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Biodegradation of a high molecular weight aliphatic ether – indications of an unusual biodegradation pathway

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Abstract

An aliphatic ether (1-phytanyl-1-octadecanyl-ether) of high molecular weight was used as a sole carbon source in degradation experiments with different aerobic bacteria. The enriched culture B5, obtained from fuel contaminated soils, was able to degrade the substance for more than 90%. A culture of *Rhodococcus ruber* was similarly effective. Detailed investigation of the metabolites allowed us to characterize an unusual degradation pathway via a mid-chain oxidation mechanism ('internal oxidative pathway'). Obviously, formation of intermediate alkenes mainly at the unbranched side chain was a prerequisite for bacterial degradation of the added substrate. Degradation proceeded – in spite of the usually preferred terminal oxidation – via oxidation of the internal double bond and was followed by an ester cleavage. In turn, a series of alcohols was formed which were subsequently oxidized to the respective carboxylic acids and were further metabolized via the normal β-oxidation pathway.

Introduction

The biodegradation of water-insoluble organic compounds of high molecular weight such as waxes, lubricants, rubber, heavy oils, asphaltenes or industrial crude oil residues has been the subject of great interest for many years but has not been studied in detail. Often, these substances are only combusted or disposed of if not needed. A better understanding of the degradation pathways and yields of these organic compounds is needed especially with respect to waste disposal problems. Bacterial deterioration may ideally result in their degradation and the formation of solid material, e.g. polyhydroxyalkanoic acids (PHA) or other 'new' organic compounds of further value.

Bacteria provide some strategies to overcome the natural membrane barrier to use a variety of otherwise recalcitrant substances (see Gottschalk 1986). For the uptake of biopolymers, bacteria produce specific exoenzymes to degrade the polymers into small transportable molecules. In case of hydrocarbons, bacteria form special structures as constituents of their cell walls in which hydrocarbons are dissolved and transported through the membrane. Thus, it is probable that bacteria are also able to degrade other organic substances of higher molecular weight. Some research has been performed on the biodegradability of organic substances like rubber (Keursten & Groenevelt 1996; Tsuchii et al. 1997) and synthetic polymers (e.g. Andrady 1994). However, the biodegradation pathways are not well understood at a molecular level.

In the scope of studies about biodegradation of high molecular weight compounds, we worked with an asymmetric heavy aliphatic ether. The investigated aliphatic ether resembles common important elements of diverse geological macromolecules (Nip et al. 1986; Jenisch et al. 1990, 1997; Derenne et al. 1992; Richnow et al. 1992; Gelin et al. 1996). Thus, the investigation of its biodegradation should give a starting point to comprehend bacterial strategies for the metabolization of complex organic compounds.

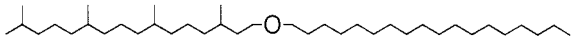

Figure 1. Structure of the aliphatic model compound: a phytanyl ether with a linear C_{18} side chain.

The ether linkage in our aliphatic substrate was considered to have an obstructive effect on biodegradation. Therefore, we expected the formation of intermediate products. Different aerobic bacteria were incubated with the substance as the sole source of carbon and energy. In this paper we present detailed data on metabolites produced during degradation and on the effectiveness of the degradation process. The obtained data are used to characterize a degradation pathway which has been ignored to date but which might not only be important for degradation of high molecular weight organic compounds but also for 'normal' alkanes.

Materials and methods

Model compound

An aliphatic ether was synthesized containing a phytanyl (isoprenoid C_{20}) and a linear (*n*-C_{18}) substituent: 1-(octadecyloxy)-3,7,11,15-tetramethylhexadecane known as 1-phytanyl-1-octadecyl-ether (Figure 1). The compound has a M_r of 550 Da.

Phytol (Merck 97%) dissolved in ethanol was hydrogenated by refluxing with an excess of Raney Nickel (Fluka) under a hydrogen atmosphere (5 hours). After extraction and filtration over silica gel (Merck H60) the resulting phytanol was heated with KOH pellets (4 eq per 1 eq of phytanol) until solubilization. Subsequently, aliquat 336 (Aldrich, 0.1 eq) and 1-octadecylbromid (Aldrich 96%, 2 eq) were added. The mixture was heated under reflux (argon atmosphere; 6 hours). The model compound was purified by column chromatography (silica gel; Merck H60) using *n*-hexane first and subsequently dichloromethane as solvents (thin layer chromatography: R_f 0.28 with *n*-hexane). Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) data indicated that the final product had a purity of 92% with one unsaturated homologue (double bond in the isoprenoidal side as in phytol) and dioctadecylether as by-products.

