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Bacterial Oxidation of Polyethylene Glycol

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The metabolism of polyethylene glycol (PEG) was investigated with a synergistic, mixed culture of *Flavobacterium* and *Pseudomonas* species, which are individually unable to utilize PEGs. The PEG dehydrogenase linked with 2,6-dichlorophenolindophenol was found in the particulate fraction of sonic extracts and catalyzed the formation of a 2,4-dinitrophenylhydrazine-positive compound, possibly an aldehyde. The enzyme has a wide substrate specificity towards PEGs: from diethylene glycol to PEG 20,000. K_m values for tetraethylene glycol (TEG), PEG 400, and PEG 6,000 were 11, 1.7, and 15 mM, respectively. The metabolic products formed from TEG by intact cells were isolated and identified by combined gas chromatography-mass spectrometry as triethylene glycol and TEG-monocarboxylic acid plus small amounts of TEG-dicarboxylic acid, diethylene glycol, and ethylene glycol. From these enzymatic and analytical data, the following metabolic pathway was proposed for PEG: $HO(CH_2CH_2O)_nCH_2CH_2OH \rightarrow HO(CH_2CH_2O)_nCH_2CHO \rightarrow HO(CH_2CH_2O)_nCH_2COOH \rightarrow HO(CH_2CH_2O)_{n-1}CH_2CH_2OH$.

Various kinds of polyethylene glycols (PEGs) are used in many fields. These compounds are water-soluble polymers of the common structural formula $HO(CH_2CH_2O)_nCH_2CH_2OH$, but they differ from each other in their average molecular weights. Much of this material reaches conventional sewage disposal systems and becomes one of the recalcitrant materials that cannot be rapidly biodegraded.

We have isolated many PEG-utilizing bacteria and divided them into five groups characterized by the ability to utilize PEG 400, 600, 1,000, 4,000, or 20,000 for growth (7). PEG 20,000 is utilized only by a synergistic, mixed culture of two strains (*Flavobacterium* and *Pseudomonas* species). Neither strain alone utilizes PEGs for growth, but when both cultures are grown together they utilize PEGs 300 to 20,000.

Payne and Todd (9) and Ohmata et al. (8) suggested that the flavin-dependent dehydrogenases are involved in the tetraethylene glycol (TEG) or PEG 400 metabolism by soil bacteria. Haines and Alexander (3) suggested that PEG 20,000 is hydrolyzed and proposed that an extracellular enzyme is responsible for conversion of PEG 20,000 to diethylene glycol (DEG) and ethylene glycol (EG) by *Pseudomonas aeruginosa*.

The present study was undertaken to establish how PEGs are metabolized by a synergistic, mixed culture of *Flavobacterium* and *Pseudomonas* species.

MATERIALS AND METHODS

Microorganisms and culture. A mixed culture of *Flavobacterium* and *Pseudomonas* species was used throughout this study. Two organisms were maintained as a mixed culture on a PEG slant, as described previously (4). The mixed culture was grown on a basal medium (pH 7.2) containing (grams per liter of tap water): PEG 6,000, 5; $(NH_4)_2HPO_4$, 3; K_2HPO_4, 2; NaH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; and yeast extract, 0.5. The bacteria were cultured with continuous shaking at 28°C in 500 ml of medium in a 2-liter flask (7 days) and also at 28°C in 30 liters of medium in a 50-liter fermentor (3 days), with the rotation of the impeller at 300 rpm and the aeration at 25 liters/min. After cultivation, the cells were harvested by centrifugation at 10,000 × *g* for 20 min at 5°C and washed twice with cold 0.01 M potassium phosphate buffer (pH 7.2). The washed cells were suspended in an appropriate amount of the same buffer.

The medium used for induction of the oligomer-utilizing enzymes contained (grams per liter of tap water at pH 7.0): a main carbon source, 10; $(NH_4)_2HPO_4$, 2; K_2HPO_4, 2; NaH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; yeast extract, 0.5; and an inducer, 0.1. The bacteria were cultured with continuous shaking at 28°C for 11 days in 5 ml of the medium.

