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Preparation and Properties of A-B-A Tri-Block Copolymer Membranes Consisting of *N*-Hydroxyethyl-L-glutamine as the A Component and L-Leucine as the B Component

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ABSTRACT: A-B-A type tri-block copolymer consisting of *N*-hydroxyethyl-L-glutamine as the A component and L-leucine as the B component as well as the corresponding random copolymers and homopolymers were prepared by carrying out an aminoalcoholysis reaction with 2-amino-1-ethanol, together with a crosslinking reaction with octamethylenediamine on membranes of the starting polymer membranes including γ-benzyl L-glutamate residue. It was shown that the effective crosslink density was proportional to the percent crosslinker in the reaction mixture. The relation between their bulk structure and membrane properties was investigated, such as the degree of swelling in water, aqueous vapor permeability, tensile properties, and enzymatic degradation behavior of the membranes in pseudo-extracellular fluid (PECF) solution. The tensile properties of the hydrophilic membranes were highly dependent on the degree of swelling in PECF, and on the hydrophobic portions in molecular chains, whose behavior was typical of an elastomer. A common relation was observed between the rate of water vapor permeation and the degree of swelling Q_w(%) of membranes in PECF in the higher Q_w region, in spite of the difference of the nature of the side chains. Biodegradation of samples *in vitro* by proteases indicated that the degradation was a bulk rather than a surface phenomenon, and that the rate of degradation was also highly dependent on the Q_w values of sample membranes in PECF.

KEY WORDS A-B-A Tri-Block Copolymer / *N*-hydroxyethyl-L-glutamine / L-Leucine / Bulk Structure / Degree of Swelling / Water Vapor Permeability / Tensile Property / Enzymatic Degradation Property / Microheterophase Structure /

Poly(α-amino acid)s and their copolymers are typical biodegradable polymers. They have been considered very useful for biomedical applications such as temporary artificial skin substitutes in burn therapy, and as temporary barriers to prevent adhesion between natural tissue planes that are damaged either by accident or as a result of surgery. On the other hand, it was pointed out that the materials exhibiting the microheterophase structures of some specified dimensions are prominent in antithrombogenicity.[1] In practice, normal vascular endotherium, which is the ideal non-thrombogenic, is considered to have a microheterophase structure composed of hydrophilic and hydrophobic microdomains.[2] In previous studies,[3-5] we have synthesized A-B-A type tri-block copolypeptides consisting of γ-benzyl L-glutamate (G) and L-leucine (L) or L-valine of high molecular weight and characterized the solid state properties. As expected, the choice of cast solvent resulted in significant effect on the solid state structure of the cast films. If hydrophilic components are partly introduced into such block copolymers, the products obtained are to be antithrombogenic

and tissue compatible.[6] For this purpose, we have prepared many types of hydrophilic–hydrophobic poly(α-amino acid) membranes.[7,8]

In the present study, we prepared A-B-A type tri-block copolypeptides consisting of γ-benzyl L-glutamate (G) as the A component and L-leucine (L) as the B component, as well as the corresponding random copolymer (GL) and homopolymers (G and L). Hydrophilic polymers were obtained by carrying out the subsequent aminoalcoholysis reaction with 2-amino-1-ethanol (E), together with crosslinking reaction with octamethylenediamine (OMDA) on the G residue of the starting polymer membranes, and investigated the relation between their bulk structures and membrane properties, such as the degree of swelling Q_w in the pseudo-extracellular fluid (PECF),[9] tensile properties in PECF, water vapor permeability, and enzymatic degradation behavior *in vitro* of the membranes in PECF solution from an applicable point of view for the biomedical materials.

EXPERIMENTAL

Materials

N-Carboxyanhydride (NCA) of γ-Benzyl L-Glutamate (G) and L-Leucine (L)

The monomers, G-NCA and L-NCA, were prepared according to the method reported in the previous paper,[3] and purified by recrystallization from an ethyl acetate solution with the addition of petroleum ether. Recrystallization was repeated more than three times.

