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Aerobic Degradation of Polypropylene Glycol by *Corynebacterium* sp.

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The aerobic metabolism of polypropylene glycol (PPG) was investigated with a strain, *Corynebacterium* sp. no. 7, which can use PPG as a sole carbon and energy source. PPG was metabolized by washed, resting cells, but it was not degraded by a culture filtrate of the organism or by the cell-free extract. The metabolic products by intact cells were studied using dipropylene glycol (DPG) as a model compound. Three structural isomers of DPG, $HOCH(CH_3)CH_2$-O-$CH_2CH(CH_3)OH$, $HOCH(CH_3)CH_2$-O-$CH(CH_3)CH_2OH$, and $HOCH_2$-$CH(CH_3)$-O-$CH(CH_3)CH_2OH$, which were separated and determined by capillary gas chromatography-mass spectrometry (GC-MS), were degraded. The metabolic products from DPG by intact cells were isolated and identified by GC-MS analysis as $OC(CH_3)CH_2$-O-$CH_2CH(CH_3)OH$, $OC(CH_3)CH_2$-O-$CH(CH_3)CH_2OH$, and 1,2-propanediol plus a small amount of $OC(CH_3)CH_2$-O-$CH_2CO(CH_3)$. On the other hand, PPG dehydrogenase activity linked with 2,6-dichlorophenolindophenol and phenazine methosulfate was found in sonic extracts of intact cells. The analytical and enzymatic data suggest that PPG is metabolized not by a hydrolytic reaction, but by an oxidative reaction.

Polypropylene glycol (PPG) which has the common structural formula, $HO[CH(CH_3)$-$CH_2O]_nH$, is widely used as a solvent for drugs and an ingredient of lubricants, inks, cosmetics, etc. and also as a component of polyurethane, nonionic and anionic surfactants etc. Much of this material reaches rivers, lakes, seas, and conventional sewage disposal systems like another polyether, polyethylene glycol. In spite of many studies on the biodegradation of polyethylene glycols,[1–8] recalcitrant materials whose biodegradation takes a long time, there has been no report on the susceptibility of PPG to biological degradation until recently. Kawai *et al.* first isolated PPG-degrading bacteria, which can use PPG as a sole carbon and energy source, from soil and activated sludge acclimated to PPG.[9] The most active bacterium was identified as *Corynebacterium* sp. no. 7 from its morphological, cultural, and physiological characteristics.

In this paper, we report on the aerobic metabolism of PPG by *Corynebacterium* sp. no. 7. Since PPG contains molecules of different mol wt., dipropylene glycol (DPG: mol wt. 134) was used as a model substrate for biodegradation.

Materials and Methods

Materials DPG was obtained from a commercial source (Nakarai Chemicals, Ltd., Kyoto, Japan). Impurities such as 1,2-propanediol, tripropylene glycol etc. were not detected on gas chromatography. Other chemicals used were of commercial grade.

Microorganism and culture *Corynebacterium* sp. no. 7[9] was used throughout this study. The bacterium was grown on a basal medium (pH 7.2) containing, in grams per liter of tap water: PPG 2000 or DPG, 7; NH_4Cl, 2; K_2HPO_4, 2; $NaHPO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.2; and yeast extract, 0.2. The bacterium was grown with continuous shaking at 30°C in 500 ml of the medium in a 2-*l* shaking flask for 5–7 days. No difference in bacterial growth rate between PPG 2000 and DPG was found, but the rate of degradation of DPG by intact cells grown on PPG was higher than that of the cells grown on DPG. After cultivation, the cells were harvested by centrifugation at 2,000 × *g* for 10 min

at 5°C and washed twice with 0.01 M potassium phosphate buffer (pH 7.0). The washed cells were suspended in an appropriate amount of the same buffer and used as intact cells.

Preparation of cell-free extract and assay of PPG dehydrogenase activity Intact cells were disrupted on ice with a 19 kHz ultrasonic oscillator (Kaijo-denki Co., Ltd., Tokyo, Japan). The disrupted cells were centrifuged at 2,000 × *g* for 10 min at 5°C to remove intact and broken cells and the resultant supernatant fluid was used as the cell-free extract.

