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Metabolism of the Plasticizer di-*n*-Butylphthalate by *Pseudomonas pseudoalcaligenes* Under Anaerobic Conditions, with Nitrate as the Only Electron Acceptor

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The metabolism of di-*n*-butylphthalate by a denitrifying strain of *Pseudomonas pseudoalcaligenes* B20b1 was studied under anaerobic conditions, with nitrate as the only electron acceptor. Thin-layer chromatography and mass spectral analysis of culture extracts (20 days at 30°C) showed mono-*n*-butylphthalate and phthalic acid as the only products, suggesting that one butanol moiety served essentially as the carbon source for growth and denitrification. N_2 and N_2O were detected by gas chromatography. In contrast to aerobic metabolism, phthalate was not degraded further if nitrate was the only electron acceptor.

Non-halogenated, aromatic phthalate esters have been widely used as plasticizers, pesticides, and cosmetics (16). The relative persistence of these compounds in the natural environment has been well documented by their general distribution in waters (10, 12, 14), sediments (2, 12, 17), soils (9, 15), and plants (11, 16). Because di-*n*-butylphthalate (DBP; molecular weight = 278,000) has been metabolized aerobically (3, 4) and has even been used as a sole carbon source (4), its relative accumulation in natural environments may be ascribed to a number of ecological conditions that affect the availability of oxygen and thus the rate of mineralization. The delayed turnover of an aromatic compound such as DBP is remarkable, since other simple aromatic moieties (benzoate, *p*-hydroxybenzoate, phenol) have been shown to be degradable under anaerobic conditions in the presence of nitrate (5). If specific denitrifying bacteria are indeed capable of cleaving the aromatic nucleus with nitrate as the sole electron acceptor, then DBP should likewise be mineralizable under such conditions. This hypothesis has been examined in the present study.

MATERIALS AND METHODS

Denitrifying organism. *Pseudomonas pseudoalcaligenes* B20b1 was enriched from the effluent of a biological sewage plant (Stuttgart-Möhringen), with DPB (0.2%) as the only carbon source and nitrate (KNO_3, 0.6%) as an electron acceptor (under a 9:1 N_2-CO_2 atmosphere in an anaerobic jar). The bacteria were isolated by repeated subculture on a synthetic mineral salt base-KNO_3-DBP agar (pH 7.4) under anaerobic conditions, and strain B20b1 was identified as *P. pseudoalcaligenes* (G. Benckiser, Ph.D. thesis, University Stuttgart-Hohenheim, Germany, 1980). During enrichment and isolation, the conditions were not completely O_2 free.

Cultural conditions. Liquid cultures were grown (in triplicate) in 200-ml Erlenmeyer flasks containing 50 ml of a synthetic mineral base (6) supplemented with nitrate (700 ppm [μg/ml] NO_3^--N) and DBP (0.2% [wt/vol]) emulsified in 0.1% (vol/vol) Tween 80 (polyethylenesorbitanemonooleate) as suggested by Engelhardt et al. (4). In control experiments Tween 80 was used as the only carbon source. Each flask was inoculated with 3 ml of 2- to 3-day-old (30°C) culture grown in the same synthetic mineral base fortified with (grams per liter) nitrate (KNO_3), 6; yeast extract (E. Merck A6), 1; sodium benzoate, 0.5; and sodium glycerophosphate, 0.5 (pH 7.4). Stock cultures were maintained on 0.4% DBP-mineral base agar (5°C). Each flask was incubated (28 days at 30°C) in an anaerobic jar (Gössner Co., Hamburg, Germany), which was modified by an additional glass tube bridge on its lid. The glass bridge was equipped with a rubber sampling cap, two valves, and a rubber ball (for mixing the gas before sampling with a syringe). Each jar was evacuated, flushed five times, and filled with helium gas (99.9%, Messer Griessheim) to establish O_2-free conditions.

Gas chromatography. The O_2-free atmosphere and the production of CO_2, N_2, and N_2O were determined by using a gas chromatograph (model F22, Perkin-Elmer, Bodenseewerke, Überlingen). The F22 was equipped with a TC detector and stainless-steel columns packed with Porapak-Q and -R (for N_2O and CO_2) as well as with a molecular sieve (Ma 5a, to separate O_2 and N_2) as described earlier (1, 8). Results were recorded and quantified by an integrator (gas chromatograph system SIP-1, Perkin-Elmer).

Isolation and identification of metabolites. Degradation of DBP was analyzed by extraction of the residual compound and its metabolites from the culture solu-

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TABLE 1. Metabolites and gaseous losses from DBP or Tween 80 (or both) mineralization by *P. pseudoalcaligenes* B20b1 under anaerobic conditions (helium atmosphere), with nitrate as the only electron acceptor (20 days at 30°C)

Substrate	Recovery of: (mg/liter)			Production of: (mg/liter)				
	DBP	Tween 80[a]	NO_3^--N	MBP	PA	CO_2	N_2	N_2O-N
DBP + Tween 80	18,884 ± 60 (89.7)[b]	nil (0)[b]	246.6 ± 55 (35.2)[b]	67 ± 29 (3.2)[b]	Traces	551	117	194
Tween 80		nil (0)[b]	348.6 ± 30.6 (49.8)[b]			481	89	139

[a] Calculated as oleic acid (68.7% of Tween 80 was originally oleic acid).
[b] The percentage of the original amount is in parentheses.

tion with chloroform (25 ml, twice) and (15 ml) petroleum ether (40 to 60°C) after acidification of the medium with 4 N HCl (pH 1) as reported previously (3, 4). The joint extracts were dried (anhydrous Na_2SO_4) and used for quantitative thin-layer chromatography on silica gel plates (0.5 mm, 60-F254, E. Merck AG), using a 9:1 ratio of benzene/acetic acid as developing solvent A. Reference substances and metabolites were detected under UV light, removed from the plates, eluted in methanol, and determined quantitatively by UV analyses (Zeiss Spectrophotometer PM-2DLPMQ) by comparison with standard solutions at 275 nm. In addition to solvent A, the joint extracts were developed in petroleum ether (40 to 60°C)-diethyl ether-acetic acid (77:20:3; solvent B) as well as in benzene-methanol (7:3; solvent C) (4). Metabolites (and references), developed in solvent C and eluted in methanol, were recorded at 76 eV with a Varian MAT-311 mass spectrometer.

