APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1985, p. 265–273
0099-2240/85/080265-09$02.00/0

Vol. 50, No. 2

Fungal Metabolism of *tert*-Butylphenyl Diphenyl Phosphate

MICHAEL A. HEITKAMP, JAMES P. FREEMAN, DAVID C. McMILLAN, AND CARL E. CERNIGLIA*

National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas 72079

Received 4 March 1985/Accepted 3 May 1985

The fungal metabolism of *tert*-butylphenyl diphenyl phosphate (BPDP) was studied. *Cunninghamella elegans* was incubated with BPDP for 7 days, and the metabolites formed were separated by thin-layer, gas-liquid, or high-pressure liquid chromatography and identified by ^{1}H nuclear magnetic resonance and mass spectral techniques. *C. elegans* metabolized BPDP predominantly at the *tert*-butyl moiety to form the carboxylic acid 4-(2-carboxy-2-propyl)triphenyl phosphate. In addition, 4-hydroxy-4′-(2-carboxy-2-propyl)triphenyl phosphate, triphenyl phosphate, diphenyl phosphate, 4-(2-carboxy-2-propyl)diphenyl phosphate, 2-(4-hydroxyphenyl)-2-methyl propionic acid, and phenol were detected. Similar metabolites were found in the 28 fungal cultures which were examined for their ability to metabolize BPDP. Experiments with [^{14}C]BPDP indicated that *C. elegans* metabolized 70% of the BPDP after 7 days and that the ratio of organic-soluble metabolites to water-soluble metabolites was 8:2. The results indicate that fungi preferentially oxidize BPDP at the alkyl side chain and at the aromatic rings to form hydroxylated derivatives. The trace levels of mono- and diaryl metabolites and the low level of phosphotriesterase activity measured in *C. elegans* indicate that phosphatase cleavage is a minor pathway for fungal metabolism of BPDP.

Triaryl phosphate esters (TAPs) are used as fire-resistant hydraulic fluids and lubricant additives and are present as plasticizers in many consumer products (21). The production of TAPs has increased steadily since 1970 as they have been used as replacements for cresylic phosphate esters and as substitutes for the restricted polychlorinatd biphenyls (1). TAPs are weak acute toxicants; however, some TAPs have been shown to induce delayed neurological effects in animal studies (7, 13). TAPs which are not produced from *o*-cresol and those which lack *ortho* substitutents on their aryl moieties generally exhibit less toxicity (14, 15). As a class, TAPs have low water solubility and are lipophilic (23); thus, the potential exists for human exposure from the uptake and bioaccumulation of TAPs or their metabolites into food chains. Since TAPs have been reported in fish, river water and sediments from several industrialized areas (20), drinking water (18), and recently as contaminants in food (6), there is some concern for the environmental disposition and metabolism of these phosphate esters.

tert-Butylphenyl diphenyl phosphate (BPDP) was first marketed in 1976 and is now a component of several commercial formulations of phosphate esters. BPDP is used primarily as a flame-retardant plasticizer, and yearly production is projected to reach 1×10^7 to 2×10^7 lbs (4,535,920 to 9,071,847 kg) by 1986 (21). The acute toxicity of BPDP has been examined via oral administration to rats and chickens and dermal exposure to rabbits and was found to be relatively nontoxic by either route of exposure (13). However, uptake and clearance studies with rainbow trout and fathead minnows have shown that BPDP does accumulate in fish tissues (24). The biodegradation of BPDP has been reported in semicontinuous activated sludge and river-die-away tests (27), but very limited information exists regarding the metabolic pathway for BPDP biodegradation.

The presence in the environment of bacteria and fungi which are able to utilize TAPs as sole carbon sources has been previously reported (22, 25). These studies were based on either the observance of growth in the presence of TAPs or the detection of P_i (believed to be released from enzymatic cleavage of TAPs by phosphatases). It has been recently reported that indigenous sediment microflora are able to completely mineralize some TAPs to CO_2 (11, 27), but metabolic pathways remain unclear because very few intermediates have been isolated and chemically identified. Fungi contribute significantly to environmental heterotrophic activity and often utilize metabolic pathways similar to those reported for mammalian enzyme systems (3). In this study, *Cunninghamella elegans* ATCC 36112, a fungus which utilizes polycyclic aromatic hydrocarbons (3), was used to determine the identification of metabolites and the pathway of BPDP metabolism. In addition, we screened 28 fungal strains for their ability to metabolize BPDP.

MATERIALS AND METHODS

Microorganisms and biotransformation conditions. The investigations were carried out with *C. elegans* ATCC 36112 (3) and 28 other strains (see Table 3). Fungal cultures were maintained at 4°C on Sabouraud dextrose agar slants (Difco Laboratories, Detroit, Mich.) and were inoculated into sterile 125-ml Erlenmeyer flasks containing 30 ml of Sabouraud dextrose broth. The cultures were incubated for 2 to 5 days at 22°C on a rotary shaker at 150 rpm. Mycelia of the filamentous fungi were aseptically filtered and suspended in 0.1 M phosphate buffer (pH 7.2), whereas cells of the yeast cultures were aseptically transferred to sterile 40-ml centrifuge tubes, centrifuged at 10,000 × *g* for 10 min, suspended in 30 ml of buffer, and aseptically transferred to sterile 125-ml Erlenmeyer flasks. All fungal cultures were resuspended twice in phosphate buffer over a 24-h interval and then exposed to BPDP. Fungal cultures were incubated with 2 mg of unlabeled BPDP or with 0.23 μCi of [^{14}C]BPDP dissolved in 30 μl of dimethylformamide. The cultures were incubated for 5 or 7 days as described above. Cell lysis from BPDP was not observed with any of the fungi tested. Control flasks consisting of either BPDP and sterile Sabouraud dextrose broth or fungal cells with no test substrate were included in the study. To isolate sufficient quantities of BPDP metabolites for structure elucidation, we conducted batch experiments in which 20 125-ml Erlenmeyer flasks containing 30 ml of Sabouraud dextrose broth, 2 mg of BPDP, and *C.*

* Corresponding author.

elegans were incubated as described above for 7 days and were extracted and analyzed for BPDP metabolites as described below.

The ability of *C. elegans* to utilize BPDP as a sole source of energy and carbon was tested by inoculation of 125-ml Erlenmeyer flasks containing 30 ml of a modified Pope and Skerman mineral salts medium (16) and 0.02% BPDP. Flasks were incubated for 4 weeks as described above, and culture growth was visually compared with that of inoculated flasks containing mineral salts and mineral salts plus 0.02% glucose.

Extraction and analysis of metabolites. Cells were removed by either filtration or centrifugation (10,000 × *g* for 10 min), and cells and culture filtrate were each extracted with 6 equal volumes of ethyl acetate. Filtrates were then titrated to pH 4.0 with a known volume of 0.1 N HCl and reextracted with 3 equal volumes of ethyl acetate to enhance the recovery of acidic metabolites. The combined organic extracts were dried over anhydrous sodium sulfate and then evaporated in vacuo at 40°C. Nonextractable ^{14}C-labeled residue in the aqueous phase was quantitated by adding a 1-ml portion to a scintillation vial containing 12 ml of Scintisol (Isolab, Inc., Akron, Ohio), and the radioactivity was determined in a Beckman model LS100 liquid scintillation counter (Beckman Instruments, Inc., Berkeley, Calif.). Nonextractable cellular ^{14}C-labeled residue was quantitated by filtering and drying extracted cells overnight at 60°C and by combustion in a Packard B306 Tri-Carb Sample Oxidizer (Packard Instrument Co., Inc., Downers Grove, Ill.). The radioactivity of combusted samples was measured as described above.