The standard reaction mixture for the assay of PPG dehydrogenase contained 250 μmol of potassium phosphate buffer (pH 8.0), 2.5 μmol of potassium cyanide, 0.25 μmol of 2,6-dichlorophenolindophenol (DCIP), 0.25 μmol of phenazine methosulfate (PMS), 0.25 μmol of PPG 2000 or 10 μmol of DPG, and an appropriate amount of cell-free extract in a total volume of 2.5 ml, and was incubated at 30°C. The reaction was started by the addition of substrate and the decrease in the absorbance at 600 nm was followed with a Shimadzu double-beam spectrophotometer UV-200 against a blank tube containing the reaction mixture without substrate. One unit of the enzyme activity was defined as the amount of enzyme that reduced 1 μmol of DCIP per min under the standard conditions. The protein content was determined by the Bio-Rad protein assay (Bio-Rad Lab., USA) with bovine serum albumin as the standard.

Reaction by a culture filtrate, cell-free extract, and intact cells with PPG 2000 or DPG as substrate The reaction mixture which contained 0.5 g of PPG 2000 or DPG, 1 mmol of potassium phosphate buffer (pH 7.2) and 1.4–1.6 g (dry wt.) of intact cells, or cell-free extract prepared from 1.4–1.6 g (dry wt.) of intact cells, or 90 ml of a culture filtrate which were all prepared from PPG 2000-grown cultures in a total volume of 100 ml, was incubated at 30°C for 20–30 h with shaking. The incubation was stopped by heating the reaction mixture for 2 min in a boiling water bath. The amount of PPG in the supernatant solution was measured by the same method as described previously.[9] DPG and its metabolites were separated on a 0.25 mm-thick silica gel 60 F_{254} plate (E. Merck) with a solvent system of chloroform-methanol (80: 20, v/v). The substrate and metabolites were located with a $K_2Cr_2O_7$-H_2SO_4 reagent.[10]

Gas chromatography An aliquot of the reaction supernatant was dried with the aid of a Taiyo Concentrator model TC-8 (Taiyo Sci. Ind. Co., Ltd., Tokyo, Japan) and the residue was dissolved in acetone, followed by analysis with a Shimadzu GC-9A gas chromatograph (Shimadzu Corp., Kyoto, Japan) with a flame ion detector. The operation conditions were as follows: column temperature, 140°C; injector temperature, 250°C; carrier gas, N_2 50 ml/min. A glass column (3 mm by 2 m) packed with 10% PEG 20 M on Chromosorb W NAW (60 to 80 mesh) was used. The amounts of substrates and metabolic products were calculated on the basis of the peak areas with a Shimadzu Chromatopac E1A.

Gas chromatography-mass spectrometry (GC-MS) analyses Structural isomers of DPG were determined with a computer-controlled Hewlett-Packard 5992B gas chromatograph-mass spectrometer (quadrapole type) with a 18947A GC-MS capillary interface and an electron capture detector. A fused silica capillary column (0.25 mm *i. d.* by 25 m) coated with Carbowax 20 M purchased from Gasukuro Kogyo, Inc., Tokyo, Japan, was used. The operational conditions were as follows: column temp, 120°C; injector temp, 200°C; carrier gas, He 2 ml/min; separator temp, automatically controlled; ionization mode, electron impact; ionizing electron energy, 70 eV; split ratio, 1/25. For the identification of isomers of DPG, the total and selective ion monitoring methods were used together with a usual mass spectral measurement. Metabolites were also analyzed with the same GC-MS system as used for the determination of substrate except that a column 50 m long was used. A reaction supernatant was concentrated to dryness *in vacuo* and dissolved in a small amount of acetone which was used for GC-MS analysis.

