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Metabolism of Diethyl Ether and Cometabolism of Methyl *tert*-Butyl Ether by a Filamentous Fungus, a *Graphium* sp.

LINDA K. HARDISON, SADIE S. CURRY, LYNDA M. CIUFFETTI, AND MICHAEL R. HYMAN*

Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331-2902

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In this study, evidence for two novel metabolic processes catalyzed by a filamentous fungus, *Graphium* sp. strain ATCC 58400, is presented. First, our results indicate that this *Graphium* sp. can utilize the widely used solvent diethyl ether (DEE) as the sole source of carbon and energy for growth. The kinetics of biomass accumulation and DEE consumption closely followed each other, and the molar growth yield on DEE was indistinguishable from that with *n*-butane. *n*-Butane-grown mycelia also immediately oxidized DEE without the extracellular accumulation of organic oxidation products. This suggests a common pathway for the oxidation of both compounds. Acetylene, ethylene, and other unsaturated gaseous hydrocarbons completely inhibited the growth of this *Graphium* sp. on DEE and DEE oxidation by *n*-butane-grown mycelia. Second, our results indicate that gaseous *n*-alkane-grown *Graphium* mycelia can cometabolically degrade the gasoline oxygenate methyl *tert*-butyl ether (MTBE). The degradation of MTBE was also completely inhibited by acetylene, ethylene, and other unsaturated hydrocarbons and was strongly influenced by *n*-butane. Two products of MTBE degradation, *tert*-butyl formate (TBF) and *tert*-butyl alcohol (TBA), were detected. The kinetics of product formation suggest that TBF production temporally precedes TBA accumulation and that TBF is hydrolyzed both biotically and abiotically to yield TBA. Extracellular accumulation of TBA accounted for only a maximum of 25% of the total MTBE consumed. Our results suggest that both DEE oxidation and MTBE oxidation are initiated by cytochrome P-450-catalyzed reactions which lead to scission of the ether bonds in these compounds. Our findings also suggest a potential role for gaseous *n*-alkane-oxidizing fungi in the remediation of MTBE contamination.

The ether bond (C—O—C) occurs in a wide range of compounds, of which many are considered to be poorly biodegradable in the environment (43). These compounds range from the natural product lignin to simpler and widely used anthropogenic chemicals, including several pesticides (2), common solvents such as diethyl ether (DEE) (2), and, more recently, gasoline oxygenates such as methyl *tert*-butyl ether (MTBE) (23, 45). Currently, very little is known about the microbial degradation of simple alkyl ethers. For instance, until very recently, there were only two reports (14, 28) of microorganisms that utilize DEE as a growth-supporting substrate. Both of these organisms were identified as actinomycetes, although little is known about the enzymes involved in DEE oxidation. The growth of methane-oxidizing bacteria on dimethyl ether has also been previously reported (13, 44). Methane-oxidizing bacteria are known to oxidize and O-dealkylate several alkyl and aromatic ethers in vivo (7, 18). In vitro, the purified soluble form of methane monooxygenase oxidizes DEE to equimolar concentrations of ethanol and acetaldehyde and dimethyl ether is oxidized to methanol and formaldehyde (30), two intermediates in the methane oxidation pathway. However, studies claiming the growth of methane-oxidizing bacteria on dimethyl ether have been criticized because of contamination of this compound by the growth substrate methanol (39).

Unfortunately, the possibility of growth-supporting contaminants or the effects of ethers on enzyme induction have not been adequately considered in previous studies of DEE-utilizing bacteria (14, 28), and the importance of these factors has been well illustrated by two recent studies (16, 41). For example, it has been demonstrated that the growth of *Ancyclobacter aquaticus* in the presence of 2-chloroethyl vinyl ether is supported by abiotic hydrolysis products of ether rather than by the ether compound itself (41). Similarly, it has been recently reported that *Burkholderia cepacia* G4 cannot grow on DEE, whereas a mutant strain, G4/PR1, that constitutively expresses the di-iron toluene 2-monooxygenase grows well on this compound (16). Interestingly, the purified toluene 2-monooxygenase from strain G4 oxidizes DEE to equimolar concentrations of ethanol and acetaldehyde (16). This observation suggests that the different abilities of G4 and G4/PR1 to grow on DEE are due to the inability of DEE to induce the correct enzyme systems for its metabolism in strain G4.

Unlike DEE, which is used as an industrial solvent, MTBE is widely used in many modern gasoline formulations. MTBE acts both as an octane enhancer and as an oxygenating compound, thereby allowing both the elimination of alkyl-lead antiknocking agents and reductions in automobile carbon monoxide emissions (19, 36). MTBE consumption in the United States, the world's largest MTBE consumer, was recently estimated at approximately 2.0×10^{10} gallons/year (1). The long-term human health effects of MTBE exposure are unclear. The U.S. Environmental Protection Agency has issued a draft drinking water lifetime advisory for MTBE of 20 to 200 μg/liter (40), a range of values which reflects the current uncertainty about the carcinogenicity of this compound. Recently, MTBE has been detected in many urban groundwater supplies, most likely as the result of gasoline spills and leaking storage tanks (37). Recent studies also indicate that MTBE is poorly biodegradable in groundwater under a variety of redox conditions (23, 45). The slow metabolism of MTBE has been described for mixed microbial cultures in processes thought to involve *tert*-butyl alcohol (TBA) as an intermediate (29, 34). There has also been one recent report of MTBE metabolism

* Corresponding author. Mailing address: Department of Botany and Plant Pathology, 2082 Cordley Hall, Oregon State University, Corvallis, OR 97331-2902. Phone: (541) 737-2468. Fax: (541) 737-3573. E-mail: hymanm@bcc.orst.edu.

by pure bacterial cultures (22), but the rates of MTBE degradation were also slow and the organisms appeared to require the presence of yeast extract to effect MTBE consumption. Another recent report (38) indicates that several propane-oxidizing bacteria, including *Mycobacterium vaccae* JOB5, are capable of oxidizing high concentrations of MTBE during growth in the presence of propanol.

