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Biodegradation of nitrobenzene by a sequential anaerobic-aerobic process

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Abstract

Nitrobenzene was completely degraded by mixed cultures using a sequential anaerobic-aerobic treatment process. Under anaerobic conditions in a fixed-bed column aniline was formed from nitrobenzene through gratuitous reduction by cells of sewage sludge. This reaction was accelerated by the addition of glucose. Complete mineralization of aniline was accomplished by subsequent aerobic treatment using activated sludge as inoculum. The maximum degradation rate of nitrobenzene (4.5 mM) in the two-stage system was 552 mg l^{-1} d^{-1}, referring to 154 mg of nitrobenzene per gram of glucose. In a second experimental phase glucose as cosubstrate and H-donor was replaced by synthetic waste containing ethanol, methanol, isopropanol and acetone. Again, nitrobenzene (1.9 mM) was completely degraded (maximum degradation rate of 237 mg l^{-1} d^{-1}, referring to 251 mg per gram of solvents). The major advantage of the described two-stage process is that the reduction of nitrobenzene by anaerobic pretreatment drastically reduces emission by stripping during aerobic treatment.

Abbreviations: HRT – hydraulic retention time, OD_{546} – optical density at 546 nm

Introduction

Many pesticides, dyes, plastics, explosives and pharmaceuticals are manufactured on the basis of nitroaromatic compounds. The majority of nitroaromatics are of exceptional ecological concern because of the amounts produced and/or their release into the environment. Due to their widespread use these chemicals are prominent contaminants of soil and groundwater (Hallas & Alexander 1983; Zoeteman et al. 1980; Haas & von Löw 1986; Lenenberger et al. 1988; Rippen et al. 1987).
In general oxidative biodegradation of nonpolar nitroaromatic compounds like nitrobenzene(s) or nitrotoluenes is rendered more difficult due to the electrophilic character of the nitrogroups. Although mineralization of 1,3-dinitrobenzene and 2,4-dinitrotoluene was described recently (Dickel & Knackmuss 1991; Spanggord et al. 1991) most nonpolar nitroaromatics persist in the environment under aerobic conditions.
Nitrobenzene is a compound of crucial importance within this group of nonpolar nitroaromatics. The world wide production of nitrobenzene alone has been estimated at some 0.2 million tons (Hallas & Alexander 1983). Analysis of 2600 waste water samples from different industrial categories resulted in a 2% frequency of occurrence of nitrobenzene as a contaminant (Leisinger & Brunner 1986). Nitrobenzene harbours a highly toxic potential when ingested, inhaled or is even absorbed through the skin leading to cyanosis, affection of the central nervous

system, liver damage, and anaemia (Wirth et al. 1971; Patil & Shinde 1989). Nitrobenzene as a component of industrial waste streams can strongly inhibit bacterial growth in the activated sludge of biological treatment systems (Gomolka & Gomolka 1979). Moreover, due to its high vapor pressure at least the major part of the nitrobenzene is stripped from the activated sludge tank by aeration. This volatilization results in air pollution and strong odor nuisance. In several studies disappearance of nitrobenzene in wastewater treatment plants was observed but not very much is known about the fate of the xenobiotic (Patil & Shinde 1988 and 1989; Gomolka & Gomolka 1979; Pitter 1976; Haigler & Spain 1991; Nishino & Spain 1993). Reduction of nitrobenzene to aniline by sewage effluent under anaerobic conditions has been described but no degradation was observed during aerobic treatment of nitrobenzene (Hallas and Alexander 1983).
Under reductive conditions nitroaromatics are gratuitously converted to the corresponding aromatic amines. Due to the electron donating character of the NH_2-substituents these reduction products in contrast to the parent compounds are readily oxidized and attacked by dioxygenases (Beunik & Rehm 1990). Based on these observations the present paper describes a sequential anaerobic-aerobic process for complete mineralization of nitrobenzene.

Materials and methods

Determination of nitrobenzene and aniline

Quantitative determinations of nitrobenzene and aniline were made by reversed-phase high-pressure liquid chromatography as described previously (Bruhn et al. 1987). The solvent system contained 1 ml of 85% H_3PO_4 and 450 ml of methanol per liter of H_2O when nitrobenzene and aniline were quantified simultaneously. Where only aniline was measured the methanol content was reduced to 250 ml l^{-1}.

