inhibition studies described above support the Bi Uni Uni Bi ping-pong Ter Ter system for malonyl-CoA synthetase reaction. However, it is neceassary to eliminate the possibility of other terreactant ping-pong system, the Theorell-Chance system or the terreactant ping-pong system with rapid equilibrium segment. First, the possibility of Theorell-Chance type case (Bi Uni Uni Bi ping-pong Ter Ter, Theorell-Chance B-P) can be eliminated by looking at the result of product inhibition in which PPi is a mixed-type inhibitor with respect to malonate at unsaturating ATP and CoA concentrations (Figure 2C). Secondly, in the Bi Uni Uni Bi ping-pong Ter Ter system in which the first substrate binds in either direction and the respective complexes that are formed are at equilibrium, the intial velocity equation in the absence of products and the product inhibition patterns is changed differently from the full steady state system.

When A is varied:

$$v = VA/(K_{ia}K_b/B + A(1 + K_b/B + K_c/C)) \qquad (10)$$

Compared to the equivalent velocity equation for the full steady-state system, K_aAB term is missing in Eq. (10). Then slope $1/A$ versus $1/B$ replots go through the origin. Secondary replot using the slopes of lines obtained when initial velocity data of Figure 1C were plotted with ATP as variable substrate eliminates the possibility of this system.

As a consequence, we propose that *R. trifolii* malonyl-CoA synthetase catalyzes the reaction in the manner of an ordered Bi Uni Uni Bi ping-pong Ter Ter mechanism as follows:
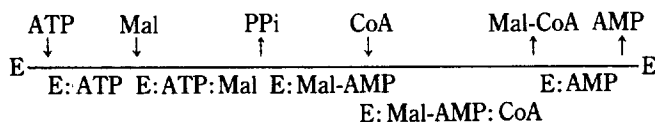
Where Mal, Mal-AMP, and Mal-CoA represent malonate, malonyl-AMP, and malonyl-CoA, respectively.

This catalytic mechanism is further supported by the isolation of first product, PPi, and by the identification of the key intermediate, malonyl-AMP.

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Molecular Conformation and Non-Newtonian Viscosity Behavior of Poly(L-proline) in Various Solvent Systems

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The non-Newtonian viscosities (the specific or intrinsic viscosities) of poly (L-proline) (PLP, $M_v = 19,000$ and 32,000) in various mixed-solvent systems like water-propanol and acetic acid-propanol of varying compositions were measured during the reverse mutarotation (Form II→Form I) by the application of external pressure (up to 4.5 psi). The non-Newtonian viscosity effect was found to be larger in acetic acid-propanol system than in water-propanol system and to somewhat decrease during the reverse mutarotation at a given solvent system. The non-Newtonian viscosity behavior of PLP in aqueous salt (CaCl₂) solution was also studied, from which it was found that the degree of the non-Newtonian effect decreased with increasing salt concentration, and increased with increasing PLP molecular weight. These findings could be explained in terms of conformational changes of PLP in solution (like the helix-helix or helix-coil transition) involved.

Introduction

Poly(L-proline) (PLP) is known as one of the most interes-

ting biopolymers in that not only it posesses a stable helical structure, despite the lack of the amino proton group, owing to the steric effect of the pyrrolidine rings but it can form

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ordered structures in dilute solutions[1-5] as well as in the solid state.[1,6-9] PLP has two distinct helical structures: one, more common crystalline form in the trans configuration (Form II) and the other form in the cis (more compact) configuration (Form I). The two forms are readily characterized by their optical rotations, and are reversibly interconvertible by change of solvent composition.[5,8,10] That is, the Form II is stable in good solvents such as acetic acid (HOAc), formic acid, and water; the transition Form II→Form I (reverse mutarotation) is induced by dilution of these solvents with an excess of either *n*-propanol or *n*-butanol, the rate of transformation being followed by polarimetry.

