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Methanogenic Fermentation of Benzoate in an Enrichment Culture

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Abstract. Enrichment cultures inoculated with black mud fermented benzoate according to the stoichiometric equation:

$$4\ C_6H_5CO_2H + 18\ H_2O \rightarrow 15\ CH_4 + 13\ CO_2.$$

Trans-2-hydroxycyclohexanecarboxylate, 2-oxo-cyclohexanecarboxylate, pimelate, caproate, butyrate, acetate, and molecular hydrogen were shown to be regular components of the culture fluid occurring in low concentrations. Inhibition of methanogenesis by chloroform, 4-chlorobutyrate, or 2-bromooctanoate resulted in a cessation of the benzoate breakdown after all intermediates had accumulated. It is proposed that benzoate is fermented via a direct reductive pathway to butyrate, acetate, H_2, and CO_2, whereafter butyrate is converted to acetate and H_2, and the latter substrates are fermented to CH_4 and CO_2 by methane producers.

Introduction

Aromatic substrates can be degraded in methanogenic fermentations by mixed bacterial cultures under anaerobic conditions. A number of studies on these methanogenic fermentations of benzoate by enrichment cultures inoculated with rumen contents, sewage sludge, or black mud have been published (3,7,8,12,13), and the knowledge gained from these studies has recently been summarized by Balba and Evans (1).

Experiments carried out by Balba and Evans (1) showed that in anaerobic enrichment cultures inoculated with sheep rumen contents or black sewage sludge, benzoate is fermented to CH_4 and CO_2 in the absence of O_2, light, nitrate, or sulfate. The compounds cyclohexanecarboxylate, cyclohex-1-enecarboxylate, and adipate are possible intermediates, suggesting the operation of the direct reductive pathway as found in *Rhodopseudomonas* (5).

In this communication we describe the methanogenic fermentation of benzoate by a bacterial consortium established in enrichment cultures inoculated with black mud from a small polluted river and the occurrence of 2-hydroxycyclohexanecarboxylate, 2-oxo-cyclohexanecarboxylate, pimelate, caproate, butyrate, and acetate as intermediates in the culture.

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Materials and Methods

Cultures

Enrichment cultures were started in 500-ml serum bottles fitted with butyl rubber stoppers by adding a 10% inoculum to 270 ml of an anaerobic mineral solution containing (in g/liter): KH_2PO_4 0.75; K_2HPO_4 0.75; NaCl 1.5; $(NH_4)_2SO_4$ 0.75; $CaCl_2 \cdot 2H_2O$ 0.15; $MgSO_4 \cdot 7H_2O$ 0.15; and sodium benzoate 2.0. Acetate and butyrate enrichment cultures were started simultaneously and were prepared in the same way as described for benzoate. Substrate concentration was 0.2% (wt/vol). The pH of the solution was adjusted to 6.7. The gas phase in the flasks consisted of oxygen-free CO_2. The flasks were incubated unshaken at 39°C in the dark. For most experiments subcultures were prepared by removing 4- or 6-ml quantities from the enrichment culture and incubating these under CO_2 in 15 × 160 mm roll-tubes (Bellco, Inc., Vineland, N.J.) fitted with recessed butyl rubber stoppers. During all procedures strict anaerobic conditions were maintained using the Hungate technique. The pH in the experiment subcultures with benzoate as the substrate needed no major adjustments but varied only between 6.7 and 6.9.

The CO_2 used as the gas phase in these cultures was exposed to heated copper prior to passage of the gas into the tubes.

Analytical Methods

The rate of gas production was determined by the syringe method (6), and the composition of the gas was analyzed by gas chromatography on a Becker "Permalyzer" (column Porapak Q, 80-100 mesh, 1000 × 4 × 6) using pure N_2 as the carrier gas at a flow rate of 30 ml/min. Volatile fatty acids (VFA) were measured as described by Cottyn and Boucque (4).

Benzoate concentration was estimated directly by measuring the absorbance of the culture supernatant at 222 nm after centrifugation, but a more precise analysis of benzoate and benzoate derivatives was obtained by gas chromatography. Known aliquots of culture medium were freeze-dried and the dried residue extracted with pyridine. The trimethylsilyl derivatives were made by reacting an aliquot of this solution with 100 μl of N,O-bis-(trimethylsilyl)-trifluoroacetamide with 1% of trimethylchlorosilane (Pierce, Rockford, Ill.) for 45 min at room temperature. Injection volume was 1 μl. A 6-foot glass coiled column (4 mm i.d.) was packed with 5% SE-30 on chromosorb-W (AW-DMCS, 80 to 100 mesh) and connected to a hydrogen flame ionization detector in a Packard Model 417 gas chromatograph. The column temperature was 110°C and the carrier gas was pure nitrogen at a flow rate of 15 ml/min. The temperature of the injection block was kept at 225°C.