Culture conditions and harvest of cells

For experiments with whole cells or the preparation of cell-free extracts, cells from the aerated tank of the two-stage system were grown in mineral salts medium as described by Bruhn et al. (1987). Unless otherwise indicated the culture was supplemented with 20 mM glucose as source of carbon and energy and 1 mM nitrobenzene as the sole source of nitrogen.
For experiments with resting cells the organisms were cultivated and harvested as described previously (Dickel & Knackmuss 1991). The pellet was then resuspended in 50 mM phosphate buffer (pH 7.4) to an optical density (546 nm) of 2–3. Cell suspensions were supplemented with 0.8 to 2.0 mM aniline and incubated at 30° C on a water bath shaker (New Brunswick, Edison, NJ, USA).

Preparation of cell-free extracts

For the preparation of cell-free extracts, cells of the aerobic part of the system were cultivated as described above. The organisms were harvested during late exponential growth phase and suspended in 50 mM phosphate buffer (pH 7.4). Cell suspensions were disrupted using a French-press (Aminco, Silver Spring, MD, USA) and cell debris was removed by centrifugation at 100,000 g for 1 h at 4° C. Cell-free extracts were kept on ice until used.

Determination of protein

Protein in the cell-free extracts was quantified by the Bradford procedure (1976). The protein concentration of whole cells was determined according to Schmidt et al. (1963).

Determination of immobilized biomass

In order to quantify biomass on the supporting material in the fixed-bed column, decomposition of the

immobilized cells was carried out and the released protein was determined. For this purpose about 500 mg of the sintered glass beads were taken from the accessible upper part of the anaerobic column, washed with 10 ml NaCl (0.95%) and dried for ≥ 1 h at 60° C. In a reaction tube 200 mg of the dried beads were mixed with 910 µl NaOH (0.2 M) and incubated for 5 min at 95° C. The reaction was stopped on ice. After 1 min centrifugation the protein in the supernatant was quantified by the method of Sedmak & Grossberg (1977).

Enzyme assays

Quantification of glucose
Determination of glucose in the media was carried out by means of an enzymatical test using hexokinase and glucose-6-phosphate-dehydrogenase (Bergmeyer et al. 1974).

Quantification of ammonia
Standard test methods for the determination of ammonia are usually disturbed by aromatic amines. Consequently, ammonia in the media was quantified enzymatically by the method of Schmidt & Schmidt (1983) using glutamate dehydrogenase.

Quantification of ethanol
Ethanol was measured enzymatically by the method of Beutler (1984) using alcohol dehydrogenase and aldehyde dehydrogenase.

Determination of ring-cleavage activities
Catechol-2,3-dioxygenase (C230, EC 1.13.11.2) was measured by the procedure of Nozaki (1970). Catechol-1,2-dioxygenase (C120, EC 1.13.11.1) was assayed by the method of Dorn & Knackmuss (1978a, b). Extinction coefficients were those reported by Dorn & Knackmuss (1978b). If C230 was simultaneously induced, this enzyme was inactivated by treating the extract with 0.01% H_2O_2 for 10 min before adding the test substrate (Nakazawa & Yokota 1973). Specific activities are expressed as micromole of substrate converted or product formed per minute per gram protein at 25° C. Protein was determined as described above.

Measurement of oxygen-uptake

Oxygen-uptake rates were measured polarographically with a Clark-type oxygen electrode. Freshly harvested cells were washed and resuspended in 50 mM phosphate buffer (pH 7.4) to an optical density (546 nm) of about 25. Of this suspension 200 µl were added to 2.7 ml phosphate buffer (50 mM, pH 7.4) saturated with air. After 5 min of constant endogenous oxygen-uptake, the reaction was started by adding the assay substrate to a final concentration of 0.5 mM. Uptake rates were determined at 25° C and corrected for endogenous consumption in the cell suspension. Activities are expressed as millimole of O_2-uptake per minute per gram protein.

Chemicals

All chemicals were of the highest purity commercially available. Nitrobenzene was obtained from Aldrich (Steinheim, Germany) and aniline was purchased from Fluka (Neu-Ulm, Germany). All enzymes used were from Boehringer (Mannheim, Germany).