Because of these unique characteristics, many investigations[11-14] have been performed on the PLP system in solution with more exphasis, mainly, on the effects of temperature, mixed-solvent composition, pH, etc. on conformational changes of PLP molecules *via* viscometric and spectroscopic measurements. Recently, Yang[15] and Jang[16] have reported that the non-Newtonian viscosity behavior (*i.e.* the shear rate-dependent viscosity) of biopolymer solutions in the helix-coil transition region was markedly affected by the molecular conformational change, *i.e.* the more rigid the conformation, the larger the non-Newtonian effect.

Generally, viscometric quantities like the specific (η_{sp}) or intrinsic ($[\eta]$) viscometric have been most frequently used in studies of conformational changes of biopolymers. In particular, the non-Newtonian viscosity behavior of biopolymer solutions in the transition region under external (shear) force can provide a better insight into the shape (or size) of the polymer at different stages of conformational transition together with the effects of deformation and orientation of molecules on viscosity change during the flow process.

On the other hand, it is also reported that certain polymers like PLP or poly(L-lycine) undergo the helix-coil (or order-disorder) transition at highly concentrated aqueous salt solutions,[13,17,18] which will greatly affect the non-Newtonian viscosity behavior.

Hence, this work aims at investigating the effects of shear stress, solvent composition, polymer molecular weight (M), and salt concentration on the non-Newtonian viscosity behavior of PLP solutions in the helix-helix and/or helix-coil transition regions for a better understanding of the molecular conformation of PLP in shear flow.

Experimental

Materials. Two types of PLP samples (Form II) having the viscosity-average molecular weights $M_v = 19,000$ and 32,000 were purchased from Sigma Chemical Co., Ltd., and identified by FT-IR and polarimetry.[1-9] A type of $M_v = 32,000$ was preferably used throughout the study in view of the non-Newtonian viscosity effect. The glacial HOAc and *n*-propanol (solvents) and $CaCl_2$ (salt) were all reagent grade and used without further purification. Triply distilled water was also used as solvent for PLP.

Sample Preparation. The conformational transition[1-4] of PLP Form II→Form I in mixed-solvents, water-propanol or HOAc-propanol, of a given composition (1 : 9 and 2 : 8 v/v for both systems) was initiated by adding a required volume of propanol to solutions of PLP-II in water or HOAc. The aqueous salt solutions of PLP-II were prepared by dilu-

Table 1. Dimensions of the Capillary Tube Used in This Study

Capillary number	Length L (cm)	Inner diameter 2R (mm)	L/R
O_a	9.25	0.53	349.06
I	9.25	0.63	293.65
I.	9.25	0.84	220.24
II	9.25	1.13	163.72
III	9.25	2.01	92.04

tion of a stock solution whose concentration was already determined. It should be noted that the very dilute concentration (0.1 g/dL) of PLP-II in solution was employed for all experiments to avoid the aggregation problem.

Measurements. All the measurements here were conducted at least more than twice using the instruments equipped with circulating thermostats, and the results obtained proved to be highly reproducible within small experimental errors. Viscosity measurements were performed at 25 ± 0.05 °C with an automatic Ubbelohde-type viscometer (Schott-Gerate) conneted to an external pressure (99.9999% dry nitrogen gas) regulator necessary for creasing the non-Newtonian viscosity effect. Table 1 shows the dimensions of the capillary tubes used in this study. The relationship between external pressure Δp and shear stress f for the capillary (Poiseuille) flow is given by[16,19]

$$f = \Delta p (R/2L) \tag{1}$$

where f is the (maximum) shear stress at the capillary wall, R the radius, and L the length of the capillary. The Δp in Eq. (1) is equal to applied pressure p minus atmospheric pressure p_o plus hydrostatic pressure Δp_h developed in the capillary tube. Actually, $\Delta p \approx p - p_o$ since $\Delta p_h \ll p - p_o$. For convenience sake, we used Δp instead of f in this paper.