Synthesis of GLG Block Copolymers

To make a symmetrical tri-block copolypeptide, the center block was initially prepared with the use of hexamethylenediamine initiator, followed by simultaneous polymerization of the end blocks. A typical example of the GLG synthesis is as follows: L-NCA, 0.03 mol, was dissolved in 100 ml of benzene–dichloromethane (1 : 2), and 2×10^{-4} mol of 1,6-hexamethylenediamine in dichloromethane was added to initiate polymerization. The polymerization reaction was monitored by the carbon dioxide evolved.[3] The time required was dependent on the ratio of the anhydride to the initiator. On completion of the L polymerization, G-NCA in the same solvent mixture as that for the polymerization was added through a filter. The total polymerization time was from 3 to 7 days.

Related random copolymers (GL) and the corresponding homopolymers, poly-γ-benzyl L-glutamate (G) and poly-L-leucine (L) were also synthesized by the NCA method. The polymerization was initiated with triethylamine at an NCA-to-initiator molar ratio of 50. All starting polymers were purified and fractionated as described in the previous paper.[3] The results of all the polymerization are summarized in Table I.

Preparation of Hydrophilic Polymer Membranes

The partial aminoalcoholysis of the G-portion of these polymer membranes by reacting with 2-amino-1-ethanol (E) was performed as follows: A polymer membrane of *ca.* 100 μm in thickness was prepared by casting from a chloroform (CF)–trifluoroethanol (TFE) mixture, and was immersed in a mixture of 2-amino-1-ethanol (E) and 1.0 to 6.0 mol% of 1,8-octamethylenediamine (OMDA), the crosslinker, at 58°C for an appropriate time (4 to 72 h). The hydrophilic polymer membranes

Table 1. Preparative data of original samples

Sample Code	γ-BLG	[η]	M_w
	mol%	dl g^{-1}	
PBLG-1	100.0	1.94	371,000
PLLeu-1	0.0	1.55	
GL-1	85.0	0.88	138,000
GL-2	68.5	1.70	317,000
GL-3	48.5	1.67	312,000
GLG-1	84.8	0.53	61,000
GLG-2	66.8	0.56	68,000
GLG-3	49.8	0.67	80,500
LGL-1	50.4	0.38	

were washed with water and ethanol and stored in ethanol at 5°C.

Measurements

Molecular Characterizations of the Starting Polymers

The molar composition of GLG and GL copolymers was determined by amino acid analysis carried out in The Peptide Institute, Inc. (Minoh-shi, Osaka). The results are also summarized in Table I.

The molecular weight of the starting polymers was determined in *N*,*N*-dimethylformamide (DMF) at 25°C by the sedimentation equilibrium method using a MOM-3170-b ultracentrifuge equipped with a Reyleigh interference optical system and a 12 mm double sector cell.[10] The viscosity numbers were also obtained in a dichloroacetic acid (DCA) solution, using an Ubbelohde type viscometer at 25°C. These data are listed in Table I.

Conformation and Structure of Starting Polymer Membrane

The chain conformation of starting polymers in membranes was estimated from infrared (IR) spectra measured by a Hitachi 260—30 IR spectrophotometer. Wide-angle X-ray diffraction (WAXD) profiles of membranes were obtained with a Rigaku Geigerflex Type DF-3 X-ray generator equipped with an automatic diffractometer by using Ni filtered Cu-K_α radiation, setting a flat surface of the membranes normal to the beam.

Physical Properties of Hydrophilic Membranes

The degree of swelling Q_w (%) in PECF was determined by equilibrating the membrane in PECF solution at 37.0°C. The membrane was removed, blotted to remove surface PECF and weighed until a constant weight was achieved. The membrane was then dried in a vacuum oven. The Q_w (%) is defined as the ratio of the amount of PECF to weight of the dried crosslinked membrane.

The tensile properties of membranes were measured in PECF by a Tensilon UTM-II-20 (Toyo-Boldwin Co.) using the standard techniques at 25°C. All the samples were tested at an elongation rate of 40% per minute.

To water vapor permeation through the membranes was measured with a cylindrical glass cell[11] at 37.0°C. The exposed membrane area was 12.57 cm^2.

