

## Degradation of Dehydrodivanillin by Anaerobic Bacteria from Cow Rumen Fluid

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**Dehydrodivanillin (DDV; 0.15 g/liter) was biodegradable at 37°C under strictly anaerobic conditions by microflora from cow rumen fluid to the extent of 25% within 2 days in a yeast extract medium. The anaerobes were acclimated on DDV for 2 weeks, leading to DDV-degrading microflora with rates of degradation eight times higher than those initially. Dehydrodivanillic acid and vanillic acid were detected in an ethylacetate extract of a DDV-enriched culture broth by thin-layer, gas, and high-performance liquid chromatographies and by mass spectrometry.**

Degradation of lignin and related compounds by microorganisms has been studied extensively with an expanding range of organisms known to have this property. In particular, fungi classified as white rot (*Basidiomycetes* and a few *Ascomycetes*), brown rot (*Basidiomycetes*), and *Fungi Imperfecti* are well known to degrade lignin. Some bacteria are able to degrade lignin completely, acting synergistically with fungi (13). The oxidative pathways of lignin degradation by such aerobes have been reported earlier (13).

On the other hand, anaerobic lignocellulose degradation has been studied mainly in relation to the digestion by ruminants of the cellulose present in grass and fodder (1). Porter and Singleton (19) reported that about 10% of lignin in fodder is digested by ruminants.

Mixed cultures from sludges in anaerobic tanks of sewage digestion plants ferment a range of aromatic compounds, yielding a biofuel consisting of CO<sub>2</sub> and CH<sub>4</sub> (20). Clark and Fina (2), using mixed cultures from a similar source, confirmed that benzoate was degraded to CO<sub>2</sub> and CH<sub>4</sub>. Fina and Fiskin (7) demonstrated the production of <sup>14</sup>CH<sub>4</sub> from [<sup>14</sup>C]benzoate added during fermentative growth of mixed cultures enriched by rumen fluid or sludge from anaerobic digestion tanks. Healy and Young (8, 9) reported methanogenic enrichments in a range of 11 simple lignin-related compounds, yielding CH<sub>4</sub> and CO<sub>2</sub> under strictly anaerobic conditions with a serum bottle modification of the Hungate technique. Healy et al. (10) proposed a pathway of methanogenic degradation of ferulic acid by mixed anaerobic bacteria. Several other reports have described the anaerobic degradation of lignin-related compounds (3, 5, 6, 17, 18, 20). Little attention has been paid to the compounds of the biphenyl type such as dehydrodivanillin (DDV). This paper presents evidence that anaerobic bacteria from cow rumen fluid degrade DDV.

### MATERIALS AND METHODS

**Chemicals.** DDV was synthesized by the method of Elbs and Lerch (4) and used as a carbon source for microorganisms. Dehydrodivanillic acid (DDVA) was synthesized by the method described before (4). Vanillic acid (VA) was purchased from Tokyo Kasei Co. and purified if necessary. Authentic volatile fatty acids (VFAs) such as acetic, propi-

onic, butyric, isobutyric, valeric, and isovaleric acids and other reagents used were analytical grade.

**Microorganisms.** Fresh cow rumen fluid was obtained from the Nagoya City Meat Inspection Center; cow manure and pig manure composts were provided from Aichi-ken Agricultural Research Center; mud from a pond and soil from under a rotting tree were collected on the Nagoya University campus. All of them were inoculated into anaerobic media each containing DDV under an O<sub>2</sub>-free CO<sub>2</sub> gas atmosphere.

**Media.** Yeast extract medium (pH 6.8, 100 ml) mainly was used. The composition was as follows: 0.045% K<sub>2</sub>HPO<sub>4</sub>, 0.045% KH<sub>2</sub>PO<sub>4</sub>, 0.09% NaCl, 0.09% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.009% MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.009% CaCl<sub>2</sub>, 0.1% yeast extract, 0.45% Na<sub>2</sub>CO<sub>3</sub>, 0.0001% resazurin sodium, 0.025% L-cysteine hydrochloride, 0.025% Na<sub>2</sub>S · 9H<sub>2</sub>O, and DDV. The concentrations of DDV in the media ranged from 0.015 to 0.040%.

