CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Polyethylene glycol (PEG)

2.1.1 Chemical properties of PEG

Polyethylene glycol is a condensation polymers of ethylene oxide and water with the general formula $H(OCH_2CH_2)_nOH$, where n is the average number of repeating ethylene oxide groups typically from 4 to about 180.

Figure 2.1 Polyethylene glycol chemical structure

The low molecular weight members from n=2 to n=4 are diethylene glycol, triethylene glycol and tetraethylene glycol respectively, which are produced as pure compounds. The low molecular weight compounds up to 700 are colorless, odorless viscous liquids with a freezing point from -10°C (diethylene gycol), while polymerized compounds with higher molecular weight than 1,000 are wax-like solids with melting point up to 67°C for n=180. The abbreviation (PEG) is termed in combination with a numeric suffix which indicates the average molecular weights. One common feature of PEG appears to be the water-soluble. It is soluble also in many organic solvents including aromatic hydrocarbons. They are used to make emulsifying agents and detergents, and as plasticizers, humectants, and water-soluble textile lubricants. The wide range of chain lengths provide identical physical and chemical properties for the proper application selections directly or indirectly in the field of;

- Alkyd and polyester resin preparation to enhance water dispersability and water-based coatings
- Anti-dusting agent in agricultural formulations
- Brightening effect and adhesion enhance in electroplating and electroplating process
- Cleaners, detergents and soaps with low volatility and low toxicity solvent properties
- Coupling agent, humectant, solvent and lubricant in cosmetics and personal care bases
- Dimensional stabilizer in wood working operations
- Dye carrier in paints and inks
- Heat transfer fluid formulation and defoamer formulations
- Low volatile, water soluble and non-corrosive lubricant without staining residue in food and package process
- Mold release agent and lubricant in fabricating elastomers
- Paper coating for anti-sticking, color stabilizing, good gloss and free flow in calendering operations
- Plasticizer to increase lubricity and to impart a humectant property in ceramic mass, adhesives and binders
- Softener and antistatic agent for textiles
- Soldering fluxes with good spreading property

(http://www.chemicalland21.com/arokorhi/industrialchem/organic/POLYETHYLENE%20GLYCOL.htm)

Table 2.1 General properties of polyethylene glycol

(http://www.chemicalland21.com/arokorhi/industrialchem/organic/POLYETHYLENE%20GLYCOL.htm)

Property	**Molecular weight**	**Appearance**	**Color, Apha**	**Moisture**	**Hydroxyl value**	**pH**	**Specific gravity**	**Oral rat LD_{50}**	**Viscosity**	**Melting point**
PEG 200	190-210	clear oily liquid	10 max	0.2% max	535-590 (mg KOH/g)	5-7	1.12-1.13	28 g/kg	50 cP at 25°C	-45°C
PEG 300	280-320	clear oily liquid	10 max	0.2% max	355-395 (mg KOH/g)	5-7	1.12-1.13	27.5 g/kg	70 cP at 25°C	-15°C
PEG 400	380-420	clear oily liquid	10 max	0.2% max	265-295 (mg KOH/g)	5-7	1.12-1.13	30.2 g/kg	90 cP at 25°C	4°C
PEG 600	570-630	clear oily liquid	10 max	0.2% max	175-195 (mg KOH/g)	5-7	1.12-1.13	30 g/kg	135 cP at 25°C	17°C

Property	Molecular weight	Appearance	Color, Apha	Moisture	Hydroxyl value	pH	Specific gravity	Oral rat LD_{50}	Viscosity	Melting point
PEG 1000	950-1050	white paste	10 max (50% aqueous solution)	0.1% max	105-120 (mg KOH/g)	5-7	1.08-1.09 (50% aqueous solution)	32 g/kg	20 cP (50% aqueous solution)	25°C
PEG 2000	1800-2200	white flake	10 max (50% aqueous solution)	0.1% max	50-65 (mg KOH/g)	5-7	1.08-1.09 (50% aqueous solution)	15 g/kg	40 cP (50% aqueous solution)	50°C
PEG 4000	3500-4500	white flake	10 max (50% aqueous solution)	0.1% max	25-35 (mg KOH/g)	5-7	1.08-1.09 (50% aqueous solution)	50 g/kg	100 cP (50% aqueous solution)	58°C

Property	Molecular weight	Appearance	Color, Apha	Moisture	Hydroxyl value	pH	Specific gravity	Oral rat LD_{50}	Viscosity	Melting point
PEG 6000	5000-7000	white flake	10 max (50% aqueous solution)	0.1% max	25-35 (mg KOH/g)	5-7	1.08-1.09 (50% aqueous solution)	more 50g/kg	100 cP (50% aqueous solution)	60°C
PEG 8000	7500-8500	white flake	10 max (50% aqueous solution)	0.1% max	25-35 (mg KOH/g)	5-7	1.08-1.09 (50% aqueous solution)	more 50g/kg	100 cP (50% aqueous solution)	65°C

Table 2.2 Characteristics of polyethylene glycol

(http://www.surfactant.co.kr/surfactants/peg.html)

Characteristics	**Overview**
Highly compatible to various kinds of organic compounds	PEG is compatible with most organic solvents, and has excellent water-solubility.
High boiling point	Effective as a non-volatile solvent because of its high boiling point.
Easy control of the degree of condensation	As the degree of condensation is properly governed, PEG has a broad spectrum of products ranging from rigid solids to oily liquids.
Controllable hygroscopic property	Every PEG type surfactant has excellent hygroscopic property, and this is controllable by adjusting the degree of condensation. As the degree of condensation increases, the hygroscopic property is degreased.
Less toxicity	PEG is characterized by less toxicity and less skin irritation. There is no damage in case of contact with skin or lips.