Experimental set-up

Design of the sequential anaerobic-aerobic system

The sequential anaerobic-aerobic system mainly consisted of a fixed-bed column (anaerobic part) and an agitated tank (aerobic part) as shown in Fig. 1. From a storage bottle the substrate was pumped into the up-flow fixed-bed column. For pressure compensation the storage bottle was provided with an argon-filled balloon. The anaerobic part consisted of a glass column (length 50 cm, diameter 6 cm) with a glass filter inserted at the bottom. In order to allow immobilization of the anaerobic biomass the column was filled with sintered glass beads as bacterial support. The surface of the spherical material (type Sikug 035/10/300 A, Schott, Mainz, Germany) was not modified. The porosity of the beads was 60% and the active surface 74 $m^2 l^{-1}$. The interstitial

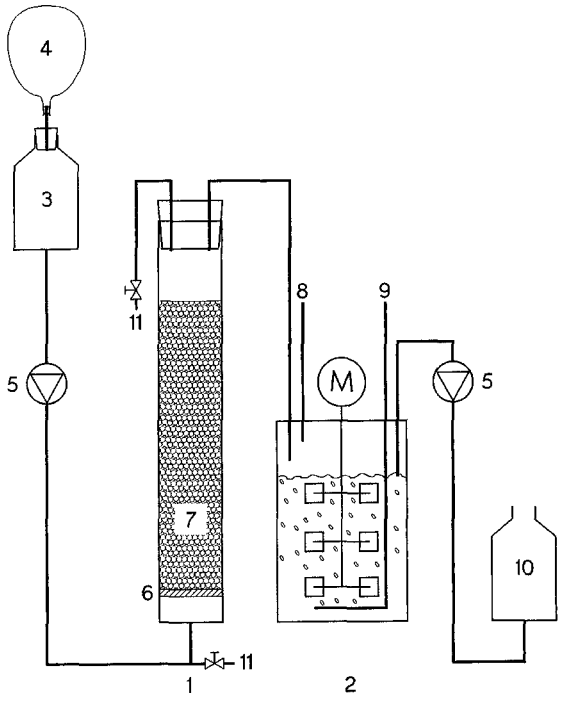

Fig. 1. Schematic diagram of the anaerobic-aerobic treatment system. (1) fixed-bed column, (2) agitated tank, (3) feed, (4) argon, (5) peristaltic pump, (6) glass filter, (7) carrier bed, (8) outlet air and sampling point, (9) air supply, (10) waste, (11) sampling point.

fluid volume was 1 l. The efflux at the top of the anaerobic column was transferred into an aerated laboratory fermenter vessel (Biostat M, Braun Diesel Biotech, Melsungen, Germany) and agitated by a stirrer (type IKA/RW 20 DZM, Janke and Kunkel, Staufen, Germany). The culture volume again was 1 l and the rate of air supply 1 l min^{-1}. By means of a second peristaltic pump the excess of the aerobic culture was conveyed into a waste tank. The flow rate of the whole system was 21 or 42 ml h^{-1} resulting in hydraulic retention times (HRT) of 4 and 2 days, respectively. In order to avoid loss of nitrobenzene teflon material was used where tubings were necessary.

Media and inoculum

The inocula for the anaerobic and aerobic part of the two-stage system were from the sewage treatment plant of the University of Stuttgart, Germany. Anaerobic sewage digester sludge and activated sludge of the treatment plant were used directly as inocula without enrichment of a specific bacterial population. For the anaerobic column 500 g of the sintered glass beads were seeded with 20 g of sewage sludge and preincubated for 48 h at 30° C with 1 l nutrient broth under an argon atmosphere. The supporting material was then transferred into the column and the entire two-stage system was filled with mineral salts medium supplemented with 20 mM glucose, 1 mM $(NH_4)_2SO_4$ and 2 mM NH_4Cl. The aerobic tank was inoculated with 50 ml of activated sludge. In order to equilibrate the sequential bioreactor mineral medium containing glucose (20 mM) was pumped from the storage bottle through the system (flow rate 21 ml h^{-1}, HRT = 4 days). After 1 week nitrobenzene was added to the feed (0.1 mM). During an adaptation period of 4 weeks the nitrobenzene concentration was increased to 0.5 mM by a linear gradient. Then, after 2 more weeks the flow rate was enhanced to 42 ml h^{-1} (HRT = 2 days) and the concentration of nitrobenzene was raised by monthly intervals to 0.8, 1.0, 1.6, 2.8, 5.0, and finally 5.5 mM. The whole system was run at ambient temperature.