For the detection of 2-oxo-cyclohexanecarboxylate, silylation was carried out with Sylon BTZ reagent (Supelco Inc. Bellefonte, Pa.) for 1 hour at 80°C, after the keto-group was allowed to react with O-methylamine HCl for 1 hour at 60°C (10). The column temperature was raised to 230°C. Cyclohexanecarboxylate, cyclohex-1-enecarboxylate and cyclohex-3-enecarboxylate could not be separated by gas chromatography and hence a peak occurring at this time on the chromatograms will be referred to as *reduced benzoic acids*.

Radioactivity in CO_2 and CH_4 produced in the subcultures from labeled benzoate was determined as follows. At the end of the incubation, subcultures in roll-tubes were inactivated by injection of H_2SO_4 5N (final concentration ± 0.5N) through the rubber stopper. The total volume of the gas phase in the tube including the dissolved CO_2 and bicarbonate released by the acidification was fed slowly into a combustion-absorption train by connecting the tube to the train and washing the gas from the tube with a slow stream of N_2 from a cylinder. The complete train consisted of three gas washers each containing 4 ml of 10 N KOH for the complete absorption of CO_2, connected to a CuO column in quartz tubing and heated to 750°C to combust the CH_4 to CO_2, which was then absorbed in another set of three gas washers with KOH. Scintillation counting of 1-ml samples from the gas washers after addition of 15 ml Unisolve-1 (Koch-Light Labs, Ltd., Colnbrook, U.K.) was performed in a liquid scintillation counter (Packard Tricarb 2425B).

Benzoate and volatile fatty acids were separated by partition chromatography on cellulose powder columns (Whatman CF 11) eluted with mixtures of acetone in n-hexane (9), and aliquots of the eluted fractions were assayed for radioactivity by scintillation counting. Benzoate could not be separated from butyrate on the cellulose columns, but the presence of radioactivity in butyrate as well as in other VFA was also determined by using a gas proportional counter (Packard Model 894) connected to the gas chromatograph.

Results

Stoichiometry of the Benzoate Fermentation

Successful enrichment cultures were established by inoculating 270 ml of the anaerobic salts solution containing a final concentration of 0.2% (wt/vol) of sodium benzoate as the only carbon and energy source with 30 g of black mud sampled from a small polluted river (de Grift) running through the city of Utrecht. At 7-day intervals 10% of the culture volume was removed and replaced with an equal volume of the autoclaved anaerobic salts solution containing 2% (wt/vol) of sodium benzoate. It took about 2 months for the cultures to reach a steady state in C balance as judged from the rates of gas production and the rate of benzoate disappearance.

The 300-ml enrichment culture produced on average 103 ml of gas daily with an average composition of 52% CH_4 and 48% CO_2. The gas produced accounted for 93.5% of the C in the substrate used. Thus, the fermentation of benzoate occurred according to the following stoichiometric equation:

$$4\ C_6H_5COOH + 18\ H_2O \rightarrow 15\ CH_4 + 13\ CO_2$$

Rate of Benzoate Degradation

From the rates of methane production in a number of experiments carried out with 4-ml subcultures, rates of benzoate degradation were calculated at different substrate concentrations, using the stoichiometry of the benzoate fermentation shown above. These data were accumulated from the simultaneous adaptation studies as well as from the experiments with labeled benzoate (see below), and despite the fact that the incubation times differed widely, the inhibition of the fermentation rate at substrate concentrations higher than 18 mM was clearly suggested.

Therefore, the influence of the benzoate concentration on the fermentation rate was studied in a number of separate experiments with 4-ml subcultures, and the relation found between these parameters is shown in Fig. 1. The rate of benzoate disappearance was determined by directly assaying the benzoate concentration. It is clear that benzoate in concentrations higher than about 18 mM inhibited its own fermentation. The concentration of 18 mM is just somewhat higher than the benzoate concentration in the enrichment culture at the start of each 7-day cycle.

