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Study on Ozone Treatment of Water-Soluble Polymers. II. Utilization of Ozonized Polyethylene Glycol by Bacteria

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Synopsis

A bacterium capable of utilizing polyethylene glycol of low molecular weight (less than 300) was isolated from soil and identified as *Pseudomonas aeruginosa* by biologic characteristics (named *P. aeruginosa* PEG-K). The effect of ozone degradation on the utilization of polyethylene glycol of high molecular weight by the bacterium was studied on the basis of the measurement of oxygen uptake by Warburg manometer and of bacterial growth. The polyethylene glycol, which can never be utilized at all because of high molecular weight, became utilizable by the bacterium as a result of ozonization, while the formaldehyde produced by the ozonization inhibited the utilization of the ozonized polyethylene glycol by the same bacterium. However, such inhibition disappeared by treating the aldehyde with hydrogen peroxide. From the results of gas chromatography and measurement of chemical oxygen demand, *P. aeruginosa* PEG-K was found to utilize ethylene glycol, diethylene glycol, and triethylene glycol, which were produced by the ozonization.

INTRODUCTION

Polyethylene glycol (PEG) of high molecular weight can never be utilized by bacteria at all. With respect to oligoethylene glycol, however, bacterial utilization was reported by Fincher and Payne in 1962.[1] Therefore, ozone degradation of PEG was carried out in order to enable PEG to be utilized by bacteria. The mechanism and the products of the ozone degradation were described in a previous paper.[2] The present paper is concerned with the utilization of the ozonized PEG by a bacterium isolated from the soil.

EXPERIMENTAL

Bacterium

The bacterium used throughout this study was isolated from the soil according to the procedure of Fincher and Payne[1] and was identified as a strain of *Pseudomonas aeruginosa* (*P. aeruginosa* PEG-K) according to biologic characteristics. The bacterium was cultured in stock culture on nutrient agar slant containing triethylene glycol 1%, and stored at 4°C.

For experimental studies, the bacterium was precultured for two days at 37°C on a basal salt medium (pH 7.4) containing K_2HPO_4 (9.28 g), KH_2PO_4 (1.81 g), NH_4Cl (0.5 g), $(NH_4)_2SO_4$ (0.5 g), and 0.1 g $MgSO_4 \cdot 7H_2O$ per liter of

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distilled water and triethylene glycol (1%) as sole carbon sources. The bacteria were harvested by centrifugation, washed aseptically twice in the basal salt medium without carbon source, and resuspended in the basal salt medium. The turbidity of the suspension was adjusted to 1.0 optical density at 650 mμ with a nephelophotometer in order to keep constant the number of bacteria. This bacterial suspension was used for the studies of growth and manometry.

Ozonization of PEG

The ozonization of PEG (MW 8000) was carried out in 2% aqueous solution which was prepared to pH 12 with 1*N* sodium hydroxide at the beginning of the experiment. The other ozonization techniques and the measurements of the viscosity and the formic ester in the ozonized PEG were the same as those described previously.[2] In addition, formaldehyde was determined by the chromotropic acid method.[3]

The aldehyde group was oxidized by hydrogen peroxide in alkaline solution in the following way in order to determine the effect of the aldehyde upon the bacterial growth. One milliliter each of 0.3% H_2O_2 and of 4*N* NaOH were added to the ozonized solution (10 ml), and the solution was heated for 30 min and then neutralized with 4*N* HCl.

Growth Study

The ozonized polyethylene glycols or those treated with hydrogen peroxide were mixed with the basal salt medium. The culture media containing the ozonized PEG (1%) as sole carbon source were filtered through a membrane filter with a pore size of 0.2 μ to remove various germs. These culture media (10 ml of each) were placed in a shaking test tube (25 ml) and the suspension of *P. aeruginosa* PEG-K was added. Then the samples were cultured with continual shaking at 37°C. The growth of bacteria was determined turbidimetrically at 650 mμ with a Otake colorinephelophotometer. The grown bacteria were separated by centrifugation, and the chemical oxygen demand (C.O.D.) and the viscosity of the supernatant were determined. The oligoethylene glycol (ethylene glycol monomer, dimer, and trimer) in the supernatant was determined by gas chromatography using tetraethylene glycol dimethyl ether as internal standard.

Measurement of Oxygen Uptake

The oxygen uptake of *P. aeruginosa* PEG-K in the substrate of ozonized PEG was measured at 37°C with a Mitamura Warburg manometer. Compositions of vessels were as follows: main room contained 4 ml of the bacterial suspension, side arm contained 2 ml of the substrate (2% in concentration), and central well contained 0.2 ml 20% KOH.

