Microbial Ecology of Pectin Decomposition in Anoxic Lake Sediments

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Anaerobic digestion of pectin by bacteria was examined in two freshwater lakes in Wisconsin and in defined laboratory cultures of species prevalent in the lake sediment. The turnover times for pectin biodegradation to methane in sediments incubated at *in situ* temperature were much longer (100 h in Lake Mendota and 185 h in Knaack Lake) than either that observed for glucose (12 h in Lake Mendota) or previously reported for acetate (0·22 h in Lake Mendota). The numbers of pectinolytic anaerobes varied seasonally in both sediments (10^2–10^5 and 10^3–10^5 ml^{-1} in Knaack Lake and Lake Mendota, respectively), and were highest during the fall after sedimentation of algal blooms and/or leaf detritus. *Clostridium butyricum* was identified as a prevalent pectinolytic anaerobe in both lakes. In mono-culture pectin fermentations, *C. butyricum* produced methanol, H_2/CO_2, acetate, ethanol and butyrate; growth stopped in the presence of excess energy source when the pH fell to 4·3. In co-culture pectin fermentations of *C. butyricum/Methanosarcina barkeri*, H_2/CO_2, methanol and acetate were detected as intermediary metabolites, and pectin was completely degraded to CH_4 and CO_2, the pH remaining neutral. ^{14}C-radiotracer analysis substantiated the simultaneous conversion of H_2/CO_2, methanol and acetate to CH_4 by *M. barkeri* as these metabolites were generated from pectin hydrolysis by *C. butyricum*.

INTRODUCTION

Pectin is a polymer of galacturonic acid linked by 1,4-β bonds and methoxylated to a varying extent at the carboxyl moieties. Pectin is a component of photosynthetic biomass (Kertesz, 1951) and is found in the plant leaves and algal cell walls that are commonly deposited into freshwater lake sediments. Pectin can be abundant in cyanobacterial sheaths (Desikachary, 1959; Wolk, 1973) and green algal walls (Gooday, 1971; Green & Jennings, 1967; Prescott, 1968; Sikes, 1978). Quantitative data on the total input of pectin into a lake ecosystem from algal blooms or plant detritus are not available. Nonetheless, pectin is undoubtedly decomposed by anaerobic micro-organisms in association with the annual sedimentation of plant litter and algal cells.

Ecological studies on microbial pectin degradation are scant and have generally been related to plant–microbe interactions in disease or symbiosis, or to crop and food spoilage. Our previous studies (Winfrey *et al.*, 1977; Winfrey & Zeikus, 1977, 1979*a*, *b*; Zeikus & Winfrey, 1976) on the microbial ecology of anaerobic decomposition processes have characterized the metabolism of immediate methanogenic precursors in Knaack Lake and Lake Mendota, Wisconsin. The methanogenic population of Lake Mendota was characterized in a general way; it was composed primarily of H_2/CO_2-metabolizing *Methanobacterium* species and *Methanosarcina barkeri* which fermented H_2/CO_2, methanol and/or acetate (Weimer & Zeikus, 1978*a*, *b*; Zeikus & Winfrey, 1976). Carbon input in these lake sediment ecosystems varies tremendously because Knaack Lake is a small meromictic lake that receives considerable litter from surrounding trees (Winfrey & Zeikus, 1979*b*), whereas in the deep portions of Lake Mendota the non-limnic contribution to the total

photosynthate decomposed is negligible and cyanobacteria and green algae make up the greater part of the sedimented primary production (Fallon & Brock, 1979).

Pectin was chosen as a model biopolymer for studies on anaerobic biomass decomposition because of its presence in sedimented organic matter of the lakes, its unique chemical structure (i.e. a methoxylated polysaccharide), its commercial availability as a ^{14}C-tracer, and its described decomposition by pectinolytic, anaerobic bacteria (Avrova, 1975; Lund & Brocklehurst, 1978; Miller & Macmillan, 1970; Rexová-Benková & Markovič, 1976). The purpose of the present paper was to characterize the decomposition of pectin and the anaerobic pectinolytic population in two freshwater lake sediments and to document the metabolic interactions between prevalent pectinolytic bacteria and other sediment bacteria that account for complete anaerobic mineralization of the polymer.

METHODS

Sites and sampling procedures. Lake Mendota is a moderately eutrophic, hard-water lake of glacial origin in southern Wisconsin, U.S.A. It covers about 3900 ha, has an average depth of about 12 m and a maximum depth of 24 m, and is anaerobic below 10·5 m during stratification. Two sampling sites (18 and 24 m in the deepest portions of the lake) were studied. Knaack Lake is a eutrophic, meromictic lake in central Wisconsin and covers about 1·1 ha. Anaerobic sediment was obtained from the deepest portion of the lake (22 m).

