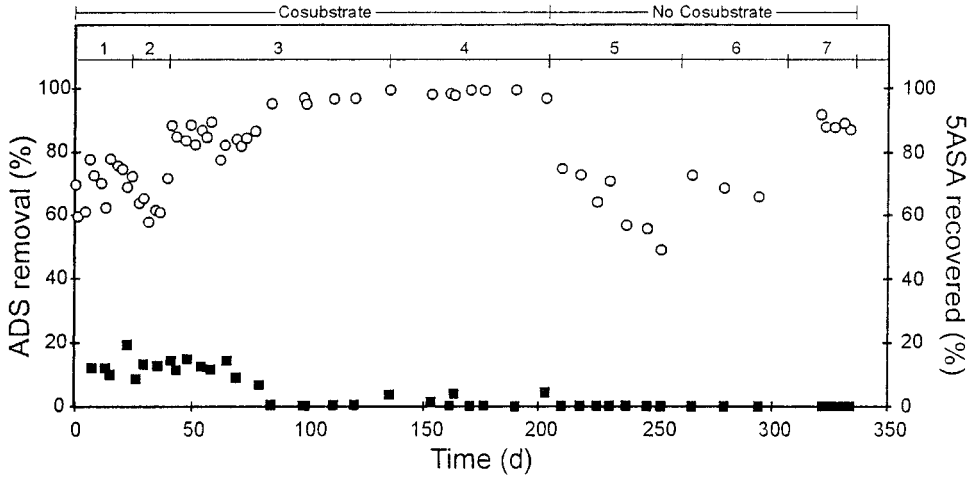


**FIGURE 2. Treatment efficiency of ADS degradation with glucose (periods 1−4) or no cosubstrate (periods 5−7) in UASB bioreactor (R2); % ADS removal (○), % 5ASA recovered (as a molar yield of ADS removed) (■). Numbers in the top of the figure indicate the experimental periods (see Table 2).**

electron donor. Consequently, it was of interest to examine the role of two cosubstrates; a VFA mixture and glucose. Tables 1 and 2 indicates that both VFA and glucose supported azo dye reduction. The glucose bioreactor was found to handle higher ADS loading rate with a higher reduction efficiency (up to 99%), indicating that glucose was a better cosubstrate for azo dye reduction.

Many reports indicate that electron-donating cosubstrates are required for azo reduction by bacteria (*6*, *24*, *25*). It has been postulated that the presence of cosubstrates enhances the reduction rate of the azo compounds by increasing the rate of formation of reduced enzyme cofactors which are able to fortuitously and nonspecifically reduce the azo dyes. Gingell and Walker (*26*) found that soluble flavins played an important role in azo dye reduction in *Streptococcus faecalis*. Reduced flavins, acting as two electron donors, rapidly reduced Red 2G nonenzymatically, and the reduced flavins can act as electron shuttles from NADPH-dependent flavoproteins to the acceptor azo compound. Methanogenic and acetogenic bacteria in the granular sludge contain unique reduced enzyme cofactors, such as $F_{430}$ and vitamin $B_{12}$, that could also potentially chemically reduce azo bonds, similar to what has been found for the reductive dechlorination of the chlorinated aliphatics (*27*). It is, therefore, not surprising that azo reduction rates are sensitive to the amount of available fermentation substrate in an anaerobic system, since catabolism of these substrates is ultimately responsible for the production of reduced enzyme cofactors.

It is also likely that cosubstrates could act as donors of reducing equivalents (e.g., via NAD(P)H) to specific azo reductases. Roxon et al. (*28*) reported that both NADH and NAD(P)H are active electron donors for the reduction of tartrazine in whole-cell suspensions of *Proteus vulgaris*. In the same way, Zimmermann et al. (*29*) have also shown that certain specific oxygen-insensitive azoreductases of *Pseudomonas* sp. have NAD(P)H dependency to catalyze the reductive cleavage of the azo group of carboxy-Orange I and carboxy-Orange II under aerobic conditions.

**5ASA Degradation.** In this study, the extent of azo dye reduction was rather high in both continuous bioreactors. However, the recovery of 5ASA was very low after prolonged reactor operation (end of period 3 and onwards in both R1 and R2 bioreactors). The 5ASA recovered in the effluent accounted for less than 1% (molar yield of ADS removed) of the expected value, indicating that the aromatic amine was being mineralized or being degraded to another product. On day 206 (period 5), the cosubstrate was no longer added to R2. If 5ASA was being mineralized, we hypothesized that the reducing equivalents needed for the azo bond reduction could be generated. The hypothesis was confirmed since more than 60% ADS removal was obtained without any cosubstrate. ADS was used as a sole substrate for more than 100 days. According