The occurrence of volatile ^{14}C-labeled metabolites and $^{14}CO_2$ resulting from biodegradation of BPDP by *C. elegans* was determined with an enclosed, flowthrough test apparatus (12). Cultures were exposed to [^{14}C]BPDP or 2 mg of unlabeled BPDP for 7 days in a flask which was continuously purged with compressed air. The gaseous effluent from each culture was directed through Teflon tubing into glass Michel-Miller chromatographic precolumns (inside diameter, 2 cm) containing 7 cm of polyurethane foam and 0.5 g of 20/35-mesh Tenax (Alltech Associates, Inc., Deerfield, Ill.). The efficiency of polyurethane foam and Tenax for trapping volatile organics has been previously described (19, 28). The gaseous effluent was then bubbled through a test tube (250 by 25-mm inside diameter) containing 40 ml of monoethanolamine-ethylene glycol (3:7 vol/vol) to trap evolved $^{14}CO_2$.

The polyurethane foam from each volatile-organic-trapping column was sliced into 1-cm sections, and radioactivity was measured in scintillation vials containing 12 ml of Scintisol as described above. Radioactive residues in the Tenax were determined by combustion as previously described for cellular ^{14}C-labeled residues, and $^{14}CO_2$-trapping columns were analyzed by placing 1-ml samples of the trapping solution into scintillation vials containing 7.5 ml of Beckman Fluoralloy dissolved in toluene and 7.5 ml of methanol. The presence of volatile metabolites from *C. elegans* cultures exposed to unlabeled BPDP was determined by back-flushing polyurethane foam with 3 ml of ethyl ether and by chemical analysis by capillary column gas chromatography and mass spectrometry (GC-MS).

Thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) were used for the separation of metabolites. All TLC analyses were performed with 500-μm Silica Gel GF plates (Analtech, Newark, Del.), and separation was achieved with a hexane-acetone-acetic acid (80:20:1, vol/vol/vol) solvent system. XAR-2 X-ray film (Eastman Kodak Co., Rochester, N.Y.) was exposed to TLC plates of [^{14}C]BPDP extracts for 2 weeks and developed to produce autoradiograms. Metabolites and undegraded BPDP were scraped from TLC plates, eluted with acetone, and further purified by HPLC. All HPLC analyses were performed with a Beckman system with two model 100A pumps and a Tracor model 970A variable-wavelength absorbance detector adjusted to 254 nm. A 5-μm C_{18} Ultrasphere ODS column (4.6 mm by 25 cm; Altex Scientific, Berkeley, Calif.) was used, and separation was achieved with an acidic (1% acetic acid) acetonitrile-water linear gradient (25 to 100% vol/vol; 30 min) at a flow rate of 1 ml/min. In experiments with [^{14}C]BPDP, 0.5-ml fractions were collected in scintillation vials; 10 ml of Scintisol was added to each vial, and radioactivity was determined as described above.

A GC-MS system (model 4023; Finnigan Instruments, Sunnyvale, Calif.), equipped with a quadrupole mass filter and a DB5 capillary column (0.25-mm inside diameter by 30 m; J and W Scientific, Rancho Cordova, Calif.) was used to analyze metabolites of BPDP after methylation with diazomethane (26). Analyses were performed in the electron impact mode with an electron energy of 70 eV. Samples were injected into the GC at 60°C, held isothermally for 2 min, and programmed to 210°C at 20°C/min. The program was then reduced to 5°C/min to 250°C and held isothermally at 250°C for 15 min. Direct-probe MS was performed on extracted and purified samples which were dissolved in 3 μl of methanol and dried under argon in glass sample cups. Spectra were recorded as the probe temperature was increased ballistically from 35 to 200°C while the ion-source temperature was maintained at 270°C.

The ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WM500 spectrometer in acetone-d_6 and methylene chloride-d_2. The data were acquired under the following conditions: data size, 32,000; sweep width, 7,042 Hz; filter width, 17,800 Hz; temperature, 305 K; flip angle, 68°. The chemical shifts are reported in parts per million (ppm) (δ) downfield from the internal standard tetramethylsilane. Assignments were made via homonuclear decoupling experiments and by consideration of substituent effects.

Phosphatase activities. Cultures of *C. elegans* were assayed for phosphatase activities by a method modified from that of Eivazi and Tabatabai (8) which involves the measurement of *p*-nitrophenol released after incubation at 37°C for 1 h. Cultures were filtered and suspended in 0.05 M Tris buffer, and 4-ml portions were assayed in sterile, capped, 50-ml centrifuge tubes containing 0.25 ml of toluene and 1 ml of Tris buffer. The buffer contained 1, 3, or 5 mM *p*-nitrophenyl phosphate disodium for phosphomonoesterase assays or bis-*p*-nitrophenyl phosphate for phosphodiesterase assays. Since tris-*p*-nitrophenyl phosphate is insoluble in water, 23 mg (10 mM) was weighed and directly added for phosphotriesterase assays. Cultures were chilled on ice after incubation, and *p*-nitrophenol was extracted with 0.5 ml of 0.5 M $CaCl_2$ and 4 ml of 0.1 M Tris buffer (pH 12). The cultures were centrifuged at 10,000 × *g* for 10 min. The *p*-nitrophenol in the supernatants was measured colorimetrically at 400 nm, and concentrations were calculated from a standard curve prepared with *p*-nitrophenol standards. Controls were extracted as described above, and values were subtracted as background from all samples. Excess supernatant was removed from each tube, and cellular pellets were weighed after drying overnight at 60°C. Phosphatase activities are reported as micrograms of *p*-

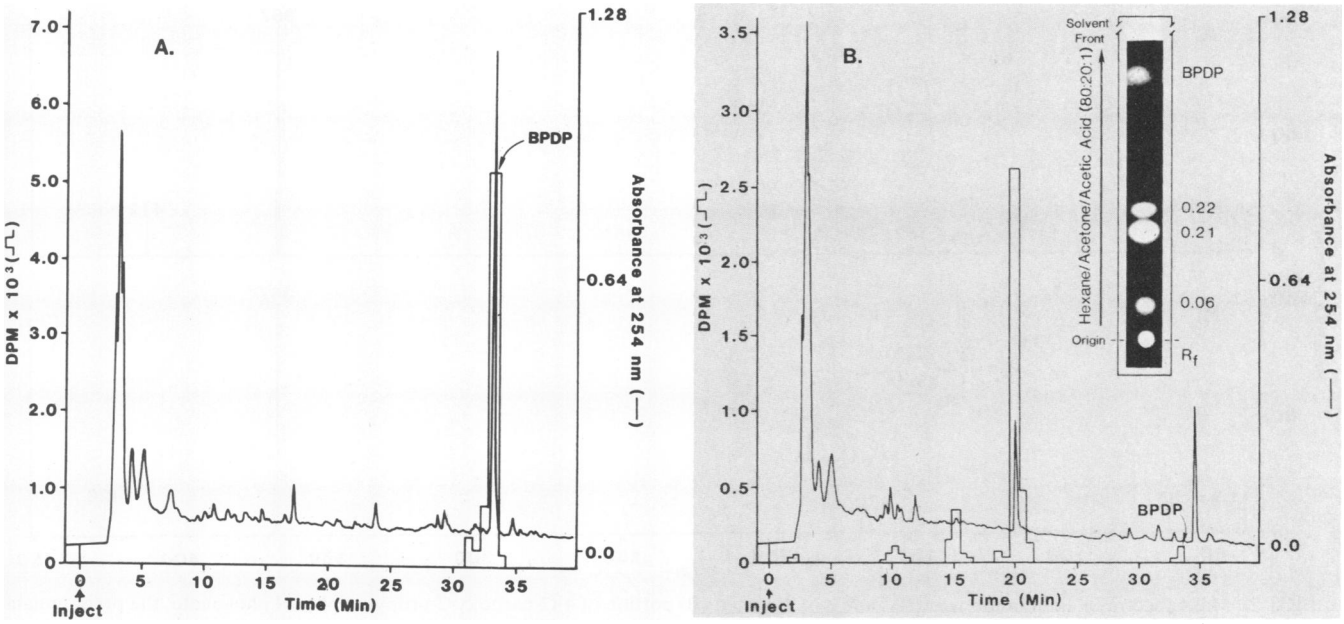

FIG. 1. UV and radioactivity elution profiles from analyses of the organic-extractable residues from *C. elegans* cultures which were extracted immediately (A) or after 5 days (B) of exposure to BPDP and [^{14}C]BPDP. Inset of panel B shows an autoradiogram from preparative TLC analysis of an extract from *C. elegans* after 5 days of exposure to [^{14}C]BPDP.