Sediment samples were taken with an Eckman dredge. The overlying water was carefully removed and the upper 1–2 cm of the sediment was collected and transferred into 200 ml glass bottles. These were gassed continuously with O_2-free N_2, anaerobically sealed with rubber stoppers and transferred to the laboratory in insulated chests (Zeikus & Winfrey, 1976).

Growth media and culture methods. All media and culture methods were appropriate for stringent anaerobes (Zeikus, 1977; Zeikus *et al.*, 1979). The low-phosphate buffered basal medium (LPBB medium) described by Zeikus *et al.* (1979) was used as a minimal medium for enumeration, enrichment and isolation of strains. Pectinolytic bacteria were enumerated and grown in LPBB medium containing 0·1% (w/v) yeast extract and 0·5% (w/v) pectin. Pectin solutions were prepared as described previously (Schink & Zeikus, 1980). The pectin used had a degree of methoxylation of 66% and was a gift from Sunkist Growers (Corona, Calif., U.S.A.). Total heterotrophic bacteria were enumerated on LPBB medium containing 0·3% (w/v) yeast extract, 1% (w/v) tryptone, and 0·5% (w/v) glucose. Methanogenic bacteria were enumerated on LPBB medium containing 0·05% yeast extract, with either a H_2/CO_2 (80:20, v/v) gas phase (for H_2 oxidizers) or 0·5% (v/v) methanol and a N_2/CO_2 (95:5) gas phase (for methanol oxidizers). Mixed culture studies with *Methanosarcina barkeri* strain UBS used the carbonate buffered basal medium (CBB medium) described by Zeikus & Wolfe (1972). Strain UBS was isolated from Lake Mendota sediment (Zeikus & Winfrey, 1976) and was growth adapted and cultivated for one year in CBB medium containing 0·5% (w/v) acetate and a N_2/CO_2 (95:5) gas phase. All media were reduced with 0·05% (w/v) sodium sulphide before inoculation. Except for co-culture experiments, all cultures used anaerobic culture tubes (18 × 142 mm, Bellco Glass Co., Vineland, N.J., U.S.A.) sealed with black rubber stoppers and containing 10 ml medium. Co-culture experiments were performed in 60 ml Wheaton serum bottles (Millville, N.J.) closed with flanged black rubber bungs (Bellco) and aluminium crimp seals and containing 25 ml medium. Enrichments and enumerations were incubated at 30 °C without shaking, and co-culture experiments were incubated at 37 °C on a rotary shaker [previous experiments had shown the optimum temperature for various metabolic processes of bacteria from the lake sediments to be in the range 30–40 °C (Winfrey & Zeikus, 1979b; Zeikus & Winfrey, 1976)]. Growth was measured turbidimetrically at 660 nm in a Spectronic 20 colorimeter (Bausch and Lomb).

Enumeration procedures. Bacteria were enumerated in sediment samples by the three-tube most probable number (MPN) technique. Most probable numbers were calculated from standard tables (American Public Health Association, 1969) after 2–3 weeks incubation. Positive tubes for non-methanogens had an $A_{660} > 0.2$; those for methanogens produced >1% (v/v) methane in the culture headspace. Controls for enumeration of pectinolytic bacteria and methylotrophic methanogens used radioisotopic tracer analysis (see below) and were based on the production of $^{14}CO_2$ or $^{14}CH_4$ from labelled substrate.

Isolation of pectinolytic anaerobes. From the last positive tube in each enumeration series, pectinolytic bacteria were purified by streaking on to plates of the same pectin medium containing 2% (w/v) agar in an anaerobic glove box. Single colonies were picked, purified by streaking, and then transferred to liquid medium for preparation of stock cultures. Purity was checked by uniformity of colonies on streak plates and by phase-contrast microscopy. For identification procedures, API 20A and 20E biochemical assay kits (Analytab Products, Plainview, N.Y., U.S.A.) were used.

Radioactive tracer techniques. Degradation of pectin in mud samples and in enumeration series was studied

with [U-^{14}C]pectin (110 µCi mg^{-1}; 4 MBq mg^{-1}; ICN Pharmaceuticals Inc., Irvine. Calif.). [^{14}C]Pectin (0·5–1 µCi) was added to the tubes from a stock solution (55 µCi ml^{-1}) and the appearance of ^{14}CO$_2$ and ^{14}CH$_4$ measured in the gas phase by the combined gas chromatography and gas proportional counting procedures described by Nelson & Zeikus (1974). The same analysis techniques were used for tracer studies with [U-^{14}C]glucose, [^{14}C]methanol, [1-^{14}C]acetate and [2-^{14}C]acetate (obtained from New England Nuclear Division, Boston, Mass., U.S.A.). The turnover time constant for [^{14}C]pectin decomposition in sediment was determined by calculating the first-order rate of degradation to ^{14}CH$_4$ (100% biodegradation represents a recovery of 50% of the ^{14}C-labelled substrate as ^{14}CH$_4$).