Although many eukaryotes are known to utilize long-chain hydrocarbons as carbon and energy sources, *Graphium* sp. strain ATCC 58400 is one of the few eukaryotes known to grow on gaseous *n*-alkanes (10–12, 21). Substrate utilization studies with this species and other physiologically related fungi suggest a conventional *n*-alkane oxidation pathway that involves alcohol, aldehyde, and fatty acid intermediates (12, 25, 46). Again analogous with other fungi, the initial hydroxylation of *n*-alkanes by the *Graphium* sp. is likely catalyzed by an inducible cytochrome P-450 enzyme (35). Support for this is also provided by our observation (8) that acetylene, ethylene, and other unsaturated gaseous hydrocarbons all prevent the growth of the *Graphium* sp. on gaseous *n*-alkanes but do not affect its growth on substrates which do not require *n*-alkane-oxidizing activity, such as potato dextrose broth (PDB), or likely *n*-alkane oxidation products, such as ethanol or acetate. While many microbial non-heme-containing oxygenases are known to be inactivated by acetylene and other *n*-alkynes (17), the only monooxygenases currently known to be inactivated by both acetylene and gaseous *n*-alkenes, including ethylene, are cytochrome P-450-type monooxygenases (27).

Previous studies have shown that mammalian cytochrome P-450s can hydroxylate gaseous *n*-alkanes (33) and can O-dealkylate both DEE (5, 6) and MTBE (4, 24). In view of the likely involvement of a cytochrome P-450 in *n*-alkane oxidation by the *Graphium* sp., we were interested in determining whether this fungus also oxidizes DEE and MTBE. A demonstration of these activities in a microorganism would provide important insights into possible environmental fates for these compounds. Here, we describe the results of a study of the degradation of DEE and MTBE by the *Graphium* sp. Our results demonstrate that DEE is utilized as a growth-supporting substrate but that MTBE is only partially oxidized in a cometabolic reaction. Our results also suggest that the initial oxidation of DEE, MTBE, and gaseous *n*-alkanes are all catalyzed by the same cytochrome P-450 enzyme and that alcohol dehydrogenase activity is involved in the further transformation of the initial oxidation products of each substrate.

MATERIALS AND METHODS

Materials. *Graphium* sp. strain ATCC 58400 was obtained from the American Type Culture Collection (Rockville, Md.). DEE (99.9%; spectrophotometric grade), MTBE (99.8%), TBA (>99%), *tert*-butyl formate (TBF; 99%), and calcium carbide (ca. 80%; for acetylene generation) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis). All gases were of the highest purity available, and all other chemicals were at least of reagent grade.

Growth of the *Graphium* sp. Stock cultures of the *Graphium* sp. were maintained on potato dextrose agar plates at 25°C under constant illumination. Conidia were harvested from mycelia grown for 6 to 9 days on potato dextrose agar plates and were used to inoculate both liquid suspension cultures and filter-attached cultures (see below). Liquid suspension cultures were grown axenically in 600-ml glass bottles (Wheaton Scientific, Millville, N.J.). The growth medium (100 ml) was either PDB (24 g/liter) or, for growth on hydrocarbons, a mineral salts medium (MSM) (8). Bottles were inoculated with conidia (10^6) and then sealed with screw caps fitted with butyl rubber septa (Wheaton Scientific). DEE was added to cultures from a saturated aqueous solution made with sterile MSM. The solubilities of DEE and MTBE in saturated aqueous solution at 25°C were taken as 0.815 (42) and 0.544 (45) M, respectively. Gaseous hydrocarbon substrates and inhibitors were added to the bottles as an overpressure by using syringes fitted with sterile filters (0.25-μm pore size). Liquid suspension cultures were incubated for 4 (PDB) or 7 (MSM) days at 24°C in an orbital shaker (125 rpm). For experiments with liquid suspension-grown mycelia, cultures were harvested by gentle vacuum filtration and washed with MSM (three times with 100 ml each). Then mycelia were placed in a fresh culture bottle and resuspended with fresh MSM (50 ml). The bottles were then sealed with screw caps fitted with butyl rubber septa.

A complication observed with cultures grown in liquid suspension was that it was difficult to completely remove the residual growth medium during vacuum-based washing without damage to mycelia. We found that we were able to completely eliminate residual growth substrates by culturing the *Graphium* sp. attached to a solid substrate. For this method, an aqueous suspension (400 μl) of conidia (2.5×10^6/ml) was pipetted onto sterile glass fiber GF/A filters (7 by 4 cm) (Whatman Ltd., Maidstone, England) that had been wetted with either MSM or PDB. The inoculated filters were then placed on a sterile blotting paper wick saturated with either MSM or PDB. The filter and wicks were placed in sterile petri dishes, which were then put in sealed containers (ca. 2 liters). For mycelia grown on MSM and gaseous hydrocarbons, substrates were added to the gas phase of the sealed containers to an initial concentration of approximately 10% (vol/vol; gas phase). For mycelia grown on PDB, the gas phase in the sealed containers was air. Regardless of the growth substrate, the containers were incubated for 4 days at 25°C under constant illumination. As required for experiments, filter-attached mycelia were removed from the growth container and shaken gently in air to remove residual gaseous substrates. The filters were placed directly into glass serum vials (120 ml), which were then sealed with butyl rubber stoppers and aluminum crimp seals (Wheaton Scientific).

Cell extracts and enzyme assays. Cell extracts of mycelia were prepared by the method of Onodera et al. (26). Two to six grams of mycelia from 5-day liquid suspension cultures was harvested and washed by vacuum filtration. Mycelia were transferred to an Omni-mixer chamber (Sorvall, Newtown, Conn.) and homogenized for 90 s with 5 ml of buffer (10 mM K_2HPO_4 [pH 7.0]) containing mannitol (0.3 M), β-mercaptoethanol (4 mM), and $MgCl_2$ (2 mM) at 4°C. The homogenate was filtered through Miracloth (Calbiochem, La Jolla, Calif.) and centrifuged for 10 min at $1,000 \times g$ at 4°C. The supernatant was centrifuged again for 20 min at $20,000 \times g$ at 4°C, followed by ultracentrifugation in a Beckman Ti 70.1 rotor for 90 min at $105,000 \times g$ at 4°C. The alcohol dehydrogenase activity of the final supernatant was determined spectrophotometrically by monitoring acetaldehyde-dependent NADH oxidation (31).

Analytical procedures. The degradation of *n*-alkanes, DEE, and MTBE and the accumulation of products were monitored with a gas chromatograph (model GC-8A; Shimadzu, Kyoto, Japan) fitted with a flame ionization detector. A stainless steel column (0.3 by 122 cm) filled with Porapak Q (60/80 mesh) (Waters Associates, Framingham, Mass.) was utilized at a temperature of 170°C, with a detector temperature of 200°C. Nitrogen was used as the carrier gas. The gas chromatograph was interfaced to an integrator (model C-R3A; Shimadzu). In all experiments, gas-phase (100-μl) or liquid-phase (3-μl) samples were taken and analyzed directly without further sample preparation. The products generated from DEE degradation and MTBE degradation were identified by coelution with authentic standards. The identities of MTBE oxidation products were further confirmed by computer matching of the product mass spectra with a reference library. The mass spectra of MTBE oxidation products were obtained by using a Finnigan 4000 gas chromatograph-mass spectrometer with a Varian 3400 gas chromatograph, coupled to a Galaxy 2000 data system. The mass spectrometer was operated in electron impact mode (70 eV) with a source temperature of 140°C. A DB-1 column (0.32 mm by 30 m) was used with a temperature ramp of 2°C/min from 50 to 200°C and a detector temperature of 250°C.