The degree of the transition Form II→I during the reverse mutarotation of PLP in a given mixed-solvent system at 25°C was determined by optical rotation measurments through use of a Rudolph automatic polarimeter (Autopor III) equipped with a sodium D line (589 nm) light source. The fraction f_I of Form I (cis fraction) of PLP at a given trsansition stage in mixed solvents was estimated by the following relationship:[15,16]

$$f_1 = ([\alpha]_{II} - [\alpha]) / ([\alpha]_I) \tag{2}$$

where $[\alpha]_{II} = -540$ (for pure Form II), $[\alpha]_I = -15$ (for pure Form I), and $[\alpha]$ is the specific rotation of the sample under transition.

Results and Discussion

Viscosity Changes of PLP during Reverse Mutarotation. The variations in the reduced viscosities (in η_{sp}/c with c being the polymer concentration) of PLP ($M_v = 32,000$) solutions in various mixed-solvent systems ($c = 0.1$ g/dL) during the reverse mutarotation (from $[\alpha]_D = -420$ to $[\alpha]_D = -20$) under no external force at 25°C are illustrated graphically in Figure 1. In fact, the intrinsic viscosity is the most adequate quantity in expressing the conformational state of
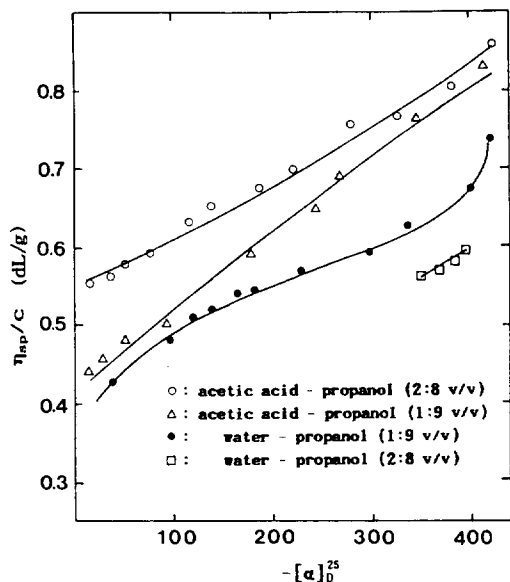
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Figure 1. Zero-shear reduced viscosity changes of PLP (M_v= 32,000) solutions in various mixed-solvent systems (c=0.1 g/dL) during the reverse mutarotation at 25℃.

an isolated polymer molecule in solution.

However, it was nearly impossible to get the correct [η] data corresponding to a given transition stage (or [α]_D value) for each solvent system covered because of the noticeable transition speed. In addition, the initial decrease in specific rotation from [α]_D= −540 to [α]_D≈ −400 caused by transferring the polymer (PLP-II) to the alcoholic medium was essentially instaneous for each mixed-solvent system. As shown in Figure 1, the viscosity of PLP solutions during the reverse mutarotation decreased in a monotonous fashion (*i.e.* η_sp/c decreasing with decreasing −[α]_D value) for the respective mixed-solvent systems, in accord with the results reported by Steinberg *et al*[2]. This tendency may be ascribed to the fact that PLP-II has the more extended structure than Form I. Figure 1 also shows that the viscosity of PLP in the water-propanol system at a given transition stage is lower than that of the same substance in the HOAc-propanol system over the entire transition region, suggesting that PLP molecules have more compact conformation in water-propanol than in HOAc-propanol. In order to understand this point more clearly, zero-shear (reduced) viscosities of PLP-II (M_v= 19,000 and 32,000) in water-HOAc mixtures (c=0.1 g/dL) at 25℃ are plotted as a function of the vol.% of HOAc in the solvent mixture for two kinds of PLP molecular weights in Figure 2.