Microorganisms

Several enriched 'mixed' cultures of microorganisms were obtained from soil samples of sites in Northern Germany contaminated by diesel fuel. The samples were slurried in a carbon-free mineral medium (but with vitamins) according to Brunner (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig; DSMZ No. 457). An aliquot of this suspension was used as an aerobically grown inoculum using the aliphatic ether as the sole carbon source (mineral medium with vitamins and 1.56 mg/ml aliphatic ether; 30 °C). The culture with the highest turbidity by macroscopic and microscopic observation was obtained from a soil polluted with diesel fuel. The hydrocarbon fraction of this soil had already been intensively biodegraded *in situ* as determined by GC-MS measurements. This 'mixed' culture (called B5-enrichment) was used in further degradation experiments.

Monocultures of *R. ruber* (DSMZ No. 7512) or *Pseudomonas putida* (DSMZ No. 6755) were first grown on TSA (trypticase soy agar, DSMZ No. 220) and later on the aliphatic ether with a mineral medium (DSMZ No. 457).

Degradation experiments

The bacteria grown on the aliphatic ether as the sole carbon source were used as inoculum for the degradation experiments. Sterile 100-ml flasks were inoculated with 1 ml of the aliphatic ether dissolved in diethyl ether (stock solution: 30 g aliphatic ether/l). The bottles were covered with cotton-wool to avoid contamination with bacteria and dust and placed under a fume hood. The solvent was allowed to evaporate overnight until dryness and diethyl ether could no longer be smelled. Subsequently, 30 ml of the mineral medium (DSMZ No. 457) containing vitamins and 10 μl of the preculture were added. Each data point was obtained by running the experiments in duplicate. Control experiments were run without bacteria. The flasks were incubated aerobically in the dark at 30 °C in a shaking incubator. At the end of each degradation experiment, the flasks were frozen at −18 °C.

Protein determination

One set of samples from the degradation and control experiments was treated in an ultrasonification bath and then used to determine the protein content according to Bradford (1976). One ml of homogenized

sample was centrifuged in a Beckman Mikrofuge E^{TM} for 10 min at maximum speed. The pellet was suspended in 1 ml 1N NaOH and heated to 90 °C for 5 min. The sample was then centrifuged again, and 100 μl of the supernatant were added to 1 ml Coomassie-reagent (40 mg Serva blue G-250 dissolved in 50 ml ethanol and 100 ml 85% orthophosphoric acid and diluted to 1 l with distilled aqua). The extinction was measured at 595 nm, and the protein content was determined by comparison with a calibration curve performed with bovine serum albumin (0.1 mg/ml in 1 N NaOH).

BIOLOG system

Microorganisms isolated from contaminated soils were identified using the methods and techniques given in the BIOLOG instruction manual (MicroStation^{TM} System, 1993, Biolog, Inc., Hyward, USA).

Derivation

Hydrogenation of double bonds was achieved by stirring the substance dissolved in ethyl acetate in the presence of catalytic amounts of Pd on charcoal under a hydrogen atmosphere for 2 hours. The resulting compound was filtered over silica gel and then subjected to GC and GC-MS.

Positions of double bonds could be determined after dissolving the compound (about 0.5 mg) in 200 μl *n*-hexane, using 200 μl dimethyldisulfid (H_3C–S–S–CH_3) and 40 μl of a J_2 solution (60 mg J_2/1 ml diethyl ether). After 48 hours at 50 °C, about 1 ml of a 5% Na-thiosulfate solution was added and the obtained derivatives were extracted with *n*-hexane, dried over sodium sulfate and subjected to GC and GC-MS.

Quantification of metabolites

For structural characterization and quantification of the organic compounds, the contents of the flasks were filtered over glass fiber filters (No. 6, S & S). The filters were extensively washed with water, acetone and subsequently with diethyl ether, dried (at 40 °C, 50 mbar) and weighed. The solid material remaining on the filters (corrected for associated metabolites and educt which were determined in fraction A as described below) was analyzed by optical means and consisted mainly of whole cell remnants.

To examine the metabolites and the residual substrate associated with the solid material, the filter material was transesterified using *p*-toluolsulfonic acid and methanol (>12 hours, 1 h ultrasonication). The released carboxylic acid methyl esters and alcohols were extracted with ether (fraction A).

The filtrate from the first step was separated into an organic and a water phase; the water phase was repeatedly extracted with diethyl ether (water phase: see below). The combined and dried diethyl ether phases were treated with 1n-trimethylchlorosilane in methanol (12 h) to esterify free carboxylic acids and in a following step with bis(trimethylsilyl)trifluoroacetamide (1 h, 60 °C) to convert alcohols to TMSi-derivatives (fraction B).

The combined water phases were reduced until total dryness (40 °C; 50 mbar). Subsequently, a transesterification using *p*-toluolsulfonic acid and methanol was performed (stirring, 2 days at room temperature) and the organic compounds containing carboxylic acid methyl esters and alcohols were again extracted with diethyl ether. Alcohols present in the mixture were converted to TMSi-ethers (fraction C).

The compounds in fractions A, B and C were subjected to GC and GC-MS.

