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# Metabolism of Di- and Mono-*n*-Butyl Phthalate by Soil Bacteria

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Di-*n*-butyl phthalate and other dialkyl phthalates are used as carbon sources by three *Nocardia* sp. isolates; mono-*n*-butyl phthalate is used as a carbon source by an *Arthrobacter* sp. isolate and a *Pseudomonas* sp. isolate. The compounds were metabolized in these organisms by hydrolysis to the corresponding monoesters and free phthalic acid. Phthalic acid was then metabolized via protocatechuic acid by 3,4-dioxygenative ring cleavage.

Biodegradation of phthalate esters by activated sludge and river water has been shown to proceed with the evolution of more than 85% of the theoretical amount of $CO_2$ (9). Recently, we have selected a number of microorganisms from soil enrichment isolations and stock cultures, which use dialkyl phthalates or free phthalic acid as a source of carbon (3). Six different bacterial isolates were found to convert the diesters to the corresponding monoesters, which were not further attacked. One fungus, *Penicillium lilacinum*, was obtained which formed various metabolites when grown on dialkyl phthalates (2). In addition, three *Nocardia* isolates metabolized different dialkyl phthalates as well as the monoesters and free phthalic acid. Phthalic acid was oxidized by all bacteria via the 3-oxoadipate pathway (4, 8).

Phthalate esters were also used by strains of *Micrococcus* (P. Keyser, B. G. Pujar, R. W. Eaton, and D. W. Ribbons, Environ. Health Perspect., in press). When grown on dimethyl phthalate, these bacteria, however, cleaved the protocatechuate, arising from phthalate oxidation by 4,5-dioxygenative ring cleavage. Phthalate esterase activity is a constitutive property of this microorganism.

The present study provides a more detailed description of the metabolism of di- and mono-*n*-butyl phthalate (DBP, MBP) by three *Nocardia* isolates. In addition, the hydrolysis of MBP by whole cells and extracts of an *Arthrobacter* and a *Pseudomonas* isolate was examined.

## MATERIALS AND METHODS

**Chemicals.** Dialkyl phthalates were obtained from Chemical Service, Media, Pa., and were of chemical grade. Monoalkyl phthalates were synthesized by heating equimolar amounts of phthalic anhydride with the respective alcohol. The monoalkyl phthalates formed were purified by preparative thin-layer chromatography (TLC) in solvent system A (see below) and crystallized from chloroform. All other compounds are commercially available and were of analytical grade.

**Organisms and culture conditions.** The experiments were conducted with *Nocardia* sp. DSM 43250, *Nocardia* sp. DSM 43251, *Nocardia* sp. DSM 43252, *Arthrobacter* sp. DSM 20389, and *Pseudomonas* sp. DSM 5030. These bacteria had been isolated from soil by enrichment with phthalic acid as source of carbon (3, 4). They have been identified on the basis of morphological and physiological criteria. In addition, the composition of the cell wall of the gram-positive bacteria as well as the lipid composition of the *Nocardia* isolates had been investigated (4).

Liquid cultures were grown in 100-ml Erlenmeyer flasks containing 30 ml of Hegeman's mineral base (6) with addition of 0.05% yeast extract and 0.1 to 0.2% phthalate ester or 0.4% phthalic acid as the main carbon source on a rotary shaker (New Brunswick G 10) at 30°C and 140 rpm. The mixture was adjusted to pH 7.2. After the addition of 0.05% Tween 80, the dialkyl phthalate-containing media were transformed to a stable emulsion by vigorous shaking. The inocula were from 24- to 48-h cultures in 0.4% glucose-mineral base. Unadapted cells were also grown in 0.4% glucose-mineral base. Stock cultures were maintained on 0.4% phthalic acid-mineral base agar slopes.