Shaking of the cultures did not affect the rate of benzoate degradation nor the rate of methane formation.

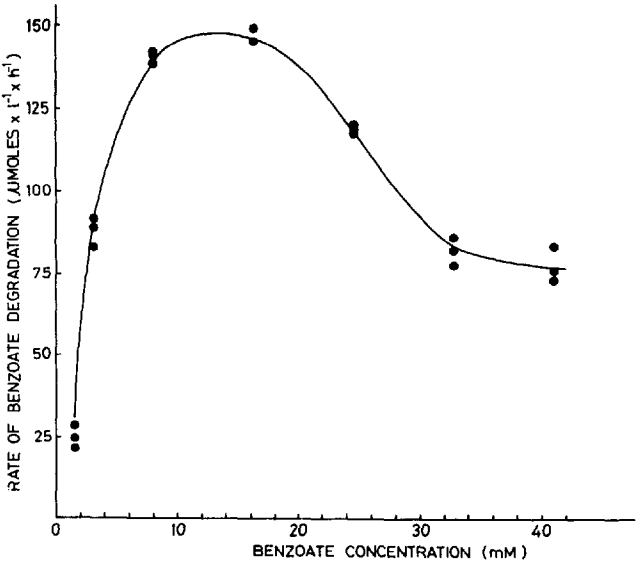

Fig. 1. Rates of gas production from benzoate by the mixed culture at different substrate concentrations; ••• experimental data.

Simultaneous Adaptation Studies

Just before the end of a 7-day incubation cycle, when the substrate was exhausted, 4-ml quantities were removed from the enrichment culture and incubated anaerobically with different concentrations of benzoate, or with other substrates such as the substituted benzoic acids 3-hydroxybenzoate, 4-hydroxybenzoate, or with possible intermediates in the benzoate fermentation pathway as well as with some short-chain acids. Rates of gas production in these cultures were measured after 24 and 48 hours of incubation. These studies showed that benzoate-adapted cells (without a lag) (Fig. 2) readily fermented caproate, butyrate, and acetate to CO_2 and CH_4. The benzoate derivatives 3-hydroxybenzoate and 4-hydroxybenzoate, the reduced acids cyclohexanecarboxylate and cyclohex-1-enecarboxylate, and also the short-chain acids propionate, pimelate, adipate, and malonate were not or were only weakly attacked. From analyses by gas chromatography, it appeared that succinate was decarboxylated to propionate and carbon dioxide.

Mixed cultures adapted to butyrate or acetate readily fermented caproate, butyrate, and acetate to CO_2 and CH_4 but not benzoate or any of the other substrates tested.

Influence of Sulfate

The mineral medium used in the experiments reported above contained sulfate, and this was apparently partly reduced to sulfide as judged from the smell of the culture and the black precipitate which always was formed during incubation.

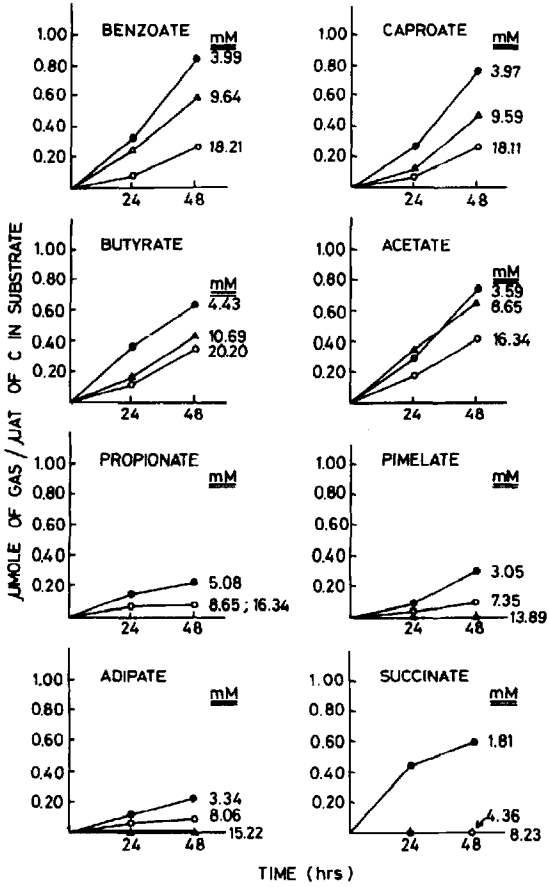

Fig. 2. Rates of gas production from different substrates by mixed bacterial cultures adapted to benzoate as the only carbon and energy source. CO_2 and CH_4 were the only gases detected.

