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Degradation of unsaturated hydrocarbons by methanogenic enrichment cultures

(Aromatic compounds; alcohol degradation; hexadecene; squalene; hydratation; carboxylation)

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1. SUMMARY

The biodegradability of hydrocarbons under anaerobic conditions was studied in enrichment cultures using mineral media inoculated with sewage sludge or sediment samples of limnic and marine origin. No indication of methanogenic degradation was obtained with either *n*-hexane, *n*-hexadecane, *n*-heptadecane, 1-hexene, *cis*-2-hexene, *trans*-2-hexene, isoprene, 1-hexine, benzene, toluene, xylene, cyclohexene, cycloheptatriene, cyclopentadiene, styrene, naphthalene, azulene, or β-carotene. Squalene was incompletely converted to methane and carbon dioxide. Complete degradation was observed with 1-hexadecene. Methanogenic subcultures were maintained on 1-hexadecene and squalene. Both enrichments contained after several transfers *Methanospirillum hungatei* and *Methanothrix soehngenii* as prevalent methanogenic bacteria. Acetate ($\leqslant 80$ μM) was the only intermediary product detected indicating that degradation proceeded via hydrogen-dependent syntrophic β-oxidations. Short rods on hexadecene and cocci on squalene were found to be associated with substrate degradation. The results indicate that terminal double bonds can be sufficient to allow methanogenic degradation of hydrocarbons whereas branching and terminal ring closures may significantly contribute to hydrocarbon stability in anoxic environments.

2. INTRODUCTION

Disastrous oil spills always raise the question to what extent oil constituents such as hydrocarbons can be degraded by natural microbial communities. Whereas aerobic degradation of hydrocarbons has been studied in great detail, relatively little attention has been paid so far to anaerobic hydrocarbon degradation. The literature contains contradictory reports on the 'ifs' and 'hows' of hydrocarbon biodegradation in the absence of oxygen [1–5]. Earlier reports claiming degradation of saturated hydrocarbons by, e.g., sulphate-reducing bacteria are probably a consequence of poor anaerobic techniques together with poorly defined culture conditions, and usually deal with mixed cultures of aerobic and anaerobic bacteria or with growth of anaerobes on substrate contaminations [6–9]. Until the present work, no stoichiometric anaerobic conversion of hydrocarbons to reduced end products has been published, and the general theory on anaerobic hydrocarbon degradation is

that it is—if occurring at all—an extremely slow process [10,11].

From a thermodynamic point of view, saturated hydrocarbons, e.g. ethane, may easily be converted to methane,

$$8C_2H_6 + 6H_2O \rightarrow 14CH_4 + 2HCO_3^- + 2H^+ \quad (1)$$

$$\Delta G'_0 = -34.4 \text{ kJ} \cdot \text{mol}^{-1}$$

and this reaction becomes even more exergonic with increasing chain length of the substrate [2]. However, the initial attack on the alkane has, as far as we know today, to be catalyzed by a monooxygenase enzyme which needs molecular oxygen as a cosubstrate [3]. A pathway of desaturative attack on saturated alkanes by a dehydrogenase enzyme has been proposed [12–15], but this was probably a misinterpretation of the monooxygenase action mechanism [16].

Unsaturated hydrocarbons do not necessarily need a monooxygenase enzyme for degradation. The methanogenic degradation becomes far more exergonic (calculations of free energy changes after [17]):

$$2C_2H_4 + 3H_2O \rightarrow 3CH_4 + HCO_3^- + H^+ \quad (2)$$

$$\Delta G'_0 = -102.1 \text{ kJ} \cdot \text{mol}^{-1}$$

$$4C_2H_2 + 9H_2O \rightarrow 5CH_4 + 3HCO_3^- + 3H^+ \quad (3)$$

$$\Delta G'_0 = -208.7 \text{ kJ} \cdot \text{mol}^{-1}$$

Concerning the mechanism of reaction (2), one could speculate on a hydration of ethylene to ethanol and subsequent syntrophic degradation via acetate to methane and CO_2:

$$C_2H_4 + H_2O \rightarrow CH_3CH_2OH \quad (4)$$

$$\Delta G'_0 = -12.7 \text{ kJ} \cdot \text{mol}^{-1}$$

$$2CH_3CH_2OH + H_2O \rightarrow 3CH_4 + HCO_3^- + H^+ \quad (5)$$

$$\Delta G'_0 = -89.5 \text{ kJ} \cdot \text{mol}^{-1}$$

Evidence for anaerobic degradation of acetylene via acetate has recently been provided [18], and pure cultures of acetylene-fermenting anaerobes were isolated in the author's lab (Schink, in preparation). During attempts to enrich for ethene-degrading anaerobes, ethene was found to strongly inhibit methane formation, probably by direct action on the methanogenic bacteria [19]. With some higher alkenes, on the other hand, substrate-dependent methane formation was observed. The present paper reports unsuccessful and successful methanogenic enrichment experiments with hydrocarbons and related compounds as sole organic substrates, and provides the first conclusive evidence for stoichiometric conversion of unsaturated hydrocarbons to methane.

3. MATERIALS AND METHODS

3.1. Sources of inocula

Black anoxic sediment samples were taken from polluted freshwater creeks near Konstanz and Hannover, F.R.G. Anaerobic sewage sludge was obtained from the municipal sewage treatment plants in Göttingen and Konstanz, F.R.G. Anoxic marine sediments were sampled near Cuxhaven, F.R.G. and in the Canal Grande, Venice, Italy. Screw-cap bottles were filled with sampling material and stored under nitrogen gas at 4°C.

3.2. Media and cultivation conditions

All procedures for cultivation and chemical analysis were essentially identical with those described earlier [20,21]. The basal medium had the following composition (values in g/l): KH_2PO_4, 0.2; NH_4Cl, 0.25; KCl, 0.5; $CaCl_2 \cdot 2H_2O$, 0.15. Freshwater medium contained in addition 1.0 g NaCl and 0.4 g $MgCl_2 \cdot 6H_2O$; salt water medium 20.0 g NaCl and 3.0 g $MgCl_2 \cdot 6H_2O$. Sodium bicarbonate, sodium sulphide, trace element solution SL 10 [22] and vitamin solution [23] were added to the autoclaved, cooled medium from stock solutions as previously described in detail [21].

The pH was adjusted to 7.2–7.3. The medium was dispensed per 50 ml into 100 ml serum bottles gassed with N_2/CO_2 mixture (80%/20%) and sealed with butyl rubber stoppers.

Liquid hydrocarbons (50 μl) were added with Eppendorf pipettes to 50 ml aliquots of medium. Solid substrates were added as a dry powder (50 mg per 50 ml medium). Enrichment cultures were incubated at 28°C either with or without shaking (100 strokes $\cdot$ min^{-1}). Development of gas pressure was checked by periodic cautious release of

the stoppers, avoiding any contamination with air.

Quantitative determination of methane was carried out by injecting samples of 100 μl gas from the culture headspaces with pressure-lok syringes into a Perkin–Elmer Sigma 4B gas chromatograph equipped with a molecular sieve column (0.5 nm, 60–80 mesh, 0.5 m, 1/8″) and a flame ionization detector [20].

Acetate and other volatile fatty acids were assayed by gas chromatography on Porapak QS, 100–120 mesh, 2.0 m × 1/8″, in a Perkin–Elmer Sigma 3B gas chromatograph with flame ionization detector and a Perkin–Elmer M 3B integrator. Samples were acidified prior to injection with formic acid from 10 M stock solution to 0.5 M final concentration.

3.3. Chemicals

All chemicals used were of reagent or analytical grade quality and obtained from Merck, Darmstadt, F.R.G., and Baker Chemical Co., Deventer, The Netherlands. Hydrocarbons were purchased from Fluka AG, Buchs, Switzerland.