nitrophenol released · hours^{-1} · grams of cellular dry weight^{-1}. The Michaelis-Menten enzyme constant (K_m) and the maximum enzyme reaction velocity (V_{max}) were calculated from the classical Lineweaver-Burk plot (10) for phosphomonoesterase and phosphodiesterase enzymes.

Chemicals. Unlabeled BPDP was isolated from Fyrquel GT (lot no. 4833-1; Stauffer Chemical Co., Westport, Conn.) by open-column silica gel chromatography. Approximately 1 g of Fyrquel GT was loaded on a glass column (inside diameter, 2 cm) packed with 36 cm of silica gel (32 to 63 μM; Universal Scientific, Inc., Atlanta, Ga.) in hexane. The column was sequentially eluted with 50 ml of hexane, 400 ml of 1% acetone in hexane, and 400 ml of 2% acetone in hexane. The progress of the separation was monitored by TLC (Silica Gel IB2-F; J. T. Baker Chemical Co., Phillipsburg, N.J.) with 5% acetone in hexane. The BPDP eluted in the 630 to 780-ml fraction, and purity exceeded 99% as determined by GC-MS.

Radiolabeled BPDP was purchased from California Bionuclear Corporation, Sun Valley, Calif., and was uniformly ring labeled on the butylphenyl moiety. The specific activity was 4.1 mCi/mmol, and analysis by TLC indicated that purity exceeded 99%. The radiolabeled diphenyl phosphate was uniformly ring labeled and had a specific activity of 5.1 μCi/mg. It was purified by reverse-phase TLC, and purity exceeding 99% was verified by GC after methylation. Both isotopes were generous gifts from the Columbia National Fisheries Research Laboratory, Columbia, Mo.

RESULTS

Preliminary experiments. When *C. elegans* cells were inoculated into flasks containing mineral salts medium and BPDP, no significant growth was observed after 30 days. These results indicate that *C. elegans* cannot utilize BPDP as a sole energy and carbon source. However, when *C. elegans* cultures were grown for 48 h in Sabouraud dextrose broth and then suspended in buffer containing BPDP, approximately 70% of the BPDP was converted into metabolites after 7 days of exposure. Metabolism of BPDP was almost 50% greater from *C. elegans* cells suspended in buffer than from those exposed to BPDP in Sabouraud dextrose broth.

Ethyl acetate extracts from *C. elegans* cultures exposed to both unlabeled BPDP and [^{14}C]BPDP were analyzed by HPLC after 0 and 5 days of exposure. Figure 1A shows the HPLC elution profile from a *C. elegans* culture which was extracted immediately after the addition of BPDP. The recovery of BPDP is apparent from the UV trace at 254 nm and the presence of radioactivity in the collected fractions at an elution time of 33 min which is identical to the elution time for authentic BPDP. Figure 1B shows the disappearance of BPDP after 5 days and the presence of radioactive-metabolite peaks with HPLC elution times of 3 (void volume), 10.5, 15.5, and 20 min. The relative intensity of the peak observed at 20 min with both the UV and the radioactive trace demonstrates its relative abundance in comparison to the other metabolite peaks. The UV peak in Fig. 1B with an elution time of 35 min was apparent as a smaller peak in Fig. 1A; it was nonradioactive and presumed to be biogenic in origin.

Isolation and identification of BPDP metabolites. Batch cultures of *C. elegans* were exposed to BPDP for 7 days to obtain sufficient metabolites for structure elucidation. Preparative TLC was used to enrich the metabolites which eluted at R_f values of 0.0, 0.06, 0.21, and 0.22. The TLC elution order correlated directly with their sequential elution pattern from HPLC analysis (Fig. 1B, inset). The material which eluted from R_f 0.21 gave one chromatographic peak with an HPLC retention time of 20 min. Mass spectral analysis of this predominant metabolite (Fig. 2A) gave a molecular ion (M^+) at *m/z* 412 and a fragment ion at *m/z* 367 (M^+-45, COOH loss). Mass spectral analysis of a methylated derivative of this compound (Fig. 2B) gave a 14-unit increase (M^+) at *m/z* 426 and a fragment ion at *m/z* 367 (M^+-59, $COOCH_3$ loss). The mass spectral fragmentation patterns indicated that this compound was a carboxylic acid derivative of BPDP. The ^{1}H NMR spectroscopic analysis of

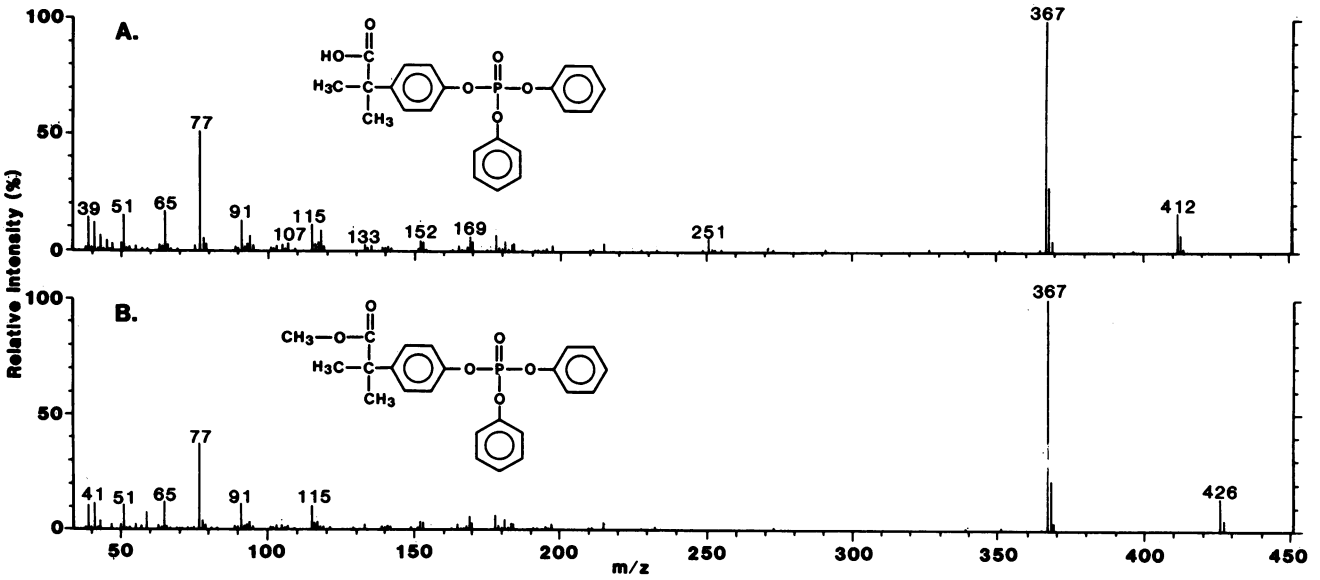

FIG. 2. Mass spectra of an unmethylated (A) and a methylated (B) portion of 4-(2-carboxy-2-propyl)triphenyl phosphate, the predominant metabolite of BPDP produced by *C. elegans*, which had an HPLC elution time of 20 min.

