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BIODEGRADATION OF COPOLY(N-HYDROXYPROPYL-D,L-GLUTAMINE) *IN VITRO*

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Abstract—Random copolypeptides (PHPDLG) consisting of N-hydroxypropyl-D-glutamine (HPDG) and N-hydroxypropyl-L-glutamine (HPLG) covering the whole range of D,L-copolymer composition were prepared by carrying out aminolysis reactions with 3-amino-1-propanol (P) on starting random copolymers consisting of γ-benzyl-D-glutamate and γ-benzyl-L-glutamate (PBDLG). The effects of copolymer composition and sequential distributions on the rate of hydrolysis by papain in a pseudo-extracellular fluid (PECF) at pH 7.4 and 37.0°C were studied to simulate *in vivo* polymer degradation. Degradation data for these samples followed the Michaelis–Menten rate law, being of the first order to the enzyme concentration. It was shown that the rate of degradation by papain was controlled by the comonomer composition as well as sequential distributions of comonomers in the copolymer chains.

INTRODUCTION

Poly(α-amino acid)s and their copolymers have a potential for biodegradable medical applications such as temporary artificial skin substrates in burn therapy, temporary barriers to prevent adhesion between natural tissue planes damaged either by accident or after surgery between the pericardium and heart wall during open-heart surgery, polymer carriers for protein conjugates and drug delivery systems [1]. Especially, water-soluble poly(α-amino acid)s are typical biodegradable polymers, and thus these controlled release systems offer the distinct advantage such that no residual polymer remains following drug release or polymer biodegradation. The degradation was attributed to cleavage of the poly(α-amino acid) main chains by proteolytic enzymes, such as endopeptidase cathepsin B, released during acute and chronic stages of the inflammatory response. For practical use of poly(α-amino acid)s in biomedical materials, it is worthwhile to investigate the partial modification of side chains to understand how to control the rate of degradation by proteolytic enzymes in detail. On the other hand, Gill *et al.* [2] demonstrated that a poly-D-glutamic acid was capable of eliciting an antibody response in rabbits, and that part of the D-polypeptide was degraded and excreted in the urine, whereas the majority of the retained D-polymer was in the kidney and a small amount was in the liver [3]. In view of that finding, it is very interesting to determine whether D-peptide is capable of being degraded or not by proteases from the standpoint of the practical use in biomedical material fields.

In this study, the preparation of a water-soluble random copolypeptide (PHPDLG) consisting of N-hydroxypropyl-D-glutamine and N-hydroxypropyl-L-glutamine was performed to clarify the effect of the optical isomer of the comonomer and copolymer composition on the rate of degradation by papain in a pseudo-extracellular fluid (PECF) [4] at pH 7.4 and 37.0°C to simulate *in vivo* polymer degradation. The composition of PECF is listed in Table 1. Papain is a well characterized plant thiol endopeptidase [5–7] with a broad range of specificity. It is closely related to cathepsin B, a thiol endopeptidase isolated from mammalian spleen, liver, kidney, and lung, and is released by cells in response to inflammation [8].

EXPERIMENTAL

Materials

Synthesis of starting polymers. N-carboxyanhydrides (NCA) of γ-benzyl-D-glutamate (γ-BDG) and γ-benzyl-L-glutamate (γ-BLG) were prepared by the phosgenation of the corresponding amino acids in tetrahydrofuran (THF) and purified by multiple recrystallization.

Solutions (0.1 M, *c* 2 wt%) of the mixture of γ-BDG-NCA and γ-BLG-NCA in 1:1 (v/v) mixture of dioxane: methylene dichloride were prepared and polymerization was initiated with triethylamine (TEA) at a total of the NCA monomers: TEA equals to 25:1.

The copolymerization reaction was followed by CO_2 evolution according to the method of Patchornik and Shalitin [9]. Solvents used for synthesis and TEA were purified by the usual method described in the literature. All starting polymer (PBDLG) samples were precipitated by adding about four times the amount of methanol in volume including 5 vol.% of 0.1 N HCl to the polymer solution at 4°C. The precipitation products were washed in methanol and dried under reduced pressure at 50°C. The D,L-composition of these PBDLG samples was determined from optical

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Table 1. Pseudo-extracellular fluid (PECF) ($NaHCO_3$, K_2HPO_4, NaCl, KCl)

	Concentration (meq/l)	
Ion	Physiological	PECF
Na	145	145
K	5	5
Cl	113	118
HCO_3	30	30
HPO_4	2	2

rotatory dispersion (ORD) in a random solvent, dichloroacetic acid (DCA).