Preparation of cell extract and particulate fraction. Washed cells from 500 ml of culture were suspended in 20 ml of 0.01 M potassium phosphate buffer (pH 7.2) and disrupted for 3 min on ice with a 19-kHz ultrasonic oscillator (Kaijo-denki Co., Ltd., Tokyo, Japan). The disrupted cells were centrifuged at 10,000 × *g* for 20 min at 5°C, and the resultant supernatant fluid was used as the cell extract. The cell extract was further centrifuged at 100,000 × *g* for 2 h

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at 4°C. The resultant pellet was used as the particulate fraction.

Estimation of PEG. PEG was assayed by the Stevenson method (10).

Determination of protein. Protein was estimated by the method of Lowry et al. (5).

Manometry. Oxygen uptake by intact cells and partially purified PEG-oxidizing enzymes was measured with a Gilson differential respirometer (Gilson Medical Electronics, Inc., Middleton, Wis.). When intact cells were used, the main compartment of the manometric flask contained 0.5 ml of cell suspension and 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.2). The side arm contained 0.5 ml of 0.01 M substrate, and the center well contained 0.3 ml of 20% KOH. When the partially purified enzyme was used, the main compartment contained 0.2 ml of the enzyme, 0.5 ml of 0.5 M potassium phosphate buffer (pH 8.0), 0.7 ml of water, and 0.1 ml of 5 mM 2,6-dichlorophenolindophenol (DCPIP). The side arm contained 1.0 ml of 10% PEG 6,000, and the center well contained 0.3 ml of 20% KOH. The substrate in the side arm was transferred to the main compartment, and then the measurement of oxygen uptake was initiated at 30°C with reciprocal shaking.

Assay of PEG-oxidizing activity. For assay of PEG-oxidizing activity, the reaction mixture contained 80 mg of PEG 6,000, 250 μmol of potassium phosphate buffer (pH 8.0), 0.6 μmol of flavine adenine dinucleotide, 5 μmol of KCN, 0.25 μmol of DCPIP, and enzyme preparation in a total volume of 2.50 ml and was incubated at 30°C. Enzyme activity was determined by measuring the initial rate of DCPIP reduction at 600 nm and by measuring the absorbance at 580 nm of the 2,4-dinitrophenylhydrazone of the product formed in a 20-min reaction period (2,4-DNPH assay) (6). In the 2,4-DNPH assay, KCN was omitted from the reaction mixture. The change in absorbance of various electron acceptors was assayed at 340 nm for nicotinamide adenine dinucleotide (phosphate), 420 nm for ferricyanide, 570 nm for 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, and 550 nm for cytochrome *c* and calculated as nanomoles of electron acceptor reduced per minute. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the reduction of 1 nmol of DCPIP per min under the standard assay conditions, and the specific activity is defined as the units of enzyme contained per milligram of protein.

Thin-layer chromatography. PEGs and their metabolites were separated on a 0.25-mm-thick silica gel 60 F_{254} plate (E. Merck). The chromatograms were developed with methanol-water (60:40, vol/vol) for PEG 6,000 and its metabolites and with 1-propanol–ethanol–water (50:30:20, vol/vol) for TEG and its metabolites. PEGs were detected with Dragendorff reagent (1). Oligomers and their metabolites were detected with a $K_2Cr_2O_7$-H_2SO_4 reagent (11).

Reaction of intact cells with TEG. The reaction mixture contained 5 g of TEG, 5.8 g (dry weight) of intact cells, and 5 mmol of potassium phosphate buffer (pH 7.2) in a total volume of 500 ml. Incubation was carried out at 28°C for 2 h with shaking and was stopped by centrifuging the mixture at 10,000 × *g* for 20 min; intact cells were then removed.