this compound gave the following chemical shifts: (CD_2Cl_2) δ 1.60 (s, 6, CH_3) and 7.15 to 7.42 (m, 14, aromatic H), and confirmed that this compound was 4-(2-carboxy-2-propyl)triphenyl phosphate. NMR analysis also indicated a minor component in this sample. Multiple development of a preparative TLC plate containing 4-(2-carboxy-2-propyl)triphenyl phosphate indicated another compound (Fig. 1B, inset) which had an R_f value of 0.22. This compound, which eluted at 20 min from the HPLC column, represented 10% of the total concentration (Fig. 1B). Mass spectral analysis of this compound (Fig. 3) gave an M^+ at *m/z* 326 and mass spectral fragmentation pattern and chromatographic properties identical to those of authentic triphenyl phosphate (TPP).

The mass spectrum of the compound (Fig. 4A) with an R_f value of 0.06 (Fig. 1B, inset) and an HPLC retention time of 15.5 min gave an M^+ at *m/z* 428 and fragment ions at *m/z* 383 (M^+-45, COOH loss) and *m/z* 366 (M^+-62, COOH, OH loss). The mass spectral fragmentation pattern indicated that this compound was a phenolic derivative of BPDP carboxylic acid. Mass spectral analysis of a methylated sample of this compound (Fig. 4B) gave an M^+ at *m/z* 456, which indicated that both the carboxylic and hydroxyl moieties were derivatized. The position of the hydroxyl group on the aromatic ring was determined by ^1H NMR analysis. The following are the NMR spectral parameters obtained from first-order measurements: (acetone-d_6) δ 1.56 (s, 6, CH_3), 6.85 (d, 2, H_3'), 7.12 (d, 2, H_2'), 7.25 (d, 2, H_2), 7.29 (d, 2, H_2''), 7.43 (dd, 2, H_3'') 7.47 (d, 2, H_3), 7.54 (t, 1, H_4''), and 10.43 (bs, 1, COOH) ppm; $J_{2',3'}$ = 8.6 Hz; $J_{2,3}$ = 9.0 Hz; $J_{2'',3''}$ = 8.2 Hz; $J_{3'',4''}$ = 8.6 Hz.

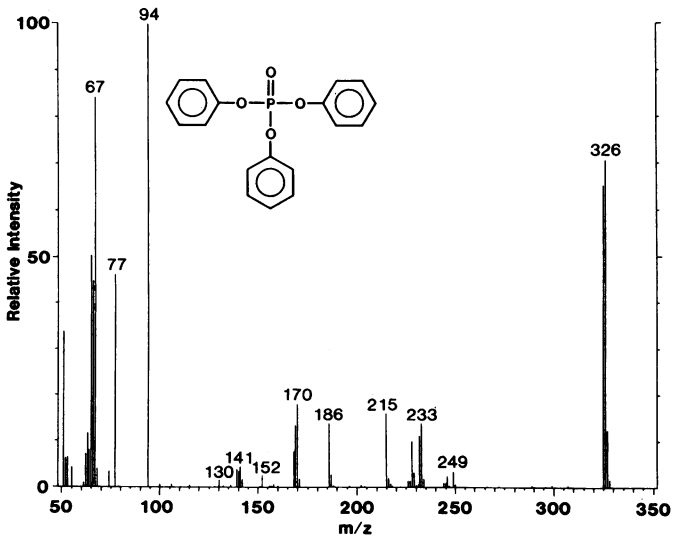

FIG. 3. Mass spectrum of triphenyl phosphate produced from BPDP by *C. elegans* which eluted from the HPLC at 20 min but was isolated by TLC (see Fig. 1B, inset).

The assigned chemical shifts and coupling constants were indicative of three different benzene ring systems. The furthermost upfield doublet at 6.85 ppm was consistent with a two-proton doublet adjacent to a hydroxyl group. The doublet at 6.85 ppm and 7.2 ppm is consistent with a *para*-disubstituted ring. The pair of doublets at 7.25 and 7.47 ppm are also consistent with a *para*-disubstituted aromatic ring system. The NMR data confirmed that this compound was 4-hydroxy-4'-(2-carboxy-2-propyl)triphenyl phosphate. The minor compound with an HPLC retention time of 10 min (Fig. 1B) was analyzed by MS. Direct-probe MS analysis suggested that it was a trihydroxylated BPDP metabolite. Insufficient material was available for further chemical characterization.

Polar metabolites which remained at the origin during preparative TLC (Fig. 1B, inset) were methylated with diazomethane (26) and analyzed by GC-MS. The GC elution profile for these metabolites is presented in Fig. 5, and the mass spectral fragmentation patterns and molecular characteristics are shown in Table 1. The two metabolites with GC retention times of 3.19 and 3.51 min had M^+s at *m/z* 108 and 94, respectively, which indicates methylated and unmethylated phenol arising from phosphatase cleavage of an unsubstituted phenyl from BPDP. The mass spectral frag-

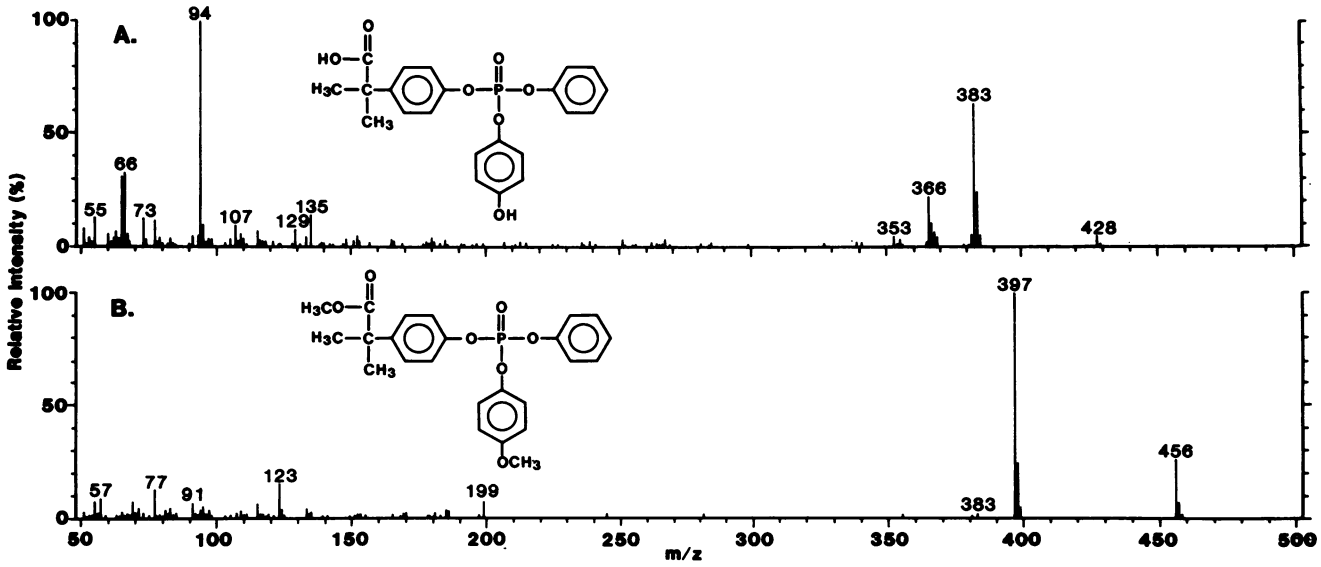

FIG. 4. Mass spectrum of an unmethylated (A) and a methylated (B) portion of 4-hydroxy-4'-(2-carboxy-2-propyl)triphenyl phosphate produced from BPDP by *C. elegans* which had an HPLC elution time of 15.5 min.