The preparative data of the PBDLG samples is listed in Table 2.

Preparation of hydrophilic samples. The aminolysis reaction of the side chains of γ-benzyl glutamate residues was carried out by 3-amino-1-propanol [10]. The completion of the aminolysis reaction was ascertained by using a u.v. spectra measurement. After the aminolysis reaction, the aqueous PHPDLG polymer solution was dialysed exhaustively against distilled water, filtered through a Millipore filter and lyophilized.

Papain (3.5 m Anson μ mg^{-1}, No. 7144) was purchased from Nakarai Tesque Co. (Kyoto, Japan), and used without further recrystallization. Papain was activated in the PECF solution as proposed by Homsey at pH 7.4 with 0.01 M cystein and 0.01 M EDTA.

Measurements

Molecular characterizations. Intrinsic viscosity [η] (dl/g) of the starting polymers (PBDLG) was determined in dichloroacetic acid (DCA) at 25°C using an Ubbelohde-type viscometer and is listed in Table 2. [η] of the water-soluble samples after the aminolysis reaction was measured with an Ubbelohde-type viscometer at pH 7.4 and 37°C in PECF. Molecular weights of the PHPLG and PHPDLG (D/L = 1/1) series, as well as the molecular weight distribution of the samples, were investigated by gel permeation chromatography (GPC) on a Toyo-Soda high-speed liquid chromatograph HLC-803D equipped with TSK-gel type G-4000SW, C-No. SW46A0015 in PECF at 25°C. The preparative data of these water-soluble samples are summarized in Table 3.

All kinetic measurements of the enzymatic hydrolysis of the water-soluble samples were achieved using an Ubbelohde type viscometer in PECF. The initial rate of degradation, V, was calculated by measuring the time necessary for the molecular weight to drop to half its initial value. A viscosity–kinetic measurement was performed as follows. An aliquot of the polymer solution (10 ml unless otherwise stated) was pipetted into the viscometer. After determining the viscosity of a solution, 0.2 ml of the stocked papain solution was added and viscosity was measured periodically with stirring. Unless otherwise stated, the papain concentration was controlled to $[E] = 5.10 \times 10^{-6}$ M after being mixed with the polymer solution in the viscometer.

To convert the reduced viscosity to intrinsic viscosity, the Huggins expression [11], $\eta_{sp}/C = [\eta] + k'[\eta]^2 C$, relating reduced viscosity to concentration was followed in the concentration range of interest and the constant in the Huggins expression was within the same error as that for molecular weight. [η] values as a function of digestion time may thus be calculated in this way.

Table 2. Copolymerization data of starting copolypeptides

Sample code	F_L (mol%)	f_L (mol%)	P (%)	[η] (dl/g) (DCA, 25°C)
PBLG-1	100.0	100.0	79	1.25
PBLG-2	100.0	100.0	82	0.87
PBLG-3	100.0	100.0	75	0.64
PBLG-4	100.0	100.0	72	0.33
PBDLG-91	90.0	90.2	81	1.38
PBDLG-82	80.0	80.6	81	1.18
PBDLG-73	70.0	69.3	80	1.14
PBDLG-64	60.0	60.1	77	1.14
PBDLG-55-1	50.0	50.6	72	1.26
PBDLG-55-2	50.0	50.2	68	0.88
PBDLG-55-3	50.0	50.5	70	0.39
PBDLG-46	40.0	39.8	75	1.39
PBDLG-37	30.0	29.5	77	1.35
PBDLG-28	20.0	19.0	78	1.27
PBDLG-19	10.0	9.0	80	1.24
PBDG-1	0.0	0.0	82	1.61