**Manometry.** Manometric experiments were performed in the conventional Warburg constant-volume apparatus at 30°C. Organisms were harvested in the late-logarithmic growth phase by centrifugation, washed in 0.05 M phosphate buffer (pH 7.5), and resuspended in 0.1 M phosphate buffer (pH 7.5) to give a final optical density of 0.8. Warburg flasks with one side arm contained: 2 ml of cell suspension in the main compartment of the flask, 0.5 ml of a 0.5% substrate solution in 0.1 M phosphate buffer, pH 7.2 (with addition of 0.05% Tween 80 in the phthalate diester samples) in the side arm, and 0.3 ml of 10% aqueous NaOH in the center. In the control vessels the substrate solutions were replaced by phosphate buffer with addition of 0.05% Tween 80. Endogenous respiration was always measured over a period of 30 min before tipping the substrates into the main com-

partments. All data are corrected for values of endogenous respiration.

**Estimations.** Degradation of phthalate esters was assayed by extraction of residual compounds and metabolites from the media followed by quantitative ultraviolet light (UV) analysis with a Beckman spectrophotometer, model DB. The whole cultures or incubation mixtures were acidified with 4 N HCl to pH 1 and extracted three times with the same volume of ethyl acetate. The extracts were dried over anhydrous $Na_2SO_4$, and 2- to 10-ml portions were chromatographed on TLC plates coated with silica gel (150G/LS254, 0.25-mm thickness; Schleicher and Schüll, Dassel, Germany) in solvent system A as the developing solvent (see below). Starting materials and metabolites were visualized with a Camag UV lamp ($\lambda_{max}$ = 254 nm). Areas carrying the compounds were removed from the plates by scraping, and the compounds were eluted with methanol and determined by quantitative UV analysis by comparison with standard solutions. For co-chromatography of metabolites the following solvent systems were used: (A) benzene-acetic acid (9:1); (B) benzene-dioxan-acetic acid (90:25:4); (C) benzene-methanol (7:3). UV spectra were recorded on a Beckman model DB spectrophotometer. Melting points were determined using a Kofler hot stage.

**Enzyme assays.** Di- and monoalkyl phthalate esterase activity was assayed by incubating a suitable amount of crude extract, prepared by the alumina grinding technique, for 1 to 20 h with 12.5 to 50 μmol of DBP or MBP, respectively, in 10 ml of 0.1 M phosphate buffer (pH 7.5) with addition of 1 nmol of Tween 80 in sealed vials at 30°C. Enzymatic hydrolysis of phthalate esters was estimated by extraction of substrates and metabolites with ethyl acetate followed by quantitative UV analysis of the compounds. Protocatechuate 3,4-dioxygenase activity was estimated by the method described by Gibson (5).

Specific activity of crude extracts was calculated as micromoles of substrate degraded per minute (1 U) per milligram of protein under assay conditions. Protein was determined by the method of Lowry et al. (7), using bovine serum albumin as standard.

## RESULTS AND DISCUSSION

**Bacterial growth on dialkyl phthalates and detection of intermediates.** *Nocardia* sp. DSM 43250, *Nocardia* sp. DSM 43251, and *Nocardia* sp. DSM 43252 grew well in mineral base with the addition of 0.2% DBP as the carbon source. MBP accumulated as a transient intermediate in the culture solution (Fig. 1). The compound was identified on the basis of its melting point, UV absorption spectrum, mass spectrum, and behavior on TLC by comparison with authentic material as described (3). Neither free phthalic acid nor protocatechuic acid, which accumulates during phthalate metabolism by these organisms (4), could be detected in the media by means of ethyl acetate extraction and TLC of the extracts when the bacteria were grown on MBP or DBP.

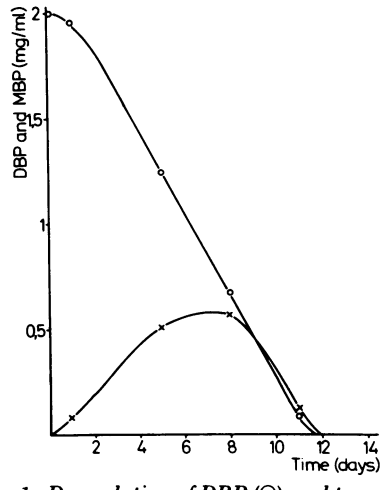


FIG. 1. *Degradation of DBP* (○) *and transient accumulation of MBP* (×) *during growth of Nocardia sp. DSM 43251 in DBP-mineral base.*

With increasing length of alkyl side chains, degradation rates of phthalate esters decreased significantly. Diethyl phthalate, di-*i*-butyl phthalate, di-2-ethylhexyl phthalate, and di-*n*-octyl phthalate were used by all three isolates as sources of carbon, whereas dimethyl phthalate was metabolized only by *Nocardia* sp. DSM 43250 and DSM 43251. The corresponding monoesters accumulated as transient intermediates in the culture solution (3), and no other degradation products could be detected.