Gas chromatography-mass spectrometry. The reaction products of intact cells with TEG were fractioned by column chromatography as described in Results. Each fraction was evaporated to dryness and dissolved in an appropriate amount of chloroform. Chloroform solutions containing 1 to 2 mg of product were placed in glass-stoppered tubes and evaporated to dryness. A 100-μl volume of 25% *N,O*-bis(trimethylsilyl)acetamide in acetonitrile (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) was added to each sample, and the reaction was completed within a few minutes at room temperature. A mixture of authentic compounds (EG, DEG, triethylene glycol, and TEG, 2 mg each) was treated by the same procedures described above. The trimethylsilylated products were subjected to gas chromatography-mass spectrometry carried out with a Hitachi RMS-4 mass spectrometer-Hitachi M-5201 gas chromatograph equipped with an electron capture detector. Operational conditions were as follows: column temperature, 180 or 200°C; injector temperature, 230°C; ion source temperature, 160°C; separator temperature, 300°C; carrier gas, He; ionizing electron energy, 20 eV at 80 mA total emission. A glass column (3 mm by 1 m) packed with 5% OV-1 on Chromosorb W (80 to 100 mesh) was used.

Materials. Average molecular weights and sources of PEGs were described previously (7).

RESULTS

PEG 6,000 was not degraded by the supernatant fluid of a culture but was metabolized by washed, resting cells, and Dragendorff-positive material disappeared from the medium. EG and DEG, postulated hydrolytic products of PEG 6,000, were not utilized for growth, a result that is at variance with the data of Haines and Alexander (3).

The oxygen uptake of intact cells was increased by the addition of PEGs; the uptake rate increased with the increasing polymerization up to PEG 400 (Fig. 1). The oxygen uptake with EG was lower than that with PEG 6,000.

Presence and properties of PEG-oxidizing activity. Essentially all of the PEG-oxidizing activity was present in the cell extracts. They were centrifuged at 100,000 × *g* for 2 h, and the PEG-oxidizing activities of the resultant supernatant and particulate fractions were then assayed (Table 1). Approximately 60% of the initial activity was found in the particulate fraction, and the specific activity was 5.8 times higher than that of the supernatant fraction. The enzyme in the particulate fraction was solubilized by Anhitol 24B (lauryl betaine; KAO-ATLAS Co., Ltd., Tokyo, Japan), and the solubilized enzyme was purified approximately sevenfold by 30 to 80% ammonium sulfate fractionation and diethylaminoethyl-cellulose column chromatography. The enzyme (109 mg as protein) fractioned by 30 to 80% ammonium sulfate satura-

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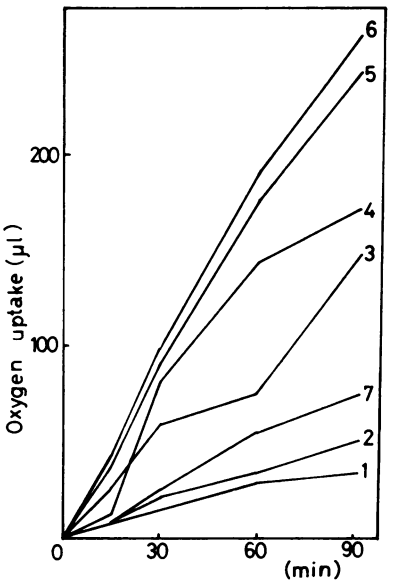

FIG. 1. *Oxygen uptake by resting cells. The optical density (610 nm) of the cell suspension used was 5.14. (1) Endogenous, (2) EG, (3) DEG, (4) triethylene glycol, (5) TEG, (6) PEG 400, (7) PEG 6,000.*

TABLE 1. *Localization of PEG-oxidizing activity in cell extract*

Enzyme prepn	Sp act[a]	Total activity[b]
Cell extract	5.74	3,690
After centrifugation[c]		
Supernatant fraction	2.15	1,100
Particulate fraction	12.4	2,130

[a] Units of enzyme contained per milligram of protein.

[b] Total units contained in enzyme preparation.