The yields of mycelia were determined from dry weight measurements (8). Liquid suspension cultures were vacuum filtered onto paper filters which had been previously dried and weighed. Mycelia and filters were dried at 65°C for 24 h and reweighed. For filter-attached mycelial cultures, the GF/A filters were similarly dried and weighed.

The protein concentrations of mycelial cell extracts were determined by the method of Bradford (3) with bovine serum albumin as the standard.

RESULTS

We considered it likely that DEE would support the growth of the *Graphium* sp., as an initial O-dealkylation reaction would be expected to produce ethanol and acetaldehyde (6), the same intermediates thought to be sequentially generated during metabolism of the established growth substrate ethane. We observed that mycelial biomass production correlated well with the time course of DEE consumption in batch cultures of the *Graphium* sp. when DEE was present as the sole source of carbon and energy (Fig. 1). In contrast, neither biomass accumulation nor DEE consumption occurred in the presence of acetylene (Fig. 1). Similar experiments established that the growth of the *Graphium* sp. on DEE was also completely inhibited by the same concentrations of ethylene, propylene, propyne, and *n*-butyne (0.5% [vol/vol]; gas phase) which inhibit the growth of the *Graphium* sp. on gaseous *n*-alkanes (8) (data not shown). In contrast to the abundant growth on DEE, we

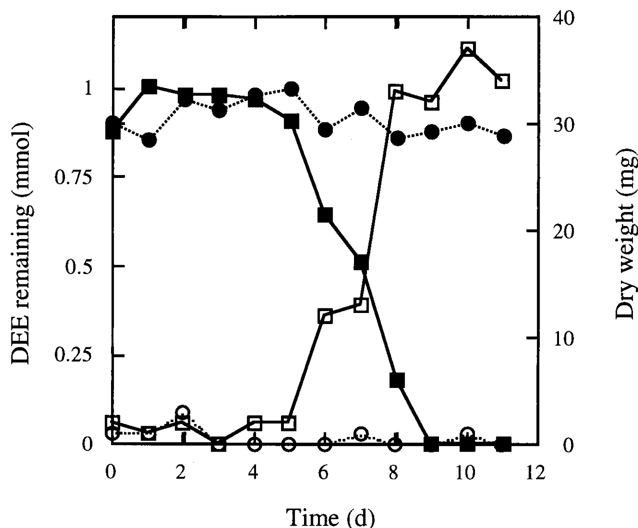

FIG. 1. Time course of DEE consumption and biomass production by liquid suspensions of the *Graphium* sp. Cultures of the *Graphium* sp. were grown in 24 glass bottles (600 ml) sealed with butyl rubber-lined screw caps. Each bottle contained MSM (100 ml), DEE (1 mmol), and conidia (10^6). Acetylene (0.5% [vol/vol]; gas phase) was added to 12 bottles. At the indicated times, the DEE contents for one bottle with and one bottle without acetylene were determined by gas chromatography. The mycelia from these bottles were then harvested to determine biomass production. Shown are the time courses of DEE consumption (closed symbols) and biomass production (open symbols) for conidia incubated with DEE in the presence (circles) and absence (squares) of acetylene. d, days.

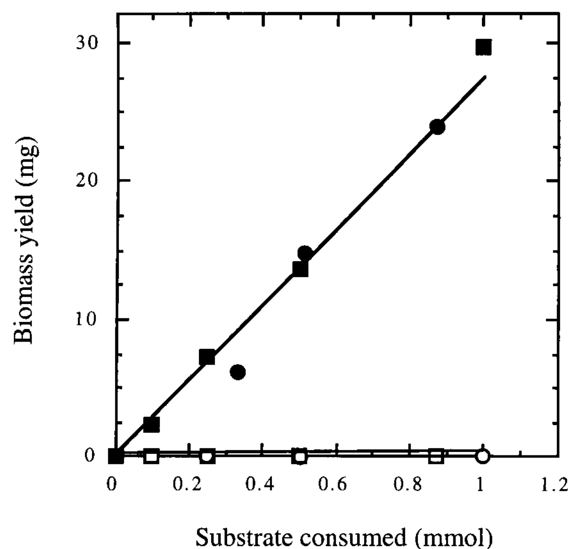

FIG. 2. Growth yield of the *Graphium* sp. on DEE and *n*-butane. The yields of mycelia from conidia incubated with DEE (■) and *n*-butane (●) were determined from dry weight determinations made after complete consumption of the added growth substrate had been confirmed by gas chromatography. The biomass yields obtained for conidia incubated with acetylene (0.5% [vol/vol]; gas phase) and either DEE (□) or *n*-butane (○) are also shown.

were unable to grow the *Graphium* sp. on other ether-bonded compounds, including 2-ethoxyethanol, *n*-propyl ether, and MTBE.

The molar growth yield for the *Graphium* sp. on DEE was determined by using carbon-limited batch cultures in which the biomass was determined after the complete consumption of added DEE had been confirmed by gas chromatography. This yield (28.5 g of biomass/mol of substrate consumed) was indistinguishable from that determined for *n*-butane (Fig. 2). We also observed that filter-attached *n*-butane-grown mycelia were able to immediately oxidize DEE and that this reaction was inhibited by acetylene (Fig. 3). The maximum rate of DEE oxidation in this experiment was 85 nmol of DEE oxidized/h/mg (dry weight). Aqueous suspensions of *n*-butane- and DEE-grown mycelia also oxidized DEE (10 μmol) at very similar initial (0 to 1 h) rates of 92 ± 24 and 90 ± 35 nmol/h/mg (dry weight), respectively. Similar levels of alcohol dehydrogenase activity were also detected in cell extracts of these mycelia (1,065 ± 15 and 590 ± 160 nmol of NADH oxidized/min/mg of protein for DEE- and *n*-butane-grown mycelia, respectively). In contrast, DEE was not oxidized by PDB-grown mycelia and extracts of these mycelia did not exhibit significant alcohol dehydrogenase activity ($<$20 nmol of NADH oxidized/min/mg of protein). In experiments with aqueous suspensions of DEE-oxidizing mycelia, we did not detect the extracellular accumulation of any organic oxidation products from DEE when liquid samples were analyzed by gas chromatography. However, we occasionally observed the transient appearance of low concentrations of both ethanol and acetaldehyde in gas-phase samples taken during reactions in which filter-attached, *n*-butane-grown mycelia were exposed to DEE (data not shown).