Increasing the sulfate concentration up to 10 mM or replacement of the sulfates by sulfides did not affect the rate of benzoate degradation nor the composition of the gas produced in short-term incubations in the subcultures. In all further experiments sulfates were replaced by the respective sulfides, although the sulfate content had no influence on the overall stoichiometry.

Tracer Experiments

Figure 3 shows the results obtained from incubation studies with carboxyl[^{14}C] benzoate (5 μC; spec. act. 0.18×10^6 dpm/μmole) and ring[U-^{14}C] benzoate (5μC; spec. act. 0.18×10^6 dpm/μmole, respectively, in 6-ml subcultures. Within 4 days of incubation benzoate was almost completely fermented to CO_2 and CH_4. Of the total radioactivity added, 95.9% was recovered in these two gases after 96 hours of incubation with ring[U-^{14}C] benzoate and 92.3% with carboxyl[^{14}C]

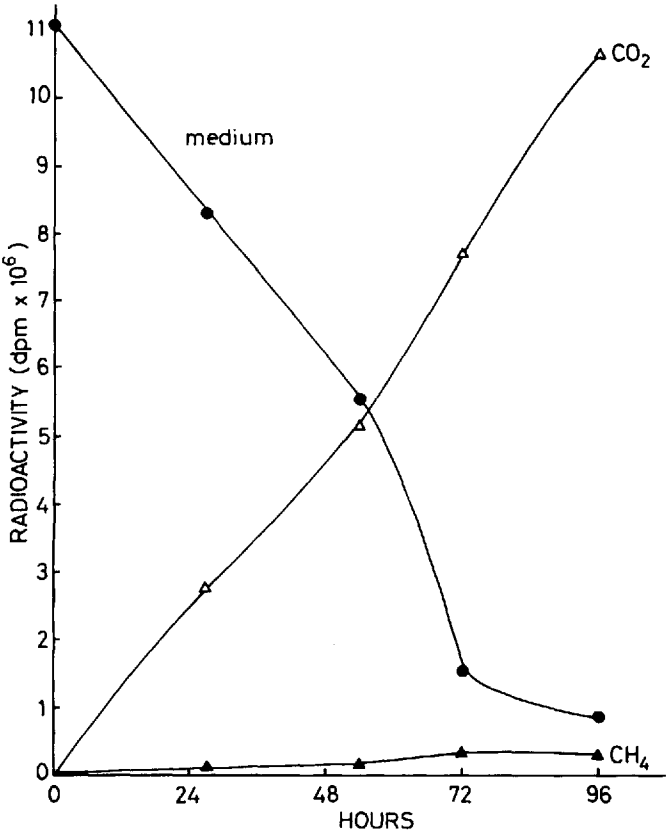

Fig. 3.(A): Formation of CO_2 and CH_4 during the degradation of carboxyl[^{14}C]benzoate. Counts in medium were determined after removal of the gases.

benzoate. It could be demonstrated by using a gas chromatograph with a gas proportional counter as well as by partition chromatography on cellulose columns that butyrate and acetate were the only volatile fatty acids that became labeled with either ring- or carboxyl-labeled benzoate. This means that both acids were extracellular intermediates occurring in the mixed culture during the fermentation of benzoate. There was a transient accumulation of these acids (shown for acetate in Fig. 3b), and concentrations of about 2.3 mM of acetate and 1.2 mM of butyrate were reached between 24 and 48 hours of incubation, whereas butyrate disappeared thereafter followed by the disappearance of acetate. None of the following short-chain acids became labeled: formate, propionate, valerate, branched-chain volatile fatty acids, pyruvate, lactate, succinate, or fumarate. The ratio of radioactivity in the gases CO_2:CH_4 was higher initially (2.00 at 27 hours) than at the end of the incubation (0.93 at 96 hours) with ring[U-^{14}C] benzoate. With carboxyl[^{14}C] benzoate this ratio CO_2:CH_4 did not change much during the 96 hours incubation, being 31 at 27 hours and 29 at 96 hours of incubation.