Gas chromatography and gas chromatography-mass spectrometry

Quantification of individual compounds was performed by gas chromatography (GC) by peak area integration and comparison with an internal reference compound (a defined amount of squalane or phenanthrene-d_{10} was added to the respective fraction prior to GC measurements). GC conditions were used as follows: on-column injector; flame ionization detector; fused silica capillary column: DB5 J&W Scientific, 60 m × 0.32 mm × 0.25 μm. The carrier gas was H_2 and the temperature program was: 80 °C 3 min isothermal, 80–320 °C 6 °C/min, 320 °C 30 min isothermal.

Identification was achieved by comparison of retention times and mass spectra of known and synthesized compounds using gas chromatography-mass spectrometry (GC-MS). The GC-conditions were as follows: on-column injector, fused silica capillary column: DB5-HT J&W Scientific, 60 m × 0.25 mm × 0.25 μm; carrier gas: helium; temperature program: 80 °C 5 min isothermal, 80–320 °C, 4 °C/min, 320 °C 30 min isothermal. The MS conditions were: ionization energy: 70 eV; source temperature: 250 °C; and electron multiplier voltage: 2200 μA.

Results and discussion

Characterization of the mixed culture

The different bacteria of the mixed culture (designated as B5-enrichment) were isolated on sodium acetate as the sole carbon source. Six out of the 10 isolates could be identified by Gram-staining and the application of the BIOLOG-system: three of these were Gram-negative, coccoid, and showed the highest similarity to the *Delftia acidovorans* species – a member of the Comamonadaceae (Willems et al. 1991; Wen et al. 1999), formerly *Comamonas acidovorans* (Tamaoka et al. 1987). The other three isolates were Gram-positive rod-shaped Actinomycetes exhibiting the highest similarity to the *Rhodococcus erythropolis* species as obtained by the BIOLOG-database. According to their morphology and BIOLOG-system data, the remaining microorganisms most probably also belonged to the two bacterial genera mentioned above. The morphology of the isolated bacteria differed little when grown on the aliphatic ether; cells were often slightly smaller than those grown on acetate.

The content and composition determined as the weight%$_{\text{solid material}}$ of hydrolysable sugars (2.8–3.4%), protein amino acids (30.8–41.4%), and membrane fatty acids (1–3%) of the B5-enrichment were determined. Based on our data on *P. putida* (hydrolysable sugars: 0.3–0.6% and protein amino acids: 14%) and *R. ruber* (hydrolysable sugars: 3.3–5.8% and protein amino acids: 21–35%) and by comparison of the respective distributions, the B5-culture was dominated by the Actinomycetes species.

Degradation of the aliphatic ether by the mixed culture

The substrate (educt) was a high molecular weight aliphatic ether (1-phytanyl-1-octadecyl-ether). It was used as a sole carbon and energy source in the biodegradation experiments. The water insoluble compound adhered to the flasks or floated on the mineral medium. With increasing time of incubation, the aliphatic ether became more and more finely dispersed.

Protein amount was used as a simple parameter to follow the bacterial growth (Table 1). The weight of solid material increased rapidly, indicating that the B5-enrichment was able to degrade the substrate without a lag-phase (Figure 2; Table 1). This might be due to the fact that the bacteria were precultured on the aliphatic ether. Within the first 4 days, 44% of the substrate

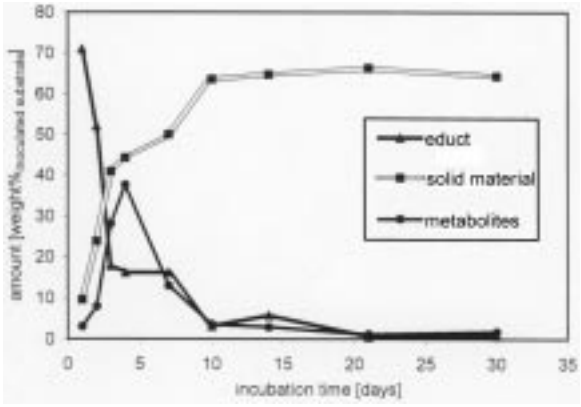

Figure 2. Amounts of educt (▲), solid material (■), and metabolites (●) after inoculation of the aliphatic ether with the enriched mixed culture B5 [% of initially inoculated substrate].

was converted into solid material. After 10 days, the stationary phase was reached, as documented by fairly stable values of solid material ranging between 64 and 66% of the initial substrate.

Increasing amounts of solid material paralleled the decrease in substrate. During the first 4 days, the educt was rapidly reduced to only 16% of initial substrate. Thereafter, biodegradation continued more slowly but steadily. After 10 days, the educt was degraded to values below 6%. In turn, the amount of metabolites increased to a maximum of 37.5% after 4 days and decreased thereafter. In the longest degradation experiment, educt and metabolites nearly vanished.