One notices that the viscosity of PLP in water-HOAc mixtures increases with an increase either in HOAc content or in PLP molecular weight, supporting the previous result. In fact, many investigators[20,21] have recently reported that the presence of only a small fraction (*e.g.* 2 or 3%) of cis residues within a PLP-II molecule, as in water, will lead to the considerably decreased molecular dimension, and hence lowered viscosity, for these cis residues may be distributed nearly randomly along the chain, producing points of flexibility which have marked effects on hydrodynamic properties of PLP. Thus, although both solvents, water and HOAc, have
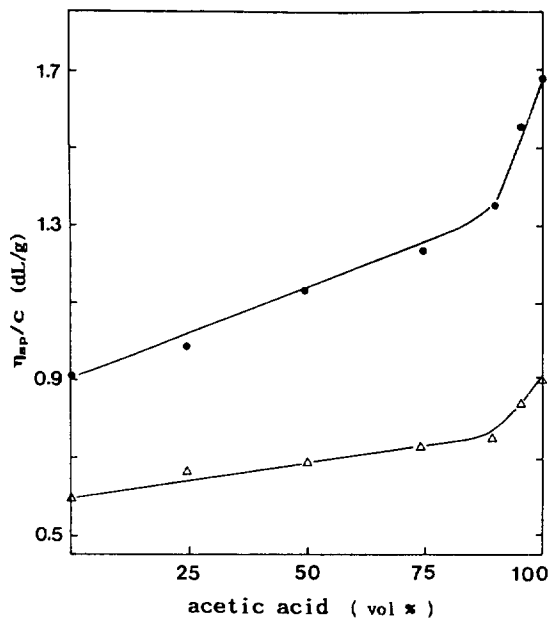

Figure 2. Zero-shear reduced viscosities of PLP-II in HOAc-water mixtures (c=0.1 g/dL) at 25℃ as a function of the vol.% of HOAc for two different molecular weights of PLP: (△) M_v= 19,000, (●) M_v=32,000.

PLP in Form II stabilized, chain dimensions of PLP in water and HOAc are different from each other. More precisely, PLP will take the more compact conformation (and hence leading to the lower viscosity) in water than in HOAc, and the same thing applies to chain dimensions of PLP in water-propanol and HOAc-propanol of the same composition. This could be more easily interpreted in terms of either one or both of the following two well-known (intrinsic) viscosity-molecular dimension relationships. The first one is the so-called Mark-Houwink (M-H) equation:[22]

$$[\eta] = KM^\nu \qquad (3)$$

where K and ν are empirical parameters depending on the polymer-solvent pair and temperature. In particular, with flexible polymers the expondent ν assumes values between 0.5 and 0.8, depending on "goodness" of the solvent, while with rigid polymers ν is higher ($1<\nu<2$) and less dependant on the solvent. The other, more formal one is due to Flory:[22]

$$[\eta] = \Phi <r^2>^{2/3}/M \qquad (4)$$

where Φ is the universal constant and $<r^2>$ is the mean-square end-to-end distance of the polymer. Eq. (4) implies that [η] M is a relative measure of the "hydrodynamic volume" of a polymer molecule in solution. Thus, the results shown in Figure 2 may be equivalent to saying that the M-H exponent ν (Eq. (3)) or the hydrodynamic volume (Eq. (4)) will have the smaller values for the water (or water-propanol)-PLP system than for the corresponding to the HOAc (or HOAc-propanol)-PLP system.