Table 2.3 Applications of polyethylene glycol

(http://www.surfactant.co.kr/surfactants/peg.html)

Industries	**Overview**
Rubber industries	As PEG has good water-solubility even in its solid state, it is used as a releasing agent for foam rubber, latex rubber, etc. PEG is also used as an air-pack releasing agent, inner releasing agent, and lubricant in the tire industry.
Textile industries	Because PEG has a broad range of applications, it is used as a softener, antistatic agent, scouring agent, sizing agent, dyeing auxiliary, etc. in the textile industry.
Paper industries	PEG has a softening effect on paper.
Metal industries	PEG improves grinding effects by being added to the grinding powder and it is used as a raw material for anticorrosion and the cleaning of metals.
Wood industries	By dipping wood in a PEG solution in advance, contraction and cracking of wood can be avoided.
Phamaceutical industries	PEG is used as a base materials of oinments.
Cosmetic industries	PEG can be used in shampoos, hand creams, lotions, etc.
Resin and Paint industries	PEG is used as the raw material of paints and resins.

2.1.2 Environmental fate of PEG

No environmental fate data are available for PEGs but there is the environmental fate data for ethylene glycol.

Ethylene Glycol:

Ethylene glycol's production and uses such as a coolant and antifreeze may result in its release to the environment through various waste streams.

If released to air, a vapor pressure of 0.092 mm Hg at 25°C indicates ethylene glycol will exist solely as a vapor in the ambient atmosphere. Vapor-phase ethylene glycol will be degraded in the atmosphere by reaction with photochemically-produced hydroxyl radicals; the half-life for this reaction in air is estimated to be 2 days. Ethylene glycol is not expected to be susceptible to direct photolysis by sunlight since it does not contain functional groups that are expected to absorb light with wavelengths more than 290 nm.

If released to soil, ethylene glycol is expected to have very high mobility based upon an estimated partitioning coefficient of 1 (Koc, or how well a substance will bind to organic carbon in the soil). Volatilization from moist soil surfaces is not expected to be an important fate process based upon a Henry's Law constant of 6.00×10^{-8} atm-cu m/mole. The Henry's Law constant is a measure of how well a substance will enter the air, once dissolved in water. The higher the number, the more that will enter the air. Ethylene glycol is not expected to volatilize from dry soil surfaces based upon its vapor pressure. Ethylene glycol is biodegraded in soil 97-100% in 2-12 days.

If released into water, ethylene glycol is not expected to adsorb to suspended solids and sediment based upon the estimated partitioning coefficient. In a river die-away test, degradation was complete within 3 days at 20°C and 5-14 days at 8°C. Volatilization from water surfaces is not expected to be an important fate process based upon this compound's Henry's Law constant. A bioconcentration factor (BCF) of 10, reported for ethylene glycol in fish, Golden ide (*Leuciscus idus melanotus*), after 3 days of exposure suggests the potential for bioconcentration in aquatic organisms is low. Hydrolysis is not expected to be an important environmental fate process since this compound lacks functional groups that hydrolyze under environmental conditions.

(Center for Disease Control Hazardous Substance Database

http://toxnet.nlm.nih.gov/cgibin/sis/search/f?./temp/~N3bLf8:1)

2.2 Polypropylene glycol (PPG)

2.2.1 Chemical properties of PPG

Polypropylene glycol (PPG) is the polymer of propylene glycol with the general formula $HO(C_3H_6O)_nH$, where n is the average number of repeating propylene oxide groups. Chemically it is a polyether. The PPG is reserved for low to medium range molar mass polymer when the nature of the end-group which is usually a hydroxyl group, still matters. The oxide is used for high molar mass polymer when endgroups no longer affect polymer properties.

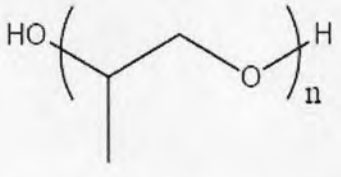

Figure 2.2 Polypropylene glycol chemical structure

Polypropylene glycol is produced by anionic ring-opening polymerization of propylene oxide. The initiator is an alcohol and the catalyst a base, usually potassium hydroxide. When the initiator is ethylene glycol or water the polymer is linear. With a multifunctional initiator like glycerine, pentaerythritol or sorbitol the polymer branches out.

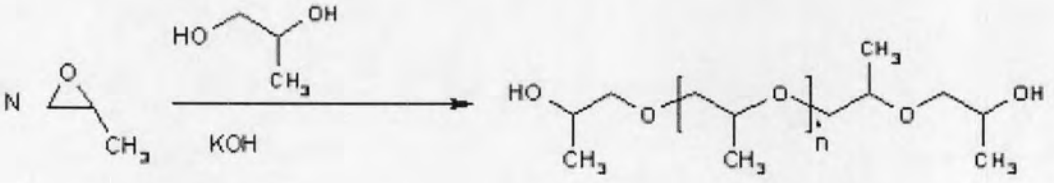

Figure 2.3 Polymerization of propylene oxide

PPG having an average molecular weight of less than approximately 700 (triol type) or 1000 (diol type) is readily water-soluble, but one having a higher molecular weight is oily and insoluble in water.

PPG has many properties in common with polyethylene glycol. The polymer is a liquid at room temperature. Solubility in water decreases rapidly with increasing molar mass. Secondary hydroxyl groups in PPG are less reactive than primary hydroxyl groups in polyethylene glycol. PPG is used in many formulations for polyurethanes. It is used as a rheology modifier.

Table 2.4 General properties of polypropylene glycol

(http://www.huntsman.com/base_chemicals/Media/PPGAust.pdf)

Product	**Color (Max Pt-Co)**	**Hydroxyl value (mg KOH/g)**	**Melting point (°C)**	**Viscosity (cP) at 25°C**	**Flash point (°C) (coc)**	**Specific gravity (at 20°C)**	**Refractive index (n_D^{20})**	**Surface tension (mN/m)**
PPG 400	100	220-250	-26	82	204	1.009	1.445	31.1
PPG 1000	100	106-118	-36	196	227	1.006	1.448	31.3
PPG 1650	-	67-71	-32	330	230	1.006	1.449	31.8
PPG 2250	50	45-47	-29	482	240	1.005	1.449	32.1
PPG 4000	250	26-30	-30	1,232	240	1.004	1.45	32.2