**Medium preparation.** A mixed solution of minerals and yeast extract was stirred well together with DDV and resazurin, an oxidation-reduction indicator, adjusted to pH 7.0 with NaOH, and kept at around 50°C for 15 min after addition of Na<sub>2</sub>CO<sub>3</sub>. Then the medium was bubbled with O<sub>2</sub>-free CO<sub>2</sub> gas for 15 min or more until the resazurin color in the medium turned pink. Cysteine was added, with the pH dropping to 6.7 to 6.8. The volume was made up to 100 ml, and Na<sub>2</sub>S was added finally to enhance the reductive state. The bubbling of O<sub>2</sub>-free CO<sub>2</sub> gas was continued until the pink color of resazurin faded. Thereafter, the medium (5 ml each) was dispensed into test tubes (1.5 by 15 cm), plugged with butyl rubber stoppers (no. 2) under an O<sub>2</sub>-free CO<sub>2</sub> gas atmosphere, and autoclaved at 110°C for 10 min in a pressed rack to avoid the release of stoppers. The procedure was fundamentally the same as the Hungate method (11).

**Cultivation. (i) Test tube.** By the Hungate technique (11), five inocula (3 drops each) were added separately under an O<sub>2</sub>-free CO<sub>2</sub> gas atmosphere to the yeast medium (5 ml) in test tubes, plugged with butyl rubber stoppers, and cultivated anaerobically at 37°C for a given period. Enrichment cultivation was performed by repeatedly feeding DDV to the appropriate culture broth until maximal degrading activities and bacterial growth were achieved. Bacterial growth was determined by measuring turbidity increments at 570 nm.

**(ii) Jar fermentor.** Rumen microflora acclimated on DDV was cultivated in a jar fermentor (MD-150, L. E. Marubishi Co. Ltd.; working volume, 500 ml). The main components of

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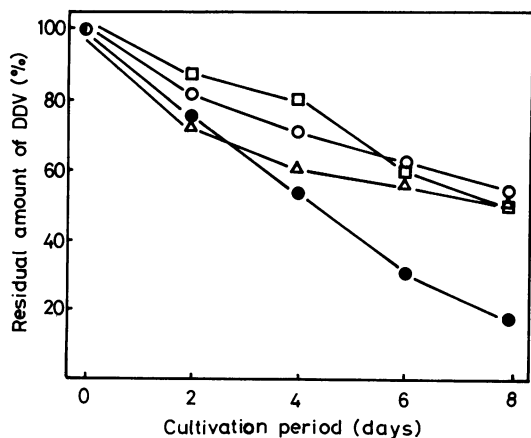


FIG. 1. Degradation of DDV in yeast extract medium at 37°C by anaerobes from cow rumen fluid (●), cow manure (Δ), pig manure compost (○), and mud from a pond (□). Three drops of each microbial source was inoculated into yeast extract medium (5 ml). Sampling was performed under an  $O_2$ -free  $CO_2$  gas atmosphere on every other day.

the yeast extract medium (minerals, yeast extract, DDV, and resazurin) were mixed well, the medium pH was adjusted at 7.0 in the jar, and the mixture was then autoclaved at 115°C for 15 min. Sterilized  $Na_2CO_3$  was added. Thereafter, bubbling of  $O_2$ -free  $CO_2$  gas into the hot medium was initiated and continued until inoculation. Soon after the resazurin color became pink, sterilized cysteine was added. After 15 min, sterilized  $Na_2S$  was added as the final step for the anaerobic medium preparation. This preparation technique has been established in a previous report (16). About 1 h later, 10 ml of seed culture was inoculated and cultivated in a completely closed system.

**Analysis.** After adequate dilution of culture broths with 1 N NaOH and distilled water, DDV concentrations in diluted samples (pH 12) were estimated by measuring the absorbance at a specific UV wavelength (360 nm) with a dual-beam spectrophotometer (model UVIDEC-610B [Jasco] equipped with an integrating sphere unit [model TIS-341] which can collect almost all the light beams once subjected to diffuse reflection or transmission by the sample and introduce them to the detector).