Table 3. Preparative data of water-soluble samples

Sample code	f_L (mol%)	[η] (dl/g) (PECF, 37.0°C)	k'	M_w
PHPLG-1	100.0	0.32	0.39	69,400
PHPLG-2	100.0	0.27	0.40	44,900
PHPLG-3	100.0	0.21	0.40	30,900
PHPLG-4	100.0	0.17	0.41	22,800
PHPDLG-91	90.2	0.34	0.39	72,300
PHPDLG-82	80.6	0.33	0.40	74,500
PHPDLG-73	69.3	0.31	0.39	82,300
PHPDLG-64	60.1	0.29	0.40	84,000
PHPDLG-55-1	50.6	0.30	0.40	92,800
PHPDLG-55-2	50.2	0.21	0.39	49,500
PHPDLG-55-3	50.5	0.14	0.41	27,900
PHPDLG-46	39.8	0.34	0.39	93,300
PHPDLG-37	29.5	0.36	0.40	89,700
PHPDLG-28	19.0	0.38	0.41	91,500
PHPDLG-19	9.0	0.41	0.39	82,500
PHPDG-1	0.0	0.38	0.40	78,300

RESULTS AND DISCUSSION

Relation between intrinsic viscosity and molecular weight

The dependence of the intrinsic viscosity of PHPDLG as well as PHPLG samples on molecular

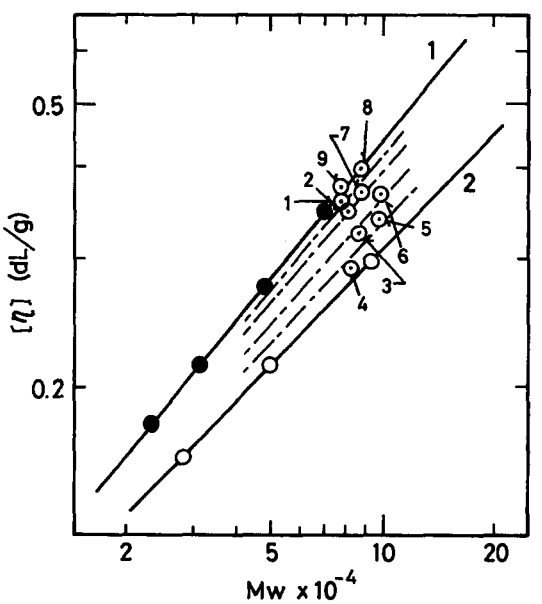

Fig. 1. The intrinsic viscosity [η] vs molecular weight (M_w) for (1) PHPLG and (2) PHPDLG-55 in PECF at pH 7.4 and 37.0°C. Plots are experimental data for copolymers for 1: PHPDLG-91; 2: PHPDLG-82; 3: PHPDLG-73; 4: PHPDLG-64; 5: PHPDLG-46; 6: PHPDLG-37; 7: PHPDLG-28; 8: PHPDLG-19; and 9: PHPDG-1.

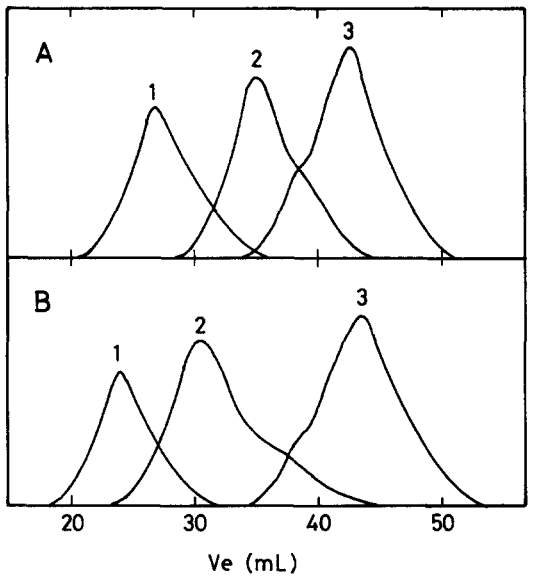

Fig. 2. GPC elution curves for reaction products of copolypeptides in PECF solution by papain. (A) PHPLG-1 for (1) original, (2) 30 min, and (3) 150 min of papain digestion, and (B) PHPDLG-55-1 for (1) original, (2) 50 and 240 min of papain digestion. Papain concentration; $[E] = 5.10 \times 10^{-6}$ M.

weight is shown in Fig. 1. A straight line is drawn to fit the experimental points for each case of PHPLG and PHPDLG (D/L = 1/1), respectively. From this straight line, the empirical parameters, K' and α, in the equation $[\eta] = K'M^{\alpha}$ for each case were obtained. In regard to the copolymer samples other than PHPDLG (D/L = 1/1), it would be impossible to obtain different molecular weight fractions with exactly the same copolymer composition and their molecular weight distributions. Thus, an imaginary straight relation was used to distinguish the molecular weights of the copolymers from the intrinsic viscosity experimental data.