4. RESULTS

4.1. Enrichment of hydrocarbon-utilizing bacteria

50-ml enrichment cultures, with carbonate-buffered freshwater or saltwater medium and hydrocarbons as substrates, were each inoculated with 5 ml of anoxic mud. With every substrate, 6 parallels were run, 2 of which were inoculated with sewage sludge, 2 with freshwater creek sediments, and 2 with marine sediments. Control cultures for endogenous methane formation contained the same amount of inoculum but no substrate. All enrichments were checked for methane formation over a period of at least three months by weekly observation and release of gas pressure. Active methane formation could often be detected by appearance of gas bubbles rising from the sediment after cautious shaking or knocking of the bottles on a wooden shelf.

No significant formation of methane was observed in enrichment cultures containing *n*-hexane, *n*-hexadecane, *n*-heptadecane, 1-hexene, *cis*-2-hexene, *trans*-2-hexene, isoprene, 1-hexine, benzene, toluene, *o*-, *m*-, *p*-xylene, cyclohexene, cycloheptatriene, cyclopentadiene, styrene, naphthalene, azulene or β-carotene during 14 weeks of incubation. Enrichments with 1-hexadecene and squalene exhibited methane formation after 4–12 weeks of incubation. Active cultures were transferred after about 4 weeks of methane formation. Cultures were shaken vigorously before transfer, and 5 ml was transferred in every case to make sure that sediment or surface-associated bacteria were also included. In enrichments from freshwater sources, methane formation started again after a lag period of about 2–4 weeks, whereas in marine enrichments methane formation in subcultures resumed only very weakly. These cultures were discarded after the second attempt at transfer.

4.2. Characterization of enrichment cultures with hexadecene and squalene as substrates

Freshwater enrichments with hexadecene and squalene were transferred 8–12 times on the same medium thus diluting out all remnants of sediment contents. After the fifth transfer, measurements of the stoichiometry of substrate-dependent methane formation were initiated. Fig. 1 shows the time course of methane formation from hexadecene-1 in 2 different enrichment cultures. Methane forma-

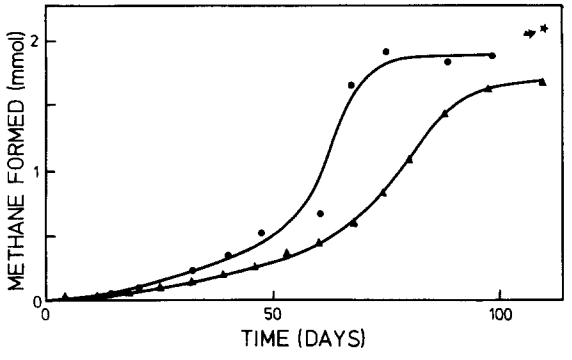

Fig. 1. Formation of methane from 1-hexadecene by enrichment cultures. Experiments were carried out in 120 ml serum bottles containing 50 ml of mineral medium under an atmosphere of 80% N_2 and 20% CO_2. 50 μl (174 μmol) of hexadecene was added as substrate. ●, Enrichment culture Ko 16en from sewage sludge transferred 5 times; ▲, enrichment culture Wo 16en from creek sediment transferred 6 times. Arrow points to the amount of methane calculated for complete methanogenic degradation.

tion increased slowly but continuously during 2–3 months of incubation, and ceased after 3–4 months. By this time, the methane formed amounted to 78–91% of the theoretically possible value calculated by the equation:

$$C_{16}H_{32} + 12H_2O \rightarrow 12CH_4 + 4HCO_3^- + 4H^+$$

Acetate was the only short-chain fatty acid detectable in the medium by gas chromatographic analysis, and was found in concentrations of 30–80 $\mu mol \cdot l^{-1}$.

Enrichments with squalene as substrate exhibited slow but continuous methane formation which ceased after 3–4 months of incubation (Fig. 2). Cultures only reached about 50% of the theoretically possible amount of methane which was calculated by the following equation:

$$4C_{30}H_{50} + 105H_2O \rightarrow 85CH_4 + 35HCO_3^- + 35H^+$$

In these cultures, acetate was formed intermediately in trace amounts (25–65 $\mu mol \cdot l^{-1}$).

Other intermediate products could not be detected in the aqueous phase of the medium by conventional gas chromatography which would detect alcohols and volatile fatty acids up to octanoate. Droplets of an oily residue remained in the culture bottles and indicated that the missing products of squalene degradation were probably water-insoluble hydrocarbons.