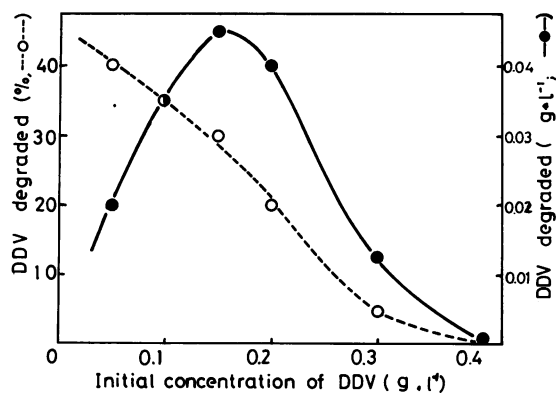


FIG. 2. Effect of initial concentration of DDV on its degradation by rumen anaerobes during a 2-day cultivation in yeast extract medium at 37°C. Symbols: ●, amount of DDV degraded; ○, percentage of DDV degraded.

**Isolation and identification of metabolites.** Some derivatives of DDV were extracted from acidified entire culture broths of the 0- and 5-day cultivations (pH 2 to 3, 500 ml) of rumen microflora by refluxing ethyl acetate for more than 7 h at 95°C in a Soxhlet liquid-liquid extractor. Acidic extracts in ethyl acetate were completely dehydrated with an excess amount of  $Na_2SO_4$  and evaporated to remove ethyl acetate under reduced pressure at 40°C. The residue was methylated with diazomethane. Methylated samples were analyzed by gas chromatography (JEOL TGC-20K [Jasco]) equipped with a glass column (2 by 1,000 mm) containing 1.5% silicon OV1 (100 to 200 mesh) as a carrier. The column temperature was 170°C. Each fractionated sample was further analyzed by mass spectrometry (JEOL TMS-D100). The eluent from the gas chromatograph was monitored with successive mass spectral scans with an ionization energy of 23 eV. The mass spectra were acquired under resolution ( $R$ ) conditions with  $R = 1,000$ . Methylated samples were also analyzed by thin-layer chromatography (precoated plates with Silica Gel 60F 254; developing solvent, ether-hexane [1:1]). The compounds were detected as dark spots when the plates, which had been sprayed with 15%  $H_2SO_4$  solution, were heated. High-pressure liquid chromatography (Jasco, Twincle, Dev-elosil 60-5) was carried out (column, 4 by 250 mm; eluting solvent, ether and hexane; flow rate, 2 ml/min; pressure, 40 kg/cm<sup>2</sup>; UV at 256 nm).

Samples for gas chromatography of VFAs such as acetic, propionic, *n*-butyric, isobutyric, and isovaleric acids were prepared by mixing 1 ml of the 5-day culture broth supernatant and 0.5 ml of 1 M  $H_3PO_4$  solution with 0.5 ml of 2% valeric acid as an internal standard. The amounts of acetic, propionic, isobutyric, *n*-butyric, and isovaleric acids in the culture broth with and without DDV were determined with a gas chromatograph (Hitachi 163) equipped with an integrator (model 3390A, Hewlett-Packard).

## RESULTS

Time courses of DDV degradation by microflora from cow rumen fluid, cow manure, pig manure compost, or mud in the pond are shown in Fig. 1. Organisms from rumen fluid could degrade DDV at almost a constant rate during an 8-day cultivation with 80% of the DDV being degraded. The extent of degradation was only around 40% by microflora from other sources. Accordingly, rumen fluid was used in all

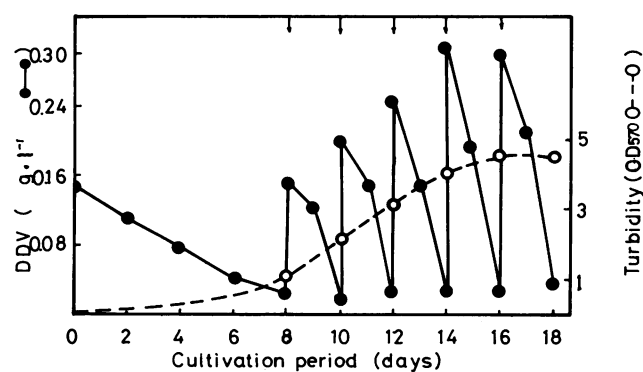


FIG. 3. Enrichment cultivation of anaerobes from rumen fluid by addition of DDV to yeast extract medium when consumed at 37°C. Symbols: ○, growth of bacteria (optical density [OD] at 570 nm); ●, amount of DDV; ↓, addition of DDV as powder under an  $O_2$ -free  $CO_2$  gas atmosphere (dissolved by shaking well).