Table 2.5 Characteristics of polypropylene glycol

(http://www.dow.com/polyglycols/ppgc/na/rc/sharprop.htm)

Characteristic	Overview
Excellent solvency	Polypropylene glycols are compatible with many performance additives and are miscible in various organic liquids.
Lubricity	Polypropylene glycols exhibit excellent film forming properties and low coefficient of friction.
High viscosity indices	Polypropylene glycols have great viscosity versus temperature profiles making them an excellent choice as base stocks for synthetic lubricants.
Range of viscosity	Polypropylene glycols cover a broad range of viscosity from very low to very high viscosity.
Low color and odor	Polypropylene glycols are typically water white, with little or no odor.
Low ash, non-varnishing	In high temperature applications and when burned, polypropylene glycols do not leave black carbon or sticky residues.
Toxicological and ecotoxicological profile	Polypropylene glycols and polyglycol copolymers exhibit a low order of acute toxicity by ingestion or skin exposure. Aquatic toxicity is typically low and many of the products are biodegradable. Consult individual safety data sheets for more specific information.

2.2.2 Environmental fate of PPG

No environmental fate data are available for PPGs but there is the environmental fate data for propylene glycol.

Propylene glycol:

Propylene glycol's production and use as an emollient in cosmetics and pharmaceutical creams, a corrosion inhibitor, the manufacture of resins, an additive to paint to provide freeze-thaw stability, solvent in food colors and flavors and as an airplane de-icing fluid may result in its release to the environment through various waste streams.

If released to air, a vapor pressure of 0.13 mm Hg at 25°C indicates propylene glycol will exist solely as a vapor in the ambient atmosphere. Vapor-phase propylene glycol will be degraded in the atmosphere by reaction with photochemically-produced hydroxyl radicals; the half-life for this reaction in air is estimated to be 32 hours.

If released to soil, propylene glycol is expected to have very high mobility based upon an estimated partitioning coefficient (Koc) of 8. Volatilization from moist soil surfaces is not expected to be an important fate process based upon an estimated Henry's Law constant of $1.3X10^{-8}$ atm-cu m/mole. Propylene glycol is not expected to volatilize from dry soil surfaces based upon its vapor pressure. Propylene glycol was mineralized 73-78% in laboratory studies conducted using an agricultural soil over a 51 day incubation period, suggesting biodegradation will be an important environmental fate process in soils.

If released into water, propylene glycol is not expected to adsorb to suspended solids and sediment based upon the estimated partitioning coefficient (Koc). Volatilization from water surfaces is not expected to be an important fate process based upon this compound's estimated Henry's Law constant. Numerous screening studies using wastewater or sewage inoculum as seed, suggests that propylene glycol will be degraded readily in aqueous environments. Propylene glycol is not expected to undergo hydrolysis since this compound lacks functional groups that hydrolyze under environmental conditions. An estimated bioconcentration factor (BCF) of 3 suggests the potential for bioconcentration in aquatic organisms is low.

Occupational exposure to propylene glycol may occur through inhalation and dermal contact with this compound at workplaces where propylene glycol is produced or used. Propylene glycol is contained in a number of consumer products including cosmetics, pharmaceutical creams and some food additives. The general population may be exposed to propylene glycol through dermal and ingestion pathways when products containing this compound are used. Propylene glycol is also used in aerosol mists that are commonly employed in hospitals and public buildings for disinfection purposes; therefore, the general population may be exposed to this compound by inhalation routes.

(Center for Disease Control Hazardous Substance Database

http://toxnet.nlm.nih.gov/cgibin/sis/search/f?./temp/~N3bLf8:1)

2.3 Assimilation of microorganisms

2.3.1 Polyethylene glycol (PEG)

2.3.1.1 Bacteria

(1) Aerobic bacteria

Microbial degradation of polyethers has been studied extensively on PEGs using aerobic microorganisms (Kawai, 1987). Since the first report of Fincher and Payne (1962) on a PEG 400-utilizing bacterium, many reports have been made on aerobic PEG-utilizing bacteria which assimilate PEGs with a variety of molecular sizes (Table 2.6) (Kawai, 2002). For the bacteria, diversity exists with respect to the maximum size of the PEG molecule which can be degraded (Kawai, 1995). PEG with molecular weight up to 4000 was assimilated by various bacteria and by mixed culture in activated sludges. PEG with a molecular weight of more than 4000 was assimilated by a limited number of species such as *Pseudomonas aeruginosa* (up to 20,000) (Haines and Alexander, 1975), soil bacteria (up to 6000) (Hosoya *et al.*, 1978), *Pseudomonas stutzeri* (up to 13,500) (Obradors and Aguilar, 1991) and *Sphingomonas* species (up to 20,000). 6000-Dalton-PEG or longer was completely metabolized by consortia of *Sphingomonas terrae*, a group of concomitant associates (*Rhizobium*, *Agrobacterium* and *Methylobacterium* sp.) (Takeuchi *et al.*, 1993) and by pure cultures of *Sphingomonas* sp. (Kawai and Takeuchi, 1996).

Sphingomonads were defined in 1990 as a group of gram-negative, rod-shaped, chemoheterotropic, strictly aerobic bacteria that posses ubiquinone 10 as the major respiratory quinine, contain glycosphingolipids (GsLs) instead of lipopolysaccharide in their cell envelopes, and typically produce yellow-pigmented colonies. The sphingomonads are widely distributed in nature, having been isolated from many different land and water habitats, as well as from plant root systems, clinical specimens, and other sources. Due to their biodegradative and biosynthetic capabilities, sphingomonads have been utilized for a wide range of biotechnological applications, from bioremediation of environmental contaminants to production of extracellular polymers such as sphingans (e.g. gellan, welan and rhamsan) used extensively in the food and other industries. Various lipophilic xenobiotic-assimilating bacteria were included in this genus: (poly)chlorophenol-, polycyclic aromatic hydrocarbons-, γ-hexachlorocyclohexane-, 2,4-dichlorophenoxyacetic acid-, dibenzo-*p*-dioxin and diphenyl ether-utilizing bacteria (Nohynek *et al.*, 1996; Kawai, 1999).