Type of degradation

To determine whether random degradation of the main chain of a polypeptide is dominant in the reaction with papain, GPC analyses of partially degraded polypeptides were carried out. Figure 2 illustrates some examples of GPC curves for polypeptide samples. From Fig. 2, it may be concluded that each sample is dominantly degraded by a random main chain cleavage as in the case of the degradation of poly(N-hydroxyethyl-L-glutamine) by endopeptidases such as papain, chymotrypsin, or pepsin [12, 13].

Rate of degradation

Typical plots of $1/M$ against the papain digestion time are shown in Fig. 3. The procedure in this study for converting reduced viscosity to the molecular weight is strictly valid only if the molecular weight distribution during degradation differs but little from that in the original sample. Even if starting polypeptides have rather narrow molecular weight distributions, the aminolysis reaction of side chains of γ-benzyl glutamate residues will break a few peptide bonds which both theoretically [14] and experimentally [15] widens the molecular weight distribution considerably. Thus, original samples should have very nearly random or Gaussian molecular weight distribution. Theoretically, a plot of $1/M$ against degree of polymerization should be linear for the random degradation of an initially random distribution. For random degradation of a random distribution, the molecular weight will drop to one half its initial value when one bond has been broken per initial molecular weight. Since the GPC profiles in Fig. 2 and plots of $1/M$ against papain digestion time in Fig. 3 indicate random degradation, and since the original samples have random distributions, the procedure used to convert reduced viscosity to molecular weight is completely justified. As shown in Fig. 3, linear relations were obtained for all samples, but when the reaction proceeded over a rather long period, its rate dropped off slightly, indicating loss of enzyme activity. This and the order of rate of degradation will be discussed as follows.

Rate law

It is known that all proteases yield data which can be analysed in the framework of the Michaelis–Menten mechanism. The rate of degradation of samples was expected to be first order in papain concentration. It was necessary to confirm this since the papain concentration varied over a wide range so that the rate could always be measured. Figure 4 shows the expected experimental results for PHPLG-1 and PHPDLG-55-1; they indicate the first order behaviour and are typical of endopeptidase degradation.

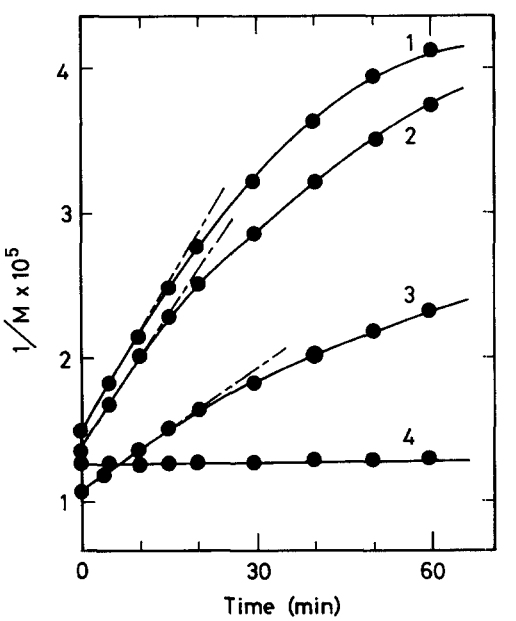

Fig. 3. Typical plots of 1/M for samples vs papain digestion time. Papain concentration; $[E] = 5.10 \times 10^{-6}$ M at pH 7.40 and 37.0°C. Numerals in the figure denote: 1, PHPLG-1; 2, PHPDLG-82; 3, PHPDLG-55-1; and 4, PHPDG-1.