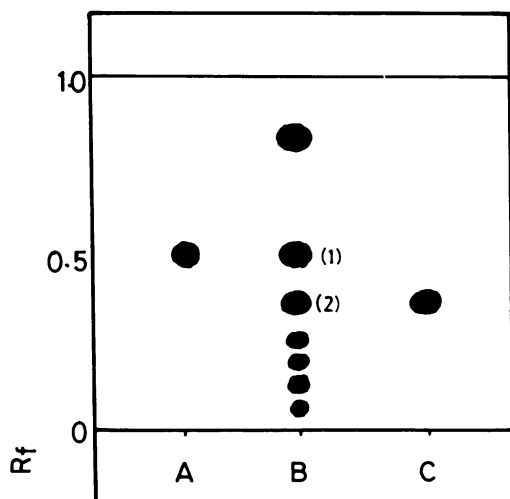


FIG. 4. Thin-layer chromatography of a methylated acid fraction from a DDV-containing culture broth of rumen microflora. A, Authentic methylated VA; B, methylated acid fraction of DDV metabolites; C, authentic methylated DDVA. Spots 1 and 2 had the same  $R_f$  values as those of methylated VA and DDVA, respectively.

further studies as the source of microflora with highest activity of DDV degradation.

The percent degradation of DDV by microflora decreased sigmoidally with an increase in initial concentrations of DDV in the medium (Fig. 2). The total amount of DDV degraded during cultivation increased with an increase in initial concentration. This value was maximal at an initial concentration of 0.15 g/liter. When the DDV was increased to 0.4 g/liter, degradation was inhibited completely, and negligible growth of microflora was observed.

Enrichment cultivation with DDV of rumen bacteria was performed as shown in Fig. 3. DDV (0.15 g/liter) was almost completely degraded during an 8-day cultivation. Thereafter, an equal amount of added DDV was degraded within

only 2 days. The acclimation of the microflora was continued by adding sequentially amounts of DDV greater than 0.15 g/liter yielding finally a maximum of 0.3 g of DDV per liter being degraded within 2 days. This provided an eightfold increase in degradation. During cultivation, the population of microorganisms gradually increased with the repeated additions of DDV as seen by the turbidity increments (Fig. 3). The microflora consisted of four major bacteria as manifested by four separate morphologies: rods, spheres, spirals, and needles.

Cow rumen microflora acclimated on DDV was cultivated with DDV (0.2 g) in 500 ml of yeast extract medium in a jar fermentor. Inoculum size was 2% of the working volume. The cultivation continued until the amount of DDV was reduced by at least one-half. During cultivation for about 1 week, the culture system was tightly closed for maintaining anaerobic conditions since no gaseous compound was generated. The methylate metabolites of DDV were analyzed by thin-layer chromatography, revealing seven compounds (Fig. 4B). Two spots (1 and 2) were identified as VA and DDVA by using standards (Fig. 4A and C, respectively). Both compounds 1 and 2 were extracted from the preparative thin-layer plates and analyzed by high-pressure liquid chromatography. Single peaks (Fig. 5B and D) with the retention times of authentic VA (Fig. 5A) or DDVA (Fig. 5C) were detected. Further analysis of the compounds detected by thin-layer chromatography was done with gas chromatography-mass spectrometry on methylated samples of whole DDV metabolites extracted from the 0- and 5-day culture broths. A typical total ion chromatogram from the 5-day culture broth is shown in Fig. 6, from which seven large fractions were analyzed by mass spectrometry, whereas only one (fraction 5) was mainly detected in the extract of the 0-day culture broth. The fractions were identified as follows: 1, VA; 2, 5-carboxyvanillin; 3, 5-carboxyvanillic acid; 4, 5-carboxymethylvanillin; 5, DDV; 6, DDVA semi-aldehyde; 7, DDVA. Their structures are shown in Fig. 7. VFAs such as acetic, propionic, isobutyric, *n*-butyric, and isovaleric acids were identified from the 5-day culture broth containing DDV by gas chromatography as shown in Fig. 8.