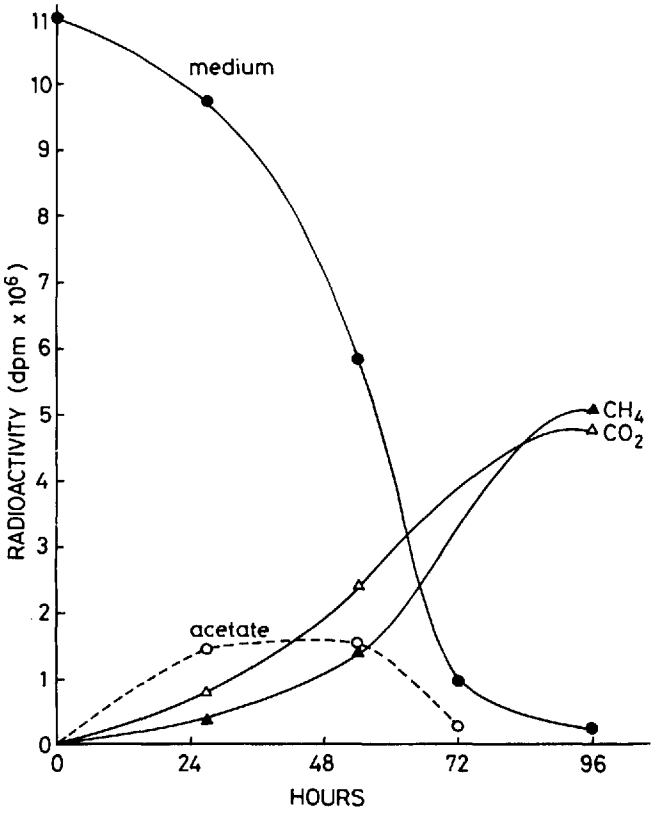

Fig. 3.(B): Formation of CO_2 and CH_4 as well as the transient accumulation of acetate during the degradation of ring[U-^{14}C]benzoate. Counts in medium were determined after removal of the gases.

When [U-^{14}C] butyrate was fed to the benzoate enrichment culture, labeled acetate started appearing in the culture fluid after several hours of incubation. Most of the label finally was recovered in CH_4 and CO_2.

Inhibition Experiments

For these experiments 4-ml amounts of the benzoate enrichment culture were subcultured immediately following the weekly feeding of benzoate, and several additional compounds were added in order to study their possible interference with the fermentation of the benzoate. Gas production was measured for three consecutive intervals of 24 hours. Butyrate in concentrations $\geq$ 4 mM appeared inhibitory to the fermentation of benzoate, whereas acetate acted inhibitory in 8 mM concentrations (Table 1). Likewise, 4-hydroxybenzoate and 3-hydroxybenzoate were inhibitory, especially the former compound.

Some incubations were carried out with halogenated compounds that might interfere with the degradation of benzoate to CO_2 and CH_4 at one or more stages

Table 1. Effect of the addition of some organic compounds on the gas production in benzoate enrichment cultures

Additions		Average Daily Gas Production[a] ml ± SD (*N*)	
None (16 mM benzoate)		1.94 ± 0.20 (21)	
4-Hydroxybenzoate	3.5 mM	1.26 ± 0.16 (7)	$p < 0.01$
4-Hydroxybenzoate	8.5 mM	1.17 ± 0.19 (7)	$p < 0.01$
4-Hydroxybenzoate	16.1 mM	1.13 ± 0.33 (7)	$p < 0.01$
3-Hydroxybenzoate	3.5 mM	1.91 ± 0.21 (7)	
3-Hydroxybenzoate	8.5 mM	1.50 ± 0.17 (7)	$p < 0.01$
3-Hydroxybenzoate	16.1 mM	1.24 ± 0.37 (7)	$p < 0.01$
Cyclohexanecarboxylate	3.8 mM	1.93 ± 0.24 (7)	
Cyclohexanecarboxylate	9.2 mM	1.89 ± 0.32 (7)	
Cyclohexanecarboxylate	17.4 mM	2.04 ± 0.28 (7)	
Cyclohex-3-enecarboxylate	3.9 mM	1.89 ± 0.13 (7)	
Cyclohex-3-enecarboxylate	9.3 mM	1.73 ± 0.11 (7)	
Cyclohex-3-enecarboxylate	17.6 mM	1.77 ± 0.11 (7)	
Caproate	4.0 mM	2.06 ± 0.31 (7)	
Caproate	9.6 mM	2.49 ± 0.48 (7)	
Caproate	18.1 mM	1.70 ± 0.41 (7)	
Butyrate	4.4 mM	1.49 ± 0.22 (7)	$p < 0.025$
Butyrate	10.7 mM	1.59 ± 0.21 (7)	$p < 0.05$
Butyrate	20.2 mM	1.47 ± 0.37 (7)	$p < 0.05$
Acetate	3.6 mM	1.83 ± 0.12 (7)	
Acetate	8.7 mM	1.29 ± 0.16 (7)	$p < 0.01$
Acetate	16.3 mM	0.97 ± 0.26 (7)	$p < 0.01$
Pimelate	3.1 mM	1.96 ± 0.27 (7)	
Pimelate	7.4 mM	1.84 ± 0.32 (7)	
Pimelate	13.9 mM	1.97 ± 0.35 (7)	