Chemical analysis. Fatty acids and alcohols were analysed by flame ionization detection in a Packard 419 gas chromatograph as described by Zeikus *et al.* (1979). All gases were quantified by gas chromatography (Nelson & Zeikus, 1974). Lactate was determined spectrophotometrically with D- or L-lactate dehydrogenase according to Bergmeyer (1965). Reducing sugars in growth media were assayed by the dinitrophenylsalicylic acid method of Miller *et al.* (1960).

Chemicals. All chemicals were reagent grade and obtained from Sigma or Mallinckrodt, Inc. (Paris, Kentucky, U.S.A.). Agar, yeast extract and tryptone were from Difco; all gases were from Matheson (Joliet, Illinois, U.S.A.).

RESULTS

Biodegradation of pectin

The decomposition of pectin into CO$_2$ and CH$_4$ was compared in anoxic sediments collected at several times throughout the sampling years. Biodegradation was monitored while sediments were incubated at *in situ* temperature in the presence of trace amounts of radioactive pectin. Representative results for pectin degradation in sediments from Knaack Lake and Lake Mendota are shown in Fig. 1. Pectin appeared completely degraded after 3–4 d in Lake Mendota, whereas in Knaack Lake sediment, total observed degradation required 10–11 d. The differences in degradation patterns observed between the two lakes did not change significantly during the course of a sampling year. The turnover rate constants for pectin decomposition to CH$_4$ in Lake Mendota and Knaack Lake were calculated to be 0·01 h^{-1} and 0·0054 h^{-1}, respectively. For comparison, biodegradation of [U-^{14}C]glucose had a turnover rate constant of 0·083 h^{-1} in Lake Mendota sediment treated under the same experimental conditions. The optimal temperature for [^{14}C]pectin degradation in both lake sediments was 30–40 °C.

Enumeration of pectinolytic anaerobes

The variation of total numbers of pectin-degrading anaerobic bacteria during the course of a sampling year in both lake sediments is shown in Fig. 2. The trend observed for increasing numbers from spring to fall was repeated in a second sampling year. In Lake Mendota, the minimum number of pectin-degrading bacteria varied between 10^3 (ml sediment)$^{-1}$ in early summer and 10^5 ml^{-1} in October/November. In Knaack Lake, minimum total numbers were about an order of magnitude lower than in Lake Mendota and varied between 10^2 ml^{-1} in May and 10^5 ml^{-1} in September/October. However, both lake sediments contained pectinolytic bacteria in numbers of the same order of magnitude when the results are compared on the basis of sediment dry matter. By comparison, the minimum numbers of pectinolytic anaerobes, total heterotrophic anaerobes, H$_2$/CO$_2$-metabolizing methanogens and methylotrophic methanogens per ml of Lake Mendota sediment throughout a two-year sampling period were 10^3–10^5, 10^5–10^7, 10^3–10^4 and 10^1–10^2, respectively. It is worth noting, however, that methylotrophic methanogens always existed as clumps of *Methanosarcina*, each containing several hundred cells.

Isolation and characterization of the prevalent pectinolytic anaerobes

The last positive enumeration tubes from numerous MPN determinations of pectinolytic anaerobes in both lake sediments always contained Gram-positive and Gram-negative rods of various sizes. Spore-forming cells as well as free, elliptical spores were present in all end dilution tubes. The cytological and physiological properties of six different strains of prevalent
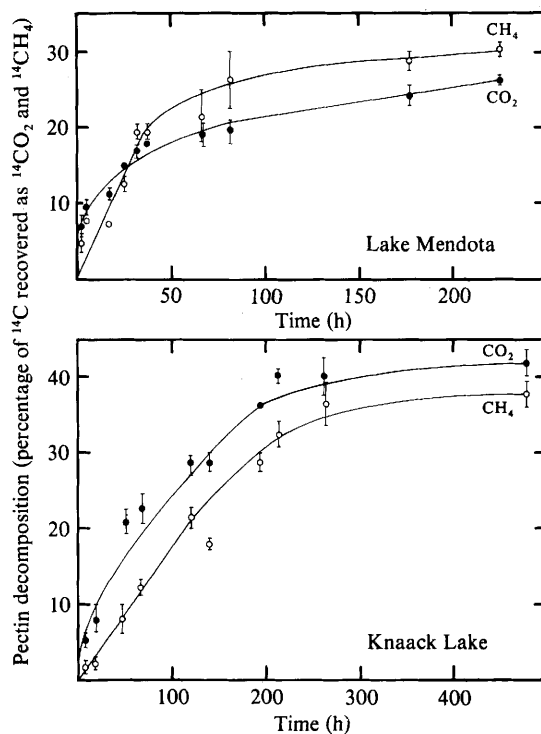
Fig. 1. Biodegradation of [U-^{14}C]pectin to ^{14}CH$_4$ (O) and ^{14}CO$_2$ (●) in anoxic lake sediments. Sediment (10 ml) was incubated at *in situ* temperature (15 °C for Lake Mendota and 4 °C for Knaack Lake) in anaerobic culture tubes that contained N$_2$ in the headspace and 1·0 × 10^6 d.p.m. [^{14}C]pectin. Pectin was not decomposed in controls that contained 0·1% formalin. Standard error bars are shown for each point.
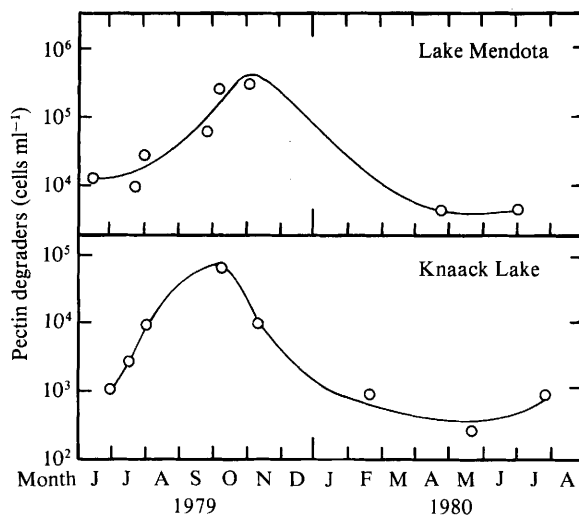