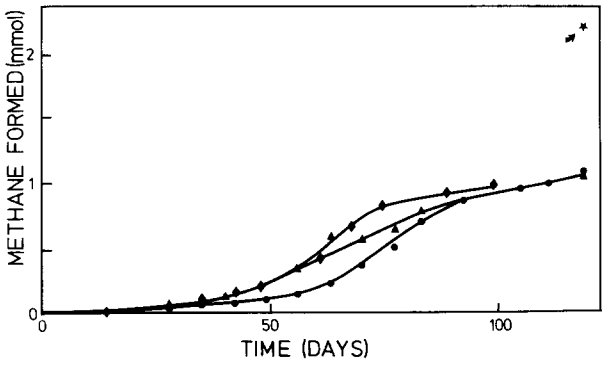

Fig. 2. Formation of methane from squalene by enrichment cultures. Experimental conditions were the same as described for Fig. 1. 50 µl (105 µmol) of squalene was added as substrate. ◆, Enrichment culture Gö Sq from sewage sludge transferred 5 times; ●, same enrichment, transferred 7 times; ▲, enrichment culture Wo Sq from creek sediment transferred 7 times. Arrow points to the amount of methane calculated for complete methanogenic degradation.

The bacteria in both types of enrichment cultures were either associated with substrate droplets at the liquid–gas interface or settled down at the bottom of the vials. Samples of the surface-associated bacteria were taken from actively methane-producing cultures by syringes. In hexadecene cultures, small straight rods predominated and were found in juxtaposition to substrate droplets (Fig. 3a, b). The only type of fluorescent methanogenic bacteria detected was a slightly curved motile rod similar to *M. hungatei*. Conspicuous aggregates of a fat, rod-shaped bacterium similar to *M. soehngenii* were also found.

In enrichments with squalene, coccoid bacteria predominated in close contact to substrate droplets (Fig. 3c). Methane bacteria similar to *M. hungatei* and *M. soehngenii* were present in these cultures, too. Morphologically similar bacterial populations were observed in parallel enrichments from other sources with the respective substrates.

All enrichment cultures were incubated up to the 6th transfer without shaking in order to allow syntrophic associations of bacteria to establish in close contact to the substrate without being subjected to excessive shearing forces. After this, the effect of shaking on methane production kinetics was studied by comparison of shaken and non-shaken cultures. Fig. 4a demonstrates that methane production from hexadecene in non-shaken cultures was largely exponentially dependent on time. An initial phase of fast exponential methane formation ($t_d \approx 9$ days) was followed by a slower but still exponential phase ($t_d \approx 15$ days). Shaking did not enhance rates of methane production significantly but again two phases of exponential methane production were observed ($t_d \approx 7$ and 13 days, respectively).

Fig. 4a also demonstrates that the cultures enriched with hexadecene converted also hexadecanol completely to methane without a significant lag phase. Palmitate, heptadecanol and pentadecanol were also converted to methane by these enrichment cultures. With the latter two substrates, propionate accumulated as an intermediary product up to 0.6 $mmol \cdot l^{-1}$ concentration. β-carotene was not utilized.