Biodegradation of Hydrophilic Membranes in Vitro

Enzymatic degradation studies *in vitro* were carried out by using papain and pronase E. These enzymes were purchased from Nakarai Chem. Co. Enzyme solutions were prepared by standard techniques[12] at 37.0°C. A series of the crosslinked membranes were exposed to the 0.1 mg ml^{-1} solution of PECF at pH = 7.4 with appropriate activators at 37.0°C. Polymer membranes were removed from the enzyme solution at appropriate time intervals, weighed, and then vacuum dried at 60°C to constant weights.

RESULTS AND DISCUSSION

Conformation and Structure of Copolymer Membranes

IR spectra were measured with polymer membranes cast from solution. In each case, bands of amides I, II, and V of polypeptide chains appeared at 1650, 1550, and 610 cm^{-1}, just at the same wave numbers for the corresponding homopolymers. It is concluded from the experimental results that the polypeptide chains in polymer membranes exist in an α-helix conformation, and moreover, the characteristic bands of ester bond at 1730 cm^{-1} as well as those of the phenyl group at 700 and 750 cm^{-1} decrease by increasing the content of *N*-hydroxyethyl-L-glutamine (E).

The WAXD patterns for the GLG block copolymers, the corresponding homopolymers, and a GL random copolymer are shown in Figure 1. The first main reflection corresponds to an intermolecular spacing of the α-helical chains and has a spacing of 12.5 Å

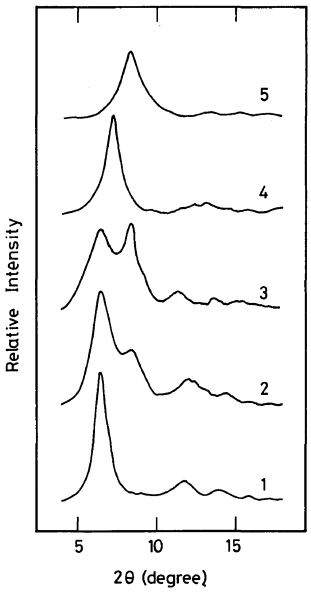

Figure 1. Wide-angle X-ray diffraction profiles of unoriented solid films cast from chloroform solutions: (1) PBLG-1; (2) GLG-1; (3) GLG-3; (4) GL-1; (5) PLLeu-1.

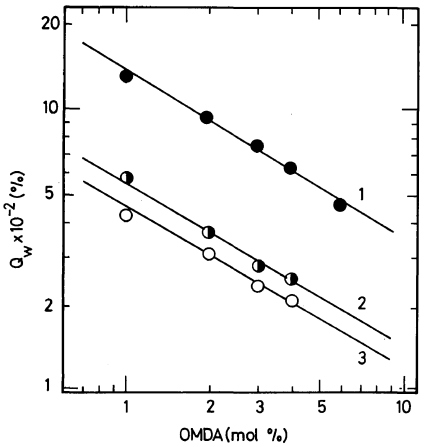

Figure 2. Degree of swelling Q_w(%) in PECF as a function of the molar percent of OMDA: (1) G(E)-1 (OH, 100 mol%); (2) GL(E)-1 (OH, 85 mol%); (3) GLG(E)-1 (OH, 85 mol%) membranes.

for PBLG-1 and 10.8 Å for PLLeu-1, respectively. GL-1 random copolymer showed a single reflection at an intermediate spacing. These two comonomer units, G and L, appeared to crystallize in random copolymers and isomorphic structures were obtained.[13]

Diffraction patterns for GLG block copolymers showed two main reflections similar to those of the two homopolymers. It appears that phase separation has occurred, and that the G and L domains assume the same structural modification as the corresponding homopolymers.

Degree of Swelling of Membranes in PECF

The degree of swelling in solvent is determined by the interaction energy between the solvent molecule and the polymer segment as well as the elastic energy (crosslink density) for a solvent-swollen polymer in a random coil conformation. The crosslinked poly(*N*-hydroxyethyl-L-glutamine) (PHEG) has a random coil conformation in water,[14] so the PHEG membrane can be expected to exhibit behavior similar to that of a swollen elastomer.

On the other hand, the precise crosslink density has not been determined because of uncertainty in the relative reactivities of 2-amino-1-ethanol and OMDA, and also because estimation of the fraction of the reacted diamine molecules which forms effective crosslinks is difficult. The effect of crosslinker (OMDA) concentration in the reaction on the degree of swelling of the crosslinked membranes in PECF is shown in Figure 2. The degree of swelling in PECF decrease with increasing the OMDA concentration (mol%) in the reaction solution.