RESULTS AND DISCUSSION

Utilization of PEG by *P. aeruginosa* PEG-K

P. aeruginosa PEG-K was cultured in the substrates of triethylene glycol, PEG-300, PEG-400, and PEG-600 (figures show the molecular weight) in

order to examine the capability of the utilization of PEG, and the C.O.D.'s of the filtrated culture solutions were determined. Figure 1 shows the growth curves and C.O.D.'s of the culture solution in each substrate. The C.O.D. of the culture solution, in the case of triethylene glycol substrate, was removed 90% after two days of incubation. The decrease of C.O.D. in PEG-300 was about 50% after five days of incubation. Although the bacteria grew slightly in PEG-400, the decrease in C.O.D. was small. The slight growth is attributed to the utilization of contaminated PEG of low molecular weight. Neither bacterial growth nor decrease in C.O.D. was observed in PEG-600. These results indicate that the limit of molecular weight of PEG which can be utilized by the bacterium is about 300.

Characterization of Ozonized PEG

Table I shows the results of the characterization of the ozonized PEG which were used for the following studies of the bacterial utilization. The values of the formaldehyde include free formaldehyde and the other compounds (e.g., glycolic acid) which are liberated formaldehyde in concentrated sulfuric acid. The formic ester in the ozonized PEG solution was confirmed by the formation of hydroxamic acid. However, the quantitative analysis was impossible owing to drastic discoloration. In the analytical result of lyophilized sample, the produced formic ester was 60 to 70 mole-% of ozone consumed.[2] The formic ester disappeared completely after the treatment

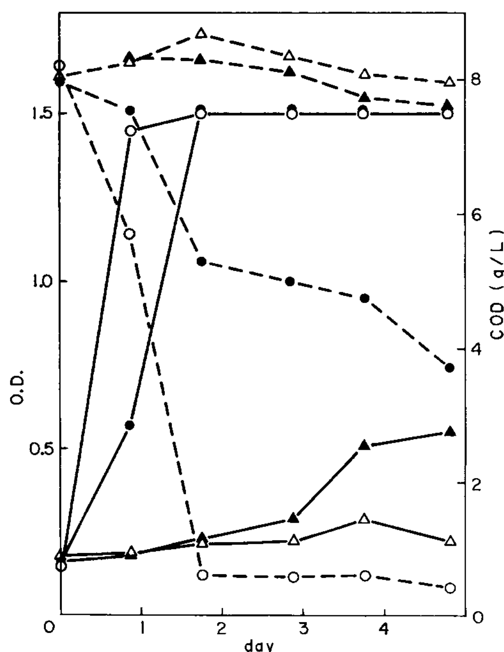

Fig. 1. Growth curves of *P. aeruginosa* PEG-K in the culture medium containing PEG of various molecular weight and decrease of C.O.D. of the culture solution: full line, growth curve; dashed line, C.O.D.; (○) triethylene glycol; (●) PEG-300; (▲) PEG-400; (△) PEG-600.

TABLE I
Model Reaction of PB with Benzylamine

Solvent	Reaction conditions Temp., °C	Time, hr	Amide I yield, %
Tetrahydrofuran	20	2	94
Dioxane	20	2	94
Anisole	20	24	94
Methanol	20	24	49
Ethanol	20	24	70
Benzyl alcohol	20	24	63
m-Cresol	20	24	79
Nitrobenzene	20	24	93
Pyridine	20	24	73
DMF	20	24	—[a]
NMP	20	24	—[a]

[a] A mixture of undefined products and a small amount of amide I was obtained.

with hydrogen peroxide. As formaldehyde is also degraded easily with hydrogen peroxide, the values in the last column in Table I, which are the data relating to the ozonized solution having treated aldehyde, seem to indicate compounds such as glycolic acid other than formaldehyde. The concentration of the aldehyde in the growth studies was one half its value of the table, as the solution and the basal salt medium were mixed.

The intrinsic viscosity of the ozonized PEG at the maximum ozone consumption was 0.044, and the number-average molecular weight was about 1000. No lowering of the viscosity caused by the hydrogen peroxide treatment was observed. This molecular weight may be too large to be utilized completely by the bacterium. As the concentration of PEG in the ozoniza-