Table 2.6 Synopsis of the microbial degradation of PEG compounds

PEG compound	***Microorganism***	***Reported***	***References***
diEG-PEG 4000	Soil bacterium	1962	Fincher and Payne
PEG 400 and 1000	Activated sludge	1967	Borstlap and Kortland
less than to PEG 400	Air-dried activated sludge	1970	Patterson *et al.*
less than to PEG 1000	Acclimated sewage sludge	1973	Sturm
EG-PEG 3500[a]	Sludges and pure culture	1973	Pitter
triEG-PEG 400	Soil bacterium (possibly *Pseudomonas*)	1974	Ohmata *et al.*
triEG or PEG 400	Sludge or soil bacterium (*Alcaligenes*)	1975	Harada and Nagashima
PEG up to 20,000	Pure cultures and symbiotic mixed cultures[b]	1975 1977	Ogata *et al.* Kawai *et al.*
PEG up to 20,000	*Pseudomonas aeruginosa*	1975	Haines and Alexander
PEG up to 4000	Adapted activated sludge	1976	Cox and Conway
EG-PEG 400	Possibly an *Actinetobacter*	1976	Jones and Watson
EG-PEG 1500	*Actinetobacter* *Pseudomonas* *Flavobacterium*	1977	Watson and Jones
PEG 200-2000	Acclimated activated sludge	1977	Suzuki and Kusunoki
PEG 400 and 6000	Soil bacteria	1978	Hosaya *et al.*
EG-PEG 200 PEG 200-4000	Bacteria	1979	Jenkins and Cook
diEG-PEG 400	*Pseudomonas*	1980	Thélu *et al.*
EG-PEG 1500 (different utilization range)	*Actinetobacter* *Pseudomonas* *Aeromonas*	1980	Pearce and Heydeman
diEG and triEG tetraEG/PEG 600	*Pseudomonas fluorescens* *Alcaligenes glycovorans*	1983/1985 1986	Schöberl
PEG 400	Activated sludge	1985	Steber and Wierich
PEG up to 10,000	*Pseudomonas stutzeri*	1991	Obradors and Aguilar
PEG 4000 and PEG up to 20,000	Sphingomonads	1996	Kawai and Takeuchi
PEG 4000 and 8000	*Pseudonocardia* sp.	2000	Kohlweyer *et al.*

[a] The growth might be caused by lower molecular PEG included in a sample.

[b] A representative mixed culture was composed of *Sphingomonas terrae* and *Rhizobium* sp.

Although a barrier for the macromolecules might possibly exist for the assimilation of polyethers, the characteristic membrane structures of this genus are possibly correlated with the uptake of large PEG by membranes. This was partly supported by the findings that *Rhodopseudomonas acidophilia* M402 is able to oxidize PEGs up to a certain size by means of its alcohol dehydrogenase, but it cannot grow on these compounds (Yamanaka, 1991) and that cell free-extracts of PEG 400-, 1000- or 4000-utilizing bacteria could dehydrogenate higher PEGs (6000 and 20,000), which could not be utilized as the sole carbon and energy sources (Kawai and Yamanaka, 1989). Recently, a Gram-positive actinomycete, *Pseudonocardia* sp. strain K1, originally isolated as a tetrahydrofuran degrader, was found to also grow on PEG 4000 and 8000 (Kohlweyer *et al.*, 2000). As this is the only Gram-positive bacterium known to grow on PEG, it is another interesting question whether the actinomycete has the same metabolic pathway and the same uptake system of large PEG into cells, as found in sphingomonads.

(2) Anaerobic bacteria

Anaerobic assimilation of PEG so far has been reported by three groups of bacteria. *Alcaligenes faecalis* var. *denitrificans* was first isolated by Fincher and Payne (1962) as an aerobic PEG 400-utilizing bacterium, but later this strain was reported to grow anaerobically as a denitrifier at the expense of several free ether glycols up to PEG 300 (Grant and Payne, 1983). Dwyer and Tiedje (1983) isolated methanogenic consortia from sewage sludge, which can degrade ethylene glycol monomer and polymers as large as PEG 20,000. Schink and Stieb (1983) also isolated an anaerobic bacterium, *Pelobacter venetianus*, which can degrade PEG up to 20,000 in size.

2.3.1.2 Fungi

Fungus assimilation of PEG has been reported as followed. The lignin peroxidase enzyme system of the white-rot fungus, *Phanerochaete chrysosporium*, was assayed for its capacity to degrade two recalcitrant aliphatic ether compounds, high-molecular-mass polyethylene glycol (PEG 20,000) and methyl *tert*-butyl ether (Kay-Shoemake and Watwood, 1996). Moreover, the brown-rot basidiomycete *Gloeophyllum trabeum* is known to cause decay in wooden structures by degrading lignocellulose. The extracellular system was produced by this fungus depolymerized [^{14}C] PEG 4000 (Kerem *et al.*, 1999).

2.3.2 Polypropylene glycol (PPG)

2.3.2.1 Bacteria

As the use of PPG is expected to increase in the future, studies of its biodegradation are equally important as those for PEG. The susceptibility of PPG to biological degradation has not been well characterized, although several groups have reported microbial assimilation of the monomer, 1,2-propylene glycol, which is supplied by the petrochemical industry at low cost.

(1) Aerobic bacteria

Fincher and Payne (1962) observed that a PEG-utilizing isolate could assimilate 1,2-propylene glycol dimer as a sole carbon and energy source. However, other PEG-utilizing bacteria did not grow on dimer or PPG (Kawai, 1987). Anaerobic PEG-utilizing bacteria did not degrade PPG either (Dwyer and Tiedje, 1986). PPG was assimilated by *Corynebacterium* sp. as a sole carbon and energy source (Kawai *et al.*, 1977). The strain was reidentified as *Stenotrophomonas*

maltophilia based on 16SrRNA homology (Tachibana *et al.*, 2002). The strain grew on monomer up to PPG 4000 (triol type) and 3000 (diol type), but did not assimilate PEGs. The strain also grew on a few PEG-PPG copolymers, which contained a larger amount of PPG than PEG, where from the weight ratio of PPG and PEG (approximately 10:1), perhaps either two or one of the terminal hydroxyl groups of PPG was not blocked by PEG and could be available to the organism. This strain must recognize a hydrophobic pendant group, such as the ethylene group, as well as a methyl group on carbon backbones.