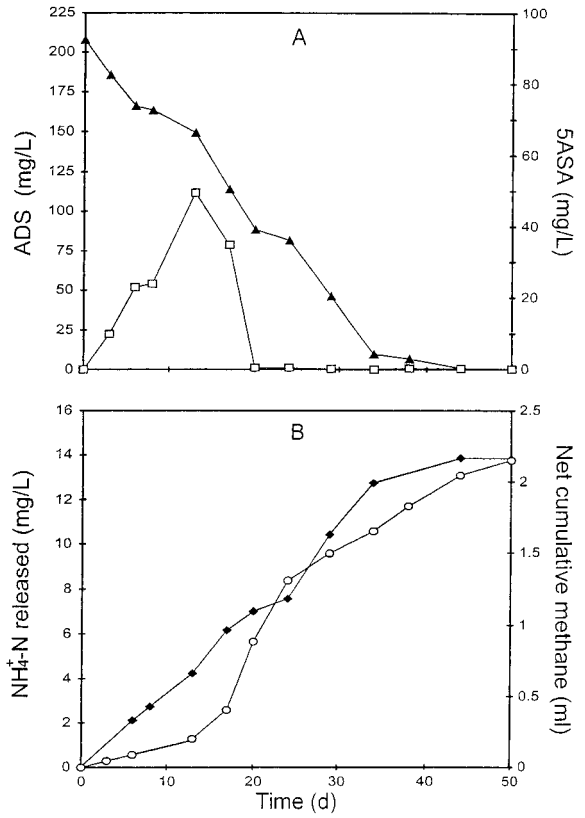


**FIGURE 3. Time course of ADS mineralization in anaerobic batch assays using ADS-adapted granular sludge withdrawn from bioreactor R2. (A) Reduction of ADS (▲) and transient accumulation of the intermediate 5ASA (□); (B) net cumulative methane production (◆), and net $NH_4^+$-N released (○) due to the ADS mineralization. The theoretical methane production expected for ADS mineralization was 2.76 mL.**

to these results, it is likely that the cosubstrate was only necessary to establish an active methanogenic consortium during the adaptation to the azo dye and that cosubstrate supplementation was no longer essential once 5ASA-degrading bacteria developed in the consortium.

**Anaerobic Biodegradation of ADS in Batch Experiments.** Results obtained in batch experiments confirmed that ADS was largely mineralized to methane and ammonium with a transient accumulation of 5ASA as a degradation intermediate. The concentration of ADS in the dye-amended autoclaved sludge remained constant, indicating that ADS was biologically degraded. This fact also excluded adsorption as a possible fate. The specific ADS degradation rate of the sludge under batch conditions (8.3 mg (g of VSS)$^{-1}$ d$^{-1}$) was high enough to account for 5ASA mineralization in the continuous bioreactors; the ADS removal rate at the end of period 7 in R2 with a VSS concentration of 21.83 g/L was 3.15 mg (g of VSS)$^{-1}$ d$^{-1}$).

The electrons required for the reductive cleavage of azo dyes by anaerobic microorganisms are known to be derived from cosubstrates. While no exogenous cosubstrates were added in the batch biodegradation assays described here, background levels of endogenous susbtrates in the sludge inoculum were most likely used to prime azo dye reduction. Thereafter, the metabolism of 5ASA released from the azo dye cleavage could provide electron donors supporting continued reduction of the dye. This hypothesis was confirmed by adding exogenous 5ASA to the sludge incubated with ADS, resulting in an enhancement of the azo dye reduction rate.

5ASA was incubated with the methanogenic consortia in the presence of the specific methanogenic inhibitor BESA.

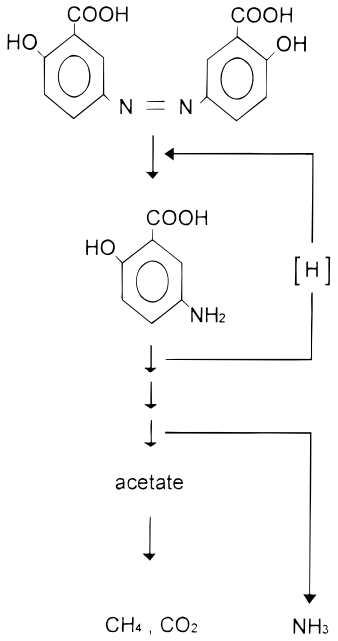


**FIGURE 4. Biodegradation pathway proposed for the complete mineralization of ADS under anaerobic conditions.**

Acetate was identified as the major intermediate formed, indicating that the degradation of 5ASA occurs via acetogenic fermentation. The results taken as a whole indicate a biodegradation pathway going from ADS, 5ASA and acetate to methane as shown in Figure 4.

This constitutes the first report indicating that an azo dye compound is completely biodegraded in the absence of oxygen. The aromatic amine cleavage products of azo dyes are generally regarded as recalcitrant compounds in anaerobic environments (*6*, *13*, *14*). The amino groups concentrate electrons into the ring, which greatly interferes with the nucleophilic strategies of aromatic compound degradation used by anaerobic microorganisms (*30*). Nonetheless, evidence is accumulating that some aromatic amines are mineralized by anaerobic microorganisms and consortia. Aromatic amines with carboxy, hydroxy, and methoxy substitutions are potentially mineralizable in methanogenic consortia. The 3 isomers of monoaminobenzoate (*16*, *31*−*33*), 2- and 4-aminophenol (*16*, *32*, *34*), and 5-aminosalicylate (*16*) were shown to be completely mineralized in anaerobic sludge. The elimination of 4,4′-diamino-3,3′-dimetoxybiphenyl was also reported in anaerobic sludge (*13*). Here we demonstrate that an azo dye constructed from anaerobically metabolizable aromatic amines is fully mineralized, supplying itself with electrons to support reductive azo bond cleavage.

Microbial communities in sediments could use alternative electron acceptors such as $NO_3^{2-}$, $SO_4^{2-}$, Mn(IV), and Fe(III) (*35*, *36*). Aniline, the simplest aromatic amine, was not degraded under methanogenic conditions but was under denitryfying and sulfate-reducing conditions (*37*, *38*), highlighting the enormous potential of microbial comunities in anaerobic environments to degrade aromatic amines. Therefore, we conclude that it should be possible for industry to design azo dyes that are fully biodegradable in anaerobic sediments of the environment. These dyes should be constructed from aromatic amines that are known to be reliably metabolized by anaerobic microbial consortia.

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