Results and Discussion

Degradation of PPG by a culture filtrate, a cell-free extract or intact cells PPG was not degraded at all by a culture filtrate or a cell-free extract of *Corynebacterium* sp. no. 7 grown on PPG 2000 as a sole carbon and energy source, but was metabolized by intact cells. This suggested that PPG was not metabolized by extracellular enzymes, but by intracellular enzymes including membrane enzymes.

To identify the metabolic products with intact cells, DPG was used as substrate instead of PPG 2000 in the following experiments since PPG contains molecules of different molecular weights.

Identification of isomers of DPG Commercially obtained DPG is a chemically synthesized compound which is expected theoretically to have several structural and optical isomers, as shown in Fig. 1. Therefore, we tried first to separate and identify

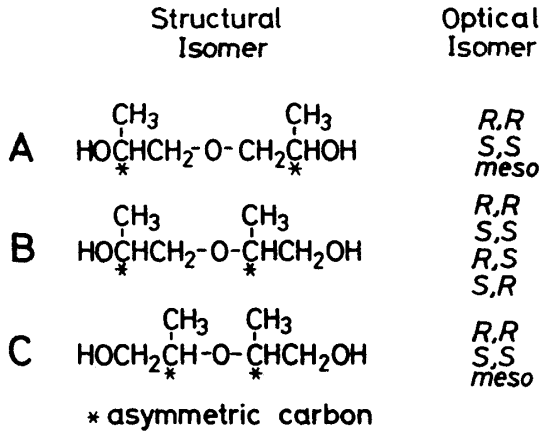

Fig. 1. Presumed structural and optical isomers contained in synthetic DPG.

these isomers by GC-MS analyses. Authentic DPG was methylated with diazomethane, acetylated with anhydrous acetic acid, or trimethylsilylated with *N,O*-bis(trimethylsilyl)acetamide, but the separation of these derivatives was unsuccessful with various columns. Then we tried to separate free isomers on several columns and found that a PEG 20M column was most suitable for the separation of free isomers. The structural isomers were separated on the column (3 mm by 2 m), but the optical isomers were separable only on a capillary column. Since the separation of *R,R*- and *S,S*- and also *R,S*- and *S,R*- optical isomers by gas chromatography is difficult, the following chromatogram is anticipated: *R,R*- and *S,S*-isomers will be eluted together as a single peak, and *R,S*- and *S,R*- isomers will be eluted together as another single peak. As shown in Fig. 2, five peaks were detected by total ion monitoring on GC-MS analysis: The area ratio of peaks II to III, and also that of peaks IV to V were almost equal. The same results were obtained by selective ion monitoring (Fig. 3). Mass spectra of these

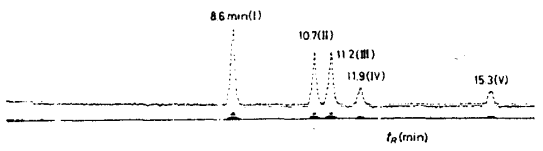

Fig. 2. Isolation of isomers contained in DPG by capillary gas chromatography-mass spectrometry (total ion monitoring).

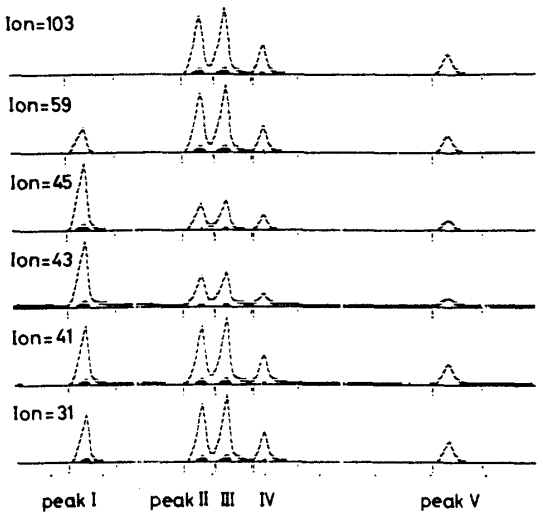

Fig. 3. Identification of isomers contained in DPG by glass capillary gas chromatography-mass spectrometry (selective ion monitoring).
Common and characteristic ions indicated above (see Fig. 4) were monitored by selective ion monitoring mode.