(2) Anaerobic bacteria

There is a lack of information about the anaerobic biodegradation of PPGs.

2.3.2.2 Fungi

There is a lack of information about the fungus biodegradation of PPGs.

2.4 Metabolism of PEG and enzymes relevant to the metabolism

2.4.1 Metabolic route for PEG

The hydrolytic degradation of PEG 20,000 to oligomers has been proposed since 1975 (Haines and Alexander, 1975). Studies on aerobic degradation of PEG later have indicated that microbial metabolism must proceed via oxidation (Kawai, 1987).

PEG is successively oxidized to an aldehyde and a monocarboxylic acid, and this is followed by the cleavage of the ether bond, resulting in PEG molecules that are shortened by one glycol unit. The simultaneous oxidation of two terminal alcohol groups of the molecule is also possible. Depolymerization might proceed *via* the same reaction observed with the monocarboxylic acid. This sequence is repeated and eventually yields depolymerized PEG. The resultant glyoxylate (GOA) may then enter into central metabolic routes by known pathways, e.g., the oxidative dicarboxylic acid cycle, tricarboxylic acid (TCA) cycle, and the glycerate pathway. In oxidative degradation, it was originally postulated that three enzymes are required:

(1) an alcohol dehydrogenase

(2) an aldehyde dehydrogenase, converting the terminal alcohol groups of PEG into carboxylic acid groups

(3) an ether-bond-splitting enzyme yielding glyoxylate (GOA) as the final product, as shown in Figure 2.4.

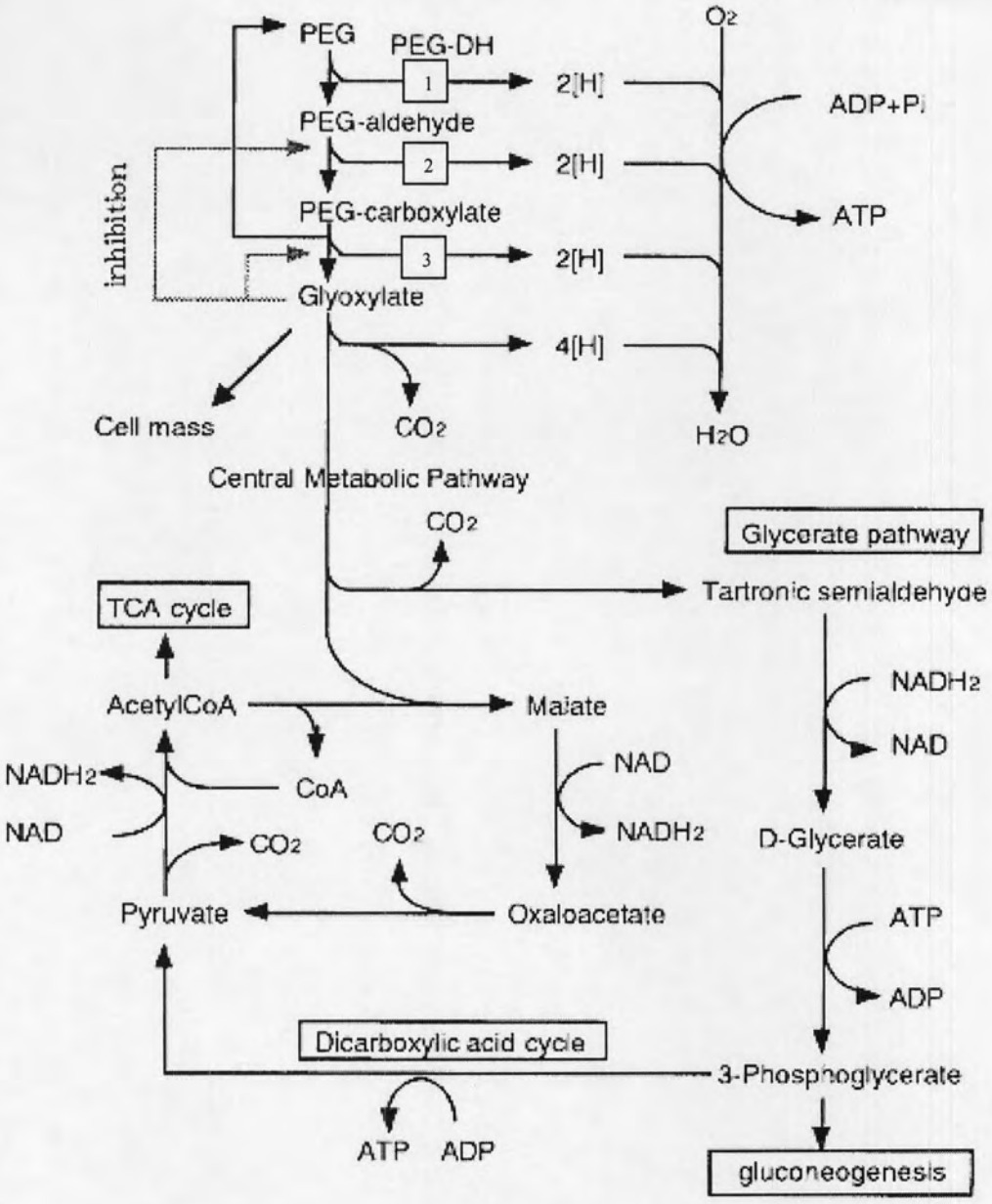

Figure 2.4 Aerobic metabolic pathway for polyethylene glycol (PEG)

[1], [2] and [3] represented alcohol dehydrogenase, aldehyde dehydrogenase and ether-bond-splitting enzyme, respectively.