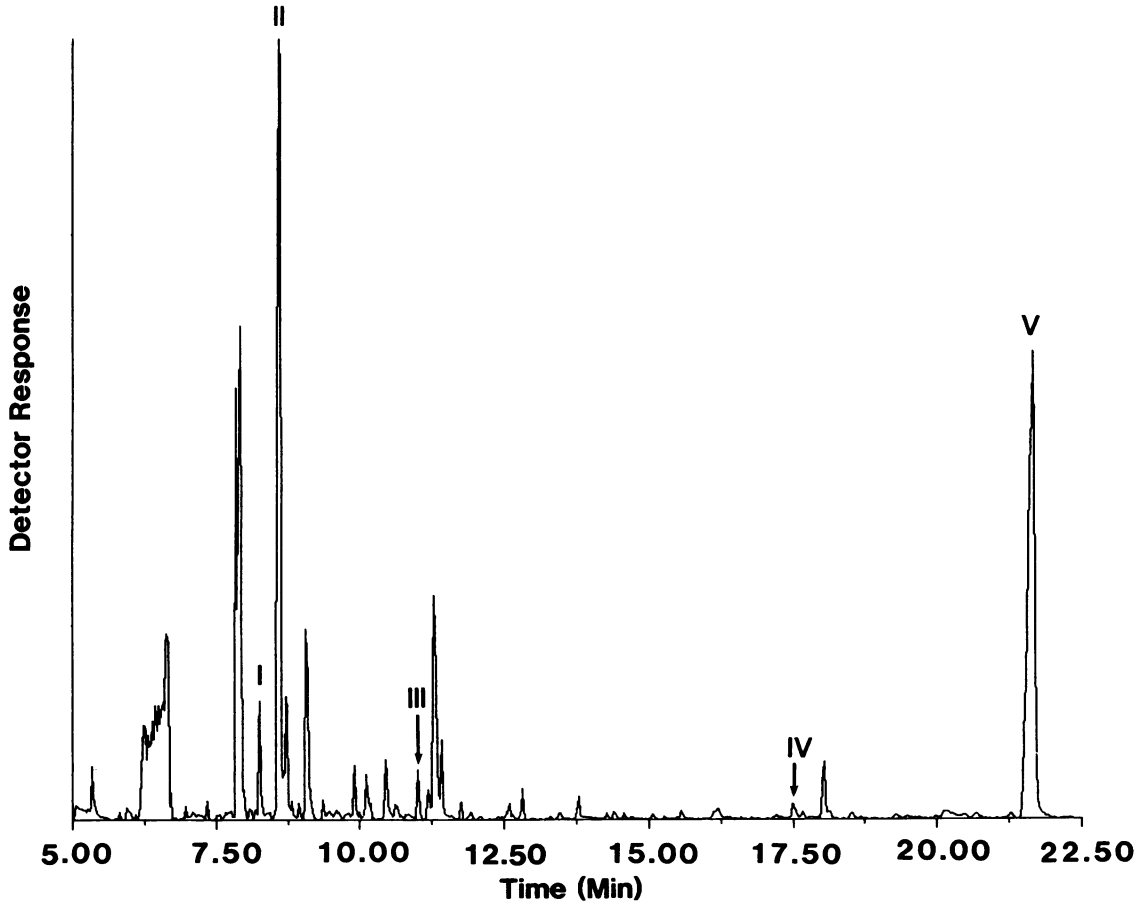

FIG. 5. Capillary column GC-MS elution profile for methylated derivatives of polar metabolites of BPDP which were extracted after 7 days of exposure to *C. elegans*. I through V, metabolites and their GC retention times.

TABLE 1. GC-MS properties of minor metabolites formed by *C. elegans* after 7 days of exposure to BPDP

Peak no.	GC retention time (min)	GC retention relative to BPDP	Mol wt	Molecular formula	Mass spectrum *m/z* (% of base peak)
—[a]	3.19	0.150	108	C_7H_8O	109 (8) 108 (100) 93 (13) 78 (63) 65 (69)
—[a]	3.51	0.164	94	C_6H_6O	95 (6) 94 (100) 66 (22) 65 (21) 55 (10)
I	8.16	0.382	208	$C_{12}H_{16}O_3$	209 (2.1) 208 (18.3) 150 (11.1) 149 (100) 121 (12.4)
II	8.36	0.397	194	$C_{11}H_{14}O_3$	195 (1.2) 194 (100) 136 (9.1) 135 (100) 119 (5.9) 107 (23.7)
III	11.03	0.510	264	$C_{13}H_{13}O_4P$	265 (9.5) 264 (100) 263 (71.8) 170 (45.9) 169 (34.1) 166 (13.8) 165 (20.2) 143 (11.6)
IV	17.30	0.807	364	$C_{18}H_{21}O_6P$	365 (1.1) 364 (6.1) 306 (16.9) 305 (100)
V	21.40	1.000	382	$C_{22}H_{23}O_4P$	383 (4.1) 382 (25.2) 368 (17.5) 367 (100)

[a] —, Data collected from another GC-MS analysis.

mentation pattern of this compound was identical to that of authentic phenol. The two metabolites with GC retention times of 8.16 and 8.36 min (Fig. 5, compounds I and II) had M^+s at *m/z* 194 and 208, respectively. These metabolites were mono- and dimethylated derivatives of 2-(4-hydroxyphenyl)-2-methyl propionic acid arising from *tert*-butyl-side-chain oxidation and phosphatase cleavage of BPDP to release the substituted phenol and diphenyl phosphate. This proposed pattern of phosphatase cleavage is further supported by the occurrence of a metabolite (Table 1, compound III) with a GC retention time of 11.03 min which was identical to that of methylated authentic diphenyl phosphate. Mass spectral analysis of this metabolite revealed an M^+ at *m/z* 264 and a fragment ion at *m/z* 170 (M^+-94), which indicates the methylated derivative of diphenylphosphate and the fragmentation loss of one phenyl group.

The metabolite (compound IV) with a GC retention time of 17.30 min had an M^+ at *m/z* 364 which indicates dimethylated 4-(2-carboxy-2-propyl)diphenyl phosphate arising from *tert*-butyl-side-chain oxidation of BPDP and removal of one unsubstituted phenyl moiety by phosphatase cleavage. Methylation of this metabolite occurred on both the butyric acid side chain and the hydroxyl group adjoining the central phosphate moiety. The fragmentation at *m/z* 305 (M^+-59) is due to the loss of the methylated carboxylic acid. BPDP (compound V) had a GC retention time of 21.40 min and is included in Table 1 for relative comparison of GC retentionand fragmentation pattern among the metabolites.