As indicated above, we were unable to grow the *Graphium* sp. with MTBE as the sole source of carbon and energy. However, filter-attached, *n*-butane-grown mycelia were capable of degrading low concentrations of MTBE (ca. 750 ppb) (Fig. 4), whereas PDB-grown mycelia did not exhibit this activity (data not shown). The consumption of MTBE by *n*-butane-grown mycelia was fully inhibited by the same concentration of acetylene (Fig. 4), ethylene, and all other unsaturated hydrocarbons which inactivate *n*-alkane-oxidizing activity (data not

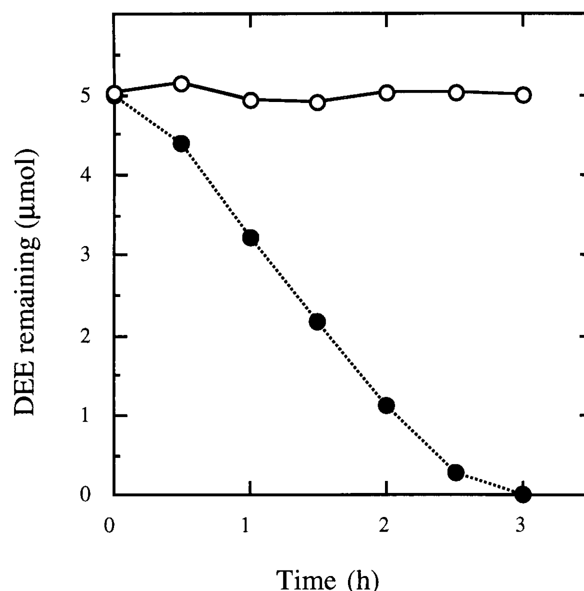

FIG. 3. Degradation of DEE by filter-attached, *n*-butane-grown *Graphium* mycelia. Filter-attached mycelia were grown on *n*-butane, as described in Materials and Methods. Mycelia (20 ± 2.5 mg [dry weight]) were incubated in a sealed glass serum vial (120 ml). The reactions were initiated by the addition of DEE (5 μmol), and 100-μl gas-phase samples were removed and analyzed by gas chromatography. Shown are the time courses of DEE consumption for mycelia incubated with DEE in the presence (○) or absence (●) of acetylene (0.5% [vol/vol]; gas phase).

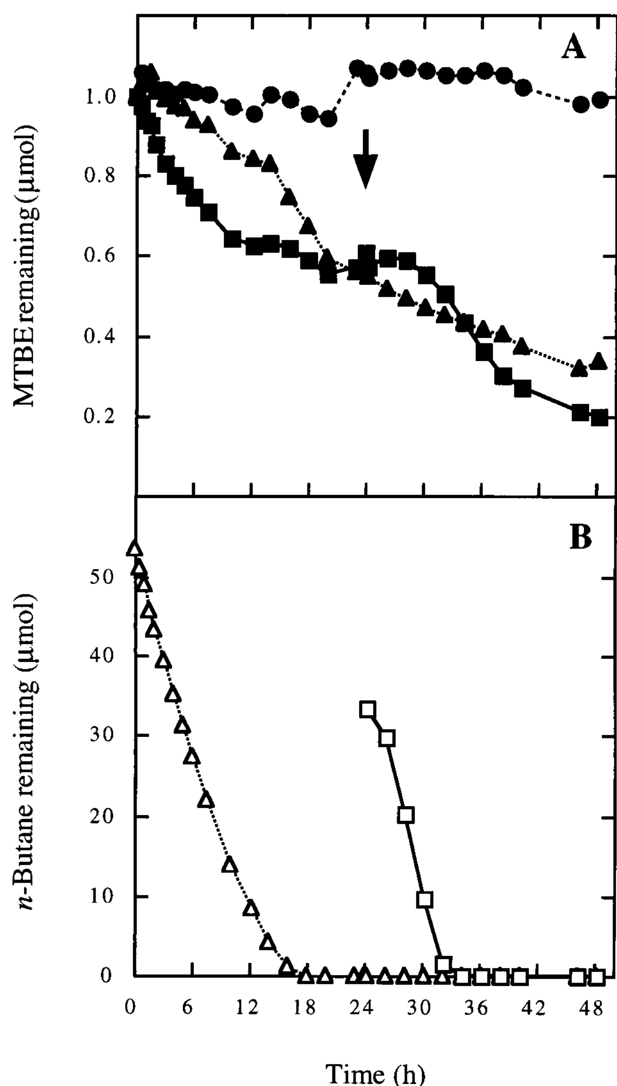

FIG. 4. Cometabolic degradation of MTBE by filter-attached, *n*-butane-grown *Graphium* mycelia. Filter-attached *Graphium* mycelia were grown on *n*-butane, as described in Materials and Methods. The filters with attached mycelia (40 ± 3 mg [dry weight]) were transferred to glass vials (120 ml), which were sealed with butyl rubber stoppers and aluminum crimp seals. The reactions were initiated by the addition of MTBE (1 μmol) from a saturated aqueous solution. (A) Time course for MTBE degradation for mycelia incubated with MTBE and acetylene (0.5% [vol/vol]; gas phase) (●), MTBE and *n*-butane (1.0% [vol/vol]; gas phase) (▲), and MTBE alone (■), with *n*-butane (0.5% [vol/vol]; gas phase) added after 24 h. The arrow indicates the point at which *n*-butane was added during incubation. (B) Corresponding time course for *n*-butane consumption for mycelia incubated with MTBE and *n*-butane (1.0% [vol/vol]; gas phase; added at 0 h) (△) and with MTBE and *n*-butane (0.5% [vol/vol]; gas phase) added after 24 h (□).

shown). These effects indicate that the consumption of MTBE can be attributed to specific enzyme activities rather than to nonspecific or abiotic processes such as absorption. The presence of *n*-butane also affected the rate of MTBE consumption (Fig. 4). For mycelia initially incubated with both *n*-butane and MTBE, the initial rate of MTBE consumption (0 to 2 h) was 0.6 nmol/h/mg (dry weight) (Fig. 4A). This rate of consumption was maintained fairly consistently throughout the experiment, even though the added *n*-butane was consumed within 18 h (Fig. 4B). For mycelia initially incubated with MTBE alone, the initial rate of MTBE consumption was faster (1.9 nmol/h/mg [dry weight]) than that observed for mycelia initially incubated with both *n*-butane and MTBE. Despite this, the rate of MTBE consumption for these *n*-butane-deprived mycelia declined to close to zero after 18 h. However, MTBE consumption was reinitiated in these mycelia by the addition of *n*-butane (Fig. 4A). This addition also led to a rapid rate of *n*-butane consumption (Fig. 4B), which indicates that the previous decline in MTBE consumption was not due to a toxic effect of this compound.