Fig. 2. Seasonal variation in total numbers of pectinolytic anaerobes in lake sediments.

pectinolytic bacteria isolated from lake sediments are shown in Table 1. The sporogenous strains MMP1, MMP2, MMP3 and KMP5 had similar properties; the asporogenous strains KMP3 and KMP4 differed from the other four – for example, they did not form significant

Table 1. *General microbiological features of prevalent pectinolytic anaerobes isolated from lake sediments*

		Cell features			Pectin fermentation products* (total μmol per tube)				
Strain	Lake	Size (μm)	Spores	Gram reaction	H_2	MeOH	EtOH	Ac	But
MMP1	Lake Mendota	1·5 × 6	+	+	20	130	61	94	17
MMP2	Lake Mendota	1·2 × 6	+	+	58	122	70	100	18
MMP3	Lake Mendota	1·5 × 6	+	+	90	148	53	136	18
KMP3	Knaack Lake	0·7 × 7	−	−	14	100	74	30	<1
KMP4	Knaack Lake	0·7 × 7	−	−	12	113	66	48	<1
KMP5	Knaack Lake	1·5 × 6	+	+	143	140	57	217	28

* Products were measured after growth for 24 h at 30 °C on LPBB medium containing 0·1 % yeast extract and 0·5 % pectin. MeOH, methanol; EtOH, ethanol; Ac, acetate; But, butyrate. Isopropanol, propanol, propionate, isobutyrate and lactate were not detectable (i.e. <0·05 μmol).

Table 2. *Relationship between energy source and fermentation products of Clostridium butyricum strain MMP3*

	Amount consumed* (μmol)	End products† (total μmol per tube)						
Substrate		H_2	MeOH	EtOH	Ac	But	Lac	CO_2
Glucose	244	118	<0·05	23	80	158	8	410
Polygalacturonic acid	112	123	<0·05	18	170	15	<0·5	224
Pectin	108	160	152	21	158	26	<0·5	197

* As determined by analysis of total reducing sugars (Miller *et al.*, 1960) before and after growth. Culture fluid (0·5 ml) was acidified with 5 M-H_2SO_4 (40 μl), boiled for 2 h and neutralized with NaOH prior to analysis for polygalacturonic acid or pectin.

† Products were measured after growth for 24 h at 30 °C on LPBB medium containing 0·1 % yeast extract and 0·5 % substrate. MeOH, methanol; EtOH, ethanol; Ac, acetate; But, butyrate; Lac, lactate.

amounts of butyrate when grown on glucose or pectin as energy source. None of the strains produced methanol from glucose. Strain MMP3 was chosen as a representative of the pectinolytic *Clostridium* species that prevailed in both lake sediments. Biochemical and physiological tests established that strain MMP3 was *Clostridium butyricum* according to the criteria of Buchanan & Gibbons (1974). Strain MMP3 fermented starch, mannan and pectin but not cellulose, xylan or arabinogalactan. It did not liquefy gelatin, nor reduce SO_4^{2-} and NO_3^-.

The fermentation products formed after growth of strain MMP3 on glucose, polygalacturonic acid or pectin are shown in Table 2. The main products of glucose fermentation were butyrate, acetate, H_2 and CO_2, with traces of ethanol and L-lactate. As compared with growth on glucose, after growth on the more oxidized substrates, polygalacturonic acid and pectin, only about half the amount of substrate was consumed, acetate was formed in higher amounts, butyrate in lower amounts, and lactate was not detectable. The average values observed for final cell density (A_{660}) after growth on glucose and on pectin or polygalacturonate were 1·05 and 0·75, respectively; the final pH was 4·3 in each case.