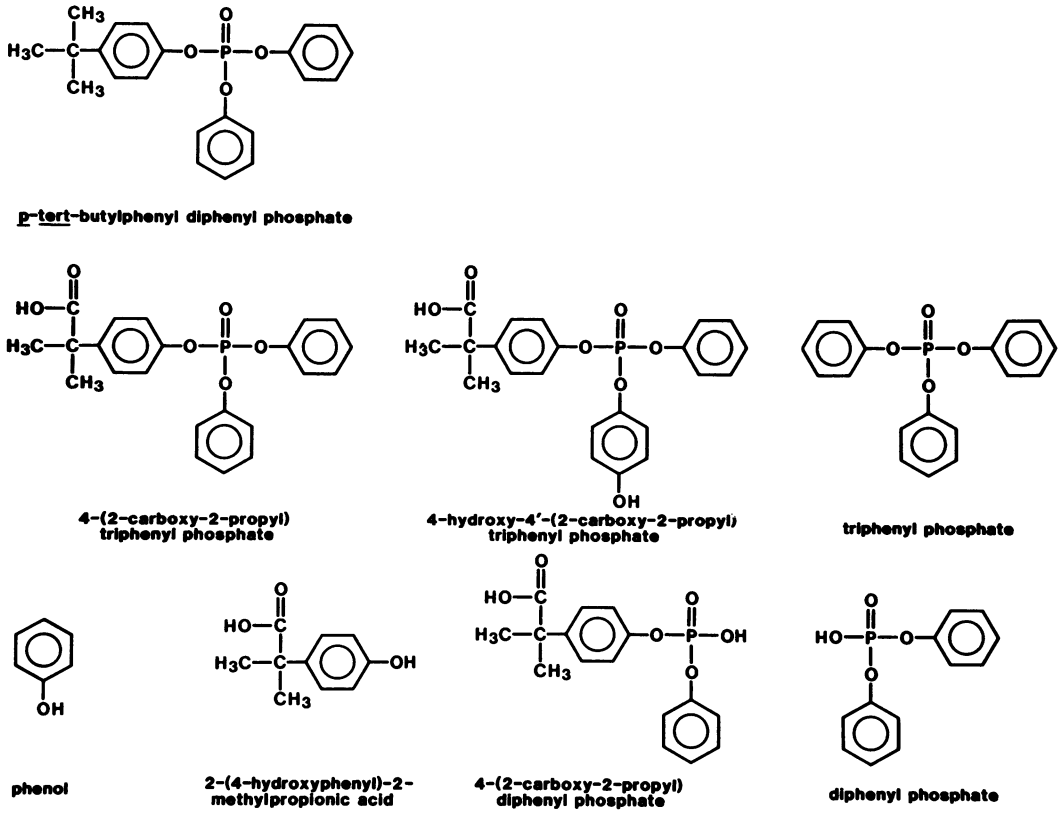

FIG. 6. Structures of identified metabolites from fungal metabolism of BPDP. Molecular weights and fragmentation patterns of the numbered metabolites are presented in Table 1.

The structures of the identified metabolites resulting from the metabolism of BPDP by *C. elegans* are shown in Fig. 6.

In a separate experiment, *C. elegans* was incubated with [^{14}C]BPDP in a flowthrough test apparatus (12) to determine if $^{14}CO_2$ or volatile ^{14}C-labeled metabolites or both were formed. Neither were detected. In addition, no abiotic degradation or hydrolysis of BPDP was observed in the control flasks employed in this study.

Time course of the metabolism of BPDP by *C. elegans*. Figure 7 illustrates the time course of BPDP biotransformation. Immediately after exposure, 62% of the radioactivity was recovered by extraction with ethyl acetate and identified by HPLC analysis as unmetabolized BPDP. However, over 20% of the [^{14}C]BPDP was already present as nonextractable cellular ^{14}C-labeled residue. This portion increased to 64% after 1 day and then decreased linearly throughout the experiment with a concomitant increase in organic extractable ^{14}C-labeled metabolites.

The ratio of organic-soluble metabolites to water-soluble metabolites throughout the experiment was approximately 8:2 (Fig. 7A). However, when the medium was titrated to pH 4.0 and extracted with 3 equal volumes of ethyl acetate, only about 3% remained as water-soluble metabolites. The enhanced extraction of organic-soluble metabolites at acidic pH supports the occurrence of the acidic metabolites reported earlier. Although ring hydroxylation is known to be an intermediate for various conjugation pathways (4), overnight incubation of the water-soluble metabolites with β-glucuronidase, aryl sulfatase, α-glucosidase, and β-glucosidase by a previously reported method (4) resulted in no increase in organic-soluble metabolites; hence, no conjugation of BPDP by *C. elegans* was detected.

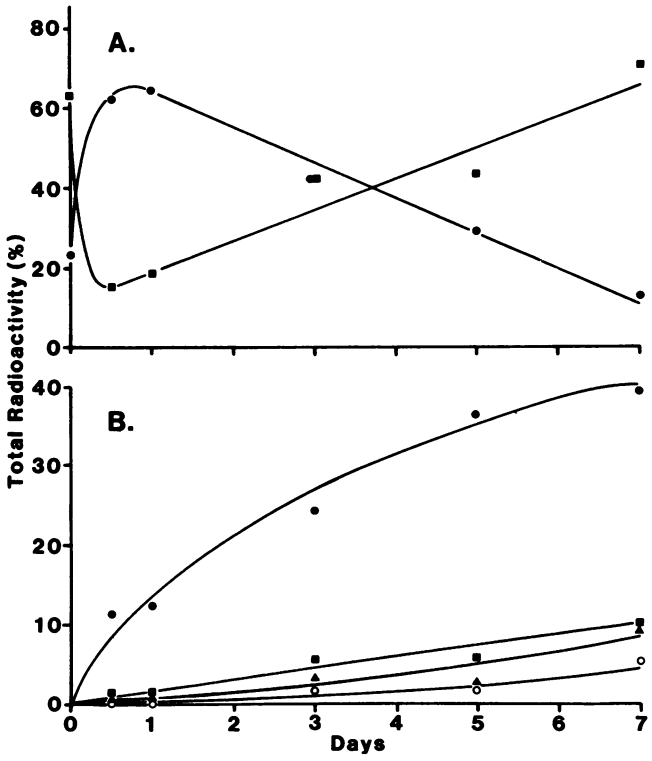

FIG. 7. Kinetic presentation of BPDP metabolism by *C. elegans* during 7 days of exposure (A) Cellular nonextractable residue (●) and organic extractable residue (■). (B) Production of 4-(2-carboxy-2-propyl)triphenyl phosphate and triphenyl phosphate (●) (9:1 ratio at day 7), 4-hydroxy-4'-(2-carboxy-2-propyl)triphenyl phosphate (■), possible triol of BPDP (▲), and combined residue of polar mono- and diaryl metabolites of BPDP (○).

TABLE 2. K_m and V_{max} for phosphatases of *C. elegans*

Enzyme	pH[a]	K_m (mM)	$V_{max} \times 10^3$[b]
Phosphomonoesterases			
Acid phosphatase	6.0	3.11	5.99
Alkaline phosphatase	10.0	1.82	4.78
Phosphodiesterase	8.0	0.36	1.18

[a] Enzymes were assayed at the optimum pH reported (2).
[b] Velocity is expressed as micrograms of *p*-nitrophenol released hours^{-1} · grams of cellular dry weight^{-1}.

The compositions of organic extracts were analyzed by HPLC at each time interval, and the rate of production of each metabolite is presented in Fig. 7B. All of the above-discussed metabolites were detected after 12 h of exposure of *C. elegans* to [^{14}C]BPDP, and their levels increased throughout the experiment. 4-(2-Carboxy-2-propyl)triphenyl phosphate accounted for about 12% of the total radioactivity after only 1 day and increased to about 40% by day 7. The slowed production of this metabolite after day 5 probably resulted from depletion of BPDP substrate as indicated by decreased cellular ^{14}C-labeled residues (Fig. 7A) as well as further metabolism of the metabolite to other products. Nonretained polar metabolites accounted for 4% of the total radioactivity after 7 days.