The amount of residual educt was well correlated with time of exposure to the bacteria though single, separate batches were investigated:

$$\text{Educt}_{\text{residual}} = 109.74\,(\text{day})^{-1.4328}\ (r^2 = 0.88). \quad (1)$$

The amount of solid material formed from the substrate by the bacteria may be calculated from Equation (2) indicating a logarithmic connection between duration of the biodegradation experiment and evolution of solid material.

$$\text{Solid material} = 16.26 + 16.99\ \ln(\text{day})\ (r^2 = 0.91). \quad (2)$$

The metabolites, educt and solid material were added to calculate percent recovery, which varied between 83 and 98% within the first 4 days of the experiment and then decreased continually to about 67% of initial substrate (Table 1). Obviously, part of the organic material was lost during the experiments. It

Table 1. Protein[1], educt, metabolites and solid material[2] contents [% of initially inoculated material] during biodegradation of the aliphatic ether by the enriched mixed culture B5, A, B and C correspond to the fractions A (compound released from solid/filter material by transesterification), B (organic solvent soluble compounds) and C (water soluble compounds)

Incubation time (days)	Protein[1]	Educt			Metabolites			Solid material[2]	Sum[3]	Loss[4]
		A	B	C	A	B	C			
1	0.18	0.07	70.76	0.05	0.04	3.03	0.00	9.61	83.57	16.43
2	0.44	0.35	51.52	0.14	1.81	6.08	0.00	23.95	83.65	16.35
3	0.66	0.64	17.20	0.04	3.58	24.49	0.00	40.80	86.76	13.24
4	1.75	0.96	14.68	0.58	7.32	29.78	0.41	44.19	97.93	2.07
7	5.12	0.43	15.68	0.05	1.82	11.18	0.00	49.90	79.07	20.93
10	5.85	0.25	2.81	0.03	1.38	2.13	0.01	63.48	70.09	29.91
14	5.94	0.19	5.53	0.05	0.89	1.90	0.10	64.69	73.35	26.65
21	4.96	0.13	0.35	0.01	0.39	0.73	0.01	66.20	67.83	32.17
30	4.95	0.09	0.69	0.03	0.46	0.95	0.33	64.23	66.78	33.22
Control										
0	0.00	nd	97.68	0.03	nd	0.51	0.00	1.62	99.84	0.16
21	0.00	0.12	88.22	0.04	0.02	0.00	0.00	4.40	92.82	7.18

[1] According to the method described by Bradford (1976).
[2] Filter material; associated educt and metabolites (determined in fraction A) are subtracted.
[3] Sum = educt (A to C) + metabolites (A to C) + solid material.
[4] Loss = 100% – sum.
nd = not determined.

might be that small polar compounds (e.g. acetic acid) may have escaped our quantification process. Most probably, part of the carbon was used as an energy source and thus was lost in the form of CO_2. In this respect, the mixed culture used the substrate as a carbon source first to form solid material and then mainly to maintain cell metabolism.

Degradation of the aliphatic ether by monocultures

The two strains isolated from the B5-enrichment, *D. acidovorans* and *R. erythropolis*, grew on the aliphatic ether but quantitative degradation experiments were not performed. In addition, the DSMZ strains *R. ruber* and *P. putida* were incubated with the aliphatic ether. These bacteria are versatile degraders of many organic compounds (Schlegel 1992; Malachowsky et al. 1994; Füchtenbusch & Steinbüchel 1999; Schumacher & Fakoussa, 1999). The high molecular weight aliphatic ether did not change when incubated with *P. putida*, indicating that the organism obviously was not able to attack the high molecular weight compound. In contrast, *R. ruber* grew well on the substrate as indicated by time dependent increasing protein contents (Table 2). About 66–70% of the initial substrate was converted into solid material – similar to the B5-enrichment. However, the aliphatic ether was less effectively degraded than by the mixed culture. Even after 28 days, about 18% of the educt was left as well as 6% of the metabolites. Consequently, monocultures seem to be less effective in degradation of the high molecular weight substrate than the mixed culture B5.

Metabolites

The highest amount of metabolites was present in fraction B corresponding to free compounds (Table 1). These were mainly derivatives of the aliphatic ether which experienced a reduction of the linear (not branched) side of the ether. The structural identifications were deduced from mass spectral data.

For confirmation, metabolites were synthesized, i.e. ethers with a phytanyl- and an alkyl-substituent (3 or 2 carbon atoms). Carboxylic acid or alcohol functional groups were introduced at appropriate sites. In Figure 3, the mass spectra of phytanyl ethers with a C_3 chain are shown. Characteristic fragments for the alcohol homologue (as TMSi-ether) were the molecular ion and the mass fragments m/z 103, 130 and 149, derived from cleavage of the functionalized side chain. The carboxylic acid derivative (as methyl ester) gave important fragments of m/z 88 and 105, typical for methyl esters with an ether substitution at the γ-position.