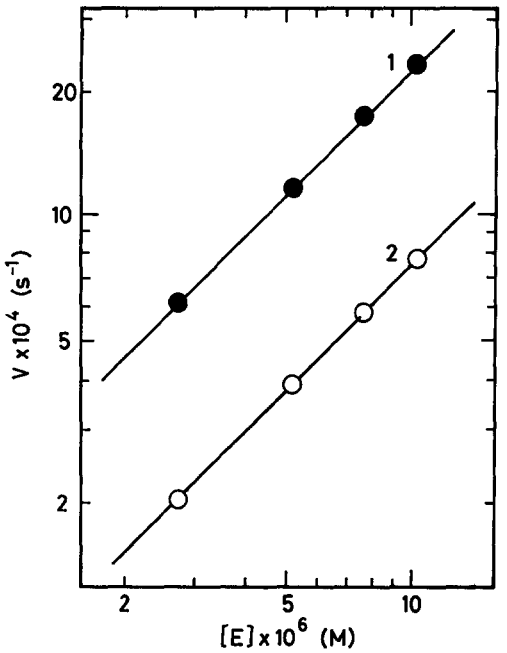

Fig. 4. Dependence of reaction rate V (sec^{-1}) on papain concentration for: 1, PHPLG-1; and 2, PHPDLG-55-1. Polymer substrate concentration; $C_s = 5.0–5.5 \times 10^{-2}$ M.

Next, the rate of degradation, V, was evaluated as a function of substrate concentration. Figure 5 illustrates the experimental results for PHPLG-1 and PHPDLG-55-1. The rate of bond breaking was calculated from the plots similar to Fig. 3, by measuring the time necessary for the molecular weight to drop to half its initial value, which corresponds to <0.5% breaking of the total bonds. Under given experimental conditions, a plot of reciprocal rate against reciprocal substrate concentration permits evaluation of the two constants (V_m and K_m) in the Michaelis–Menten rate law. Figure 6 illustrates Lineweaver–Burk plots for these experimental data obtained in Fig. 5.

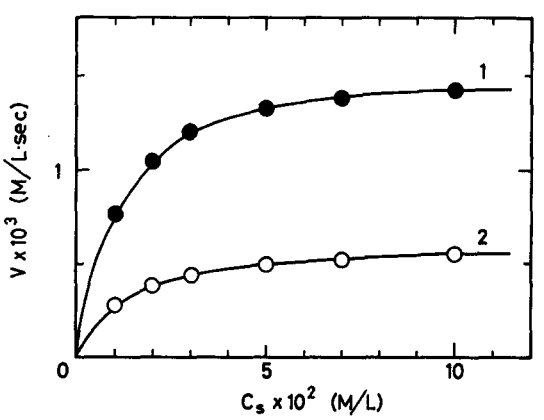

Fig. 5. Plots of reaction rate V (sec^{-1}) vs polymer substrate concentration C_s (M) in PECF solution at pH 7.40 and 37.0°C for: 1, PHPLG-1; and 2, PHPDLG-55-1. Papain concentration; $[E] = 1.2 \times 10^{-5}$ M.

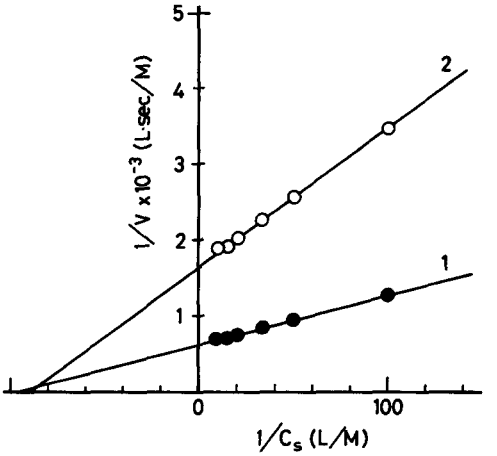

Fig. 6. Lineweaver–Burk plots for: 1, PHPLG-1; and 2, PHPDLG-55-1 with the experimental data obtained in Fig. 5.

As expected, the degradation follows the Michaelis–Menten rate law within experimental error. Since the rate of degradation, V, is dependent on the number of bonds and not macromolecules, which, for high sample molecular weights, are nearly equal to the peptide concentration. Table 4 summarizes the values of the Michaelis–Menten parameters, V_m and K_m, for samples which calculated from the experimental findings.

Effects of D-*isomer on the rate of degradation by papain*

The relation between the copolymer composition of D-isomer and the rate of degradation V by papain was investigated under the same experimental conditions and illustrated in Fig. 7. It is clearly shown in Fig. 7 that PHPDG is almost impossible to hydrolyse by papain in this experimental condition, and that the experimental values of V for D,L-copolymers do not fall on the straight line (dashed line in Fig. 7) connecting Vs for both homopolymers, PHPLG and PHPDG. This fact indicates that the sequential distribution of D- and L-isomers in copolymer chains should influence V values of D,L-copolymers.