In order to simulate conventional industrial sewage a synthetic waste water was used in a second experimental stage. Here, glucose as cosubstrate and H-donor was replaced by ethanol, isopropanol, acetone (3 mM each) and methanol (12 mM) as described by Schmidt et al. (1983). Additionally, 25 mg l^{-1} of yeast extract was added. In analogy to the first experimental stage the concentration of nitrobenzene was increased from 0.5 mM to 2.5 mM within 7 weeks.

Results

Anaerobic-aerobic process using glucose as a cosubstrate

In order to supply an appropriate carbon source and H-donor for the anaerobic reduction step 20 mM glucose was used as a cosubstrate. Over a period of 8 months the concentration of nitrobenzene in the feed was increased by monthly intervals

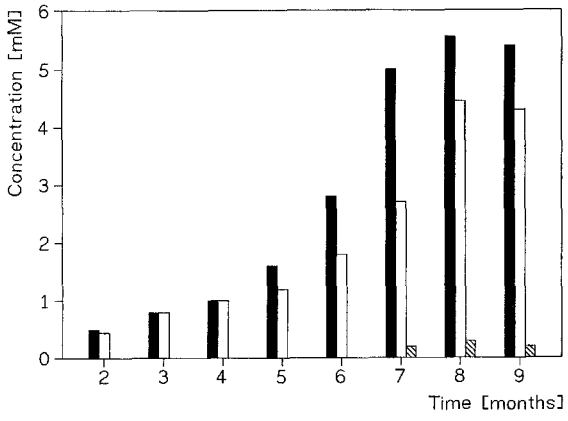

Fig. 2. Gratuitous reduction of nitrobenzene by sewage sludge using 20 mM glucose as H-donor. Nitrobenzene (■) in the influx, nitrobenzene (▨) and aniline (□) in the efflux of the anaerobic fixed-bed column.

from 0.5 mM to 5.5 mM as shown in Fig. 2. The concentrations of nitrobenzene and aniline in the in- and efflux of the anaerobic fixed-bed column were measured weekly by HPLC, those of ammonia and glucose by means of enzymatic tests.

The results show complete conversion of nitrobenzene into aniline by the anaerobic biomass during the first 4 months when the concentration of nitrobenzene was increased. A control experiment without inoculum under the same conditions, however, showed no chemical reduction of nitrobenzene by sodium sulfide added to the system. As soon as the inlet concentration of nitrobenzene was raised more drastically a distinct delay in reduction of the added nitrobenzene was observed. At ≥ 5.0 mM a break-through of nitrobenzene was measured in the efflux of the fixed-bed column, which did not disappear even after prolonged continuous cultivation (3 months). At a concentration of 5.5 mM nitrobenzene in the influx 4.5 mM (78%) was recovered as aniline in the efflux, corresponding to a maximum reduction rate of nitrobenzene of 552 mg $l^{-1} d^{-1}$ (154 mg nitrobenzene reduced per gram of glucose). The break-through concentration of nonreduced nitrobenzene was 0.3 mM (7%). No other aromatic metabolites were detected by HPLC.

During the entire experiment the concentration of ammonia in the effluent of the anaerobic column was insignificantly lower compared to the inlet concentration ($\triangle c_{ammonia}$ = 0.1 to 0.2 mM) whereas glucose was completely consumed. Ammonia was not detectable in the agitated aerobic tank. An increase of the glucose concentration in the feed to 40 mM after 9 months (5 mM nitrobenzene) lead to a pH-shift from 7.2 to 4.9 in the anaerobic column and the nitrobenzene concentration in the efflux increased to 1 mM (data not shown). According to the odour of the efflux of the anaerobic column hydrogen sulfide had disappeared and butyric and valeric acid were formed, indicating acidogenic rather than sulfidogenic conditions.

Obviously, the bulk of the immobilized biomass was at the bottom of the column. Even on the accessible supporting material in the upper part of the anaerobic column an increase of biomass could be observed after 7 months (≤ 0.2 mg protein per gram of glass beads). At the same time the optical density (OD_{546}) of the efflux of the column was 0.4 corresponding to 0.19 mg protein per ml.

