The Microbial Metabolism of Di-*n*-Butyl Phthalate and Related Dialkyl Phthalates

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In the past few years considerable attention has been paid to the analysis, environmental fate, and general toxicity of persistent organochlorine compounds such as DDT, the cyclodiene insecticides and PCBs. By comparison, little interest was shown, until recently, in non-halogenated industrial compounds as for instance phthalate esters, which are most commonly used as plasticizers and are produced in much larger quantities(ANONYMOUS, 1972) than the aforementioned organochlorine componds.

Recent results, which showed the presence of phthalate esters in environmental samples (ANONYMOUS, 1972; MAYER et al., 1972; ZITKO, 1972; WILLIAMS, 1973)and also indicated rapid biomagnification in a model ecosystem (METCALF et al., 1973), have spurred considerable amount of research in this area (for reviews see ANONYMOUS, 1972; FISHBEIN and ALBRO, 1972; AUTIAN, 1973). Although information is available on the metabolism of phthalate esters by fish (STALLING and HOGAN, 1973) and rat (ALBRO et al., 1973;ALBRO and THOMAS, 1973; CHAMBON et al., 1971) very little is known on the microbial metabolism and therefore accurate pathways of biological degradation degradation of these types of compounds in the environment.

The present study was initiated as a contribution to the understanding of biodegradation of phthalate esters in the environment with particular emphasis on structure of intermediates and selection of a number of pure cultures of microorganisms able to carry out certain reaction steps in the sequence of phthalate ester degradation.

Experimental

The organisms were selected in the following way: (a) One fungus, identified as Penicillium lilacinum, and three bacteria (2 Gram-negative short rods and 1 Gram-positive irregular branched rod)

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were isolated from soil by the enrichment method using Hegeman's mineral base (HEGEMAN, 1966) + 0.05 % yeast extract + 1.5 % agar + 0.1 % Tween 80 and 0.2 % di-n-butyl phthalate as the main carbon source. The mixture was adjusted to pH 7.2 by the addition of sodium hydroxide. The addition of Tween 80 was necessary to ensure uniform distribution of di-n-butyl phthalate in the agar plate. Active ester-degrading organisms could easily be detected by the clear zones surrounding the colonies against a milky background. (b) Three strains (Corynebacterium petrophilum ATCC 19080, Arthrobacter hydrocarboglutamicus ATCC 15583, and Mycobacterium phlei) were obtained by screening of fourty defined stock cultures for their ability to degrade di-n-butyl phthalate in the same medium.(c and d) Ten bacteria were obtained identical to(a) except that 0.4 % phthalic acid was used instead of the ester and addition of Tween 80 was not necessary. The organisms able to carry out reaction (c) (Fig. 1)were four Gram-negative motile organisms with characteristics placing them to the Pseudomonads and three Brevibacteria. Reactions (d) were carried out by three other coryneform bacteria.

For quantitative determination of DBP-degradation all microorganisms were incubated each in 30 ml portions of DBP-mineral base solution at 30° C on a gyratory shaker. After one and four weeks the whole cultures were acidified with 4 N HCl to pH 1, and extracted with (2 x 15 ml) chloroform and 10 ml petroleum ether. The extracts were dried (Na_2SO_4) and 2 ml portions were chromatographed on tlc plates coated with (Schleicher und Schüll 150 G/LS 254) silica gel using benzene: acetic acid = 9:1 as the developing solvent. Starting materials and metabolites formed were detected using a Camag UV-lamp (λ_{max}= 245nm) the zones carrying starting materials and metabolites were removed from the plates eluted with methanol and determined by quantitative UV-analysis by comparison with standard solutions at 275 nm.

Results and Discussion

The results obtained showed that within one to four weeks all isolates obtained with DBP as a carbon source for enrichment (a) and the DBP-degrading stock cultures (b) formed mono-n-butyl phthalate almost quantitatively as the only metabolite, suggesting that one butanol moiety of the molecule served as carbon source for growth of these organisms (only poor growth was observed with Tween 80 alone). The metabolite, isolated by preparative tlc, was identified by its physical characteristics (Table 1).

During growth of the three coryneform bacteria (d) on a medium containing di-n-butyl phthalate, mono-n-butyl phthalate accumulated as a transient intermediate in the culture solution. Both esters disappeared from the medium during growth, but no free phthalic acid or other aromatic degradation products could be detected as further intermediates in the culture solution. All three strains could be grown on MBP (0.2 %) and phthalic acid (0.4 %) as carbon sources as well. Cells grown on di-n-butyl phthalate immediately oxidized mono-n-butyl phthalate, phthalic acid and protocatechuic