When the degree of swelling is quite large, it is given by the following equation according to rubber elasticity theory[15]

$$Q_w^{5/3} = (\bar{v}M_c)(1-2M_c/M)^{-1}(1/2-\chi_1)/V_1 \quad (1)$$

where M_c is the molecular weight per crosslinked unit, M the primary molecular weight, $\bar{v}$ the specific volume of polymer, V_1 the molar volume of solvent and χ_1 the interaction parameter. The factor $(1-2M_c/M)$ expresses the correction for network imperfections resulting from chain ends. For a quite high molecular weight polymer chain, it reduces to unity. As

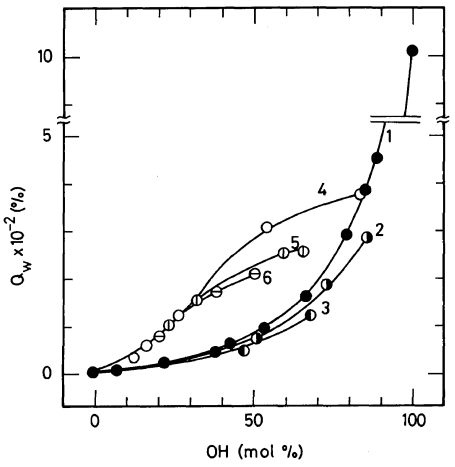

Figure 3. Degree of swelling Q_w(%) (mol%) of membranes in PECF plotted against OH: (1) G(E)-1; (2) GL(E)-1 (L, 15 mol%); (3) GL(E)-2 (L, 31 mol%); (4) GLG(E)-1 (L, 15 mol%); (5) GLG(E)-2 (L, 33 mol%); (6) GLG(E)-3 (L, 50 mol%) in 37°C.

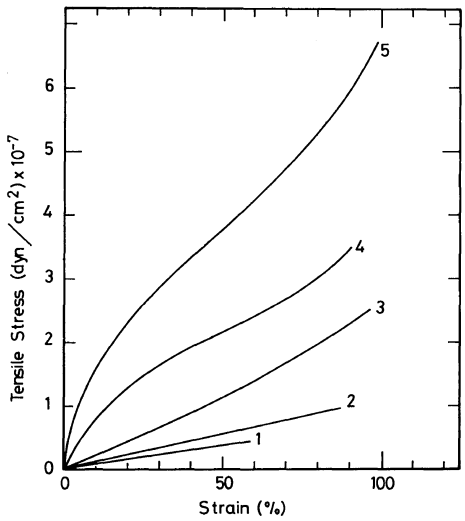

Figure 4. Stress-strain behavior of hydrophilic membranes in PECF at 25°C: (1) G(E)-1 (OH, 100 mol%); (2) GL(E)-1 (OH, 85 mol%); (3) GL(E)-2 (OH, 69 mol%); (4) GLG(E)-1 (OH, 85 mol%); (5) GLG(E)-2 (OH, 67 mol%).

the effective crosslink density, f_c, is proportional to the value of M_o/M_c where M_o is the molecular weight of the repeat unit (monomeric unit), eq 1 may be simplified as

$$Q_w^{5/3} = (\bar{v}M_o/V_1)(1/2 - \chi_1)1/f_c \quad (2)$$

The slope of log–log plots in Figure 2 has the value of $-3/5$ as predicted by eq 2. The effective cross link density f_c is proportional to the crosslinker OMDA concentration (mol%) in the reaction solution.