After 7 months the equilibrium concentration of the aerobic biomass in the stirred tank corresponded to 0.50 mg protein per ml (OD_{546} = 2.1). The number of cells which could form colonies on mineral agar plates containing aniline as sole nitrogen source was 1.5×10^7 ml^{-1} (corresponding to 60% of the viable cell count). On agar plates containing analine as sole source of carbon and nitrogen 0.2×10^7 cells per ml were counted (8% of the viable cell count). For turnover experiments 10 ml of the culture from the aerated tank were grown in mineral medium containing 0.5 mM aniline/20 mM glucose or nutrient broth. As shown in Fig. 3 aniline was

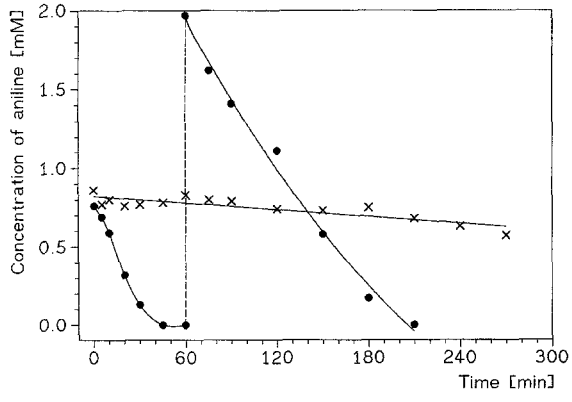

Fig. 3. Turnover of aniline by restling cells of the aerated tank after growth with 2 mM aniline/20 mM glucose (●—●), or nutrient broth (×—×).

Table 1. Oxygen-uptake rates with whole cells of the aerated tank using aniline as substrate. Concentrations of glucose and ammonia in the growth media were 20 mM and 5 mM, respectively.

Growth medium	Test substrate[3)]	Activity [mmol min^{-1} g^{-1}]
glucose/NH_4^+	glucose	71
aniline[2)]/NH_4^+	glucose	0
glucose/NH_4^+	aniline	0
aniline[1)]/glucose	aniline	57
aniline[2)]/NH_4^+	aniline	90

[1)] Aniline (0.5 mM) as sole nitrogen source.
[2)] Aniline (1.0 mM) as source of carbon and energy.
[3)] Concentrations of glucose and aniline as test substrates were 5 mM and 0.5 mM, respectively.

completely and instantaneously metabolized by induced cells (OD_{546} = 1.8). In contrast, cells grown in nutrient broth did not convert aniline at a significant rate. No activity for the degradation of aniline was measured using an oxygen electrode when cells were grown with glucose and ammonia (Table 1). In contrast, the oxygen-uptake rates were high when the cells were cultivated with aniline as sole nitrogen source or as sole source of carbon and energy.

In order to determine ring-cleavage activities experiments with cell extracts were carried out. Only low uninduced levels of catechol-2,3-dioxygenase were found after growth with glucose and ammonia (9 U per gram of protein). In contrast, high meta-cleavage activity was measured when the cells were induced after growth in aniline/glucose medium (1290 U per gram of protein). Catechol-1,2-dioxygenase activity was not found.

Solvents as cosubstrates

To investigate degradation of nitrobenzene under conditions similar to those in industrial sewage, in a second experimental phase glucose was replaced by a synthetic waste water that contained representative components such as methanol, ethanol, isopropanol, and acetone (Schmidt et al. 1983). Again, after the concentration of nitrobenzene was increased stepwise from 0.8 to 2.0 mM about 3 weeks were necessary to attain an apparent steady-state (Fig. 4). A maximum concentration of 1.9 mM nitrobenzene was completely converted into aniline by the anaerobic fixed-bed column, corresponding to 237 mg l^{-1} d^{-1} (251 mg nitrobenzene per gram of solvents). Ethanol was not detectable in the efflux of the fixed-bed column. Further increase of the nitrobenzene concentration did not lead to a higher conversion rate.