Phosphatase activity of *C. elegans*. The Michaelis constant and maximum velocity for the phosphomono- and phosphodiesterase activities of *C. elegans* are presented in Table 2. Acidic phosphomonoesterase activity was only slightly higher than alkaline phosphomonoesterase activity, but both were five to seven times greater than the activity of phosphodiesterase. Since tris-*p*-nitrophenol phosphate is insoluble in water, phosphotriesterase activity was assayed at only one concentration (10 mM), and because of low activity, the incubation time was extended to 2 h to permit detectable and reproducible activity. Phosphotriesterase activity resulted in 49.2 μg of *p*-nitrophenol released · hours^{-1} · grams of cell dry weight^{-1}, which was about 20- and 100-fold less than the activities of phosphodiesterase and phosphomonoesterase, respectively, assayed at a 5 mM substrate concentration. P_i has been reported to competitively inhibit phosphodiesterase activity (2). In the present study, the addition of 100 mM KH_2PO_4 and subsequent readjustment of the pH had no effect on acidic or alkaline phosphomonoesterase activity or phosphotriesterase activity but decreased phosphodiesterase activity by 15 to 18%.

Metabolism of BPDP by various fungal strains. A diverse group of filamentous fungi and yeasts were screened for their ability to metabolize BPDP. All of the fungi surveyed metabolized BPDP to some extent (data summarized in Table 3). Experiments with [^{14}C]BPDP indicated that the extent of BPDP metabolism ranged from 0.2 to 18.6% after 5 days of exposure.

The filamentous fungi oxidized BPDP to small amounts of polar mono- and diaryl metabolites (M-1), a possible triol of BPDP (M-2), 4-hydroxyy-4'-(2-carboxy-2-propyl)triphenyl phosphate and 4-(2-carboxy-2-propyl)triphenyl phosphate and TPP (M-5 and M-6) (Table 3). In addition, the unkown metabolite 4 (Table 3) was detected from *Aspergillus glaucus*, *Nigrospora* sp., and *Penicillium brevi-compactum*. Since only 2.2, 0.6, and 0.7% of BPDP was metabolized by

TABLE 3. Fungal survey of [^{14}C]BPDP metabolism and relative concentrations of metabolites after 5 days of exposure

Organism	% Total metabolite radioactivity[a]					Total metabolite radioactivity (cpm)	Total metabolism of BPDP (%)
	M-1[b]	M-2	M-3	M-4	M-5 + M-6		
Aspergillus flavus (ATCC 9170)	18.6	43.7	17.9		19.8	1,488	0.3
Aspergillus flavus (ATCC 15546)	3.6	19.3	13.4		63.7	4,714	0.9
Aspergillus flavus (ATCC 15548)	3.9	23.9	20.9		51.3	5,222	1.0
Aspergillus flavus (ATCC 24109)		58.6	20.6		20.8	3,036	0.6
Aspergillus glaucous			15.4	81.8	2.7	10,828	2.2
Aspergillus niger (ATCC 6275)		5.0	19.8		75.2	6,073	1.2
Aspergillus niger (ATCC 10548)			14.0		86.0	1,011	0.2
Aspergillus toxicarius		45.8	25.5		28.7	5,151	1.0
Cunninghamella elegans (ATCC 9245)	1.4	2,5	8.5		87.6	53,296	10.7
Mucor sp.		2.7	2.9		94.4	93,124	18.6
Nigrospora sp.		6.2	58.6	16.1	19.1	2,865	0.6
Penicillium brevicompactum (ATCC 10418)	5.2	5.6	39.9	34.7	14.6	3,391	0.7
Penicillium chrysogenum	4.8	6.9	38.7		49.6	87,716	17.5
Penicillium claviforme (MR 376)			20.5		79.5	2,156	0.4
Penicillium funiculosum (ATCC 11797)			18.8		81.2	7,307	1.5
Penicillium notatum		6.0	37.1		56.9	8,253	1.7
Penicillium piscarium		23.1	22.3		54.6	4,667	0.9
Syncephalastrum racemosum	7.6	29.6	21.6		41.2	37,768	7.6
Candida guilliermondii (78-006)			4.5		95.5	2,877	0.6
Candida lipolytica (37-1)			38.9		61.1	5,150	1.0
Candida lipolytica (78-003)			21.9		78.1	2,603	0.5
Candida lipolytica (78-004)			16.0		84.0	3,241	0.7
Candida maltosa (R-42)			18.3		81.7	1,229	0.3
Debaryomyces hansenii (CBS 767)			20.0		80.0	3,491	0.7
Debaryomyces sp.			13.6		86.4	2,865	0.6
Debaryomyces sp. strain NC			19.7		80.3	1,492	0.3
Saccharomyces cerevisiae			2.9		97.1	2,068	0.4
Saccharomyces cerevisiae sp.			14.6		85.4	1,809	0.4

[a] Identification and quantification of BPDP metabolites were determined by HPLC and GC-MS as described in the text.

[b] M-1, Mono- and diaryl metabolites; M-2, a possible triol of BPDP; M-3, 4-hydroxy-4′-(2-carboxy-2-propyl)triphenyl phosphate; M-4, unknown metabolite; M-5, 4-(2-carboxy-2-propyl)triphenyl phosphate; M-6, triphenyl phosphate.

these organisms, respectively, we were unable to obtain a sufficient quantity of metabolite 4 for chemical identification. However, HPLC chromatographic properties revealed that metabolite 2 is probably an oxidized triaryl metabolite of BPDP. Analysis of polar metabolites by GC-MS after methylation revealed the presence of free phenol and a mixture of partially oxidized substituted and unsubstituted diaryl phosphate metabolites similar to those detected for *C. elegans* (Table 1).

The surveyed yeasts only metabolized about 1% of the BPDP after 5 days of exposure. The yeasts oxidized BPDP to 4-hydroxy-4′-(2-carboxy-2-propyl)triphenyl phosphate and 4-(2-carboxy-2-propyl)triphenyl phosphate. Although polar diaryl metabolites were not detected by HPLC, autoradiography revealed some polar metabolites at the origin after preparative TLC. We did not detect TPP by TLC analyses or metabolites 2 or 4 by HPLC analyses of extracts from the surveyed yeasts.

DISCUSSION

The purpose of this study was to investigate the metabolism of TAPs by fungi with specific emphasis on both initial oxidation and possible further degradation via phosphatase cleavage to phenols and P_i. The fungal transformation of TAPs has not been previously reported.

The structures of the identified BPDP metabolites formed from the fungal transformation of BPDP are shown in Fig. 6. *C. elegans* converted BPDP predominately to its carboxylic acid derivative, and phenolic metabolites were minor products. The *tert*-butyl side chain of BPDP appeared to be the most susceptible position of enzymatic attack for all the fungal strains screened in this study. It appears that alkyl-side-chain oxidation is the favored pathway in comparison to aromatic-ring hydroxylation. Similar findings were previously reported for the fungal metabolism of 1- and 2-methylnaphthalene (5) and the degradation of tricresyl phosphate to *p*-hydroxybenzoic acid in activated sludge (17). Surprisingly, TPP was formed from BPDP by many of the fungi. Although the ability of microorganisms to completely degrade alkyl side chains on aromatic compounds is uncommon, TPP has been reported as an intermediate from the environmental degradation of isopropylphenyl diphenyl phosphate (11). In that report, demethylated products of isopropylphenyl diphenyl phosphate were detected, suggesting a pathway of stepwise demethylation of the isopropyl moiety. Demethylated intermediates of BPDP were not detected in our investigations, and the mechanism of *tert*-butyl group displacement by fungi is unclear. However, control experiments indicated that TPP did arise from enzymatic degradation of BPDP.