The degradability of PEG with different terminal structures was also examined, using PEG 20,000-utilizing symbiotic mixed culture E-1 (*Sphingomonas terrae* and *Rhizobium* sp.) (Kawai, 1993). It is rational that PEG-diglycolic acid was utilized, because this is a metabolite of PEG. Monoalkyl-PEG was utilized, suggesting that the degradation of PEG is exogenously started from a terminal alcohol group. This inference was supported by the observation that depolymerized products were not detected during degradation of PEG by the mixed culture. In other words, the endogenous breakdown of a polymer molecule rapidly yields depolymerized products to some extent, but the exogenous breakdown does not, because of a gradual, step-by-step depolymerization. All these data support the metabolic route shown in Figure 2.4. The culture neither grows on PPG nor PTMG. Thus, a biodegradation proceeds exogenously from a terminal group and depends strictly on the chemical structures of the monomer units.

Microbial diversity might in the future demonstrate the presence of another pathway.

2.4.2 Enzymes relevant to the metabolism for PEG

Ether-alcohol dehydrogenases showed a weak or appreciable activity on low PEGs up to 600 (Harada and Sawada, 1977; Hino *et al.*, 1981). Significant membrane-bound PEG dehydrogenase (PEG-DH) activities were found with diverse PEG-utilizing bacteria, among which PEG-DHs were purified from the symbiotic mixed culture E-1 (PEG 20,000-assimilating, inducible) and from *Sphingomonas macrogoltabidus* No. 203 (PEG 4000-assimilating, constitutive) (Kawai *et al.*, 1980; Yamanaka and Kawai, 1989). Both enzymes were solubilized with surfactants, and stabilized with 10% glycerol or ethylene glycol. The purified enzymes showed quite

similar substrate specificities toward PEGs up to 20,000, although *Sphingomonas macrogoltabidus* No. 203 cannot grow on PEGs 6000-20,000. Sequencing of PEG-DH revealed that the enzyme belongs to the group of glucose-methanol-choline (GMC) flavoproteins, with one molecule of FAD being bound to the monomer protein of the homodimeric protein enzyme.

Quinohemoprotein alcohol dehydrogenase (QH-ADHs) can be subdivided into soluble (type I) and membrane-bound (type II) forms. Type II QH-ADH has so far only been found in *Gluconobacter* and *Acetobacter* bacteria (Ameyama and Adachi, 1982), and no information exists as to whether they are able to oxidize PEG. However, type I QH-ADHs purified from *Comamonas testosteroni*, *Rhodopseudomonas acidophilia* M402 and *Ralstonia eutropha* which cannot grow on PEG act as ADHs for PEG (Duine and Kawai, 1998). The N-terminal part of the enzyme from *Comamonas testosteroni* shows high similarity with other pyrroloquinolinequinone (PQQ)-containning enzymes, in particular with those of methanol dehydrogenases (Stoorvogel *et al.*, 1996), whereas the C-terminal part does not but contains the heme *c*. Based on this and the three-dimensional structure of methanol dehydrogenase, modeling studies have shown that QH-ADH from *Comamonas testosteroni* has a large active site, which explains the enzyme's ability to oxidize PEG and secondary alcohols and to act as polyvinyl alcohol dehydrogenase (Jongejan *et al.*, 1998). However, as it has not yet been found in a PEG-utilizing organism, it is possible that QH-ADH might be an alcohol dehydrogenase by design, but a PEG dehydrogenase by accident.

PEG is known to cause a fatal toxic syndrome when absorbed, which might be due to the formation of mono- and dicarboxylated PEG (Herold *et al.*, 1982). The presence of organic acids of PEG in the blood of poisoned patients and in an

animal model suggested that PEG is metabolized *in vivo*. Oxidation of PEG homologues (n = 1~8) was catalyzed by equine liver alcohol dehydrogenase (Herold *et al.*, 1989). It suggested that sequential oxidations by ADH and aldehyde dehydrogenase were possible causes of the syndrome. Thus, PEG may be oxidized by various ADHs, the origins of which are diverse.

Studies on PEG degradation by Sphingomonads revealed an enzyme which was able to oxidize PEG-carboxylic acid under formation of GOA (Kawai, 1985). Since diglycolic acid (DGA) was also a good substrate, the enzyme was called DGA dehydrogenase (DGA-DH). In the presence of lauryl maltoside, the purified enzyme was seen to be clearly distinct from PEG-DH, as neither PEG nor any other alcohol (including diols) or aldehyde was a substrate (Enokibara and Kawai, 1997). Apparently, the presence of a terminal carboxylic acid group is crucial for oxidation to occur at the α-position in the molecule. However, the presence of an ether bond is not essential, as GOA and glycolic acid are also substrates for DGA-DH. Therefore, the oxidation mechanism might be similar to that of the peroxisomal, flavoprotein glycolate oxidase (*EC* 1.1.3.l) or to that of flavoprotein α-hydroxyl acid dehydrogenases, proceeding *via* hydride transfer or *via* deprotonation, followed by electron transfer or covalent catalysis which occurs in the adduct formed from the carbanion substrate molecule and the cofactor (Lindqvist, 1992). As no O_2 consumption has been detected in the conversion of PEG-dicarboxylic acid by DGA-DH *in vitro*, the process must be accompanied by an attack of a H_2O molecule, such that rearrangement of the oxidized ether bond can take place under foemation of a terminal aldehyde group (in GOA) and a terminal alcohol group (in the PEG_{n-1}). Elucidation of the mechanism and identification of the cofactors involved in this reaction sequence must await the purification of substantial quantities of DGA-DH.

It has been reported that DGA-utilizing *Rhodococcus* sp. 432 contains a flavoprotein DGA oxidase (Yamanaka and Kawai, 1991) which lacks a periplasmic space. As ferricyanide and dichlorophenolindophenol (DCIP) were much better electron acceptors than O_2, the enzyme might be a dehydrogenase oxidizing DGA at the cytoplasmic side of the membrane, and be able to use O_2 as electron acceptor *in vitro*. As the enzyme uses glycolic acid as well as GOA (but not PEG), similarity with glycolate oxidase might also be possible. Although the different properties of the DGA oxidase seem to exclude structural similarity with DGA-DH, further studies are required to prove this.