*Arthrobacter* sp. DSM 20389 and *Pseudomonas* sp. DSM 5030, which have also been isolated from soil by enrichment with phthalate as carbon source (4), grew well on MBP, but not on DBP. No release of free phthalic or protocatechuic acid into the medium was detected.

To demonstrate that dialkyl phthalates are metabolized via free phthalic acid, washed cells of *Nocardia* sp. DSM 43251, grown on DBP, were incubated with 75 μmol of MBP in 10 ml of 0.1 M phosphate buffer, pH 7.5 (final optical density, 1.5), under aerobic and anaerobic conditions. The MBP disappeared in both samples at the same rate, but no aromatic metabolites were found in the samples incubated aerobically. In anaerobic mixtures, however, phthalic acid was produced nearly quantitatively from MBP. Traces of protocatechuic acid were also observed. Phthalic acid was identified by comparison of its UV absorption spectrum ($\lambda_{max}$ = 282 nm) and its $R_f$ values in solvent systems A ($R_f$ = 0.15), B ($R_f$ = 0.41), and C ($R_f$ = 0.71) with authentic material. Protocatechuic acid was detected on TLC plates by spraying with the Folin phenol reagent and was identified by UV ab-

sorption spectroscopy and co-TLC with the known compound, as described (3).

**Induction experiments.** To demonstrate induction of the esterases hydrolyzing di- and monoalkyl phthalates, manometric experiments were performed with washed cells of the different isolates, grown on DBP, MBP, phthalic acid, or glucose. In addition, the specific activity of di- and monoalkyl phthalate esterase was determined using crude extracts of cells, grown on dimethyl phthalate, phthalic acid, or glucose. The results obtained showed that, when grown on DBP, cells of all three *Nocardia* isolates oxidized DBP, MBP, phthalic, and protocatechuic acid without a lag period (Table 1). Glucose-grown cells did not metabolize phthalic acid. There was a slight increase in oxygen uptake over endogenous respiration when glucose-grown cells were tested with DBP and MBP. This increased oxygen uptake was much less than that observed for DBP-grown cells.

Similarly, when grown on MBP, the bacteria immediately oxidized DBP, MBP, phthalic acid, and protocatechuic acid. Phthalate-grown cells readily oxidized phthalic acid, whereas the rates of oxidation were considerably lower with DBP and MBP. When grown on phthalate, *Arthrobacter* sp. DSM 20389 and *Pseudomonas* sp. DSM 5030 oxidized phthalic acid and MBP at high rates; DBP, however, was oxidized at a low rate. Glucose-grown cells metabolized both esters to a low extent and were inactive with phthalic acid (Table 1). These results suggest that in the two *Nocardia* isolates the esterases hydrolyzing DBP and MBP are induced by DBP and MBP. In *Arthrobacter* sp. DSM 20389 and *Pseudomonas* sp. DSM 5030, however, free phthalic acid seems to be the inducer of an esterase that hydrolyzes MBP but not DBP.

Comparison of specific activities of crude extracts of dimethyl phthalate-, MBP-, phthalic acid-, and glucose-grown cells of *Nocardia* sp. DSM 43251 with different dialkyl phthalates showed no significant differences between the extracts. Esterase activities with diethyl phthalate, dimethyl phthalate, DBP, di-*n*-octyl phthalate, and di-2-ethylhexyl phthalate were about 0.004, 0.0015, 0.0013, 0.0004, and 0.0003 U/mg of protein, respectively. Esterase activities with mono-*n*-methyl and mono-*n*-butyl phthalate were about 0.001 U/mg of protein in all extracts.