In conclusion, it was shown that papain was hardly able to digest D-amino acids *in vitro*, though it was demonstrated that the rates of elimination from the serum for poly-D-amino acids and their L-isomers were the same in rabbits, suggesting the existence of D-proteases and D-peptidases [3]. On the other hand, it was pointed out that D,L-copolypeptides, PHPDLG, were found to be degraded by

Table 4. Michaelis–Menten parameters for samples in PECF at pH 7.4 and 37.0°C

Sample code	f_L (mol%)	K_m (M/L)	V_m ($M/L \cdot sec$)
PHPLG-1	100.0	8.8×10^{-3}	1.5×10^{-3}
PHPDLG-82	80.6	9.2×10^{-3}	1.1×10^{-3}
PHPDLG-55-1	50.6	10.8×10^{-3}	0.6×10^{-3}
PHPDLG-28	19.0	14.0×10^{-3}	0.1×10^{-3}
PHPDG-1	0.0	—	—

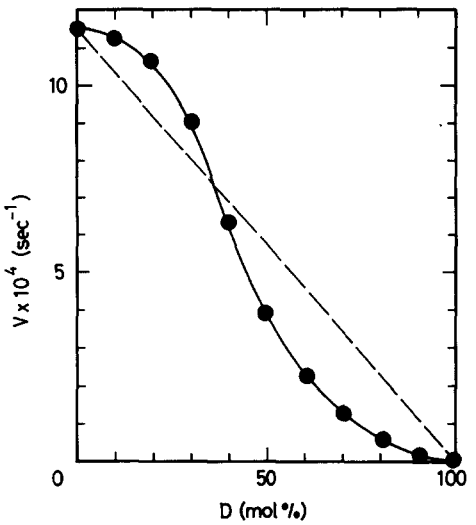

Fig. 7. Plots of the rate of papain digestion V (sec^{-1}) vs the copolymer composition of D-isomer for PHPDLG in PECF at pH 7.40 and 37.0°C. Papain concentration; $[E] = 5.10 \times 10^{-6}$ M.

random chain sission with papain, and that the degradation data for these D,L-copolymers and homopolymers followed the Michaelis–Menten rate law. Furthermore, it should be interesting to recognize that the rate of degradation was controlled by the composition of D-isomer as well as by sequential distributions of comonomers in the copolymer chains. These findings could become very important information to design biodegradable polymers whose rate of degradation is the most desirable for the biomedical applications.

REFERENCES

1. J. M. Anderson, D. F. Gibbons, R. L. Martin, A. Hiltner and R. Wood. *J. Biomed. Mater. Res. Symp.* **5,** 197 (1974).
2. T. J. Gill III, H. W. Kunz, J. H. Gould and P. Doty. *J. biol. Chem.* **239,** 1107 (1964).
3. J. T. Gill III, D. S. Papermaster and J. F. Mowbray. *Nature* **203,** 644 (1964).
4. C. A. Homsey. *J. biomed. mater. Res.* **4,** 341 (1971).
5. J. Lowbridge and J. S. Fruton, *J. biol. Chem.* **249,** 6754 (1974).
6. J. R. Kimmel and E. L. Smith. *J. biol. Chem.* **207,** 515 (1954).
7. R. Arnon. *Meth. Enzymol.* **19,** 226 (1970).
8. K. Otto. *Tissue Proteases* (edited by A. J. Barnett and J. T. Dingle), p. 1. Elsevier, New York (1971).
9. A. Patchornik and Y. Shalitin. *Bull. Res. Counc. Isr., Sect. A, Chem.* **5,** 300 (1956).
10. T. Hayashi, E. Nakanishi and A. Nakajima. *Polymer J.* **19,** 1025 (1987).
11. M. L. Huggins. *J. Am. chem. Soc.* **64,** 2716 (1942).
12. T. Hayashi, M. Iwatsuki and M. Oya. *Rep. Progr. Polym. Phys. Japan* **31,** 561 (1988).
13. W. G. Miller. *J. Am. chem. Soc.* **83,** 259 (1961).
14. A. Charlesby. *Proc. R. Soc., Lond. Ser. A.* **224,** 120 (1954).
15. C. E. Hall and P. Doty. *J. Am. chem. Soc.* **80,** 1269 (1958).