[a] SD, standard deviation; *N*, number of experiments.

of the degradation chain. The results showed that 2-chlorobenzoate (0.5 to 1.5 mM) was not inhibitory to CH_4 formation from benzoate nor to methanogenic breakdown of acetate in acetate enrichment cultures prepared similarly by inoculation with black mud. However, compounds such as 4-chlorobutyrate, 2-bromo-octanoate, or chloroform resulted in a depression or almost complete cessation of methane production from benzoate (Table 2) or acetate (Table 3). Gas chromatography showed that the inhibition of benzoate fermentation led to an accumulation of benzoate (although this was not seen with 4-chlorobutyrate), of *reduced benzoic acids,* of *trans*-2-hydroxycyclohexanecarboxylate, 2-oxo-cyclohexanecarboxylate, pimelate, caproate, butyrate, and acetate over the amounts of these intermediates present in control cultures without inhibitors. Adipate was not found. All these compounds were normally present in low concentrations (10 to 50 μM except acetate and butyrate which were present in millimolar concentrations) in the culture fluid from the benzoate fermentation flasks at all times. Only trace amounts of gaseous hydrogen accumulated in the gas phase of the cultures when methane formation from benzoate was inhibited with one of the halogenated compounds. Likewise, methanogenesis in butyrate

Table 2. Inhibition of methanogenesis and accumulation of acetate and butyrate in benzoate enrichment cultures upon addition of some halogenated compounds[a]

Additions		CH_4 Formed (μmoles)			Concentrations of Volatile Fatty Acids in the Culture at 120 hr of Incubation (mM)					
		0–48 hr	48–120 hr	Total	C_2	C_3	iC_4	C_4	iC_5	C_5
None		105.8	103.0	208.8	0.53	0.03	0.03	0.24	—	—
4-Chlorobutyrate	1.25 mM	57.4	53.0	110.4	4.59	0.04	0.03	0.84	—	—
	2.50 mM	54.7	77.3	132.0	5.62	0.04	0.03	2.94	0.05	—
	3.75 mM	22.9	106.6	129.5	10.11	0.04	0.03	6.51	—	—
2-Bromo-octanoate	4 mM	2.9	0	2.9	3.00	0.03	0.02	3.15	0.05	0.03
	8 mM	2.9	0.8	3.7	3.00	0.03	0.03	2.31	—	—
	12 mM	3.0	0.8	3.8	4.35	0.06	0.06	9.24	0.11	0.28
Chloroform	0.27 mM	1.5	15.80	17.3	4.49	0.03	0.03	9.66	0.11	—
	0.54 mM	2.9	11.30	14.2	4.03	0.03	0.03	8.76	0.08	—
	0.81 mM	2.9	0	2.9	4.21	0.02	0.02	9.14	—	—

[a] Initial benzoate concentration 18 mM; culture volume 3 ml.

Table 3. Inhibition of methanogenesis in acetate enrichment cultures by some halogenated compounds[a]

Additions		CH_4 Formed (μmoles)
None		100.7 ± 6.5[b]
4-Chlorobutyrate	(2.5 mM)	79.2 ± 8.5
2-Bromo-octanoate	(8.0 mM)	1.2 ± 0.6
Chloroform	(0.5 mM)	1.0 ± 0.6
2-Chlorobenzoate	(1.0 mM)	98.0 ± 6.0

[a] Initial acetate concentration 24 mM; culture volume 4 ml.
[b] Average of quadruplicate incubations ± SD.

enrichment cultures could completely be inhibited by chloroform (Fig. 4), but at all chloroform concentrations used only trace amounts of gaseous hydrogen accumulated in the gas phase.