We also examined the possible accumulation of MTBE oxidation products in experiments with filter-attached, *n*-butane-grown mycelia. In these experiments, we consistently detected two products which were initially identified as TBF and TBA by coelution with the authentic compounds during gas chromatography. The identities of these compounds were subsequently verified by gas chromatography-mass spectrometry by matching the mass spectra of the products with reference spectra, as described in Materials and Methods. The kinetics of TBF production and TBA production during MTBE consumption were investigated, and our results indicate that TBF production accounted for 88% of MTBE consumption during the first 2 h of the reaction (Fig. 5). The quantity of detected TBF reached a maximum after 4 h and then declined slowly over the following 24 h of incubation. In contrast, TBA was not detected during the first 3 h of incubation, but it subsequently accumulated at a nearly constant rate over the remainder of the incubation (Fig. 5). During the early stages of the experiment (0 to 4 h), the rate of MTBE consumption was 4.5 nmol/h/mg (dry weight). This rate declined to 1.3 nmol/h/mg (dry weight) toward the end of the experiment (24 to 29 h), and this rate compared to a rate of TBA production of 0.47 nmol/h/mg (dry weight) over the same period. After 29 h, the combined accumulation of TBF and TBA accounted for approximately 30% of the total quantity of MTBE consumed.

The kinetics of TBF and TBA accumulation observed in the experiment described above (Fig. 5) suggested that TBF underwent further transformations and could be a precursor to TBA. We subsequently investigated the potential for hydrolytic transformation of TBF in our experimental system. The rate of hydrolysis of TBF was determined with incubation mixtures containing either sterile filters wetted with MSM or filters with attached mycelia grown on either *n*-butane or PDB. A comparison of the wet and dry weights indicated that all of the filters contained similar quantities of water (2.2 ± 0.2 g). The reactions were initiated by the addition of TBF (10 μmol) to each vial, and the rate of TBF disappearance was monitored by gas chromatography over 8 h. In all cases, we observed that TBF was consumed and that TBA was detected as the major reaction product. In the absence of mycelia, TBF was hydrolyzed at a rate of 79 nmol/h/g of water. The presence of mycelial biomass increased this rate by 81 and 8 nmol/h/mg (dry weight) for *n*-butane- and PDB-grown mycelia, respectively.

We also investigated whether other growth substrates allowed the *Graphium* sp. to exhibit MTBE-degrading activity. Propane-grown mycelia were able to oxidize MTBE with a higher specific activity (4.5 nmol/h/mg [dry weight]) than that of *n*-butane-grown mycelia (Fig. 4) when they were incubated in gas-phase reaction mixtures with an equivalent quantity of MTBE (1 μmol = ca. 750 ppb) (data not shown). The maximum initial (0 to 3 h) rate of MTBE degradation (10.5 nmol/h/mg [dry weight]) we observed was with liquid suspensions of *n*-butane-grown mycelia incubated with 10 μmol (approximately 200 μM MTBE in solution) (Fig. 6). In this experiment, TBA was the sole MTBE oxidation product detected, although the kinetics of TBA accumulation and the total quantity of

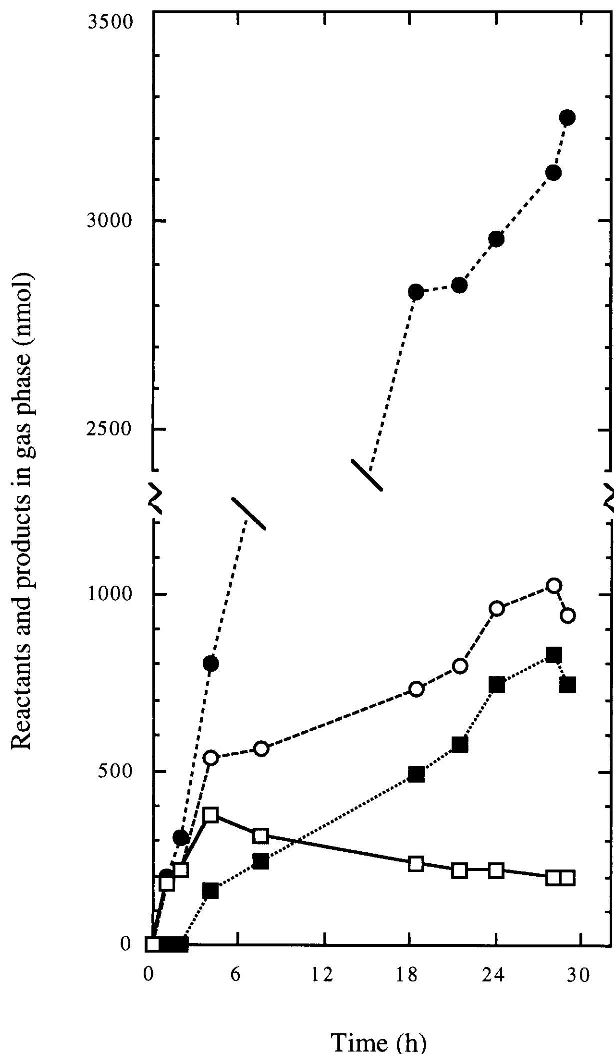

FIG. 5. Time courses for TBF production and TBA production during MTBE degradation by filter-attached, *n*-butane-grown *Graphium* mycelia. Filter-attached *Graphium* mycelia were grown on *n*-butane, as described in Materials and Methods. Mycelia (44 mg [dry weight]) were incubated in a sealed glass serum vial (120 ml). The reactions were initiated by the addition of MTBE (6 μmol) from a saturated aqueous solution. At the indicated times, samples (100 μl) of the gas phase were removed and immediately analyzed by gas chromatography. Shown are the time courses for accumulation of TBF (□) and TBA (■) in the gas phase. The combined quantities of TBF and TBA detected (○) and the total quantity of MTBE degraded (●) are also shown.