A time course for a typical pectin fermentation of *C. butyricum* is shown in Fig. 3. All the end products, except for butyrate, which was formed late in growth, were formed in relation to the increase in growth that paralleled pectin consumption. The products formed at the end of growth accounted for a balanced fermentation: the values for carbon recovery and oxidation/reduction (O/R) balance were 101·4% and 1·05, respectively. The following
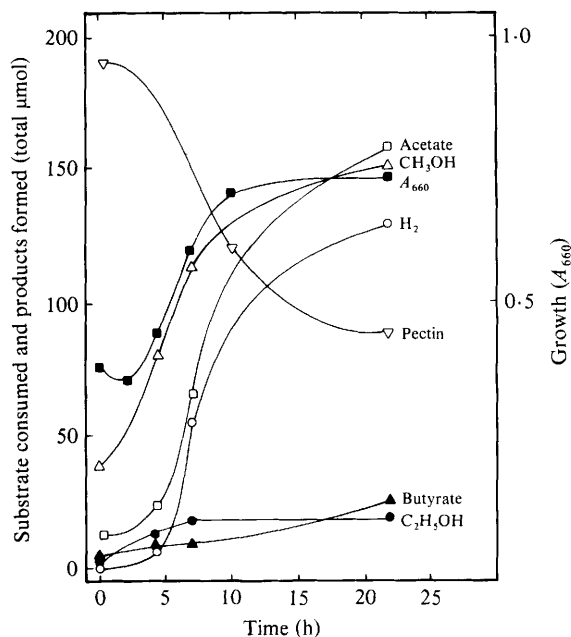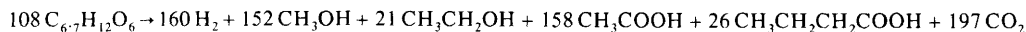
Fig. 3. Pectin fermentation time course for mono-cultures of *C. butyricum* strain MMP3. Anaerobic culture tubes contained 10 ml LPBB medium, 0·1% yeast extract, 0·5% pectin, N_2/CO_2 in the headspace and a 0·1 ml inoculum that had been grown on the same medium. The incubation temperature was 30 °C. ■, Growth (A_{660}); ▽, pectin; □, acetate; △, methanol; ○, H_2; ▲, butyrate; ●, ethanol.

balanced equation describes the stoichiometric relation observed for pectin fermentation (in total μmol):

$$108\,C_{6·7}H_{12}O_6 \rightarrow 160\,H_2 + 152\,CH_3OH + 21\,CH_3CH_2OH + 158\,CH_3COOH + 26\,CH_3CH_2CH_2COOH + 197\,CO_2$$

Methanol was formed in a ratio of 1 mol per mol methoxyl groups in the added pectin.

In order to investigate the metabolic basis for the cessation of growth of *C. butyricum* in the presence of excess energy source, the effect of different end products on the growth of strain MMP3 was examined (results not shown). In these experiments, end products were added to early/mid-exponential phase cultures at concentrations that equalled or exceeded the fermentation product concentration at the end of growth. Addition of H_2 did not inhibit growth even at concentrations up to 1 atm. Normal end product concentrations of methanol (i.e. 0·05%) did not influence growth rate or yield but at 0·2% methanol, growth yield was lowered by 14%. Addition of end product concentrations of acetate (20 mM, as sodium acetate) increased the growth rate and yield, whereas addition of 20 mM-sodium butyrate had no effect. High H^+ concentration had the greatest effect on growth: upon addition of HCl to change the culture pH to 4·5, growth ceased. Growth was not influenced in control tubes that contained 2·0% NaCl.