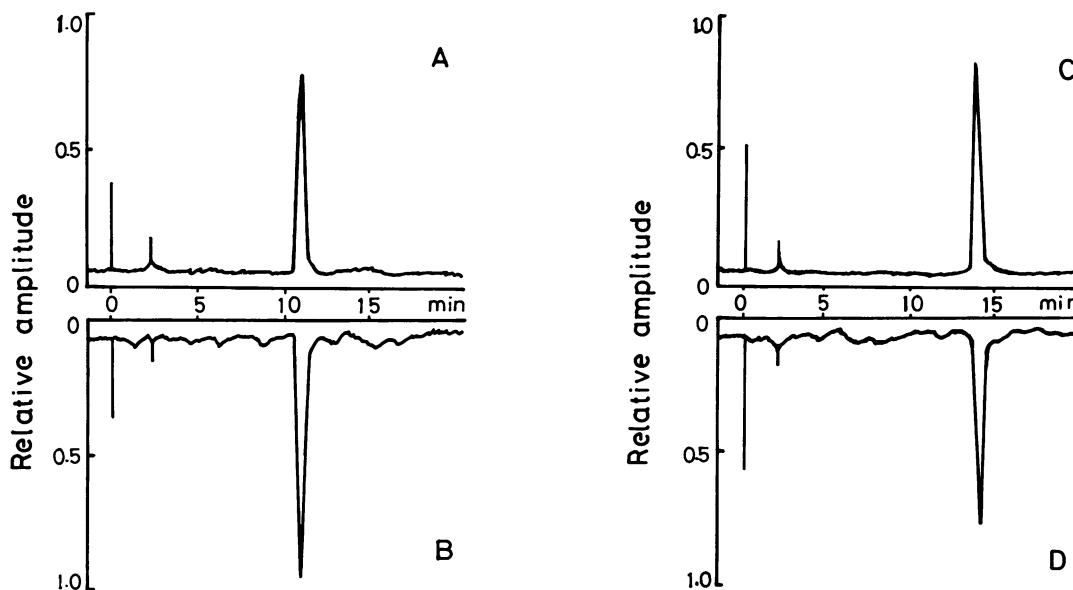


FIG. 5. High-performance liquid chromatography of extracts from spots in Fig. 4B. A and B are authentic VA and spot 1, respectively, and C and D are authentic DDVA and spot 2, respectively. The eluting solvent was ether-hexane (1:7 for VA and 1:3 for DDVA).

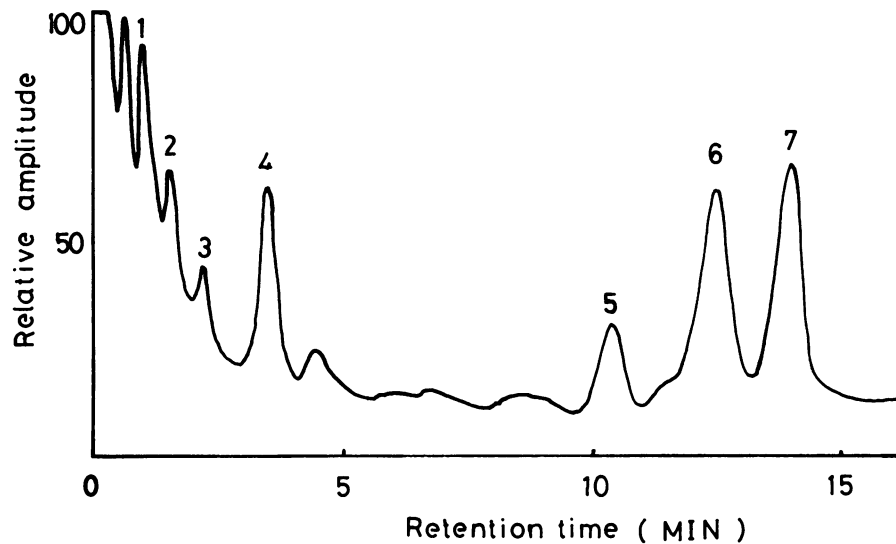


FIG. 6. Total ion chromatogram obtained by gas chromatography-mass spectrometry of the extract from DDV-containing culture broth of rumen bacteria after methylation.