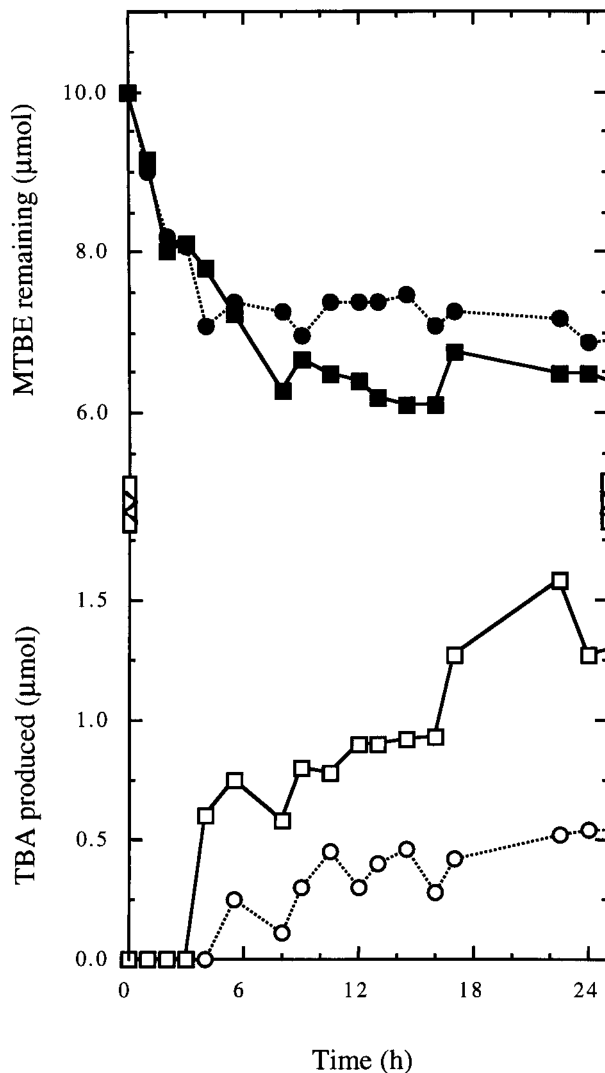

FIG. 6. Time courses for MTBE consumption and TBA production by liquid suspensions of *Graphium* mycelia grown on *n*-butane or DEE. *Graphium* mycelia were grown in liquid culture on either *n*-butane or DEE, as described in Materials and Methods. The harvested mycelia (50 and 55 mg [dry weight] for *n*-butane- and DEE-grown mycelia, respectively) were resuspended in MSM (50 ml) in glass bottles (600 ml) sealed with screw caps and butyl rubber septa. The reactions were initiated by the addition of MTBE (10 μmol) from an aqueous saturated solution. The consumption of MTBE and the production of TBA were determined from liquid-phase samples (3 μl) immediately analyzed by gas chromatography. Shown are the time course for MTBE consumption (closed symbols) and the corresponding time course for TBA accumulation (open symbols) in the reaction medium for *n*-butane-grown (squares) and DEE-grown (circles) mycelia.

degraded MTBE accounted for by TBA production were very similar to those observed in the previous experiment with filter-attached mycelia (Fig. 5). In this experiment, we also observed that mycelia grown on DEE were able to oxidize MTBE at rates very similar to those of *n*-butane-grown mycelia (Fig. 6). For DEE-grown mycelia, TBA was also detected as the sole MTBE oxidation product, although in this instance total TBA accumulation accounted for only approximately 12% of the total quantity of MTBE degraded after 24 h.

DISCUSSION

Our results with DEE and MTBE in this study suggest that these compounds are initially oxidized by the same enzyme in the *Graphium* sp. and that these two reactions are distinguished by the fact that the organism can grow on the products of one reaction, but not the other. In the case of DEE, our results suggest that this compound is used directly as a growth-supporting substrate because biomass production correlated well with both the kinetics (Fig. 1) and quantity (Fig. 2) of DEE consumption. The identical sensitivities of *n*-butane- and DEE-oxidizing activities to inhibition by unsaturated hydrocarbons and the ability of *n*-butane-grown mycelia to immediately oxidize DEE (Fig. 3) argue that both substrates are initially oxidized by very similar enzymes, if not the same enzyme. Our inability to detect the extracellular accumulation of DEE oxi-

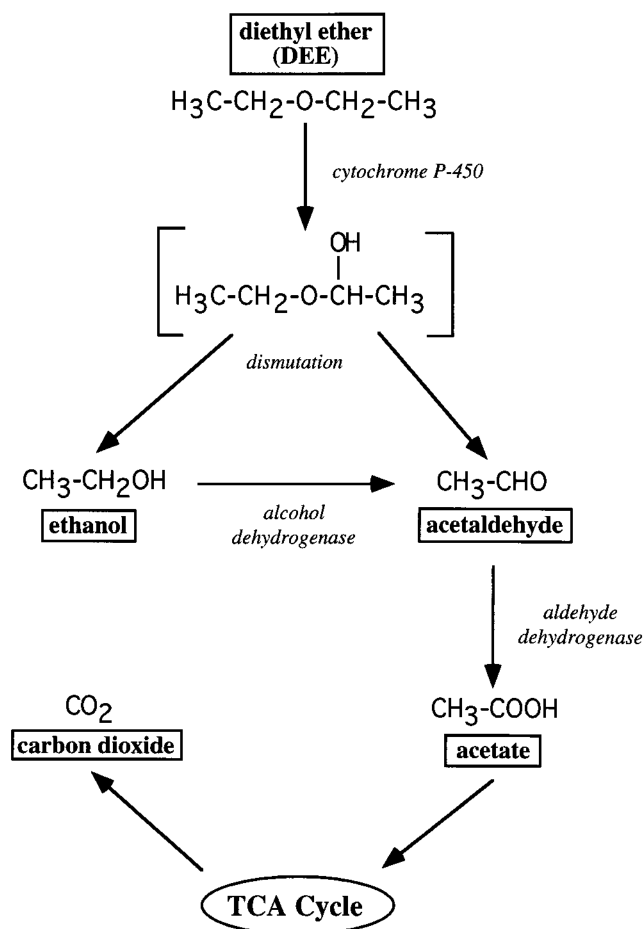

FIG. 7. Proposed pathway for the metabolism of DEE. Brackets indicate the proposed intermediate. TCA, tricarboxylic acid.

dation products in reactions involving aqueous suspensions of *n*-butane-grown mycelia also suggests that these compounds are rapidly oxidized by preexisting dehydrogenases and that the pathways of DEE oxidation and gaseous *n*-alkane oxidation are therefore very similar. The essentially identical molar growth yields (Fig. 2) and alcohol dehydrogenase activities observed for DEE- and *n*-butane-grown mycelia further support this argument. Our inability to grow the *Graphium* sp. on 2-ethoxyethanol, a hydroxylation product rather than an O-dealkylation product of DEE, also further supports our hypothesis that DEE oxidation by the *Graphium* sp., like those of several bacterial systems (16, 30), involves an initial O-dealkylation reaction which generates ethanol and acetaldehyde (Fig. 7).