peaks are shown in Fig. 4. No parent ions were detected for all peaks. The parent ions were not detectable by GC-MS at an ionizing electron energy of 20 eV, either. The parent ions of homologous compounds like tripropylene glycol (1,1'-[(1-methyl-1,2-ethanediyl)bis(oxy)]bis-2-propanol) and 1,2-propanediol have not been detected according to the EPA/NIH Mass Spectral Data Base (1980), US Department of Commerce. From its characteristic spectrum, peak I was assigned to be structural isomer A in which the *R,R*- and *S,S*- complex and the meso form were eluted as one peak. Mass spectra of peaks II, III, IV, and V showed similar patterns, but the detailed analysis of each spectrum revealed that the peak intensities of II and III and those of IV and V were almost completely equal (five experiments, standard deviation within 5.0%), indicating that peaks II and III, and also peaks IV and V have the same structure. From the reaction mechanism for the chemical synthesis of DPG, the content of the isomer B contained in DPG is anticipated to be higher than that of the isomer C. Furthermore, the comparison of chemical structures of isomers B and C suggests that isomer B is eluted next to isomer A, before isomer C. Thus, peak I seemed to

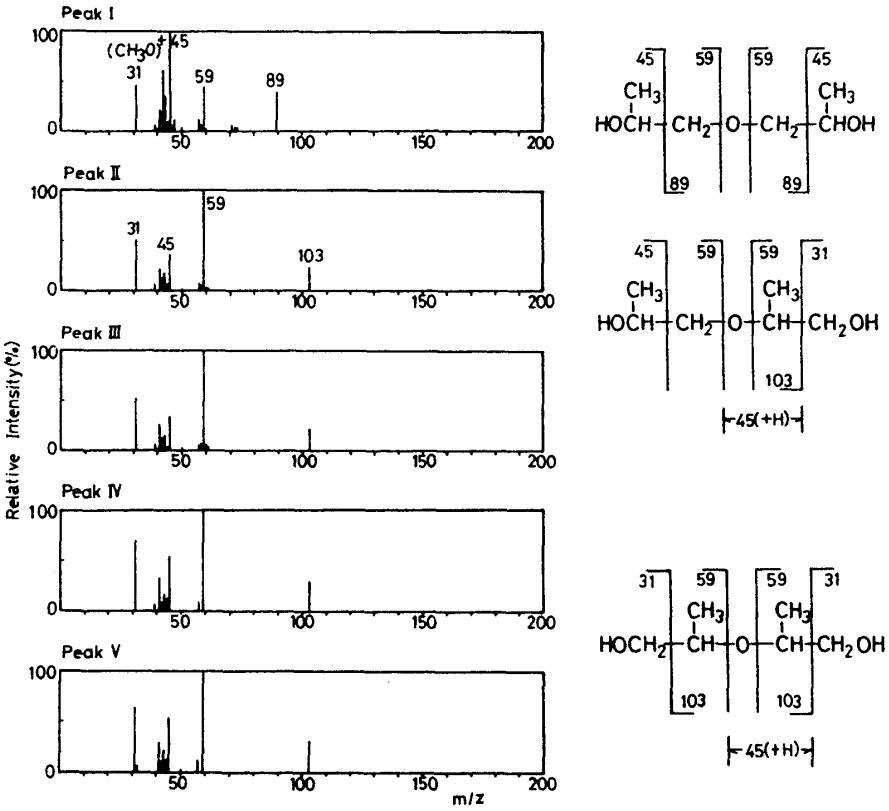

Fig. 4. Mass spectra of isomers contained in DPG.

correspond to structural isomer A, II and III to B, and IV and V to C: In peak I, optical isomers could not be separated; peaks II and III have the same structure B, but they are diastereomers (the *R,R-S,S* and *R,S–S,R* complexes); peaks IV and V have the same structure C, but they are diastereomers, too (the *R,R–S,S* complex and the meso form). The ratio of structural isomers A, B, and C was 36.3, 48.5, and 15.2%, respectively.