Estimation of Tween 80 degradation. One-milliliter chloroform-petroleum ether extract samples were saponified, transformed in oleic acid methylester, and analyzed quantitatively by gas chromatography (Fractomer F6/4, Perkin-Elmer) (13; G. Benckiser, Ph.D. thesis). A flame ionization detector and a stainless-steel column (2 m by 2 mm) packed with 2.5% ethylene glycol succinate polyester on Chromosorb G (with H_2 gas as carrier; 30 ml/min; 170°C) was used.

Nitrate. Nitrate was determined colorimetrically with brucine at 540 nm (Zeiss Spectrophotometer, PM 2DL) as outlined earlier (1, 7).

Chemicals. All chemicals used were obtained from E. Merck AG (analytical grade). Monobutylphthalate (Eastman Kodak Co.) was purified by preparative thin-layer chromatography in solvent A and crystallized from chloroform. Tween 80 was purchased from Merck-Suchardt.

RESULTS AND DISCUSSION

Denitrification with DBP. *P. pseudoalcaligenes* B20b1 developed poorly with DBP as the sole carbon source, but grew well in the presence of both DBP and Tween 80. The production of CO_2, N_2O, and N_2 after 20 days (30°C) was more intensive from DBP with Tween 80 than from Tween 80 alone (Table 1). Two metabolites (A and B) were identified and quantified by UV spectrophotometry. Apparently, mono-*n*-butylphthalate (MBP) and phthalic acid (PA) are the only metabolites produced in the culture of *P. pseudoalcaligenes* B20b1. PA was found only in traces, suggesting that essentially one butanol moiety of DBP served as a carbon source for this organism.

TABLE 2. Characterization and identification of DBP-degradation products by thin-layer chromatography and mass spectra after 28 days of incubation (anaerobic, with nitrate as the only electron acceptor and *P. pseudoalcaligenes* B20b1 as the active agent)

Compound	Mass spectrum[a] molecular iron (relative intensity)	Thin-layer chromatographic R_f values in solvents:[b]		
		A	B	C
Metabolite				
A	222 (3%)	0.34	0.55	0.75
B	166 (33%)	0.08	0.13	0.64
Reference				
DBP	278.1 (0.6%)	0.76	0.82	0.90
MBP	222 (3%)	0.37	0.58	0.78
PA	166 (32%)	0.08	0.13	0.67
p-OH-benzoate	ND[c]	0.20	0.26	ND[c]
Protocatechuic acid	154 (95%)	0.16	0.10	0.73

[a] Metabolite A showed characteristic peak at *m/e* 205, 167, 149, 122, and 105; metabolite B showed characteristic peaks at *m/e* 1, 49, 122, and 105. The relative intensity is related to *m/e* 149 or 105.
[b] Solvent B, petroleum ether (40 to 60°C)-diethyl ether-acetic acid (77:20:3); solvent A, benzene-acetic acid (9:1); solvent C, benzene-methanol (7:3).
[c] ND, Not determined.

Confirmation of metabolites by mass spectra. In Table 2 a survey is given of the physical properties recorded with metabolites A and B by thin-layer chromatography and mass spectrometry. The data are given in comparison with several reference substances. Table 2 confirms that MPB and PA are indeed the only intermediates liberated into the medium. Under anaerobic conditions in the presence of nitrate, DBP is hydrolyzed via MBP to PA. Protocatechuate, a key intermediate during aerobic degradation of DBP via MBP and PA by various *Nocardia* spp. and *Arthrobacter* spp. (5), could not be detected.

These data suggest that MBP and PA are the ultimate products of DBP hydrolysis, with nitrate as the only electron acceptor. Obviously, *P. pseudoalcaligenes* B20b1 fails to attack PA in the absence of oxygen. It should be noted here that both PA and protocatechuate are readily metabolized by *P. pseudoalcaligenes* B20b1 under aerobic conditions (G. Benckiser, Ph.D. thesis). The failure of a denitrifying bacterium to rupture simple aromatic moieties (by hydroxylation or even through reduction), with nitrate as the only electron acceptor, has been documented (7, 8). *U*-^{14}C-labeled benzoate (or *p*-hydroxybenzoate) could be utilized neither by a denitrifying pure culture of *Acinetobacter* spp. (or *Moraxella* spp.) nor by a consortium of various bacteria, if nitrate was the only electron acceptor. The complete absence of molecular oxygen in such experiments is required because even small amounts of O_2 remaining in the vessel or introduced by porous silicone tubes were sufficient to simulate anaerobic ring fission by the apparent use of nitrate as an electron acceptor (7, 8). Traces of O_2 allow a denitrifying organism to hydroxylate and rupture the benzene nucleus and to utilize the aliphatic metabolites by nitrate respiration. This apparent denitrification may have led to the misleading conclusions that simple aromatic compounds are degradable with nitrate as an electron acceptor.

In conclusion, the relative persistence of DBP in soils and waters should be ascribed to its low solubility and, therefore, high inaccessibility to microbial attack as well as to its restriction as a carbon and energy source under anaerobic conditions, even in the presence of nitrate.

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