The presence of both substituted and unsubstituted mono- and diaryl metabolites (Fig. 6) indicated that phosphatase cleavage of BPDP occurred. Significant phosphatase activities were observed with *C. elegans* in this study and have been reported for other fungi (9). Phosphotriesterase cleavage of BPDP showed no stereochemical preference, since we isolated metabolites arising from all possible reactions of initial cleavage of BPDP. The low level of phosphotriesterase activity relative to phosphodiesterase and phosphomonoesterase activities suggests that the initial

cleavage of BPDP by a phosphotriesterase occurred so slowly that BPDP was preferentially metabolized by the alkyl-side-chain and aromatic-ring hydroxylation pathways.

Bacteria in fresh water and sediments are capable of degrading some TAPs to carbon dioxide, water, and P_i (11, 27). Although fungi may not directly mineralize BPDP to any significant extent, they are ubiquitous in the environment and may facilitate environmental metabolism of BPDP by producing partially oxidized metabolites which are readily utilized as substrates by indigenous bacteria. In addition, fungal oxidation of BPDP to polar metabolites may also result in more environmental translocation of BPDP because of greater leaching from soil and greater bioavailability to aquatic organisms because of increased water solubility. The toxicity, partitioning coefficients, and bioaccumulation potential of metabolites arising from the microbial oxidation of BPDP are unknown and warrant investigation.

ACKNOWLEDGMENTS

We greatly appreciate the assistance of Dwight W. Miller for NMR spectral measurements and Virginia Calaway for the typing of this manuscript.

LITERATURE CITED

1. **Broadhurst, M. G.** 1972. Use and development of polychlorinated biphenyls. Environ. Health Perspect. **2**:81–102.
2. **Browman, M. G., and M. A. Tabatabai.** 1978. Phosphodiesterase activity of soils. Soil Sci. Soc. Am. J. **42**:284–290.
3. **Cerniglia, C. E.** 1984. Microbial metabolism of polycyclic aromatic hydrocarbons. Adv. Appl. Microbiol. **30**:31–71.
4. **Cerniglia, C. E., J. P. Freeman, and R. K. Mitchum.** 1982. Glucuronide and sulfate conjugation in the fungal metabolism of aromatic hydrocarbons. Appl. Environ. Microbiol. **43**: 1070–1075.
5. **Cerniglia, C. E., K. J. Lambert, D. W. Miller, and J. P. Freeman.** 1984. Transformation of 1- and 2-methylnaphthalene by *Cunninghamella elegans*. Appl. Environ. Microbiol. **47**:111–118.
6. **Daft, J. L.** 1982. Identification of aryl/alkyl phosphate residues in foods. Bull. Environ. Contam. Toxicol. **29**:221–227.
7. **Dollahite, J. W., and K. R. Pierce.** 1969. Neurological disturbances due to triaryl phosphate toxicity. Am. J. Vet. Res. **30**:1461–1464.
8. **Eivazi, F., and M. A. Tabatabai.** 1977. Phosphatases in soils. Soil Biol. Biochem. **9**:167–172.
9. **Garrison, R. G., and W. N. Arnold.** 1983. Cytochemical localization of acid phosphatases in the dimorphic fungus *Sporothrix schenckii*. Curr. Microbiol. **9**:253–257.
10. **Gutfreund, H.** 1972. Enzyme kinetics: steady-state, p. 128–132. *In* Enzymes: physical principles. John Wiley & Sons, Inc., Great Britain.
11. **Heitkamp, M. A., J. N. Huckins, J. D. Petty, and J. L. Johnson.** 1984. Fate and metabolism of isopropylphenyl diphenyl phosphate in freshwater sediments. Environ. Sci. Technol. **18**:434–439.
12. **Huckins, J. N., J. D. Petty, and M. A. Heitkamp.** 1984. Modular containers for microcosm and process model studies on the fate and effects of aquatic contaminants. Chemosphere **13**: 1329–1341.
13. **Johannsen, F. R., P. L. Wright, D. E. Gordon, G. J. Levinskas, R. W. Radne, and P. R. Graham.** 1977. Evaluation of delayed neurotoxicity and dose-response relationships of phosphate esters in the adult hen. Toxicol. Appl. Pharmacol. **41**:291–304.
14. **Johnson, M. K.** 1975. Organophosphorus esters causing delayed neurotoxic effects. Arch. Toxicol. **34**:259–288.
15. **Johnson, M. K.** 1975. The delayed neuropathy caused by some organophosphorus esters: mechanism and challenge. Crit. Rev. Toxicol. **34**:289–316.
16. **Jones, J. G.** 1970. Studies on freshwater bacteria: effect of medium composition and method on estimates of bacterial population. J. Appl. Bacteriol. **33**:679–686.
17. **Ku, Y., and G. H. Alverez.** 1982. Biodegradation of [^{14}C]tri-*p*-cresyl phosphate in a laboratory activated-sludge system. Appl. Environ. Microbiol. **43**:619–622.
18. **Lebel, G. L., D. T. Williams, and F. M. Benoit.** 1981. Gas chromatographic determination of trialkyl/aryl phosphates in drinking water, following isolation using macroreticular resin. J. Assoc. Off. Anal. Chem. **64**:991–998.
19. **Marcelin, G.** 1979. Use of Tenax-GC in single sample analysis of gas-liquid mixtures. J. Chromatogr. **174**:208–211.
20. **Mayer, F. L., W. J. Adams, M. T. Finley, P. R. Michael, P. M. Mehrle, and V. W. Saeger.** 1981. Phosphate ester hydraulic fluids: an aquatic assessment of Pydrauls 50E and 115E, p. 103–113. *In* D. R. Branson and K. L. Dickson (ed.), Aquatic toxicology and hazard assessment: fourth conference. American Society for Testing and Materials, Philadelphia.
21. **Mid-West Research Institute.** 1979. Assessment of the need for limitation on triaryl and trialkyl/aryl phosphates. Report prepared for the U.S. Environmental Protection Agency, contract no. 68-01-4313. Mid-West Research Institute, Kansas City, Mo.
22. **Mills, J., and H. O. W. Eggins.** 1974. The biodeterioration of certain plasticizers by thermophilic fungi. Int. Biodeterior. Bull. **10**:39–44.
23. **Muir, D. C. G.** 1984. Phosphate esters, p. 41–64. *In* O. Hunziger (ed.), The handbook of environmental chemistry. Springer-Verlag, Berlin.
24. **Muir, D. C. G., A. L. Yarechewski, and N. P. Grift.** 1983. Environmental dynamics of phosphate esters. III. Comparison of the bioaccumulation of four triaryl/phosphates by fish. Chemosphere **12**:155–166.
25. **Pickard, M. A., J. A. Whelihan, and D. W. S. Westlake.** 1974. Utilization of triaryl phosphates by a mixed bacterial population. Can. J. Microbiol. **21**:140–145.
26. **Rushing, L. G., J. R. Althaus, and H. C. Thompson.** 1982. Simultaneous determination of trimellitic anhydride and its trimellitic acid impurity by GC/FID. J. Anal. Toxicol. **6**:290–293.
27. **Saeger, V. W., O. Hicks, R. G. Kaley, P. R. Michael, J. P. Mieure, and E. S. Tucker.** 1979. Environmental fate of selected phosphate esters. Environ. Sci. Technol. **13**:840–844.
28. **Turner, B. C., and D. E. Glotfelty.** 1977. Field air sampling of pesticide vapors with polyurethane foam. Anal. Chem. **49**:7–10.