The same uncertainly concerning similarity to DGA-DH exists with respect to the DCIP-linked dehydrogenase from a *Pseudomonas stutzeri* strain, which oxidizes PEG directly under formation of GOA (Obradors and Aguilar, 1991). Based on results from polyacrylamide gel electrophoresis (PAGE) and activity staining, the researcher claimed that this involves one single enzyme oxidizing PEG as well as DGA, and purification of the enzyme did not require solubilization with a detergent. Although this suggests significant differences from the Sphingomonad system (which consists of two distinct enzymes, PEG-DH and DGA-DH, that each require detergent solubilization), in the absence of any further information on the molecular properties of the enzyme, no conclusion can be drawn at present.

The whole cells or cell-free extracts of *Pseudomonas* sp. strain SC25A that can grow on PEG dodecyl ethers yielded dodecanol as the first step of the metabolism. However, the scission mechanism has still not been studied (Tidswell *et al.*, 1996).

2.5 Anaerobic biodegradation and metabolism of PEG

2.5.1 Anaerobic bacteria capable of assimilating PEG

As for the anaerobic biodegradation of PEGs, a study by Mills and Stack (1954) succeeded in a reduction in chemical oxygen demand (COD) using two-stage continuous anaerobic sludge reactors fed with industrial waste supplemented with either DEG, triEG or PEG 400 over 40 days. Some 30 years later, three groups almost simultaneously reported the anaerobic growth of isolates or consortia on PEGs. According to the report of Schink and Stieb (1983), PEG with a molecular weight of 20,000 was anaerobically degraded in an enrichment culture inoculated with mud of limnic and marine origins. Three strains of rod-shaped, gram-negative, nonspore-forming strictly anaerobic bacteria were isolated, which were proposed as a new species, *Pelobacter venetianus* sp. nov. Dwyer and Tiedje (1983) obtained methanogenic consortia from sewage sludge, which can degrade EG, DEG and PEG with molecular weight of 400, 1000, and 20,000. The enrichments were shown to best metabolize glycols close to the molecular weight of the substrate on which they were enriched, as was found with aerobic bacteria. Fincher and Payne (1962) first isolated a PEG-utilizing bacterium, TEG-5, which was identified as *Alcaligenes faecalis* var. *denitrificans*. This organism grew aerobically, but not fermentatively, at the expense of several free ether glycols, as well as ethoxylates attached to alkyl and benzyl derivatives. However, it degraded both free ether glycols (EG and the other short-chained ether glycols and PEG 200 and 300) and several nonionic detergents while growing anaerobically as a denitrifier (nitrate-reducing), with 55% degradation of DEG being obtained (Grant and Payne, 1983).

2.5.2 PEG acetaldehyde lyase

Anaerobic bacteria must contain a different metabolic system for polymers from that in aerobic bacteria (Schink *et al.*, 1992). Extracts from PEG-degrading, anaerobic bacteria revealed a diol dehydratase and a PEG-degrading enzyme yielding acetaldehyde as product (Schramm and Schink, 1991; Frings *et al.*, 1992). Unfortunately, both enzymes appeared to be very sensitive to oxygen, and this most likely prohibited the researchers from purifying either enzymes. As the PEG-degrading activity was stimulated by the addition of certain corrinoids, it was postulated that ether bond splitting in these bacteria occurred with a PEG acetaldehyde lyase that was analogous to a diol dehydratase (Toraya *et al.*, 1979; Toraya and Fukui, 1982; Masuda *et al.*, 1999). In order to explain the formation of acetaldehyde and PEG_{n-1} as products, the researchers assumed that the terminal OH group of PEG shifts to the C_2-position (an intramolecular rearrangement which is in fact a mutase reaction) so that an unstable hemiacetal group is formed which rearranges to the products (Figure 2.5). It was suggested that at least one unmasked terminal hydroxyl group was necessary for the formation of the hemiacetal intermediate by transhydroxylation.

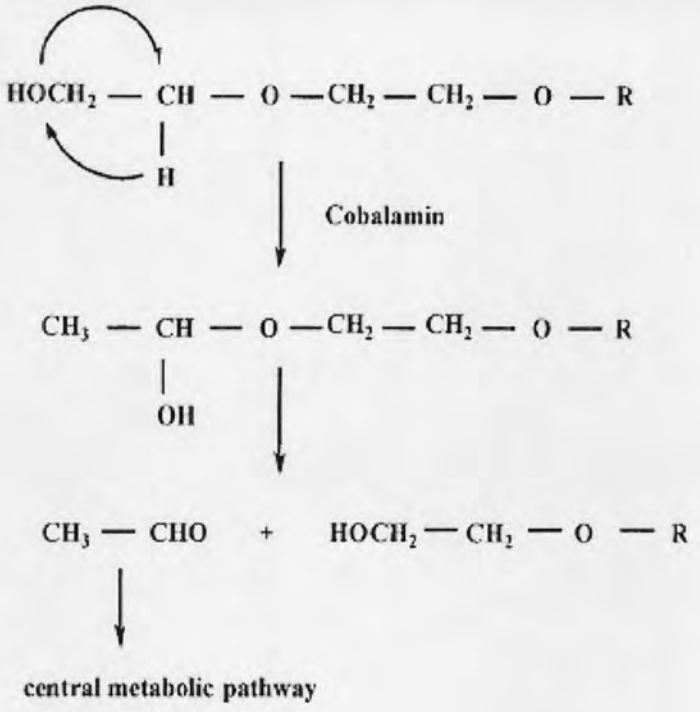

Figure 2.5 Proposed mechanism for anaerobic degradation of PEG by PEG lyase

Pearce and Heydeman (1980) first suggested this concept for the aerobic degradation of PEG. Although this seems a plausible reaction, and the concept was further supported by growth experiments with *Pelobacter venetianus* (Schink and Stieb, 1983; Strass and Schink, 1986), confirmation must wait until purification and characterization of the enzyme. Dwyer and Tiedje (1986) also suggested that acetaldehyde was a direct metabolite of PEG as they had detected the presence of a DCIP-dependent PEG-DH. The level of PEG-DH activity was too low for a primary metabolic enzyme, but that the activity might be effective on impurities such as EG and acetaldehyde. These results coincide with those obtained with aerobic bacteria- that degradation commences from the terminus of a long molecular chain and that therefore at least one free alcohol group is necessary- though both aerobic and anaerobic mechanisms appear to be different, and both ether-cleaving mechanisms require further investigation.