This result is in contrast to that obtained by the manometric experiments. Because of these very low enzyme activities of the crude extracts, it is suggested that in *Nocardia* sp. DSM 43251 phthalate esterase activity is inducible and is mainly lost during disintegration of cells.

Crude extracts prepared from phthalate-grown cells of the *Pseudomonas* sp. and the *Arthrobacter* sp., however, readily hydrolyzed MBP with the quantitative formation of free phthalic acid. Hydrolysis of MBP and formation of phthalic acid was linear with time and the amount of crude extract added. Phthalic acid was identified by comparison of its UV absorption spectrum and behavior during TLC in solvent systems A, B, and C with that of the authentic compound. Specific activities of phthalate- and glucose-grown cells of *Arthrobacter* sp. DSM 20389 were 1.7 and 0.003 U/mg of protein, respectively. This finding suggests product induction of MBP esterase activity in these organisms since it was also shown by the manometric experiments.

Protocatechuate 3,4-dioxygenase activity was below 0.001 U/mg of protein in extracts from all isolates when grown on different di- or monoalkyl phthalates. Extracts obtained from phthalate-grown cells, however, contained protocatechuate 3,4-dioxygenase activity, e.g., 0.2 U/mg of protein of extracts prepared from *Nocardia* sp. DSM 43251.

TABLE 1. *Oxidation of DBP, MBP, phthalic acid, and protocatechuic acid by Nocardia sp. DSM 43251 and DSM 43252, Arthrobacter sp. DSM 20389, and Pseudomonas sp. DSM 5030 grown with different carbon sources*

| Organism | Growth substrate | Oxygen uptake (μl/h) | | | |
|---|---|---|---|---|---|
| | | DBP | MBP | Phthalic acid | Protocatechuic acid |
| *Nocardia* sp. DSM 43251 | DBP | 93.0 | 90.2 | 12.2 | 15.9 |
| *Nocardia* sp. DSM 43252 | DBP | 52.0 | 50.2 | 37.4 | 32.2 |
| | MBP | 149.0 | 30.8 | 40.0 | 84.8 |
| | Phthalic acid | 34.2 | 14.6 | 130.6 | 83.4 |
| | Glucose | 33.8 | 1.8 | 0.0 | 0.0 |
| *Arthrobacter* sp. DSM 20389 | Phthalic acid | 5.8 | 192.2 | 233.8 | ND[a] |
| | Glucose | 7.0 | 0.0 | 0.0 | ND |
| *Pseudomonas* sp. DSM 5030 | Phthalic acid | 43.6 | 204.6 | 256.8 | ND |
| | Glucose | 36.6 | 38.8 | 0.0 | ND |

[a] ND, Not determined.

The results obtained show that dialkyl phthalates are hydrolyzed by isolates from phthalic acid enrichments to free phthalic acid with the intermediate accumulation of monoalkyl phthalates in the culture medium. Phthalic acid is metabolized via protocatechuic acid by a 3,4-dioxygenative ring cleavage. Since the tested bacteria can use aliphatic alcohols as carbon sources, the entire ester molecule seems to be mineralized.

Pure isolates from DBP enrichments, however, formed MBP as the only metabolite (3). In mammalian metabolism also, only a single hydrolysis with the accumulation of the monoesters of the phthalates is performed (1), which shows that the formation of the monoesters is the most common reaction in the total reaction sequence of phthalate ester mineralization. This is also confirmed by the strong intermediate accumulation of the compound in cultures of the *Nocardia* isolates. In the *Nocardia* isolates tested, the esterases hydrolyzing di- and monoalkyl phthalates seem to be induced by the phthalate esters. The monoalkyl phthalate esterases of the *Pseudomonas* and *Arthrobacter* isolates, however, are induced by free phthalic acid, the product of the hydrolysis. This result is in contrast to that obtained by Keyser et al. (in press), who showed that phthalate esterase activity is a constitutive property of a *Micrococcus* isolate.

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