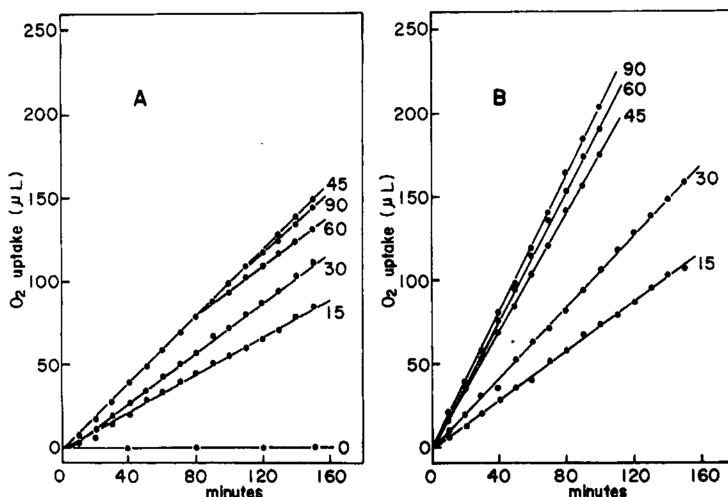

Fig. 2. Oxidation of ozonized PEG by the isolant *P. aeruginosa* PEG-K: (A) ozonized PEG; (B) ozonized PEG without aldehyde treated with hydrogen peroxide. Figures show the ozonization time.

TABLE II
Rate of Oxygen Uptake by *P. aeruginosa* PEG-K

Sample no.[a]	Rate of oxygen uptake, μl/min	
	Ozonized PEG	Ozonized PEG treated with H_2O_2
0	0	0
15	0.59	0.75
30	0.78	1.10
45	1.04	1.76
60	0.75	1.82
90	0.83	2.08

[a] Figures show the ozonization time of PEG in Table I.

tion was high for the purpose of the bacterial studies, a sufficient decrease in molecular weight was difficult.

Oxidation of Ozonized PEG by *P. aeruginosa* PEG-K

The results in Figure 2 show the oxidation of the ozonized PEG (A) and of that without aldehyde treated with hydrogen peroxide (B) by *P. aeruginosa* PEG-K. The plots of oxygen uptake versus time gave straight lines, except for two samples ozonized for 60 and 90 min, respectively. Although the lines of those two samples were curved, the reason was obscure. The rates of oxidation calculated from the slope of the lines in Figure 2 are summarized in Table II. In general, the rate of oxidation is proportional to the concentration of the substrate, cell number of bacteria, and the facility of oxidation of the substrate by the bacteria. Since the concentration of the substrates of the ozonized PEG and the number of bacteria were constant in this study, the rate of oxidation depends on the facility of oxidation of the ozonized PEG by *P. aeruginosa* PEG-K. Therefore, it is found from Figure 2 and Table II that

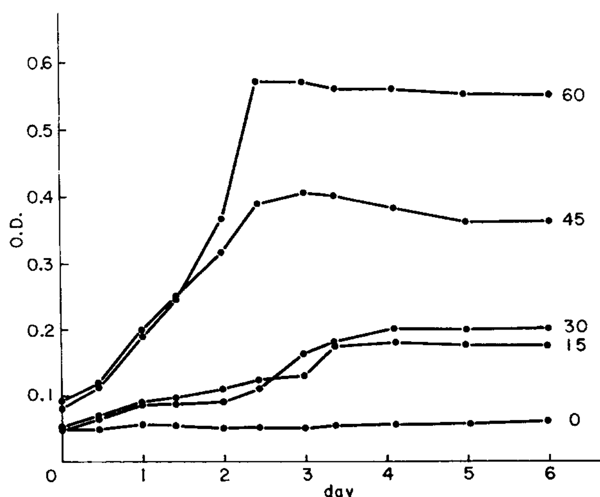

Fig. 3. Growth curves of *P. aeruginosa* PEG-K in culture medium containing ozonized PEG without aldehyde. Figures show the ozonization time.

the original PEG was not oxidized by the bacteria and that the bacterial oxidation was facilitated according to the progress of the ozonization. But excessive ozonization, on the contrary, made the bacterial oxidation difficult. On the other hand, such a phenomenon was not observed in the substrates without aldehyde treated with hydrogen peroxide. These results indicate that the aldehyde produced by the ozonization inhibited the oxidation of the ozonized PEG by *P. aeruginosa* PEG-K.

Utilization of Ozonized PEG by *P. aeruginosa* PEG-K

P. aeruginosa PEG-K was cultured in the ozonized PEG as sole carbon source. In the case of the ozonized PEG containing aldehyde, however, bacterial growth was not observed, except for a slight growth (O.D. 0.125) in the culture medium of the PEG ozonized for 15 min. On the other hand, Figure 3 shows the growth curves of the bacteria in the culture medium containing the ozonized samples without aldehyde treated with hydrogen peroxide. From the figure it was found that the growth of the bacteria was increased according to the progress of the ozonization. Since the carbon source of the culture medium was only the ozonized PEG, the growth of *P. aeruginosa* PEG-K suggests that the bacterium utilized the ozonized PEG. Such remarkable growth in the substrate without aldehyde indicates that the utilization by *P. aeruginosa* PEG-K is inhibited by the aldehyde. This aldehyde is either the free formaldehyde produced slightly by the ozonization or the formic ester which is the main product of the ozonization.