Complete decomposition of pectin in methanogenic co-culture

In order to examine the metabolic interactions associated with anaerobic mineralization of pectin to CH_4, a co-culture comprised of *Methanosarcina barkeri* strain UBS and *C. butyricum* strain MMP3 was established. Strain UBS was precultivated on 0·5% sodium acetate in CBB medium at 37 °C. An inoculum (0·5 ml) of strain MMP3 grown on pectin and pectin (1·2 mg ml^{-1}) were added to an acetate-depleted culture of strain UBS. This procedure established a stable co-culture of the two organisms that continued to degrade
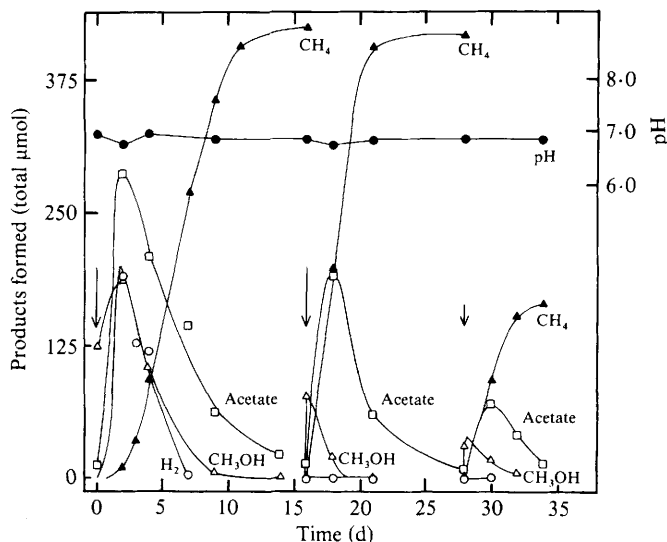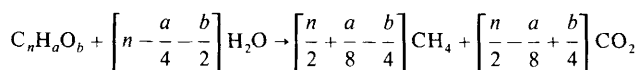
Fig. 4. Pectin fermentation time course for co-cultures comprised of *C. butyricum* strain MMP3 and *M. barkeri* strain UBS. Anaerobic serum vials contained 25 ml LPBB medium and a N_2 gas phase. Where indicated, either 30 mg (large arrow) or 10 mg (small arrow) pectin was added. Cultures were incubated at 37 °C with shaking. ●, pH; ▲, CH_4; □, acetate; △, methanol; ○, H_2.

pectin and make CH_4 upon continued addition of pectin or culture transfer into LPBB medium that contained pectin as the sole energy source. After 3 months of cultivation by semicontinuous addition of pectin, the co-culture was transferred to LPBB medium containing 1·2 mg pectin ml⁻¹ and the relationship of substrate consumption and end product formation was examined with time (Fig. 4). Methanol, acetate and H_2 appeared as the primary oxidizable end products of pectin degradation by the co-culture. These substrates were simultaneously decomposed by the co-culture during the next 15 d. After the addition of an equivalent amount of pectin on day 16, methanol and acetate appeared again as methanogenic intermediates, but in smaller amounts than before. Notably, H_2 was not detectable and the rate of pectin decomposition (as inferred from the rate of methane formation) increased over that observed during the first feeding. When pectin was added on day 28 in an amount equivalent to one-third of the initial starting concentration, pectin was completely degraded in about 6 d and the final CH_4 yield was about one-third of the amount formed during the previous feedings.

The data obtained for pectin fermentation following the first two feedings were subjected to mass balance analysis according to the following theoretical equations of Tarvin & Buswell (1934):

A. General equation for a balanced methanogenic fermentation

$$C_nH_aO_b + \left[n - \frac{a}{4} - \frac{b}{2}\right]H_2O \rightarrow \left[\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right]CH_4 + \left[\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right]CO_2$$

B. Balanced equation for methanogenic fermentation of pectin

$$C_{6·7}H_{12}O_6 + 0·67\,H_2O \rightarrow 3·32\,CH_4 + 3·32\,CO_2$$
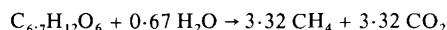
The following experimental equation was obtained at the end of the feeding experiments:

$$160\,\mu mol\ pectin \rightarrow 475\,\mu mol\ CH_4 + 480\,\mu mol\ CO_2 + 7·5\ mg\ cells$$

Thus, the results obtained account for a balanced redox fermentation. The values obtained for CH_4 (2·97) and CO_2 (3·0) per mol pectin fermented were lower than predicted in equation B as a result of the cell biomass synthesized from pectin.
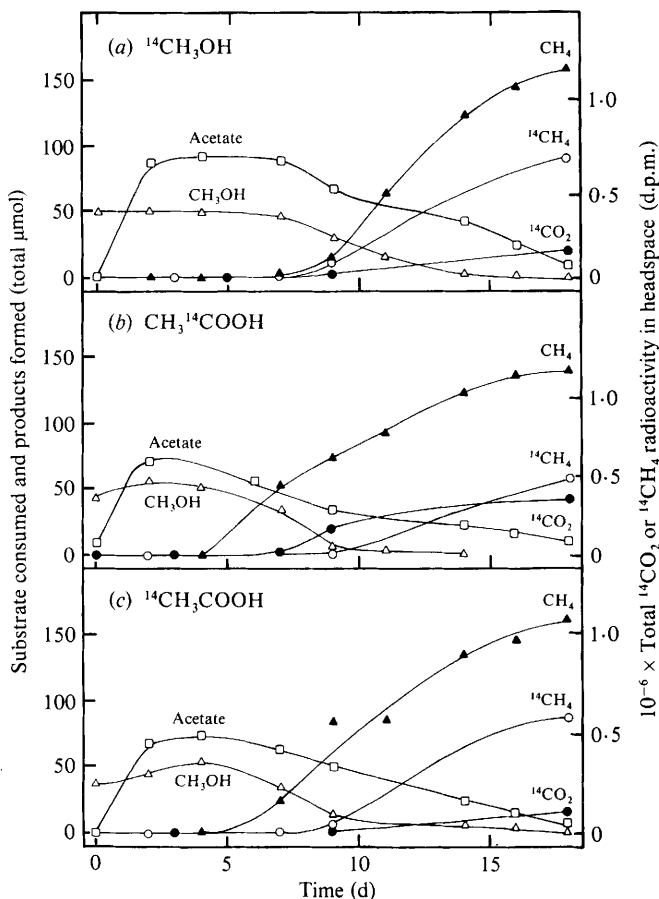
Fig. 5. ^{14}C-Radiotracer analysis of methanogenic precursors during pectin fermentation by co-cultures of *C. butyricum* strain MMP3 and *M. barkeri* strain UBS. On day 2 of the time course 1×10^6 d.p.m. of (a) ^{14}CH$_3$OH, (b) |1-^{14}C|acetate or (c) |2-^{14}C|acetate was added. Medium contents and culture conditions were the same as employed for initiation of the time course experiments in Fig. 4. □, Acetate; △, methanol; ▲, CH$_4$; ○. ^{14}CH$_4$; ●, ^{14}CO$_2$.