[c] Cell extract was centrifuged at 100,000 × *g* for 2 h.

tion was desalted with Sephadex G-25 (coarse) and applied to a diethylaminoethyl-cellulose column (2 by 16 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 8.0) containing 10% glycerol, 0.5 mM ethylenediaminetetraacetate, and 0.2% Anhitol 24B. The column was washed with the same buffer system, and then the enzyme was eluted with a step gradient of the same buffer system containing 0.1 M KCl. Eight-milliliter fractions were collected at a flow rate of 40 ml/h. The enzyme fractions showed the single elution curve, and the activity curve approximately coincided with the protein curve. From these chromatographic data, the partially purified enzyme seemed to be a single entity. The enzymatic characteristics described below were investigated with this partially purified enzyme.

(i) Electron acceptors. The reaction rate was highest with DCPIP and decreased by one-half with flavine adenine dinucleotide (Table 2). The reaction with cytochrome *c* and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide detected only a part of the activity, and that with ferricyanide, phenazine methosulfate, nicotinamide adenine dinucleotide, and nicotinamide adenine dinucleotide phosphate was negligible.

Oxygen uptake was not detected with the DCPIP-linked enzyme system.

From these results, the enzyme responsible for PEG oxidation was identified as a dehydrogenase. Since enzyme activity was also detected by the 2,4-DNPH assay, the reaction product appeared to be an aldehyde.

(ii) Substrate specificity. The PEG dehydrogenase had a low substrate specificity for PEGs (Table 3). DEG, triethylene glycol, TEG, and PEGs 400 to 20,000 were oxidized by this enzyme. The initial rate of DCPIP reduction was highest with PEGs 400 and 1,000; EG was not dehydrogenated.

TABLE 2. *Relative rates of PEG oxidation by the partially purified enzyme with electron acceptors*

Electron acceptor(s)	Relative rate[a]	
	2,4-DNPH assay[b]	Absorption[c]
None	0	—[d]
Nicotinamide adenine dinucleotide	19	0
Nicotinamide adenine dinucleotide phosphate	1	0
Ferricyanide	0	0
Ferricyanide + phenazine methosulfate	—	0
Phenazine methosulfate	2	—
Flavine adenine dinucleotide	51	—
3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide	15	8
3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide + phenazine methosulfate	—	11
Cytochrome *c*[e]	29	4
Cytochrome *c*[e] + phenazine methosulfate	—	0
DCPIP	100	100
DCPIP + phenazine methosulfate	—	104
DCPIP + flavine adenine dinucleotide	109	104

[a] The activity with DCPIP was defined as 100.

[b] Measured by 2,4-DNPH assay.

[c] Measured by the initial rate of DCPIP reduction.

[d] —, Not measured.

[e] Obtained from horse heart.

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(iii) K_m values. From the initial rate of DCPIP reduction, K_m values for TEG, PEG 400, and PEG 6,000 were calculated to be 11, 1.7, and 15 mM, respectively (Fig. 2), where the molecular weights of PEG 400 and 6,000 were postulated to be 400 and 8,400, respectively.

Identification of products. Although the mixed culture does not utilize the oligomers as sole carbon sources, oligomer utilization can be induced by PEG 400 or PEG 6,000 (Table 4). To determine the initial step in the metabolic pathway, TEG and washed cells were incubated in potassium phosphate buffer as described in Materials and Methods. The incubation resulted in formation of triethylene glycol and an unknown metabolite with an R_f of 0.31 (I). Upon thin-layer chromatography, both appeared as major products, while the third product, with an R_f of 0.17 (II), was detected only in trace amounts. For isolation, the reaction mixture was centrifuged to remove the cells, and the supernatant was passed through a Dowex 1×2 column (formate, 2.8 by 16.5 cm). The effluent was extracted with chloroform and evaporated to dryness. The column was then eluted with 500 ml of 0.1 N formate and successively with 500 ml of 0.5 N formate at a flow rate of approximately 100 ml/h, and the two eluted fractions were dried. Both the chloroform extract and the eluates were trimethylsilylated with *N,O*-bis(trimethylsilyl)acetamide and analyzed by gas chromatography-mass spectrometry. The substrate and triethylene glycol, as well as small quantities of DEG and EG, were detected in the chloroform extract. Compound I, which eluted with 0.1 N formate and had the characteristic peaks shown in Fig. 3, was identified as TEG-monocarboxylic acid. The peaks at *m/e* 352 (M) and 337 (M-15) indicated the molecular weight of compound I. Compound II was identified as TEG-dicarboxylic acid from the peak at *m/e* 351 (M-15) and other characteristic peaks (Fig. 4).