Table 2. Proteins, educt, metabolites and solid material contents [%' of initially inoculated substrate] determined in fraction B during biodegradation of the aliphatic ether by *R. ruber* (corrected by control values).

Incubation time (days)	Proteins[1]	Educt	Metabolites	Solid material[2]	Sum[3]
1	0.69	94.6	0.6	7.9	103.0
3	0.74	82.7	0.1	1.4	84.2
4	0.73	90.3	1.5	1.7	93.4
7	1.43	79.8	3.2	11.6	94.6
10	2.03	49.2	10.0	47.2	106.4
14	1.52	27.4	10.9	56.1	94.4
21	4.51	17.5	10.1	70.1	97.7
28	7.27	17.9	6.2	66.0	90.1
Control					
0	0.28	95.3	0.0	0.0	95.3
28	0.55	100.0	0.2	2.7	102.9

[1] According to Bradford (1976).
[2] Filter material; associated educt and metabolites are subtracted.
[3] Sum = educt + metabolites + solid material.

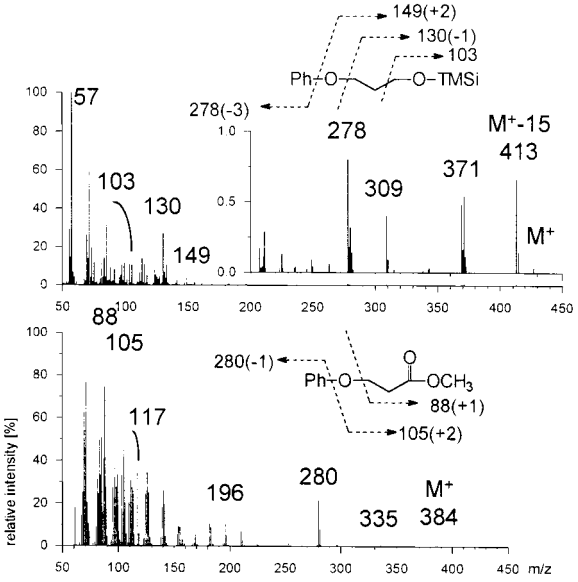

Figure 3. Mass spectra of metabolites with an alcohol and a carboxylic acid functional group at the C_3 side chain: 1-phytanyl-1-(propan-3-ol)ether (as TMSi-ether) and 1-phytanyl-1-(propanoic acid)ether (as methyl ester).

During the first 7 days of incubation, mainly alcohol metabolites were found in fraction B: the phytanyl ethers with one C_3 or C_2 alcohol substituent dominated, but phytanyl ethers with a chain up to C_8 and one alcohol group were also present. With ongoing biodegradation the amount of homologous carboxylic acids increased and phytanyl ethers with one C_2 or C_3 carboxylic acid finally predominated. The higher homologues vanished consecutively. Further metabolites were present as functionalized isoprenoids with 16–20 carbon atoms. Some of these were identified as phytanol, phytanic acid, 6,10,14-trimethylpentadecan-2-one, 5,9,13-trimethyltetradecanoic acid and 4,8,12-trimethyltridecanoic acid.

The composition of metabolites of fraction C was very similar to that of fraction B although other compounds with more functional groups were expected. In any case, the amount of this fraction was very low.

Surprisingly, a considerable quantity of metabolites and of educt was found in fraction A probably released from the solid material. As the filter material was intensively washed with organic solvents to remove free or unbound organic compounds and as the educt and the metabolites could only be analyzed after chemical deterioration of the solid material (transesterification), the metabolites and educt must have been tightly associated with the membranes. In general, the composition of fraction A and changes over time resembled those of fraction B with the exception that the higher homologues and those metabolites with a carboxylic acid functional group were relatively enriched. These differences indicate that the biodegradation of the high molecular weight aliphatic ether was mainly coupled to biochemical processes associated with bacterial membranes.

The same metabolites were produced by monocultures of *R. ruber*. Alcohol metabolites prevailed in the beginning of the biodegradation experiments and the phytanyl ether with the C_2 alcohol substitutent predominated. With ongoing biodegradation, the amount of carboxylic acid metabolites increased continuously especially for the C_3 substituted homologue. Metabolites of lower molecular weights were also discovered.

Mid-chain unsaturated metabolite

An unexpected and new metabolite was produced during the experiments run with the B5-enrichment and with *R. ruber*. After hydrogenation of the compound (Pd/H_2), mass spectral data proved that the metabolite

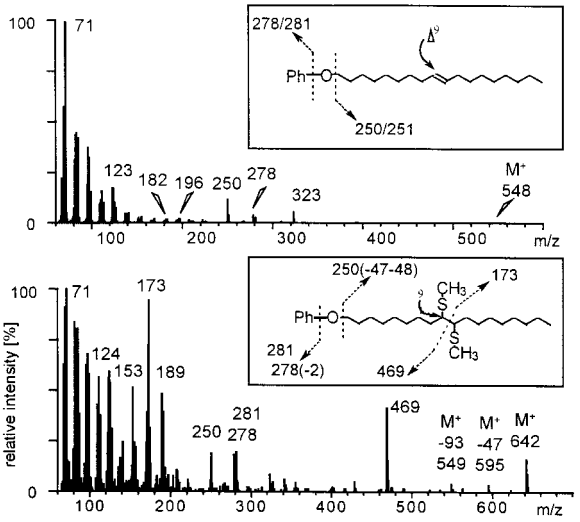

Figure 4. Mass spectra of the 1-phytanyl-1-octadec-9-enyl-ether with and without DMDS/J_2-derivatization.