The relation between the degree of swelling Q_w (%) in PECF and the molar percent of the OH at a constant OMDA concentration (2 mol%) in each case of the membranes is illustrated in Figure 3. The degree of swelling Q_w (%) in PECF for each membrane was measured after immersing membranes in PECF for 48 h at 37.0°C. The Q_w values obtained with GLG block copolymer membranes were remarkably higher than those obtained with PBLG and GL random copolymer membranes in the lower OH (mol%) region at the same OH (mol%) value. Another remarkable characteristic of the GLG block copolymer membranes compared to the PBLG or GL random copolymer membranes is that the Q_w value levels off in the higher OH (mol%) region, as shown in Figure 3. Such characteristics for the GLG membranes should be attributed mainly to the specific feature of the microheterophase structure of the block copolymers. The same characteristic behavior was observed with GAG block copolymer membranes.[16]

Tensile Properties of Membranes in PECF

The tensile properties of hydrophilic membranes are highly dependent on the degree of swelling Q_w (%) in PECF. Further, elastomeric block copolymers are highly suited to biomedical applications, such as membranes for artificial organs, reconstructive prothesis and cosmesis. Figure 4 illustrates the stress–strain curves of hydrophilic membranes in PECF at 25°C. While PHEG and GL(E) random copolymer membranes gave quite low strength, GLG(E) block copolymer membranes attained higher strength with a moderate modulus. These features are suitable for the biomedical

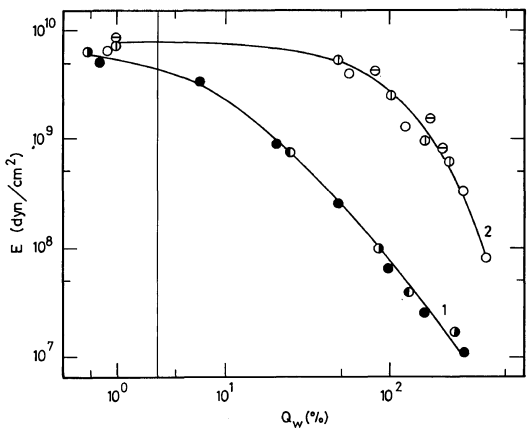

Figure 5. Young's modulus E (dyn cm^{-2}) of membranes as a function of the degree of swelling Q_w(%) in PECF at 25°C: (1) G(E)-1(●), GL(E)-1 (◑), and GL(E)-2(◐); (2) GLG(E)-1(○), GLG(E)-2(ⓘ), and GLG(E)-3(⊖).

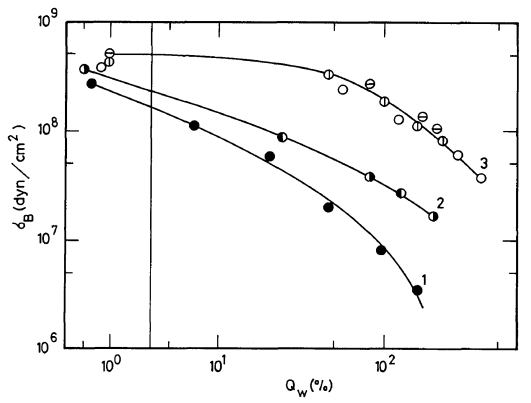

Figure 6. Tensile strength at breakage σ_B(dyn cm^{-2}) of membranes as a function of the degree of swelling Q_w(%) in PECF at 25°C: (1) G(E)-1(●); (2) GL(E)-1(◑) and GL(E)-2(◐); (3) GLG(E)-1(○), GLG(E)-2 (ⓘ), and GLG(E)-3(⊖).

applications such as an artificial skin substitute, since the mechanical properties and appearance of the human skin can be simulated by pigmented low-modulus high strength thermoplastic block copolymer elastomers.

Figures 5 and 6 show the experimental findings of Young's modulus E at an elongation of 1%, and the strength at the breakage point σ_B as a function of the degree of swelling Q_w (%) for membranes in PECF at 25°C. In Figures 5 and 6, it is shown that the PECF-swollen hydrophilic GLG block copolymer membranes tend to maintain the durability in a much broader region compared to PBLG or GL random copolymer membranes.