On the contrary, a small amount of acetic acid only was detected from the 5-day culture broth containing no DDV (Fig. 8), suggesting that these VFAs were formed by the utilization of DDV by the anaerobes. The increase in these VFA formations due to the presence of DDV in the culture broth was in the range of three to five times, with the

fractionated peak area of the culture supernatant in the absence of DDV as the criterion (Table 1).

#### DISCUSSION

According to Healy and Young (8), decomposition of ferulic acid (0.3 g/liter) by anaerobes from settled sludge

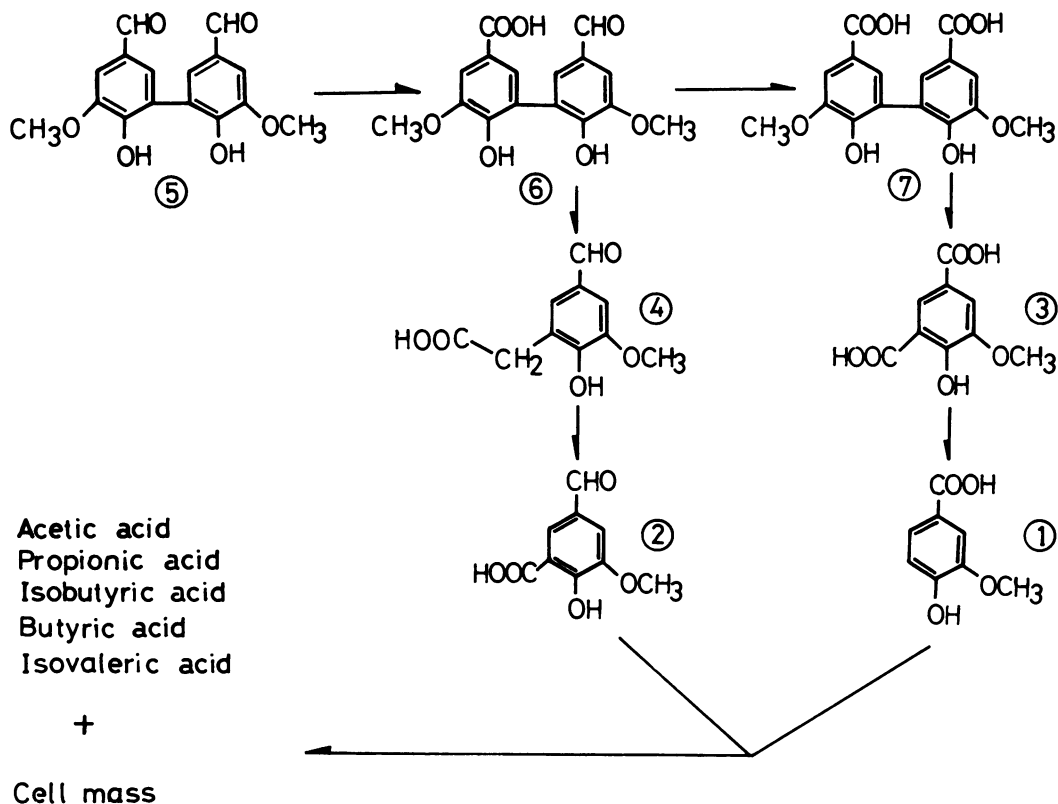


FIG. 7. Chemical structures of total ion chromatographic fractions 1 to 7 determined by gas chromatography-mass spectrometry: 1, VA; 2, 5-carboxyvanillin; 3, 5-carboxyvanillic acid; 4, 5-carboxymethylvanillin; 5, DDV; 6, DDVA semialdehyde; 7, DDVA. Arrows indicate postulated pathway of DDV decomposition by rumen anaerobes.

required an acclimation lag period of 10 days before the initiation of degradation. In contrast, rumen bacteria in the present study can degrade DDV more rapidly than microflora in sludge because of a negligible lag time. This rapid degrading property of rumen anaerobes is reasonable since the lignin-related compounds contained in grass and fodder have to be utilized in the rumen within 1 to 2 days or otherwise be excreted as manure.