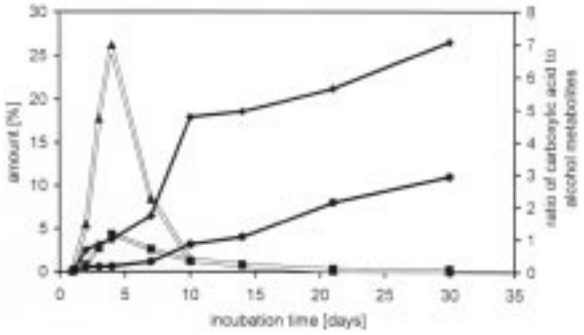

Figure 5. Amounts of metabolites [% of initially inoculated substrate] from fractions A and B with an alcohol function in the side chain (▲) and the respective carboxylic acids (■); as well as the ratios of all phytanyl-ethers with one carboxylic acid functional group to all those with one alcohol function (●) and of phytanyl-O-propylic acid to phytanyl-O-propanol (♦) during biodegradation of the aliphatic ether by the enriched mixed culture B5.

had a carbon skeleton identical to the used aliphatic ether. Therefore, the mass fragment m/z 548 suggested the presence of one double bond (Figure 4). The position of this double bond could only be localized using the DMDS/J_2 derivatization technique. The molecular ion was then shifted to m/z 642 due to the addition of two S–CH_3 groups. The mass fragment m/z 281 characterized the phytanyl side of the ether to be saturated. Therefore, the double bond must have been at the linear side chain. From the large mass fragments m/z 173 and 469 it is concluded that the double bond was positioned between the C-9 and C-10 carbon atoms (Δ^9). Co-occurring was a metabolite with a double bond between the carbon atoms C-10 and C-11 (Δ^{10}; data not shown). Consequently, the new metabolite must be a 1-phytanyl-1-octadec-9-enyl-ether which was accompanied by minor amounts of a 1-phytanyl-1-octadec-10-enyl-ether.

Alcohols

Our investigation has clearly shown that compounds with an alcohol functional group were also metabolites of the biodegraded aliphatic model compound. Alcohol metabolites first occurred in relative abundance.

The amount of metabolites with an alcohol at a C_2–C_8 side chain reached its maximum after 4 days of incubation and then decreased while the corresponding carboxylic acids increased (Figure 5). The tendency of carboxylic acids increasing at the expense of alcohols was most pronounced for phytanyl-ethers with a C_3 substituent. On average, the carboxylic acids were threefold more abundant than the alcohols after 30 days of biodegradation. This shows a close genetic relationship between alcohols and carboxylic acids. Most probably, alcohol metabolites were formed first and were then further oxidized to the corresponding carboxylic acids.

Degradation pathway

During biodegradation experiments with the mixed B5-enrichment culture and with *R. ruber*, only aliphatic ether metabolites with a degraded linear side chain were observed. An initial oxidation of organic compounds at the least branched end is a well-known feature of microbiological oxidation of hydrocarbons (Fonken & Johnson 1972). Although the metabolites from this form of oxidation were not detected, it is conceivable that the degradation of the aliphatic ether was initiated by the common terminal monooxygenase oxidation which proceeded via an alcohol and a subsequent aldehyde dehydrogenase reaction resulting in the formation of a carboxylic acid functional group at the terminal C-atom (Figure 6). This product may be further metabolized by means of the β-oxidation pathway for fatty acids (e.g. Watkinson & Morgan 1990). With ongoing degradation, the derivatives of the aliphatic ether showed a clear variation from compounds with a long side chain to compounds with a short side chain and may derive from a continuous side chain degradation via the β-oxidation pathway. Because only C_2 units are cleaved from the substrate during β-oxidation, metabolites of the aliphatic