Water Vapor Permeability of Membranes

A large variety of synthetic polymeric membranes have been investigated for developing artificial skin substitutes or effective wound closures.[17-19] Among them, for example, formation of a crosslinked polymer in a form of hydrogel appears to provide added capability for cellular migration into the graft and vascularization.[17] One of the most essential functions of skin is to keep tissue and organ water from evaporating away. Thus artificial skin substitutes or effective wound closures require maintenance of an appropriate rate of water vapor permeation through the membranes. If the rate of water vapor permeation through the membrane is excessively low, water accumulates at the interface between the woundbed and impermeable membranes, and edma results. The membrane-woundbed interfacial contact is thereby undermined. As a result, to maintain the ability to wet the woundbed appropriately and thereby maintain an air-free interface, an inner skin substitute membrane should exhibit a higher water vapor permeability value than about 500 (g m^{-2} day).[20]

Figure 7 illustrates the relation between the rate of water vapor permeation V_f (g m^{-2} day) in PECF and the degree of swelling Q_w (%) for membranes at 37.0°C. The V_f value is highly dependent on the Q_w value in PECF. It is also shown that GLG block copolymer membranes give higher V_f values than PBLG and GL random copolymer membranes in the lower Q_w region, while a common relation is obtained between V_f and Q_w of membranes in spite of the difference in the architecture of polymer chains in the higher Q_w region. The former difference may be attributed to the specific features of the microheterophase struc-

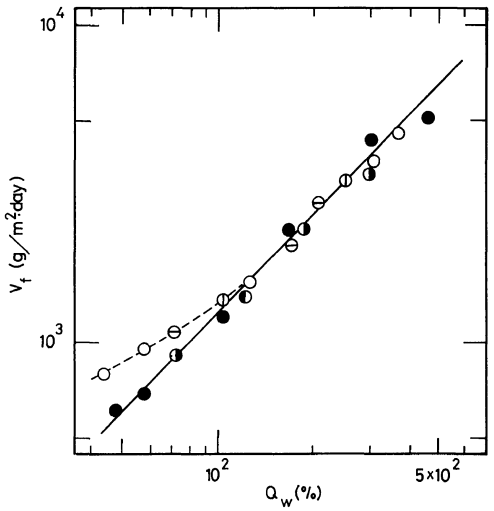

Figure 7. Rate of water vapor permeation V_f (g m^{-2} day) as a function of the degree of swelling Q_w(%) in PECF at 37°C for membranes of G(E)-1(●), GL(E)-1(◑), GL(E)-2(◐), GLG(E)-1(○), GLG(E)-2(ⓘ), and GLG(E)-3(⊖).

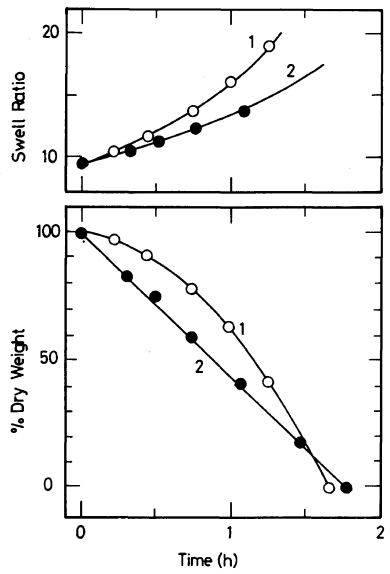

Figure 8. Dry weight ratio (%) and the swelling ratio in PECF for G(E)-1 (OH; 100 mol%) membrane as a function of enzyme digestion time (hr) at 37.0°C and pH 7.4: (1) papain; (2) pronase E.

ture in block copolymer membranes.

Biodegradation of Membranes in Vitro

Numerous proteases may be present at the wound site.[21] These proteases are divided into some classes depending on the structure of active sites. Enzymes of inflammatory response that are likely to degrade poly(α-amino acid)s include the endopeptidase Cathepsin B and the exopeptidases Carboxypeptidase and Leucine amino peptidase.[22] In the present investigation, the plant thiol endopeptidase Papain was selected as a commercially available analog of Cathepsin B as well as Pronase E as a commercially available analog of an exopeptidase released during the acute and chronic stages of the inflammatory response. Although Papain is a general plant thiol endopeptidase, it has preference for peptide bonds where the amino acid residue of the carbonyl group is arginine, lysine, or glutamine and where this amino acid is joined on either side by amino acids with hydrophobic side chains.[23,24] Pronase E obtained from *Streptomyces griseus* has been separated into eight proteases[25] of which four are serine endopeptidases.[26] These endopeptidases are not expected to hydrolyze PHEG series. The remaining four pronase enzymes are exopeptidases; two are aminopeptidases[27] and two are carboxypeptidases.[28] The pronase E activity observed in the crosslinked PHEG membranes almost certainly comes from these exopeptidases.