In considering other possible pathways to account for the growth of the *Graphium* sp. on DEE, it is important that the inhibitory effects of acetylene on mycelial growth in the presence of DEE (Fig. 1) eliminate the possibility that growth was supported by contaminants such as ethanol or acetate, since we have previously established that acetylene does not affect growth on these substrates (8). However, it is not possible for us to eliminate all possible roles for contaminants like ethanol in DEE-dependent growth of the *Graphium* sp. For example, it is known that cytochrome P-450 is induced in mammalian systems by both DEE and ethanol (5). Our present results do not allow us to determine whether the putative cytochrome P-450 activity observed in DEE-grown *Graphium* mycelia is induced by DEE itself, by trace ethanol contamination in DEE, or by ethanol generated from DEE oxidation by low levels of constitutive cytochrome P-450 activity. We considered a possible inductive effect of ethanol in other experiments in this study and demonstrated that the addition of trace levels of ethanol (2.5 μmol) to aqueous suspensions (100 ml) of conidia incubated with *n*-propyl ether (1 mmol) did not initiate growth on this ether. Notably, neither a potential inductive effect of ethanol contamination in DEE nor growth on contaminants has been addressed in most previous studies of microbial DEE utilization (14, 28).

In contrast to DEE, we conclude that MTBE degradation by *n*-alkane-grown *Graphium* mycelia is a cometabolic process (9, 15) in which MTBE is fortuitously oxidized by the same enzyme used to initiate *n*-alkane oxidation and DEE oxidation. This conclusion is supported by several results. First, MTBE consumption occurred immediately after the exposure of *n*-alkane-grown (Fig. 4) and DEE-grown (Fig. 6) mycelia to MTBE and did not occur in PDB-grown mycelia. This indicates that MTBE-consuming activity is selectively expressed when gaseous *n*-alkanes or DEE is used as the growth substrate(s). Second, MTBE consumption was inhibited by the same concentrations of unsaturated hydrocarbons which inhibit *n*-alkane oxidation and DEE oxidation (Fig. 4). As our results with DEE also suggest that DEE and gaseous *n*-alkanes are initially oxidized by the same enzyme, our overall conclusion is that DEE, MTBE, and gaseous *n*-alkanes are all oxidized by the same putative cytochrome P-450. Third, a significant portion of the products of MTBE degradation was detected extracellularly in the reaction medium (Fig. 5). This is compatible with limited catabolism of MTBE and the inability of the *Graphium* sp. to grow on this compound. Fourth, the rate of MTBE degradation by mycelia initially incubated with both *n*-butane and MTBE was slower than the rate observed for mycelia initially incubated with MTBE alone (Fig. 4). This is compatible with competitive interaction between *n*-butane and MTBE for binding to and oxidation by the same enzyme. Finally, for mycelia initially incubated with MTBE alone, the MTBE degradation rate progressively declined and was then stimulated after the addition of *n*-butane (Fig. 4). This is compatible with progressive exhaustion and subsequent replenishment of reductant required to support cytochrome P-450-catalyzed oxidations. The maximal rate of MTBE degradation observed in this study was approximately 10 nmol/h/mg (dry weight) of mycelia with liquid suspensions of the *Graphium* sp. exposed to a dissolved MTBE concentration of approximately 200 μM (ca. 18 ppm). Although we cannot accurately determine the dissolved concentration of MTBE in our experiments with filter-attached mycelia, the general increase in specific rates of MTBE transformation with increases in the quantity of MTBE added suggests that we have not yet saturated this system with MTBE or determined the maximal rate of MTBE degradation by the *Graphium* sp. This conclusion is also supported by the fact that the K_m for MTBE in mammalian microsomes ranges from 0.7 to 1.4 mM (ca. 60 to 120 ppm), depending on the compound used to induce cytochrome P-450 activities (4).

The principal difference between MTBE degradation and DEE degradation observed in this study is that the *Graphium* sp. apparently cannot utilize the products of MTBE degradation as carbon and energy sources to support growth. In mammalian microsomes, MTBE oxidation is thought to involve an O-dealkylation reaction which generates TBA and formaldehyde as products (4, 24). Like the oxidation of DEE depicted in Fig. 7, this reaction probably involves an initial cytochrome