Organisms

Microscopic examination of the enrichment culture fluid revealed that the predominant microorganisms were three distinct types of nonmotile, non-spore-forming bacteria. One organism is a long (2.5 × 7.0-μm) gram-negative rod, forming long chains. The second organism is a gram-positive coccus (diameter 1 μm), whereas the third organism is again a gram-negative rod (1 × 3 μm). Attempts to isolate these organisms in pure culture are in progress.

Discussion

The methanogenic fermentation of benzoate in the enrichment culture described in this publication is probably not the work of a single organism as once was proposed by Nottingham and Hungate (12) for the degradation of benzoate in their methanogenic enrichment cultures. It is likely that the degradation of benzoate in anaerobic environments arises from the mutual cooperation of at least two groups of organisms as has been shown by Ferry and Wolfe (7). One group will ferment the aromatic substrate to short-chain volatile fatty acids, hydrogen, and carbon dioxide, whereas another group (methanogenic organisms) is responsible for the conversion of acetate, hydrogen, and formate (which are found as extracellular intermediates in the mixed culture) to methane.

In a number of previously published studies [summarized by Balba and Evans (1)] on the anerobic fermentation of benzoate in enrichment cultures, propionate, acetate, and formate have been detected as intermediates. Balba and Evans (1) showed that in their cultures from sheep rumen contents or black sewage sludge, where propionate and acetate were shown to be intermediates, labeled adipate was formed from ring[U-^{14}C] benzoate in addition to labeled cyclohexanecarboxylate and cyclohex-1-enecarboxylate.

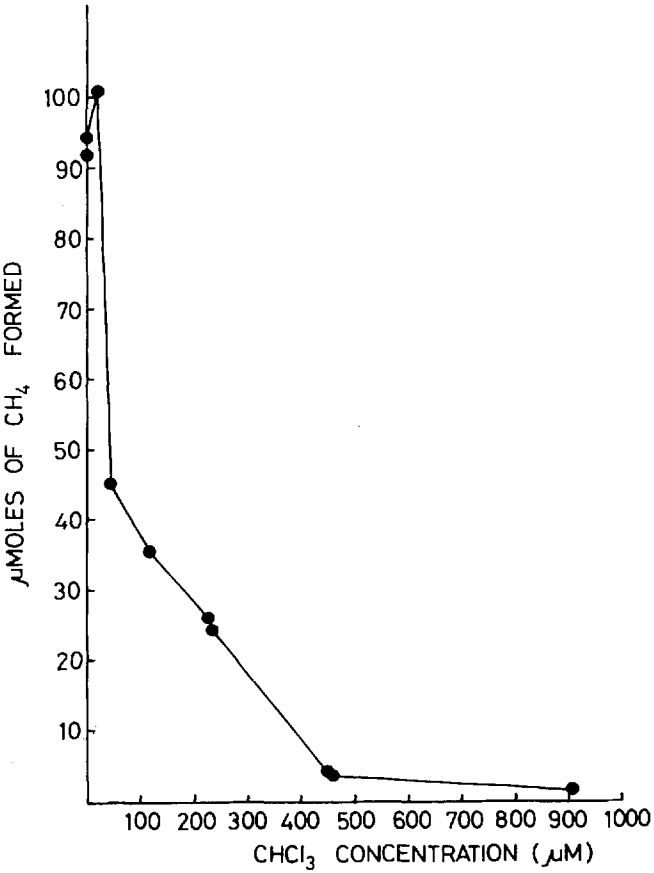

Fig. 4. Inhibition of methanogenesis in a butyrate enrichment culture by increasing concentrations of chloroform. Initial butyrate concentration 18 mM; culture volume 4 ml; incubation time 48 hours.

The results obtained in our study differ somewhat from the observation quoted above in that (a) propionate and formate were not found as intermediates, but butyrate and acetate as well as traces of caproate were found instead, (b) pimelate and not adipate was found to be an intermediate, suggesting the operation of the direct reductive pathway as discovered in *Rhodopseudomonas palustris* (5), and (c) agitation of the cultures did not influence the rate of benzoate degradation. The finding of butyrate and acetate together with the observation that labeled butyrate was converted to labeled acetate before appearing as labeled CO_2 and CH_4 suggests that these short-chain volatile acids are degraded by a process very similar to the β-oxidation of fatty acids. The electrons generated could be used in the formation of molecular hydrogen, which is converted to CH_4 by the methanogens (2,11,15). Continuous removal of dissolved hydrogen and acetate, both products of the anerobic fatty acid degradation, would allow the degradation of benzoate and butyrate to take place (for thermodynamic considerations see refs. 7 and 14). The fact that inhibition of