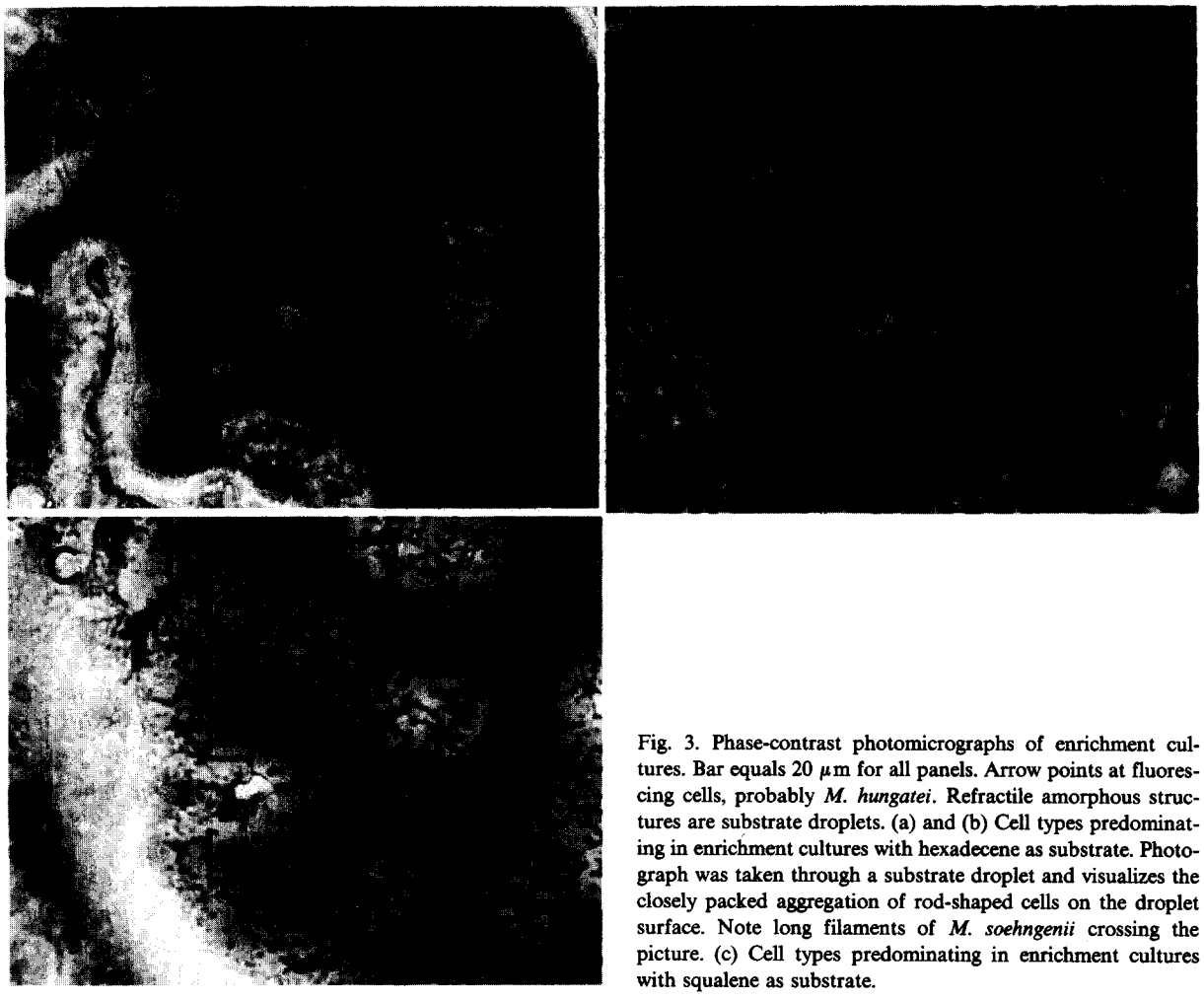

Fig. 3. Phase-contrast photomicrographs of enrichment cultures. Bar equals 20 μm for all panels. Arrow points at fluorescing cells, probably *M. hungatei*. Refractile amorphous structures are substrate droplets. (a) and (b) Cell types predominating in enrichment cultures with hexadecene as substrate. Photograph was taken through a substrate droplet and visualizes the closely packed aggregation of rod-shaped cells on the droplet surface. Note long filaments of *M. soehngenii* crossing the picture. (c) Cell types predominating in enrichment cultures with squalene as substrate.

Unshaken cultures with squalene as substrate exhibited an exponential increase of methane with time for about two months with a doubling time of about 11 days (Fig. 4b). After this time, methane production was not exponential any more. The shaken culture produced methane at the beginning significantly faster than the unshaken parallel; however, methane production was never exponential but linear with time, and no constant rate of methane production was found at any time. The squalene enrichment cultures also utilized hexadecanol, but the amounts of methane formed were equivalent to only 40–45% of the provided substrate. 3-hydroxybutyrate was quickly degraded; acetone and 2-pentanone slowly after lag phases of 3–4 weeks. No methane was formed from β-carotene.