TABLE 3. *Substrate specificity of PEG dehydrogenase*

Substrate[a]	ΔE_{600}/min (×1,000)
EG	0
DEG	7
Triethylene glycol	17
TEG	91
PEG 400	134
PEG 1,000	170
PEG 2,000	80
PEG 4,000	62
PEG 6,000	51
PEG 20,000[b]	22

[a] Approximately 10 mM.
[b] Approximately 2.5 mM, calculated from the molecular weight 20,000.

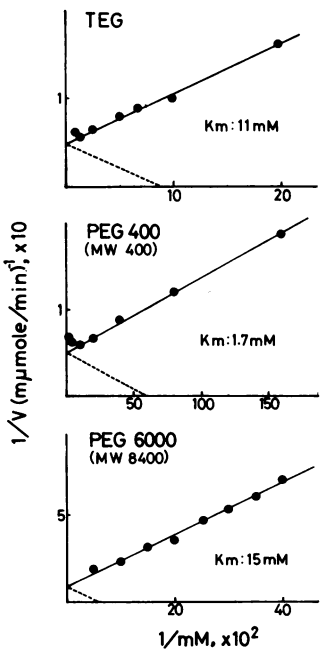

FIG. 2. *Double-reciprocal plots for PEG dehydrogenase. MW, Molecular weight.*

TABLE 4. *Induction of oligomer-utilizing activity*

Main carbon source[a]	Growth (OD_{610})[b]	
	PEG 400[c]	PEG 6,000[c]
None	0.80	0.52
EG	0.86	0.41
DEG	1.36	0.50
Triethylene glycol	2.01	1.87
TEG	1.87	1.62

[a] 1.0% (wt/vol).
[b] OD_{610}, Optical density at 610 nm.
[c] Inducer (0.01% [wt/vol]).

DISCUSSION

Payne et al. reported flavin-linked dehydrogenation of ether glycols by cell extracts of a soil bacterium (2, 9). Ohmata et al. also reported a flavin-linked dehydrogenase of PEG 400 in cell extracts of *Pseudomonas* species (8). Haines and Alexander reported that PEG 20,000 is utilized by *P. aeruginosa* and suggested that the polymer might be hydrolyzed by an extracellular enzyme (3). We found that PEG 6,000 and 20,000 are degraded by a synergistic, mixed culture of a *Flavobacterium* species and a *Pseudomonas* species (7). PEG 6,000 is not hydrolyzed by an extracellular enzyme, and the culture cannot utilize EG or DEG, which might be the hydrol-

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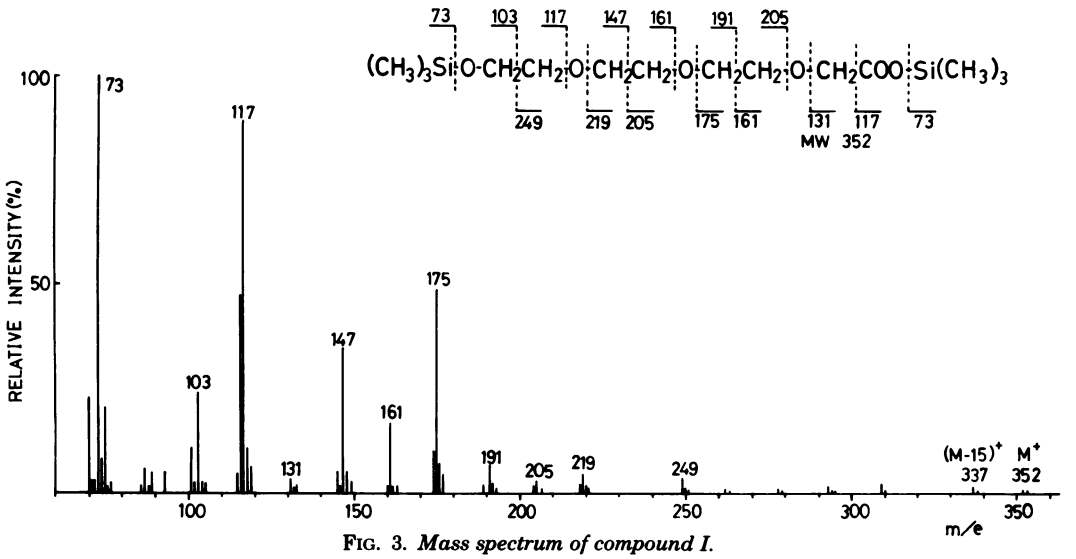