Discussion

The present investigation shows that nitrobenzene as a critical component of industrial sewage can be mineralized by a sequential anaerobic-aerobic process. In experiments with either glucose or a mixture of solvents as cosubstrates and H-donors nitrobenzene was converted into aniline under anaerobic conditions. This gratuitous reaction was followed by complete productive break-down of aniline in the aerobic part of the two-stage system. This could be demonstrated by growth of the aerobic biomass with 1 mM aniline as the sole source of carbon, energy and nitrogen in batch culture as well as on agar plates. Growth on agar plates showed that 60% of the viable cell count from the aerated tank could utilize aniline as sole source of nitrogen, whereas 8% of the organisms were able to use aniline as sole source of nitrogen and carbon. The colonies on the agar plates as well as the microscopic

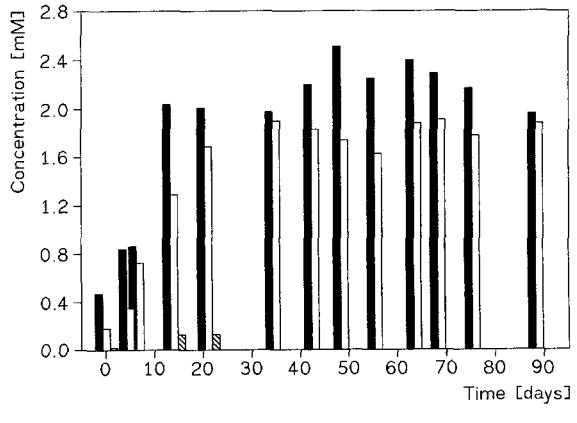

Fig. 4. Gratuitous reduction of nitrobenzene by sewage sludge using solvents as H-donor. Acetone, ethanol, isopropanol (3 mM each) and methanol (15 mM) were components of a synthetic waste water. Nitrobenzene (■) in the influx, nitrobenzene (▨) and aniline (□) in the efflux of the anaerobic fixed-bed column.

examinations of the anaerobic and aerobic biomass showed a heterogenous consortium of microorganisms. Even after several months no dominating organism was observed. Glucose was not detectable in the influx of the aerobic tank. Nevertheless, the optical density maintained at a constant level indicating that anaerobic fermentation products served as source of carbon and energy for the cells of the aerobic consortium.

Complete mineralization of aniline by organisms harbouring a productive meta-pathway is indicated by strong meta-cleavage activity in the cell extracts of aniline induced cells. Furthermore, this experiment as well as those for determination of O_2-uptake rates and turnover of aniline by resting cells showed that the enzyme(s) for aniline degradation were inducible. During the entire experimental phase aniline was not detectable in the aerobic tank. This indicates that the reduction of nitrobenzene to aniline is the rate limiting step of the entire process. Temporarily break-through of nitrobenzene was observed in the anaerobic column. This material was stripped from the aerated tank due to the high vapor pressure of the compound. Such a volatilization was not observed in the case of aniline (data not shown). In the experiment with solvents as cosubstrates and H-donors a maximum of 96% of nitrobenzene was recovered as aniline (Fig. 4). Noteworthy, no break-through of nitrobenzene in the anaerobic column was observed when the concentration in the feed was further increased from 2.0 to 2.5 mM. Probably, part of the nitrobenzene was only reduced to nitroso- and hydroxylaminobenzene due to a lack of reduction equivalents. These partially reduced compounds as well as potential intermolecular reaction products (e.g. azoxy- or azo-compounds) could have been escaped from being detected by the analytical methods used in this study. The same effect could also explain the mass balance being $\leq 85\%$ if the influx concentration of nitrobenzene exceeded 1.0 mM during the experiment with glucose as cosubstrate (Fig. 2).

To what extent this process can be applied to the purification of industrial effluents containing mixtures of nitroaromatics or higher substituted compounds (e.g. TNT) has yet to be shown. Clearly, anaerobic pretreatment of nitrobenzene may have several advantages for biological break-down under practical conditions. Firstly, reduction to aniline drastically reduces the vapor pressure so that stripping by aeration is avoided. Secondly, both the anaerobic and aerobic reaction steps are catalyzed by autochtoneous microorganisms of sewage sludge and do not require special inocula or procedures of adaptation. Generally, anaerobic pretreatment is applicable also to other xenobiotics with high electrophilic character such as azo dyes which like nitroaromatics can be reduced and thus become acceptable for aerobic mineralization (Haug et al. 1991).

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