Figure 4 shows the growth curves of the bacteria in the culture medium containing 1% triethylene glycol and 0.1–0.6 mmole/l. formaldehyde or 1–100 mmoles/l. ethyl formate. These concentrations were set on the basis of those

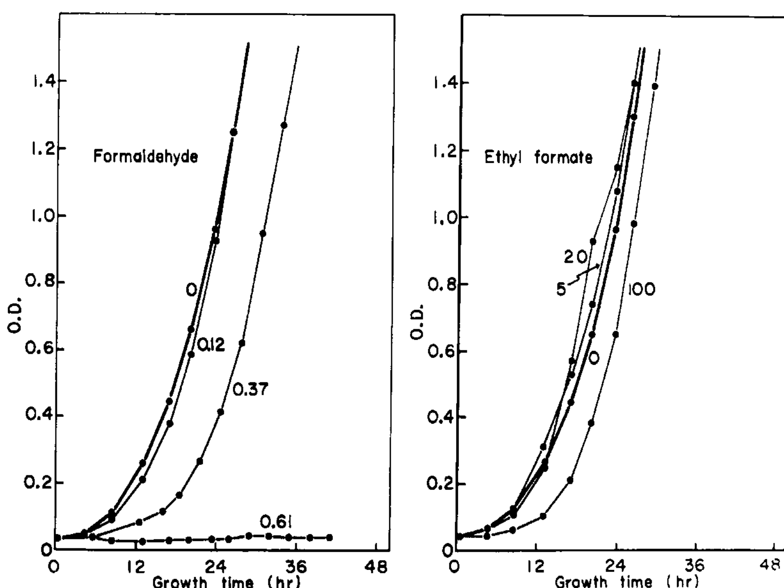

Fig. 4. Inhibitory effect of formaldehyde and ethyl formate on growth of *P. aeruginosa* PEG-K. Figures show concentration of formaldehyde and ethyl formate (mmoles/l.).

TABLE III
Utilization of EG, DEG, and TEG in Ozonized PEG[a] by *P. aeruginosa* PEG-K

Culture time, hr	Bacterial growth O.D.	EG, (moles/l.) × 10^3	DEG, (moles/l.) × 10^3	TEG, (moles/l.) × 10^3	C.O.D. removal,[b] %
0	0.05	5.62	2.65	2.62	0
20	0.23	5.75	2.63	2.48	0
27	0.39	5.65	2.38	2.26	2
44	0.66	5.10	2.00	1.62	9
68	1.05	0.50	1.46	0.76	15
91	0.80	—	0.93	0.20	19
115	0.77	—	0.76	0.20	24

[a] The PEG was ozonized for 90 min.
[b] The C.O.D. of original culture medium was 12.4 g/l.

of the aldehydes in the growth studies using the ozonized PEG. The growth of *P. aeruginosa* PEG-K was inhibited completely with 0.6 mmole/l. formaldehyde and not inhibited with ethyl formate. These results indicate that the aldehyde which inhibited the growth of *P. aeruginosa* PEG-K in the culture medium of ozonized PEG was the formaldehyde produced slightly by the ozonization.

P. aeruginosa PEG-K was cultured in the culture medium containing PEG solution ozonized for 90 min as sole carbon source, and ethylene glycol, diethylene glycol, triethylene glycol, and C.O.D. in the culture filtrate were determined in order to confirm the substrate utilized by the bacterium. These results are shown in Table III. The analytical values indicate that the *P. aeruginosa* PEG-K utilized ethylene glycol, diethylene glycol, and triethylene glycol produced by the ozonization. The C.O.D. removal calculated from the disappearance of ethylene glycol, diethylene glycol, and triethylene glycol was 8%. Accordingly, the C.O.D. removal of 24% is believed to be due to the utilization of the other polyethylene glycol of molecular weight less than 300. Such a small removal of C.O.D., 24%, is caused by the presence of the ozonization products other than oligoethylene glycol and of high molecular weight PEG which was not thoroughly degraded by ozone. This is supported also by the fact that no change in viscosity of the culture filtrate was detected.

The above-mentioned results lead to the conclusion that PEG of high molecular weight has to be degraded to MW 300 by ozone so as to be utilized by the bacteria, and that a slight amount of formaldehyde produced by the ozonization has to be treated.

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References

1. E. L. Fincher and W. J. Payne, *Appl. Microbiol.*, **10,** 542 (1962).
2. J. Suzuki, *J. Appl. Polym. Sci.*, **20,** 93 (1976).
3. C. E. Bricker and H. R. Johnson, *Ind. Eng. Chem., Anal. Ed.*, **17,** 400 (1945).

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