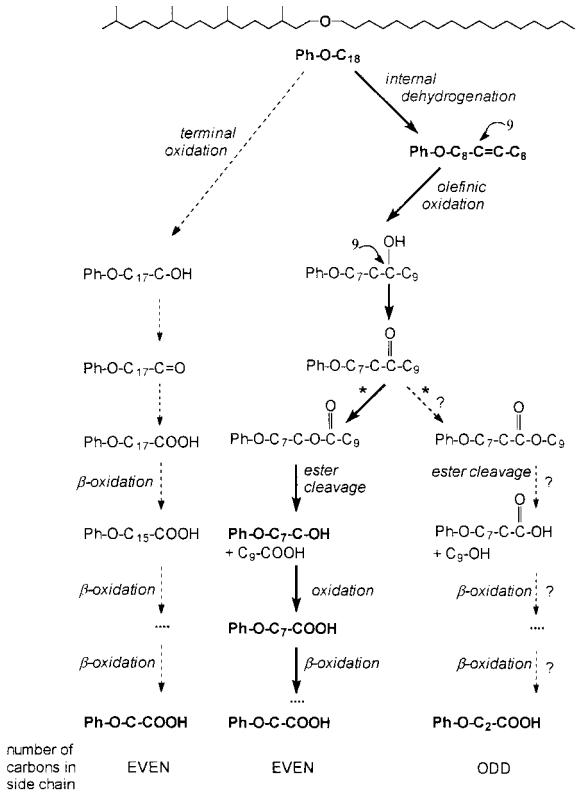

Figure 6. Possible metabolic degradation pathways of the aliphatic ether. Detected metabolites are shown in bold letters. Left side: common terminal oxidation and β-oxidation (cleavage of acetic acid units). Center: new internal oxidative pathway and β-oxidation, right side: further conceivable alternative pathway. Covalent C–H bondings are omitted for simplification. $*$ = Baeyer-Villinger oxidation.

ether should only have an even-carbon-numbered side chain. Phytanyl ethers with a C_8, C_6, C_4 and C_2 substituent and a carboxylic acid functional group may derive from such a degradation pathway.

The relatively high amount of phytanyl-O-acetic acid may be explained by an obstructive effect of the ether bond. Nevertheless, the microorganisms were able to surmount the ether bond as indicated by the presence of functionalized isoprenoids with 16–20 carbon atoms and the nearly quantitative degradation of the substrate (Tables 1 and 2).

Metabolites with an odd-carbon-numbered substituent and those with an alcohol group cannot derive from terminal oxidation followed by β-oxidation. In this case, a different mechanism must occur.

Subterminal oxidation of the aliphatic ether was suggested by identification of a metabolite having a hydroxy function at the $\omega 2$ carbon atom of the linear chain. As outlined by Gottschalk (1986), the further degradation of the compound would result also in the formation of even-carbon-numbered metabolites.

However, the finding of the mid-chain unsaturated metabolite, 1-phytanyl-1-octadec-9-enyl-ether (and minor amounts of the C-10 unsaturated homologue), points to another degradation pathway. Obviously, the bacteria were able to introduce a double bond into the saturated side chain of the aliphatic ether (Figure 6). A biological dehydrogenation of high molecular weight compounds has not been reported previously. Many obligate aerobic bacteria are able to introduce a *cis* double bond into aliphatic chains of saturated membrane fatty acids by specific dehydrogenase so that the cells can change the membrane fluidity after biosynthesis (Keweloh & Heipieper 1996). This results in formation of $C_{18:1}$ fatty acids with a double bond at carbon atom C-9. Obviously, the bacteria in the mixed culture B5 and *R. ruber* similarly introduced a double bond into the saturated aliphatic 1-phytanyl-1-octadecyl-ether independent of the presence of an ether bond and the absence of a phospholipid linkage. It might be that the enzymes responsible for this reaction are not as substrate specific as implied here. This is substantiated by data of Amblès et al. (1994) who found that alkanes (*n*-C_{19}, *n*-C_{20} and *n*-C_{22}) are dehydrogenated to alk-9-enes.

Our experiments showed that the mid-chain unsaturated metabolite tends to decrease, indicating that it is further degraded. Therefore, it can be regarded as an intermediate metabolite.

Low molecular weight alkenes may be biodegraded (discussed in Watkinson & Morgan 1990; Gottschalk 1986). Either water is added across the double bond or an epoxide is formed first, which is subsequently hydrolyzed to yield a dihydroxy alkane. Further oxidation of the alcohol gives rise to the respective ketone. For example, the double bond in octadec-9-enoic acid is eventually converted to a ketone (10-oxo-octadecanoic acid) by *Micrococcus luteus* (Esaki et al. 1994). 1-Phytanyl-1-octadec-9-enyl-ether could thus have been converted to a corresponding compound with a ketone function at the C-9 atom (Figure 6). In general, it is assumed that mid-chain oxidized compounds are further metabolized only by the normal terminal degradation pathway (Amblès et al. 1994; Esaki et al. 1994; Hita et al. 1996). However, in case of the well-known subterminal oxidation of alkanes, the secondary alcohol is oxidized to a corresponding ester (Gottschalk 1986; Watkinson & Morgan 1990). Thus, we believe that the mixed culture B5 and *R. ruber* were also able to oxidize the mid-chain