Pre-weighed 2.0%-crosslinked PHEG membranes were exposed to Pronase E or Papain and the results are illustrated in Figure 8. The dry weight of the membrane began to decrease immediately and the weight loss continued steadily thereafter for Pronase E system. Simultaneously, the swelling ratio increased steadily. On the other hand, PHEG membranes that were exposed to Papain showed a lag time with respect to the weight loss so that when the experiment was half over, *i.e.*, at half the time needed to dissolve the specimen, about 70% of the dry weight remained. The swelling ratio steadily a higher order of increasing of swelling ratio was observed with

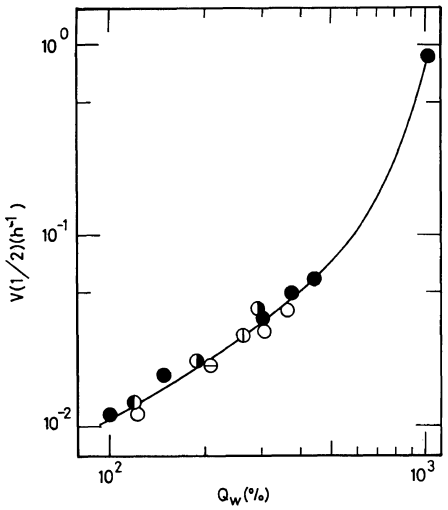

Figure 9. Rate of papain digestion $V(1/2)(h^{-1})$ of membranes as a function of the degree of swelling $Q_w(\%)$ in PECF at 37.0°C and pH 7.4 for G(E)-1(●), GL(E)-1(◑), GL(E)-2(◐), GLG(E)-1(○), GLG(E)-2(ⓘ), and GLG(E)-3(⊖).

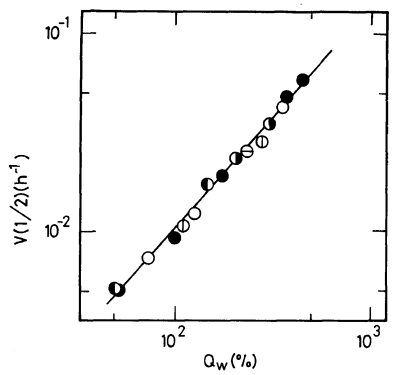

Figure 10. Rate of pronase E digestion $V(1/2)(h^{-1})$ of membranes as a function of the degree of swelling $Q_w(\%)$ in PECF at 37.0°C and pH 7.4 for G(E)-1(●), GL(E)-1(◑), GL(E)-2(◐), GLG(E)-1(○), GLG(E)-2(ⓘ), and GLG(E)-3(⊖).

the Papain system than that of the Pronase E system. Judging from the immediate increase in the swelling ratio, the degradation of the crosslinked PHEG membranes should be bulk rather than a surface phenomenon. Qualitative differences in the action of Pronase E and Papain are clearly shown in Figure 8. The immediate and steady weight loss observed with the Pronase E system is consistent with the release of monomers from the chain ends by exopeptidases. On the other hand, an endopeptidase, such as Papain, must make two incisions in a chain segment to produce a soluble fragment, but a single cleavage will decrease the effective crosslink density, resulting in the increase in the swelling ratio of membranes. Thus, the initial effect of Papain is therefore to decrease the effective crosslink density without producing soluble materials.

Figures 9 and 10 summarize the rate of Papain and Pronase E digestion $V(1/2)$ (h^{-1}), respectively, as a function of the degree of swelling $Q_w(\%)$ in PECF for hydrophilic polymer membranes. $V(1/2)$ is defined as the reciprocal of the time required for the sample weight to be reduced to one-half its initial value. A common relation was obtained between $V(1/2)$ and Q_w of polymer membranes for each case of membranes, as shown in Figures 9 and 10.

In conclusion, the microheterophase structure of a hydrophilic–hydrophobic block copolymer highly contributes the bulk properties of membranes, and that the degree of swelling in water plays an important role in membrane properties. Further, the degradation by the general proteolytic enzymes, Papain and Pronase E, is a bulk rather than surface phenomenon as shown by the immediate change in a bulk property, the swelling ratio, upon exposure to the enzymes.

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