Next, in order to investigate the effect of solvent type on viscosity behavior of PLP during the reverse mutarotation in mixed-solvents in more detail, shear rate-dependant (non-Newtonian) viscosities of PLP (M_v=32,000) solutions (c=0.1 g/dL) under external pressure applied (up to 2.5 psi) at 25℃
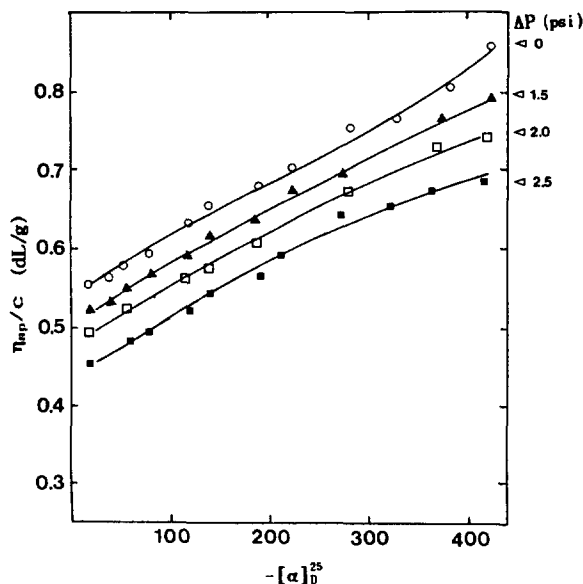
Figure 3. Non-Newtonian reduced viscosities of PLP (M_v= 32,000) solutions in HOAc-propanol (2 : 8 v/v) (c=0.1 g/dL) during the reverse mutarotation at 25℃ under various external pressures Δp (psi). The external pressures are labeled on the curves. The utmost curve at Δp=0 is the curve obtained under atmospheric pressure.
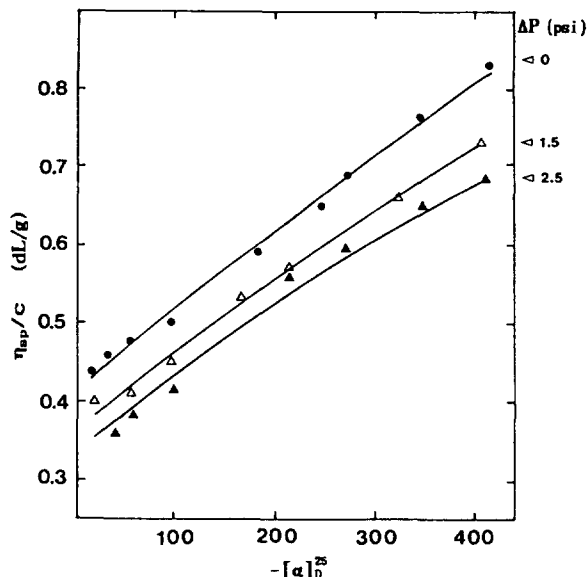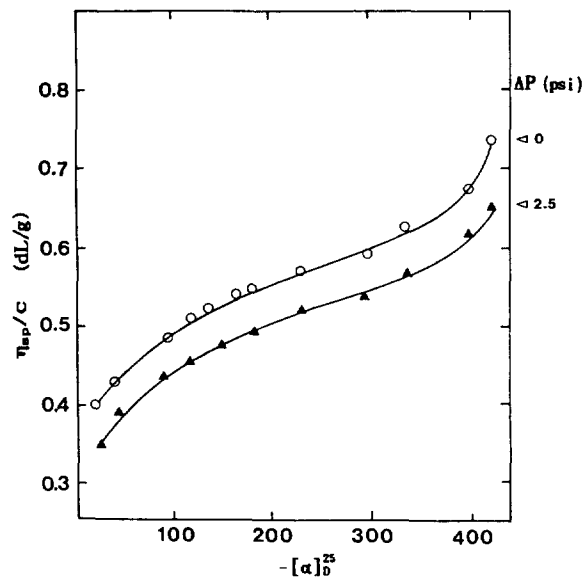
were measured in three different solvents, *i.e.* HOAc-propanol (2 : 8 v/v), HOAc-propanol (1 : 9 v/v), and water-propanol (1 : 9 v/v), whose results are displayed in Figures 3, 4, and 5, rspectively. Because of the transition behavior of PLP proceeding with time progressively, as stated before, reduced viscosities were forcibly used instead of intrinsic viscosities, and it was also impossible to obtain reduced viscosities corresponding to the same $[\alpha]_D$ values at various external pressures (or shear stresses) during the reverse mutarotation except for the water-propanol system. Inspection of these figures reveals that the reduced viscosity η_{sp}/c decreases with increasing external pressure Δp (or shear stress f) at about the same $[\alpha]_D$ for all three mixed-solvents, which is the so-called shear-thinning non-Newtonian viscosity behavior, the degree of the non-Newtonian effect being in the order of HOAc-propanol (2 : 8 v/v)>HOAc-propanol (1 : 9 v/v)>water-propanol (1 : 9 v/v) over a whole range of reverse mutarotation. Namely, this order implies that the larger the Solvent Power for PLP (*i.e.* the larger v value), the larger the non-Newtonian effect in the helix-helix transition region of PLP. Moreover, the observed difference in the non-Newtonian viscosity behavior of PLP solutions in various solvent-systems during the reverse mutarotation seems to be in line with the previous argument that PLP molecules assume more compact conformation, and hence smaller chain dimensions, in water-propanol than in HOAc-propanol. These figures also show that as the transition Form II→Form I proceeds, the non-Newtonian viscosity effect appears to somewhat decrease at a given solvent system; *i.e.*, the non-Newtonian effect assumes to be a little higher in Form II than Form I. Unfortunately, however, we cannot discover any marked differences in the non-Newtonain effects among different molecular conformations under reverse mutarotation for