ketone to an ester by a Baeyer-Villinger mechanism. Such an oxidation is well known to be performed on cyclic compounds by pseudomonads and *Corynebacterium* species (Fonken & Johnson 1972) and has most recently been reported for *R. ruber* (Schumacher & Fakoussa 1999). An ester bond in our high molecular weight aliphatic compound may easily be cleaved by an esterase. As a result, one would expect to find a phytanyl-ether substituted by a C_9 carboxylic acid or by a C_8 alcohol (Figure 6). Cleavage products would be a short-chain alcohol (e.g. nonanol) or a short-chain fatty acid (decanoic acid), respectively. The highest homologue of the found metabolites was a phytanyl-ether with a C_8 alcohol. Therefore, we believe that this compound derives from such an internal oxidation pathway.

Isolates of the mixed culture B5, identified as *D. acidovorans* and *R. erythropolis* by the BIOLOG-system, did not grow on nonanol or octanol. This indicates that short-chain alcohol by-products of the ester cleavage, such as nonanol (Figure 6), do not serve as a carbon source for these organisms. In general, these results suggest that the bacteria systematically achieve in formation of such esters which can be cleaved into a phytanyl derivative with an alcohol function and a short-chain fatty acid. The latter might easily and directly serve for metabolic purposes.

As already mentioned, different internal carbon positions were also dehydrogenated, like the C-10 carbon atom. It could be possible that additional unsaturated homologues might exist as short-lived intermediates. An oxidation and subsequent ester cleavage would explain the presence of the other phytanyl ether derivatives with a C_2–C_7 side chain containing a hydroxy functional group.

Subsequent biodegradation of the resulting phytanyl-ether with an alcohol side chain along the β-oxidation pathway would then give rise to metabolites with even- and odd-carbon-numbered side chains and a carboxylic acid functional group (Figure 6).

The alcohol metabolites are present in relatively high amounts most probably because they still must be oxidized to the corresponding carboxylic acids whereas the respective carboxylic acids may be quickly and easily degraded by the β-oxidation pathway. In this respect, one should expect a relative enrichment of alcohol metabolites initially and an increasing importance of the different carboxylic acids with prolonged incubation time. The aliphatic ether was quantitatively degraded by the mixed culture B5 (Table 1). Thus, the isoprenoidal part of the aliphatic ether served also for metabolic purposes. This was confirmed by the presence of isoprenoidal C_{20}, C_{17} and C_{16} carboxylic acids and one C_{18} isoprenoid with a ketone function at the C-2. The occurrence of these compounds can only be explained by an ongoing degradation of the phytanyl-ether metabolites. In any case, no isoprenoidal metabolites deriving from terminal oxidation of the isoprenoidal side of the aliphatic ether were found.

It has been reported that oxygenation at the saturated end of a given molecule competes significantly with oxidative attack at the olefinic center (Fonken & Johnson 1972). Our degradation experiments have clearly shown that the internal degradation pathway seems to be preferred to the terminal degradation step.

According to Watkinson & Morgan (1990) there is only some weak evidence for an *n*-alkane metabolism via alkenes. Our investigation now indicates that this initial attack is an essential mechanism for an ongoing bacterial metabolization of aliphatic structures – at least those of high molecular weight. This mechanism seems to be more widespread than so far assumed because not only the mixed culture B5 but also *R. ruber* were able to metabolize the substrate by using this pathway.

This degradation pathway might also work on other aliphatic substructures, e.g. those present in rubber, asphaltenes or residues. Similarly, Schumacher & Fakoussa (1999) assume that a cyclododecane monooxygenase is a Baeyer-Villinger enzyme able to mediate carbon-carbon fission in structures mimicking aliphatic chains or bridge structures as present in coal.

Conclusions

Within 30 days, an aliphatic ether of high molecular weight, assumed to resemble some geological and industrial macromolecules, was nearly quantitatively degraded by a mixed culture B5 enriched from a diesel fuel contaminated subsurface sample. A monoculture of *R. ruber* also used the substance as a sole carbon and energy source but less effectively.

Biodegradation started by introduction of a double bond into internal positions of the linear side chain of the aliphatic ether. After olefinic oxidation, the mid-chain alcohol intermediates were converted via a Baeyer-Villinger mechanism into esters which, in turn, were hydrolyzed. The detected metabolites may only be explained by this new internal oxidative pathway which gives rise to a series of metabolites with a

shortened chain containing an alcohol group. Cleaved products were low molecular weight fatty acids which may directly serve as an energy or carbon source. The alcohol metabolites were converted to the respective carboxylic acids in a subsequent step and then further metabolized according to the normal β-oxidation pathway.

The importance of the unusual internal oxidative pathway of aliphatic chains may have been underestimated in the past. In case of our aliphatic substrate, the ether bond restrained the bacteria from fast and complete metabolization. The resulting metabolites can easily be studied because they still have a relatively high molecular weight.

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