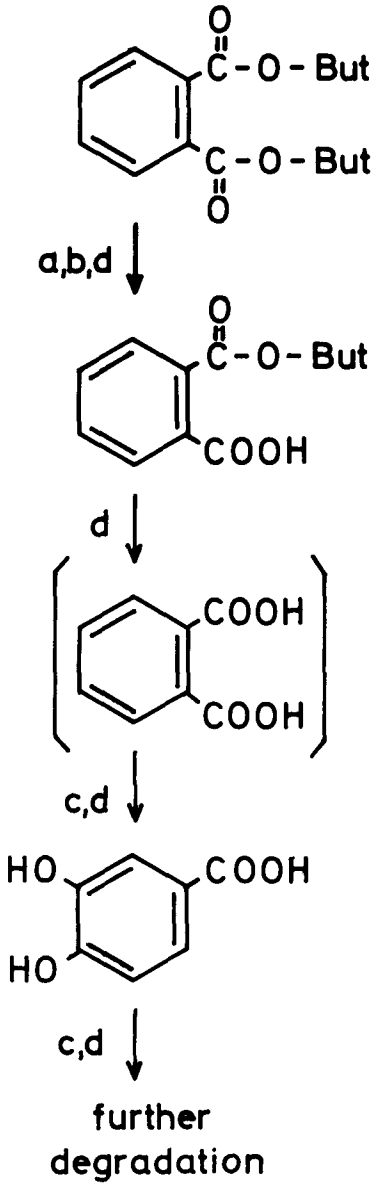

Figure 1. Degradation of di-n-butyl phthalate, mono-n-butyl phthalate and phthalic acid by different microorganisms: (a) organisms isolated with DBP as carbon source for enrichment, (b) stock cultures, (c and d) bacteria isolated from phthalic acid enrichment medium.

Table 1: Physical properties of phthalate ester metabolites

Compound	M.p.[a] °C	UV[b] λ max nm	Mass spectrum[c] Molecular ion (rel. intensity)	Tlc-data[d] R_F in solvent A	B	C
Monomethyl phthalate	82	275	180 (5 %)	0.50	0.01	0.60
Mono-n-butyl phthalate	72	275	222 (3 %)	0.60	0.04	0.85
Mono-i-butyl phthalate	63.5	275	222 (0.5 %)	0.60	0.04	0.85
Mono-(2-ethylhexyl) phthalate	oil	275	278 (3 %)	0.62	0.08	0.89
Mono-n-octyl phthalate	oil	275	278 (0.5 %)	0.62	0.14	0.89
Protocatechuic acid	195 (decomp.)	258	154 (95 %)	0.12	0.0	0.35

a All substances with melting points above room temperature show values in accordance with literature (Beilsteins Handbuch der organischen Chemie Vol. 9, Ergänzungsband III/5, p. 4098 ff.).
b Solvent: methanol.
c All phthalate esters show characteristic intense ions at m/e 148 and m/e 149. Spectra were recorded at 20 eV (protocatechuic acid at 70 eV).
d Solvent A, benzene-acetic acid, 9 : 1; solvent B, chloroform-benzene, 9 : 1; solvent C, chloroform-ethyl acetate, 1 : 1.

acid as measured by manometric experiments, whereas glucose-grown cells did not metabolize any of these aromatic compounds. Underlying STANIER's (1950) principle of simultaneous adaptaion these results suggest that DBP is degraded by these organisms either via MBP and free phthalic acid or that MBP is first oxidized by these organisms identical to free phthalic acid followed by removal of the second butanol moiety in some later degradation step.

All microorganisms isolated from phthalic acid enrichment medium (c and d) were found to grow at the expense of phthalic acid (PA), a probable product in the chemical and/or biological hydrolysis of phthalate esters in the environment, as carbon source. Early in the logarithmic phase of growth on PA-mineral base medium the cultures developed a red-brownish colour and gave transiently a strong phenol reaction, detectable by the Folin phenol reagent. When the phenol reaction was at a maximum, a culture (1 L) was centrifuged, the supernatant acidified with 4 N HCl to pH 1, concentrated to dryness under reduced pressure, and the residues dissolved in methanol. After separation by tlc in benzene: acetic acid = 9:1, the phenolic metabolite was identified as protocatechuic acid by comparison with authentic material(Table 1). Since protocatechuic acid further disappeared from the medium, it seems likely that these organisms degrade phthalic acid via 4,5-dihydroxy phthalic acid, protocatechuic acid and cis, cis-β-carboxy muconic acid, similar to different soil Pseudomonads described by RIBBONS and EVANS (1960).

The overall reaction steps of di-n-butyl phthalate degradation by different soil microorganisms are depicted in Fig. 1.

All organisms were also tested for their ability to degrade other dialkyl phthalates. Di-i-butyl phthalate, di-n-octyl phthalate and di(2-ethylhexyl) phthalate support growth of these organisms, whereas dimethyl phthalate served as a carbon source for only two of the coryneform bacteria (d). The corresponding monoesters were identified as degradation products (Table 1). Several additional, as yet unidentified metabolites with an intact phthalic acid moiety could be isolated from cultures of Penicillium lilacinum growing on di-n-butyl phthalate, di-n-octyl phthalate and di-(2-ethylhexyl) phthalate. This would indicate the existence of metabolic pathways different to those described in this paper.

From the results of this study a number of conclusions may be drawn: Since it was relatively easy to isolate phthalate ester degrading strains, such organisms are probably abundantly present in the environment; microbial degradation of phthalate esters occurs much easier than that of the " classical" persistent organochlorine compounds; although certain single organisms were shown to mineralize phthalate esters completely, the results indicate that mixed populations will be most effective in degrading these compounds; by isolating and identifying metabolites and by feeding key intermediates we have shown that not all steps in the degradation of phthalate esters are performed with equal ease; the formation of the monoesters and the degradation of phthalic acid appear to be carried out by the largest numbers of organisms, whereas the hydrolysis of the

second alcohol moiety seems to be the most difficult reaction step.

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