Radiotracer experiments with ^{14}C-labelled acetate and methanol were performed to further substantiate the simultaneous transformation of methanogenic intermediates by the co-culture. These experiments were designed in an identical manner to that described above for initiation of a co-culture pectin fermentation time course, except that replicate experimental tubes contained either ^{14}CH$_3$OH, ^{14}CH$_3$COOH or CH$_3$^{14}COOH. The results in Fig. 5 show that ^{14}C atoms in methanol and C-2 labelled acetate were simultaneously transferred to methane during simultaneous consumption of methanol and acetate by the co-culture. The origin of ^{14}C-methane from the C-1 labelled acetate was probably from the H$_2$-mediated reduction of ^{14}CO$_2$ generated from the acetate, because it was formed after the appearance of labelled CO$_2$.

DISCUSSION

Our results show that pectin is readily mineralized to CH$_4$ and CO$_2$ in anoxic sediments of Lake Mendota and Knaack Lake. In Lake Mendota the biodegradation of pectin was more rapid than that reported for cellulose (Nelson & Zeikus, 1974) and lignin (Hackett *et al.*, 1977), which appears totally recalcitrant to anaerobic mineralization at neutral pH (Zeikus,

1981). Nonetheless, the observed turnover time for decomposition of pectin to methane in Lake Mendota (100 h) was considerably longer than for either glucose (12 h), a soluble substrate metabolized by non-methanogens, or for acetate (0·22 h), the major immediate methane precursor (Winfrey & Zeikus, 1979a). These results support the general hypothesis (Wetzel, 1975; Zeikus, 1977, 1980) that degradation of biopolymers is a rate-limiting step in anaerobic digestion of biomass. Native pectin associated with biomass (in leaves or algal walls) may be degraded at a lower rate than that demonstrated here for isolated pectin. Otsuki & Hanya (1972) demonstrated that degradation of intact cyanobacteria in anoxic sediments proceeded much more slowly: after 60 d incubation, only 20% of the cells were mineralized, with 50% remaining particulate and 30% solubilized to dissolved organic matter. The reasons for differences in the rate of pectin mineralization in Knaack Lake and Lake Mendota are not known. The lower rate of biodegradation in Knaack Lake may be related to environmental differences such as lower *in situ* temperature and pH (Winfrey & Zeikus, 1979b; Zeikus & Winfrey, 1976).

The number of pectin fermenting bacteria varied seasonally by two orders of magnitude in both lake sediments studied. The highest numbers of pectinolytic anaerobes were found in the fall at a time after the main input of pectinous substrates such as sedimented leaves of higher plants and dead algal blooms. Thus, the pectinolytic clostridia isolated appear active in their environment and express their metabolic potential in response to the presence of substrate. The finding that *Clostridium* species are prevalent pectinolytic anaerobes in Lake Mendota is in agreement with the results of Monolongoski & Klug (1976), who identified clostridia as the numerically dominant fermentative micro-organism in Wintergreen Lake, Michigan. Notably, both *Clostridium* species and unidentified, Gram-negative asporogenous rods were prevalent pectin degraders in Knaack Lake. These studies extend the known niche for pectinolytic clostridia and *C. butyricum* to include anoxic freshwater sediments. The numerical significance of *Clostridium* species as important agents of pectinolysis has also been established in the retting of hemp (Avrova, 1975), the soft rot of potatoes (Lund & Brocklehurst, 1978) and the wetwood of living trees (Schink *et al.*, 1981).