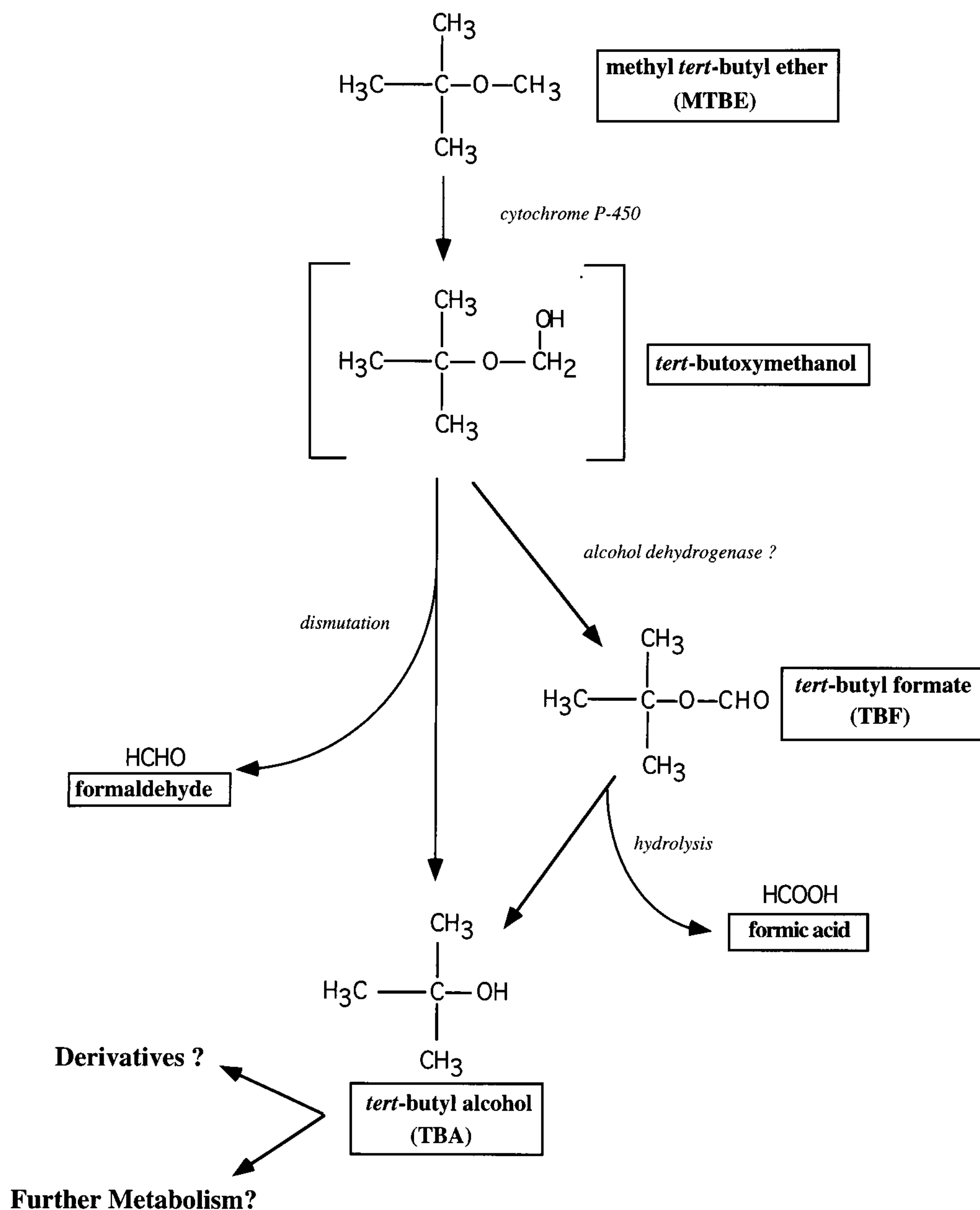

FIG. 8. Proposed pathway for the cometabolism of MTBE by the *Graphium* sp. Brackets indicate the proposed intermediate.

P-450-dependent hydroxylation of the methoxy carbon of MTBE. Analogous to the mechanism described for DEE, the resulting unstable intermediate, *tert*-butoxymethanol, would be expected to dismutate to form the products TBA and formaldehyde (Fig. 8). However, this direct pathway of TBA formation does not agree with our results (Fig. 5), which indicate that TBF, rather than TBA, is the first detected product of MTBE oxidation. A reasonable explanation of our results is that *tert*-butoxymethanol is rapidly oxidized to TBF by the alcohol dehydrogenases we have shown to be present in MTBE-degrading mycelia. We further suggest that the resulting TBF then undergoes both biotic hydrolysis and abiotic hydrolysis to yield TBA and the as-yet-undetected C_1 product formate (Fig. 8).

There are several attractive aspects of this proposed pathway for MTBE oxidation by the *Graphium* sp. First and foremost, it involves reactions which are fully consistent with the respective hydrocarbon- and alcohol-oxidizing activities of cytochrome P-450 and alcohol dehydrogenase. Second, rapid oxidation of *tert*-butoxymethanol to TBF, rather than direct dismutation to TBA and formaldehyde, would account for the delay in TBA production we consistently observed in our experiments (Fig. 5 and 6). Notably, a similarly rapid dehydrogenase-dependent oxidation of ethanol and acetaldehyde also probably explains why these predicted products of DEE oxidation usually did not accumulate extracellularly in our experiments with *n*-butane-grown mycelia. Although our present kinetic evidence certainly suggests that a direct O-dealkylation route to TBA is a minor reaction in the *Graphium* sp., it may

predominate in other MTBE-oxidizing systems, such as mammalian microsomes (4), where the levels of alcohol dehydrogenase activity involved in TBF production are likely to be considerably lower than those in *n*-alkane- and DEE-grown *Graphium* mycelia. However, we also emphasize that our ability to detect TBF as a product of MTBE oxidation was an unforeseen consequence of our use of gas-phase incubations. These reactions have very low water contents, and this may have limited the rate of hydrolysis of TBF so that it could be detected transiently in the gas phase. We also suspect that a rapid rate of hydrolysis, in conjunction with higher detection limits due to direct aqueous injections, prevented us from detecting TBF during reactions involving liquid suspensions of mycelia, even though the kinetics of TBA production were very similar to those observed in gas-phase reactions (Fig. 5 and 6). As previous studies with the degradation of MTBE by mammalian microsomes have also made use of aqueous suspensions (4), the production of TBF may have been overlooked for similar reasons. While the proposed pathway for MTBE degradation can possibly explain the role of TBF in MTBE degradation, it does not describe the fate of the remaining MTBE, which is unaccounted for by TBA accumulation (Fig. 6). Mammalian studies have demonstrated that the products of the tertiary butyl group of MTBE include TBA, 2-methyl-1,2-propanediol, and α-hydroxyisobutyric acid, whereas the products derived from the methoxy carbon include formaldehyde, formate, methanol, and carbon dioxide (24). Although some of these compounds may be generated by the *Graphium* sp., it is also possible that the products of MTBE degradation are not substantially further metabolized. Hydroxylated xenobiotics such as TBA are commonly excreted by fungi as sulfated or O-glucuronidated derivatives (32).

While questions about the proposed pathway of MTBE degradation remain, our findings suggest some approaches which may be used for the remediation of MTBE contamination. Previous studies demonstrating the degradation of MTBE by mixed cultures have stressed the use of this compound as a growth-supporting substrate and have described slow rates of MTBE consumption in the presence of substrate concentrations as high as 200 mg/liter (22, 29, 34). However, current environmental levels of MTBE are often in the microgram/liter range (37) and are unlikely to support significant microbial populations. Together, these features could limit the application of growth-based microbial processes for the remediation of environmental MTBE contamination. In contrast, microbial cometabolic activities are not significantly controlled by the concentration of the target contaminant unless toxic effects that arise from the contaminant are observed. Bioremediation schemes that involve cometabolic processes are therefore often suitable for environments with low concentrations of contaminants. Aerobic cometabolic processes catalyzed by bacteria which express nonspecific oxygenases in response to hydrocarbon growth substrates, such as methane, propane, and toluene, have been utilized for the bioremediation of chlorinated hydrocarbons such as trichloroethylene (20). As filamentous fungi which can utilize gaseous hydrocarbons as growth substrates can be readily isolated from soils (10), this present study suggests that a similar process may be envisioned for MTBE degradation by gaseous *n*-alkane-utilizing fungi either in situ or through more surface-based treatment methods.

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