Figure 4. The same interpretation as in Figure 3 except for the solvent HOAc-propanol (1 : 9 v/v).

Figure 5. The same interpretation as in Figure 3 except for the solvent water-propanol (1 : 9 v/v).

all solvent-systems covered, unlike the case for the helix-coil transition. This could be explained as follows. In general, for the systems exhibiting the shear-thinning non-Newtonian viscosity effects there are two major viscosity (or $[\eta]$-decreasing effects[16,19] arising from the external force acting on the polymer molecules in shear flow: the deformation effect, contributing more to the flexible, coil structure, and the orientation effect, playing more important role in the rigid-rod structure.

Meanwhile, the reverse mutarotation is a helix-helix transition. Hence, a PLP molecule during the reverse mutarotation in mixed-solvent preserves the rigid-rod structure, which is easy to orientation, but still hard to deformation under external (shearing) force. Consequenctly, the non-
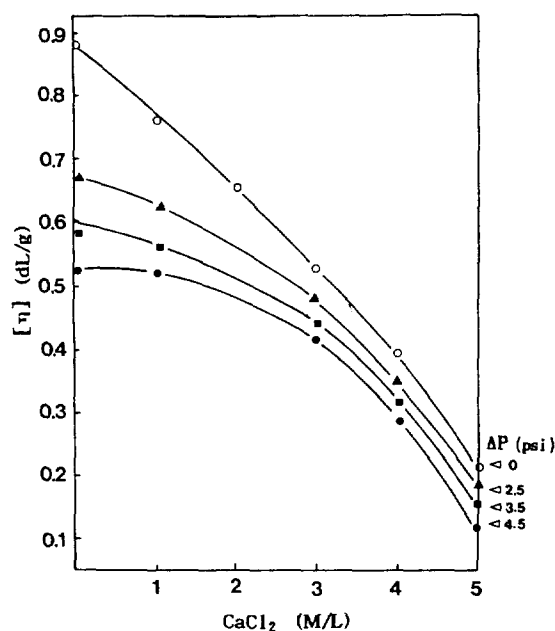
Figure 6. The salt ($CaCl_2$) concentration-dependence of the intrinsic viscosity $[\eta]$ of PLP-II ($M_v = 32,000$) in aqueous salt solutions ($c = 0.1$ g/dL) at 25℃ under various external pressures.
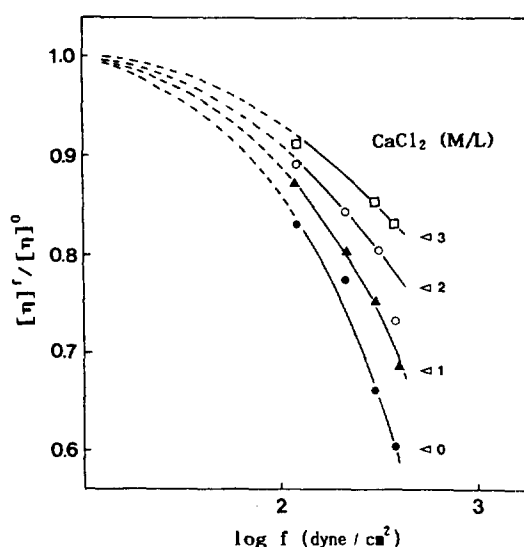