Identification of reaction metabolites from DPG with intact cells of *Corynebacterium* sp. no. 7 Next we tried to identify the metabolites of DPG with intact cells grown on PPG 2000. Degradation of DPG depended on the shaking conditions. In stationary culture, DPG was scarcely degraded. This suggested that DPG was not metabolized by a hydrolytic reaction, but by an oxidative reaction. With vigorous shaking (about 120 rpm), over 90% of DPG was consumed within 23 h, but traces of metabolites accumulated in the reaction mixture. With moderate shaking (about 65 rpm), the degradation rate was slower, but considerable amounts of metabolic products were accumulated with an R_f value of 0.36 (M1), which coincided with that of 1,2-propanediol, and an R_f value of 0.61 (M2) on thin-layer chromatography.

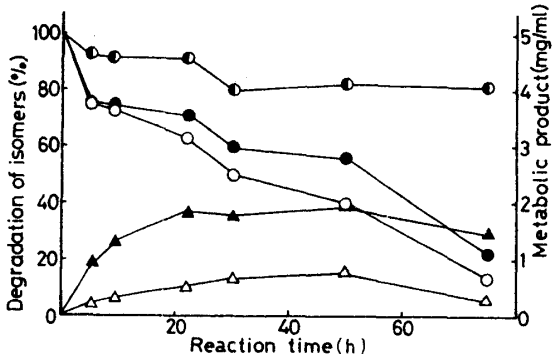

Fig. 5. Courses of the degradation of DPG and formation of metabolic products.
The degradation of DPG is expressed in terms of the residual percentage of structural isomers contained in DPG. Symbols: ○, structural isomer A; ●, structural isomer B; ◐, structural isomer C; △, M1; ▲, M2.

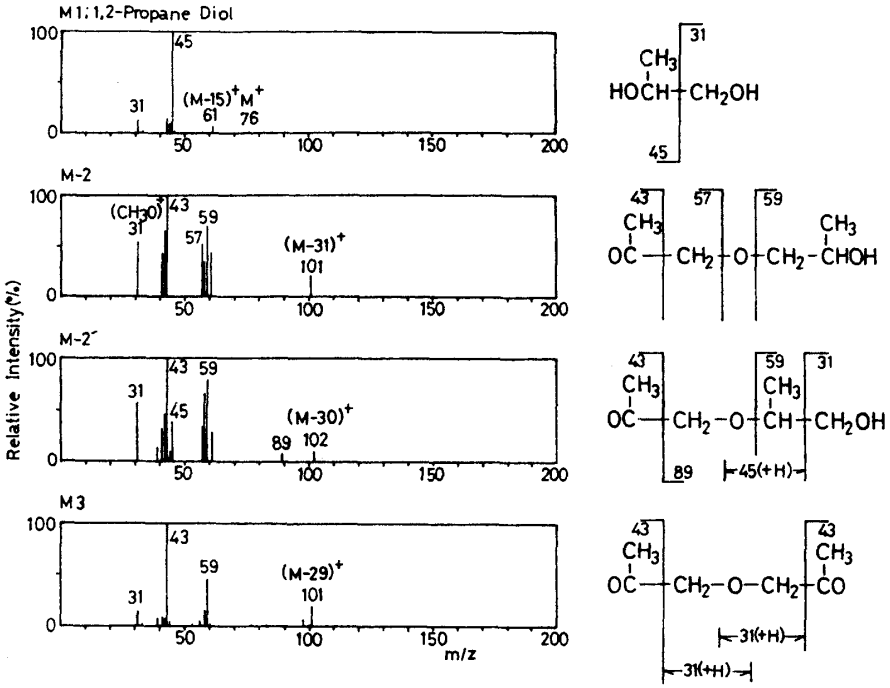

Fig. 6. Mass spectra of metabolic products.