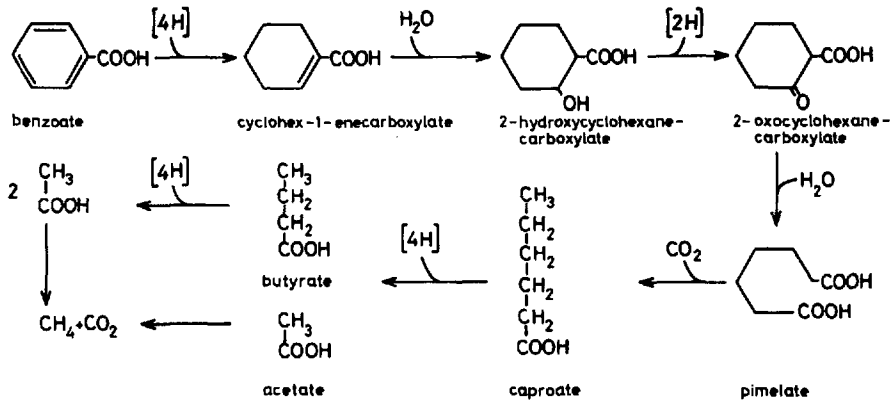

Fig. 5. Proposed anaerobic pathway for the methanogenic fermentation of benzoate in an enrichment culture inoculated with mud.

methanogenesis in butyrate cultures did not lead to an appreciable accumulation of gaseous hydrogen does not necessarily mean that hydrogen is of no importance as an extracellular intermediate, but could also be taken as evidence for the absolute dependence of butyrate degradation on methanogenesis. Inhibition of benzoate degradation by methane inhibitors immediately resulted in a rapid accumulation of acetate and butyrate in the cultures.

On the basis of the experiments reported above, the degradation of benzoate in the enrichment culture is thought to take place via the pathway proposed in Fig. 5. With the exception of cyclohex-1-ene-carboxylic acid, which by gas chromatography could not be discriminated from cyclohexanecarboxylic acid, all of the intermediates have been detected in the culture fluid. Since pimelate was detected in very low concentrations in the culture fluid during the fermentation of benzoate, it is not clear why this compound was not used in the simultaneous adaptation studies (Fig. 2). One reason could be that extracellular pimelate is not taken up by the microorganisms. In the pathway in Fig. 5, which is a conclusion from all experiments reported in this paper, it is proposed that pimelate is decarboxylated to caproate, and it can be calculated that this reaction involves a standard free energy change ($\Delta G'_0$) of -11.8 kcal/mole at pH 7.0. On the basis of the proposed pathway as well as on the stoichiometry of the fermentation, it is suggested that at least one group of organisms will ferment benzoate to acetate, butyrate, hydrogen, and carbon dioxide (reaction 1). Methanogenic organisms are required for the conversion of acetate to $CH_4 + CO_2$ or $H_2 + CO_2$ to CH_4 (reactions 3 and 4), whereas another group of organisms could be responsible for the conversion of butyrate to acetate and hydrogen (reaction 2):

benzoate + 4 H_2O → butyrate + acetate + H_2 + CO_2 (reaction 1)
butyrate + 2 H_2O → 2 acetate + 2 H_2 (reaction 2)
3 H_2 + ¾ CO_2 → ¾ CH_4 + 1½ H_2O (reaction 3)
2 acetate → 3 CH_4 + 3 CO_2 (reaction 4)

Sum: benzoate $+ 4\frac{1}{2}\ H_2O \rightarrow 3\frac{3}{4}\ CH_4 + 3\frac{1}{4}\ CO_2$
or: 4 benzoate $+ 18\ H_2O \rightarrow 15\ CH_4 + 13\ CO_2$

Acknowledgments

We are grateful to Dr. W. C. Evans of the Department of Biochemistry and Soil Science, University College of North Wales, Bangor, for his gift of both isomers of 2-hydroxycyclohexanecarboxylic acid and 2-oxocyclohexanecarboxylic acid which were used as references in the analyses by gas chromatography. Furthermore, we are indebted to Dr. S. G. van den Bergh for critically reading the manuscript.

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