The data suggest that the anaerobic microbial food chain required to degrade completely a biopolymer such as pectin may consist of only two species. This may appear surprising, but it agrees with previous artificial studies (Winter & Wolfe, 1979) that demonstrated complete fermentation of fructose to CH_4 and CO_2 by a mixed culture of *Acetobacterium woodii* and *Methanosarcina barkeri*. The association of *M. barkeri* and *C. butyricum* as prevalent methylotrophic methanogen and pectinolytic anaerobe in lake sediment may derive from a natural metabolic consequence. We recently showed that a wide variety of aerobic, facultative and obligately anaerobic bacteria produced but did not consume methanol during growth on pectin (Schink & Zeikus, 1980). Hence, the presence of pectin biodegradation in anaerobic ecosystems establishes a niche for methylotrophs. In the absence of excess electron acceptors (e.g. O_2, NO_3^- or SO_4^{2-}), methanol consumption by methanogens may be the favoured route of biodegradation that eliminates potential microbial toxicity due to methanol accumulation in lake sediments. Positive cell–cell interactions involving methylotrophic bacteria have been reported previously for aerobic species. For example, Wilkinson *et al.* (1974) demonstrated that growth of aerobic mixed cultures on methane was enhanced as the result of removal of toxic methanol by a *Hyphomicrobium* species which was produced by a methane-oxidizing *Pseudomonas* species.

Analysis of metabolic interactions between *C. butyricum* and *M. barkeri* during growth on pectin supports and further extends knowledge on the biological role of methanogens during the anaerobic digestion of organic matter in nature. As a consequence of their metabolic features, methanogens influence the decomposition of organic matter by hydrolytic anaerobes by removing toxic metabolites such as H^+, H_2 and methanol; by directing electron flow to limited reduced end products; by increasing growth rate and yields; and by supplying essential growth factors (Zeikus, 1977, 1980). The most important bioregulatory function of

the methanogen in the pectin fermentation was H^+ consumption, for the cessation of growth of *C. butyricum* in mono-culture was due to accumulation of H^+ and not H_2, methanol or acetate. H_2 was not a significant metabolic effector for growth of *C. butyricum*. H_2-producing anaerobes vary considerably as to whether H_2 inhibits growth, and this is a reflection of species differences in enzymic activities that direct electron flow within cells (Ben-Bassat *et al.*, 1981; Lamed & Zeikus, 1980). Thus, *C. butyricum* resembles *C. thermocellum* (Weimer & Zeikus, 1977), and not *C. cellobioparum*, whose growth is inhibited by low partial pressures of H_2 (Chung, 1976). Although *C. butyricum* stoichiometrically converted all the methoxyl groups of pectin to methanol, in mono-culture the final methanol concentration was not toxic to growth. The stimulation of growth observed by addition of acetate to *C. butyricum* mono-cultures was probably a consequence of circumventing the need to generate this intermediate as an electron acceptor for butyrate synthesis. In co-culture, 'normal' electron flow of *C. butyricum* was directed by *M. barkeri* to H_2 in lieu of ethanol or butyrate. This alteration of electron flow when coupled to methanogenesis enabled *C butyricum* to generate more energy in co-culture than in mono-culture from an equivalent amount of substrate metabolized. It should be noted that in the absence of yeast extract in culture medium, *C. butyricum* grows very slowly on pectin or other carbohydrates. However, growth in co-culture with *M. barkeri* was rapid even in the absence of yeast extract. This observation suggests that this methanogen may supply limiting growth factors to *C. butyricum*.

Our data support the hypothesis of Weimer & Zeikus (1978 *a, b*) that *M. barkeri* is capable of simultaneous metabolism of H_2/CO_2, acetate and methanol. This was confirmed by both analysis of immediate methanogenic precursor concentrations and ^{14}C-tracer studies during co-culture fermentation of pectin. This is contrary to other interpretations of the metabolism of *Methanosarcina* strains, which suggested that H_2/CO_2 or methanol catabolite repressed acetate utilization (Smith & Mah, 1978). Our data clearly showed that the preference for methanogenic substrate utilization by *M. barkeri* is in the order $H_2/CO_2 >$ methanol $>$ acetate. Thus, the preferential utilization of H_2/CO_2 over acetate supports the previous conclusion of Winter & Wolfe (1979) but it does not imply that H_2/CO_2 and acetate are not simultaneously converted to CH_4. Past studies on the metabolism of *M. barkeri* have been controversial, largely because of difficulties associated with growth of the organism and the enigmatic requirement for very long culture adaptation periods to obtain significant growth on acetate as an energy source (Weimer & Zeikus, 1978*a*; Zeikus *et al.*, 1975). However, significant simultaneous metabolism of acetate, H_2/CO_2 and methanol by acetate-adapted strains of *M. barkeri* requires control of individual substrate concentration in the medium. It is obvious that during co-culture growth on pectin the concentration of methanogenic substrates is limited by the rate of pectin conversion and the rate of intermediary metabolite consumption.

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