FIG. 3. *Mass spectrum of compound I.*

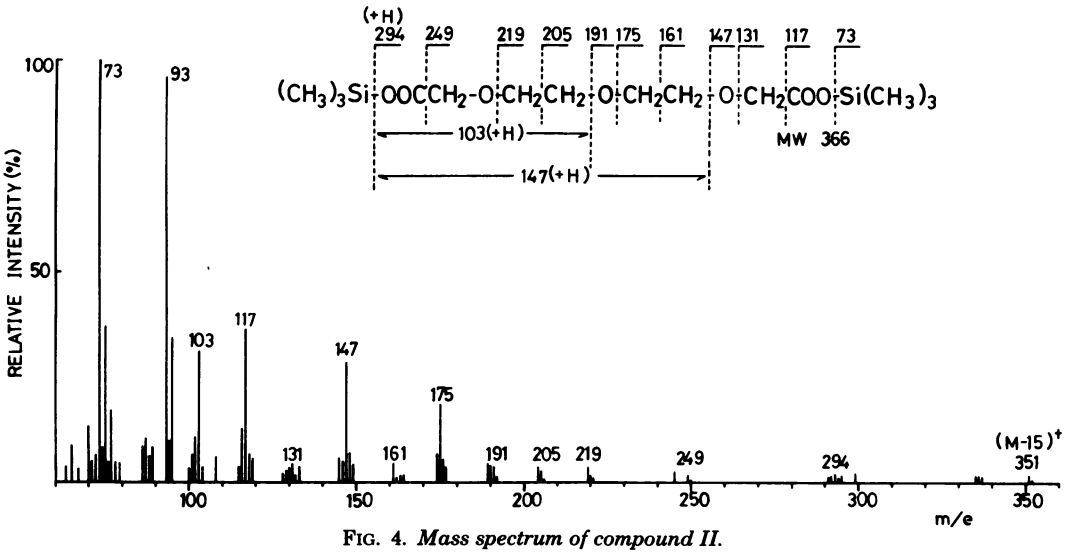

FIG. 4. *Mass spectrum of compound II.*

ysis products of PEG. Instead, PEG is oxidized to an aldehyde form by a DCPIP-linked dehydrogenase in the particulate fraction of extracts from the sonically oscillated cells. TEG-monocarboxylic and dicarboxylic acids, triethylene glycol, DEG, and EG were identified as metabolites of TEG. These results suggest that the initial steps of the PEG oxidation are as shown in Fig. 5. PEG is successively oxidized to an aldehyde, monocarboxylic acid, which is followed by the cleavage of the ether bond, resulting in PEG molecules that are reduced by one glycol unit. Simultaneous oxidation of two terminal hydroxyl groups of the molecule is also possible. Depolymerization might proceed via the same procedure as in that of monocarboxylic acid. This sequence is repeated and eventually yields depolymerized PEG.

PROPOSED PATHWAY OF PEG OXIDATON

$HO(CH_2CH_2O)_nCH_2CH_2OH$

↓

$HO(CH_2CH_2O)_nCH_2CHO$

↓

$HO(CH_2CH_2O)_nCH_2COOH$

↓

$HO(CH_2CH_2O)_{n-1}CH_2CH_2OH$

FIG. 5. *Proposed oxidative metabolic pathway of PEG.*

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