The analysis of DDV derivatives isolated from culture broth of rumen microflora acclimated on DDV, using gas chromatography-mass spectrometry, revealed the formation of DDVA via DDVA semialdehyde by possible oxidation of aldehyde groups on DDV. The detection of 5-carboxymethyl vanillin and 5-carboxyvanillic acid suggests aromatic ring cleavage of DDVA semialdehyde and DDVA. This might be caused by hydrogenation occurring on one benzene ring of DDV as shown by Taylor et al. (21) and Healy et al. (8, 9); the cleavage and decomposition of the cyclohexane product could generate compounds 2 to 4 (Fig. 8). Further modification of these compounds allowed appearance of 5-carboxyvanillin, 5-carboxyvanillic acid, and VA. Furthermore, increases in cell mass and fermented products such as acetate, propionate, iso- and *n*-butyrates, and isovalerate seem to be due to utilization by the anaerobes of these compounds generated by DDV modification (Fig. 8 and Table 1). In the metabolic pathway of DDV, oxidative modification of DDV can be easily postulated if the reactions proceed as in the Embden-Meyerhof pathway, generating 3-phosphoglycerate from D-glyceraldehyde 3-phosphate via 3-phosphoglyceroyl phosphate under anaerobic conditions in the cells (15).

The oxidative degradation of DDV also has been demonstrated by Kawakami and Shumiya (14) when DDV was modified by alkalophilic aerobes. The same pathway was demonstrated by Iwahara et al. (12) by using a *Pseudomonas* sp. under aerobic conditions. These results suggest that anaerobes and aerobes might catalyze reactions that resemble each other in DDV degradation, although their specificities against oxygen are very different.

The DDV degradation rate estimated in the present report was around 0.15 g/liter per day after acclimation (Fig. 3).

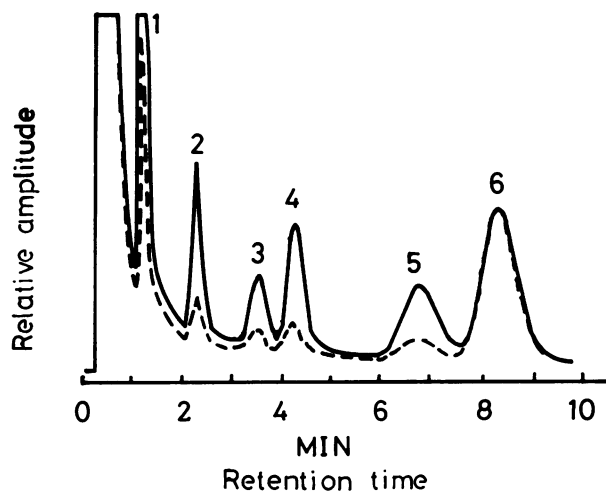


FIG. 8. Gas chromatograms of rumen bacterial 5-day-culture broth with DDV (—) and without DDV (---). A stainless steel column (2 by 1,000 mm) packed with Chromosolve 101 was used. The temperature was programmed at a constant 170°C with N<sub>2</sub> as the carrier gas.

TABLE 1. Production of VFAs in 5-day culture broth of rumen anaerobes with and without DDV

Fraction no. <sup>a</sup>	VFA	Peak area (mV) of VFA <sup>a</sup>		Area ratio (with DDV/without DDV)
		With DDV	Without DDV	
1	Acetic	165,470	59,530	2.8
2	Propionic	52,747	10,932	4.8
3	Isobutyric	34,555	8,959	3.9
4	Butyric	72,230	16,087	4.5
5	Isovaleric	82,668	22,754	3.6
6	Valeric <sup>b</sup>	164,240	166,940	1.0

<sup>a</sup> See Fig. 8.

<sup>b</sup> Valeric acid was added to the culture broth as an inner standard when analyzed by gas chromatography.

This rate was comparable to or greater than those obtained from the *Pseudomonas* culture (12) on DDV (0.13 g/liter per day) and from an alkalophilic bacterium culture (0.015 g/liter per day) (14). Judging from these results, anaerobic degradation of DDV is a rather promising method for the microbial utilization of lignin-related compounds.

The rumen microflora acclimated on DDV produced negligible amounts of gas. This property of the flora is quite different from that of the sludge anaerobes as reported by Healy et al. (8, 9). However, the production of VFAs by rumen microflora resembles that of sludge anaerobes (9).

Thus, rumen anaerobes described in this investigation could metabolize DDV and produce VFAs and cell mass but no gas, indicating that rumen bacteria seem to be very potent in converting DDV to useful compounds.

#### ACKNOWLEDGMENT

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