The incubation of stratal waters from a petroleum reservoir containing OP-10 nonionic surfactant showed that, under aerobic conditions at 32°C and pH 6.6, only 31% of OP-10 was biodecomposed in 7 days, whilst under identical anaerobic conditions, 79-85% was decomposed (Gvozdyat *et al.*, 1983).

2.5.3 Extracellular one-electron oxidation of PEG

The brown-rot basidiomycete *Gloeophyllum trabeum* is known to cause decay in wooden structures by degrading lignocellulose. The extracellular system was produced by this fungus depolymerized [^{14}C] PEG 4000 (Kerem *et al.*, 1999). The extracellular system functioned as free radical oxidants to cleave PEG rapidly by the endo route, i.e., by C-C bond cleavage: a radical oxidant abstract hydrogens from the PEG's internal methylene groups, just as the Fenton reagent (Fe^{2+}/ H_2O_2) depolymerizes PEG *in vitro*. The degradation of PEG by the fungus required 2,5-dimethoxyhydroquinone (DMHQ) and Fe^{3+}, and was inhibited by catalase (Kerem *et al.*, 1999): DMHQ was formed from 1,5-dimethoxy benzoquinone by the mycelial reductase. Direct nonenzymatic reaction between DMHQ and Fe^{3+} produced Fe^{2+} and H_2O_2. Thus, the degradation mechanism of PEG by the fungus was proved to be a biological Fenton mechanism. It was suggested that brown-rot fungi contribute not only to the degradation of lignocellulose, but also to that of recalcitrant organopollutants; that is, other polyethers as well as PEG might be depolymerized by the same system.

Hydrogen peroxide can be produced by various oxidation systems. Iron is one of most abundant metals in the Earth's crust, and is reduced to Fe^{2+} in the presence of natural reductants or under anaerobic conditions. Thus, although a biological Fenton reaction might be possible in terrestrial environments, the

metabolism of polyethers is still thought to play the major role in polymer degradation, especially in aquatic environments, where Fe^{2+} and H_2O_2 concentrations available might be insufficient for the reaction to proceed.

2.6 Aerobic metabolism of PPG

The aerobic metabolism of PPG by *Stenotrophomonas maltophilia* was studied using dimer-dipropylene glycol (DPG)- as a model substrate for biodegradation, since PPG contains molecules of different molecular weights (Kawai *et al.*, 1985).

PPG was not degraded by either a culture filtrate or a cell-free extract, but could be degraded by intact cells and/or cell debris. Hence, PPG was not metabolized by extracellular enzymes or a hydrolase, but possibly by intracellular enzymes including membrane-bound enzymes. The intracellular metabolism of PPG was supported by the finding that bacterial cells (*Stenotrophomonas maltophilia*) entrapped in polyacrylamide gels degraded PPG efficiently (Kawai, 1987). The PPG-degrading activity of the cell-free extracts prepared from DPG-grown cells was investigated, but because of clouding due to the PPG attached to the cells the activity of the cell-free extract prepared from PPG-grown cells could not be measured. DCIP and phenazine methosulfate (PMS)-dependent dehydrogenase (PPG-DH) activities were detected with cell-free extracts, and these must be linked with a respiratory chain of the organism. The effects of side or main chain structures on the growth of PPG-utilizing strain were examined (Table 2.7).

Table 2.7 Growth of *Stenotrophomonas maltophilia* on polymers (Kawai, 1987)

Substrate (0.5%)	*Growth (OD_{610})*	*pH*
None	0.12	7.0
PEG 400	NG	7.0
PPG 2000	1.34	5.8
PBO 400	1.27	5.0
2000	0.92	7.0
CoEO/glycidol 500 (*R,S*)	0.22	7.0
Diglycerin	0.66	4.6
Polyglycerin 310	0.49	4.8
500	0.32	5.6
750	0.33	5.8
Polyglycidol 13 300 (*R*)	NG	7.0
13 300 (*S*)	NG	7.0
PTMG 200	NG	7.0
PVA n = 500	NG	7.0

NG, no growth

As the bacterium grew well on PBG 400 and 2000, a methyl group in PPG was replaceable by an ethyl group in PBG. The microorganism grew on polyglycidols, PEG, PTMG or polyvinyl alcohol. These results indicated that the bacterium recognized an ether oxygen adjacent to two or three carbon chains and a hydrophobic side group such as a methyl or ethyl group.

As PEG and PPG-monoalkyl derivatives may be assimilated in a similar manner to free PEG and PPG, yet dialkyl PEG/monoalkyl PPG acetate cannot be utilized by PEG/PPG-utilizers, at least one free alcohol group is necessary for metabolism (Kawai, 1993). These results – intracellular PPG-DH, a need for the terminal free alcohol group for growth, and oxidized metabolic products – suggested that PPG is incorporated into cells at least through the outer membrane, and is oxidatively metabolized. The principal mechanism is possibly similar to that for PEG – that the terminal oxidative precedes the ether cleavage.

Several PPG dehydrogenase (PPG-DH) activities were found in PPG-utilizing *Stenotrophomonas maltophilia* (Tachibana *et al.*, 2002). During growth on PPG 2000, three PPG-DH peaks appeared in 36 hours, 7 days and 9 days: the majority (88%) of the first peak at the early logarithmic phase was localized in the cytoplasm. In the second peak (the highest) at the stationary phase of growth, activity was found in the membrane (54%), the periplasm (34%), and the cytoplasm (12%). The third peak may not contribute significantly to the assimilation of PPG, because PPG was already consumed in 9 days. As well as differing in their localization and induction times, these PPG-DHs also showed differences in their specificity towards electron acceptors.