5. DISCUSSION

Microbial degradation of hydrocarbons in the absence of molecular oxygen has been for a long time a matter of dispute among microbiologists, biochemists and geochemists. The present paper provides the first unequivocal evidence of hydrocarbon degradation under strictly anaerobic conditions, and the quantitative recovery of reducing equivalents of hydrocarbon oxidation as methane. Comparative degradation experiments with several

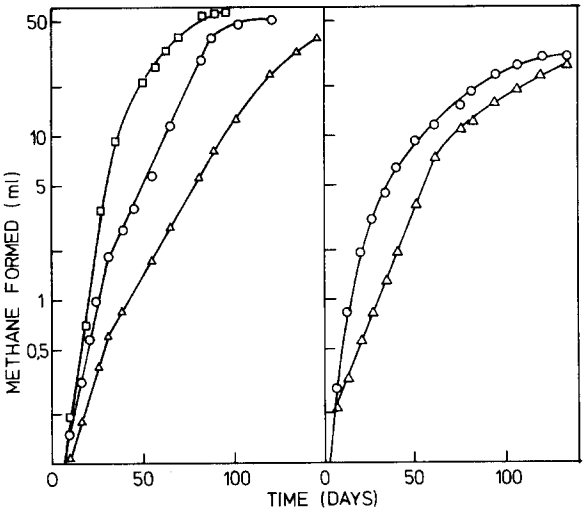

Fig. 4. Kinetics of methane formation from hexadecene and squalene by enrichment cultures. (a) Culture Gö 16en from sewage sludge after 8 transfers. ○, Shaken culture with hexadecene; △, nonshaken control culture with hexadecene; □, shaken culture with hexadecanol as substrate (50 mg or 206 μmol). (b) Culture Wo Sq from creek sediment after 9 transfers with squalene as substrate. ○, Shaken culture; △, nonshaken culture. All results were obtained in four parallel cultures each, and were representative also of methane formation kinetics in the other enrichment cultures.

different hydrocarbon substrates under identical conditions allow to define some minimum requirements to hydrocarbon structure with respect to its anaerobic biodegradability.

1-Hexadecene was chosen in the present study as a model substrate of terminally unsaturated linear hydrocarbons. Aerobic degradation of hexadecene and other long-chain α-unsaturated hydrocarbons proceeds via introduction of oxygen either at the saturated or at the non-saturated end; in the latter case, an α,β-diol is formed via epoxidation and hydrolysis [24–26]. The present study demonstrates that in the absence of oxygen 1-hexadecene was stoichiometrically converted to methane and carbon dioxide. The primary reaction was probably a hydration which led to a primary aliphatic alcohol. The presence of hydrogen- and acetate-degrading methane bacteria in the enrichment cultures indicates that the further degradation probably included oxidation to the corresponding fatty acid and subsequent syntrophic β-oxidation to acetate residues. Acetate was found as a degradation intermediate in trace concentrations. It did not accumulate to higher concentrations, probably because hexadecene hydration was the rate-limiting step among the at minimum five different metabolic processes involved. This assumption is corroborated by the observation that hexadecanol was degraded without a lag and even faster than hexadecene. Immediate utilization of palmitate and intermediate accumulation of propionate during pentadecanol and heptadecanol degradation provide further evidence for the assumed pathway of hexadecene degradation via hexadecanol, palmitate, and subsequent β-oxidation.

Squalene was first discovered in shark liver and is a minor constituent of several plant oils [27]. Its occurrence in several bacteria has been reported several times, and e.g. the Myxobacterium *Nannocystis exedens* contains squalene up to 0.4 % of its dry weight [28]. Squalene and its homologues and reduced derivatives are among the major neutral lipids of methanogenic and other archaebacteria [29]. *Arthrobacter* sp. degrades squalene aerobically by oxygenase-catalyzed splitting of internal double bonds thus forming geranylacetone and branched-chain monocarboxylic acids [30]. Oxidation of the terminal methyl groups to the corresponding α,ω-dioic acid is the primary process in squalene degradation by a *Corynebacterium* sp. [31]. Degradation of other isoprenoids by monooxygenase action on terminal methyl groups was also reported for *Vinca rosea* [32] and *Pseudomonas citronellolis* [33]. The mechanism of isoprenoid degradation has been studied with this bacterium in more detail and involves carboxylation of the methyl branches and their cleaving off as acetate [34].