Therefore, the reaction was done at 30°C for 20 to 50 h with moderate shaking (60–70 rpm). M1 and M2 were also separated from the substrate by gas chromatography. The course of degradation of each structural isomer and formation of M1 and M2 was followed by gas chromatography (Fig. 5). Each isomer was degraded to form metabolites M1 and M2. The metabolites were characterized by GC-MS analysis using a capillary glass column. M1 (t_R: 13.9 min) had the same mass spectrum as that of authentic 1,2-propanediol, which coincided well with the known data for 1,2-propanediol (EPA/NIH Mass Spectral Data Base (1980), US Department of Comerce). M2 was further separated into two peaks, M-2 and M-2′ (t_R: 20.6 min and 22.5 min). From the mass spectra of the two peaks, shown in Fig. 6, M-2 and M-2′ seemed to correspond to $OC(CH_3)CH_2$-O-$CH_2CH(CH_3)OH$ and $OC(CH_3)CH_2$-O-$CH(CH_3)CH_2OH$, respectively. Furthermore, a small peak, M3 (t_R: 7.0 min), was found on capillary gas chromatography which was considered to be $OC(CH_3)CH_2$-O-$CH_2CO(CH_3)$ from the elution position and its mass spectrum. The residual isomers in the reaction supernatant (30 h of incubation) were analyzed. Peak I was degraded by 51.4%. Peaks II and III were equally degraded by 40.7% and peaks IV and V were also equally degraded by 18.5%. This result supports the assumption that peaks II and III, and IV and V have the same structure, respectively. The results

Table 1. PPG dehydrogenase activity of the cell-free extract.

Substrate		Specific activity (unit/mg)	
		Electron acceptor (0.1 mM)	
		DCIP	DCIP+PMS
PPG 2000	0.5 mg (0.1 mM)	0.0017	0.0022
DPG	1.3 mg (4.0 mM)	0.0023	0.0038

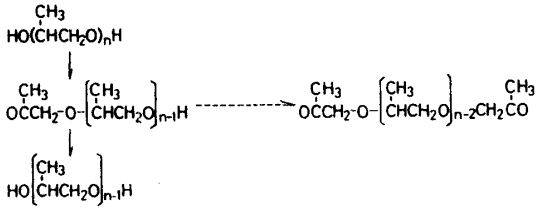

Fig. 7. Proposed metabolic pathway for PPG in *Corynebacterium* sp. no. 7.

described above also indicated that secondary hydroxy groups were preferentially oxidized.

PPG dehydrogenase activity of the cell-free extract From the analytical data of metabolic products, DPG seemed to be oxidatively metabolized by intact cells of *Corynebacterium* sp. no. 7. Because of clouds caused by PPG attached to the cells the activity of the cell-free extract prepared from PPG-grown cells was not measurable. PPG-oxidizing activity of the cell-free extracts prepared from DPG-grown cells was investigated. PPG 2000 and DPG were oxidized by the cell-free extract and the DCIP-PMS system was reduced better than the DCIP system (Table 1). Judging from the enzymatic function, the enzyme must be linked with a respiratory chain of the organism.

Based on these analytical and enzymatic results, the degradative pathway of PPG is summarized in Fig. 7.

As previously described,[1-4] we proposed an oxidative metabolic pathway for PEG in which a terminal primary hydroxy group is oxidized to an aldehyde group and then to a carboxylic acid which is followed by the cleavage of the ether bond, to form a PEG molecule that is reduced by one glycol unit. Recently we found that PEG dehydrogenase is a novel enzyme whose prosthetic group is pyrroloquinoline quinone.[11] It is interesting to clarify the mechanism of PPG metabolism because the structure is similar to PEG in the biodegradation of polyether as well as the comparative biochemistry of enzymes involved in the metabolism of polyethers. From the results described above, it seems that PPG is metabolized and depolymerized *via* the same mechanism as that for PEG: oxidation of terminal groups leads to the cleavage of the ether linkage.

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