Nothing is known so far about anaerobic degradation of squalene. Seo et al. [35] reported on hydration of squalene by resting cells of *Corynebacterium* sp. which yielded up to 5 tertiary alcohol functions per molecule. This reaction is possible also in the absence of oxygen. The alcoholic functions thus obtained could facilitate carboxylation of the terminal methyl groups and methyl branches, and the further degradation of the resulting 3-oxo-caproate and 3-oxo-butyrate residues would be no basic problem any more; however,

this hypothetical pathway has to be substantiated by enzymatic studies. The fact that the squalene enrichments readily utilized 3-hydroxybutyrate and also converted acetone and 2-pentanone to methane gives some support for this assumed degradation pathway. The reason for the observed incomplete squalene degradation in the experiments presented in this paper could be partial reduction of double bonds: the lack of α-hydroxy functions would render the important carboxylation reactions extremely difficult. Reduction of e.g. oleic acid to stearate by anaerobic estuary microbial communities has been reported [36] and similar processes have been observed with carotenoids in old anoxic marine sediments [37]. Further studies on purification and characterization of the enrichment cultures and the biochemistry of substrate degradation are in progress.

In view of the successful anaerobic degradation experiments presented it appears that anaerobic degradation of hydrocarbons is possible if unsaturated bonds exist which allow hydrations and carboxylations as primary reactions of substrate activation. No degradation of linear saturated hydrocarbons was observed in the present study. However, this and other negative results in this study may only apply to the experimental conditions employed: changing the pH, the sulfide content, or the redox potential may allow degradation also of those hydrocarbons which were resistant to methanogenic degradation in my experiments. For example, degradation of heptadecane was observed in anoxic lake sediment samples [38], however, the bacteria involved could not yet be cultivated.

Some unsaturated hydrocarbons such as hexenes, hexine, and isoprene were not significantly degraded in the present study. This may have several reasons: either suitable mechanisms for transport into the cell are lacking, or these substrates are readily reduced to form saturated hydrocarbons which resist further degradation. Also toxic effects of these substrates on anaerobic microbial communities cannot be ruled out.

The mesomeric structure of the π-electron system in benzene, toluene, xylene, and naphthalene does not allow hydration of isolated double bonds and thus probably prevents anaerobic microbial attack. Aromatic ring systems appear to need functional groups such as carboxyl or hydroxyl groups to make degradation possible in the absence of the biradical oxygen. The degradation of cyclic hydrocarbons with less symmetrically arranged double bonds such as cyclohexene, cyclopentadiene, cycloheptatriene, and azulene was tried; however, without success. Toxic effects of these substrates on anaerobic microbial communities could be one reason for this failure. Cyclopentadiene, cycloheptatriene as well as benzene and toluene partly inhibited methane formation from sediment contents [19]. Thus, the negative results of the enrichment experiments do not yet allow conclusions on the anaerobic biodegradability of these substrates.

It is also too early to speculate on the stability of carotenoids under anoxic conditions. Enrichment experiments gave no evidence of methane formation from β-carotene. Perhaps the terminal ring closures together with the comparably high molecular weight and resulting transport problems prevent anaerobic degradation which on the basis of the above mentioned considerations could only occur inside the bacterial cell. Thus, it is not unexpected that carotenoids are preserved in anoxic sediments for thousands of years [37] and can be used as tracers of lake history [39,40].

One of the arguments usually used for explaining the recalcitrance of hydrocarbons under anoxic conditions appears to be only of minor importance: low solubility in aqueous system does not prevent anaerobic degradation. The enrichment cultures with hexadecene or squalene as substrate both grew exponentially for comparably long times indicating that they were not limited by substrate availability. The kinetics of growth have also to be studied in more detail in the future. It is open at present which role the contact between substrate droplets and bacterial cells plays and if biogenic tensides are involved as found with aerobic hydrocarbon-degrading bacteria [41].

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