Figure 7. Flow curves (expressed as the relative intrinsic viscosity) of PLP-II ($M_v = 32,000$) in aqueous salt solutions ($c = 0.1$ g/dL) at 25℃. The concentration (M/L) of $CaCl_2$ is shown on each curve, and the curves for $[CaCl_2] = 4$ M and 5 M were omitted.

Newtonian viscosity effect remains about the same during the reverse mutaratation of PLP for a given solvent system, regardless of the degree of molecular asymmetry. For drawing any definite conclusions on this, however, a more extensive and systematic study on the effects of shear stress and PLP molecular weight on the non-Newtonian viscosity behavior of PLP during the reverse mutarotation in mixed-solvents will need to be carried out.

Non-Newtonian Viscosities of PLP in Aqueous Salt Solutions. Mattice and Mandelkern[11] have reported that PLP-II molecules in concentrated aqueous salt solutions undergo the conformational transition from a somewhat flexible rod-like structure to a statistical random coil, probably due to the result of cis-trans isomerization about the imide bond, as confirmed by viscometric titration (in effect, based on the use of the *M-H* exponent v). In particular, according to Tiffany and Krimm,[13] $CaCl_2$ is one of the most effective salts in inducing such structural transitions.

On the other hand, with a view to investigating the influence of molecular conformation on the non-Newtonian viscosity behavior of PLP solutions more distinctly, non-Newtonian intrinsic viscosities of PLP (Form II) in aqueous salt ($CaCl_2$) solutions of different salt concentrations were measured on two kinds of PLP samples with $M_v = 19,000$ and 32, 000 at 25℃ under various external pressures. In Figure 6, the values of the intrinsic viscosities $[\eta]$ (d/Lg) of PLP-II. With a molecular weight of $M_v = 32,000$ in aqueous salt solutions at 25℃ under various external pressures Δp (psi) (labeled on the curves) are plotted against the (molar) concentration of $CaCl_2$ (M/L). As evident from this figure, the present PLP-aq. $CaCl_2$ system exhibits the (shear-thinning) non-Newtonian viscosity behavior over a whole range of the salt concentration covered, *i.e.*, $[\eta]$ decreases with increasing Δp at a given $[CaCl_2]$, the tendency being less pronounced at higher salt concentrations. To be more concrete, $[\eta]$ for pure

water drops from 0.890 to about 0.533 dL/g whereas $[\eta]$ for a 5 M aqueous salt solution drops from 0.223 to 0.124 dL/g when Δp changes from zero to 4.5 psi. Similarly to the case of reverse mutarotation of PLP in mixed-solvents, these findings could also be explained in view of molecular conformation (*via* Eq. (3) or (4)). Namely, in case of no or little salt added (*e.g.* pure water), where PLP molecules retain the rigid, rod-like structure, of the two $[\eta]$-decreasing effects for the shear-thinning non-Newtonian flow the orientation effect is more dominant, as discussed above. Thus, the rigid molecules in solution may have the tendency to orient themselves in the flow direction in order to make the flow easier, thereby causing the decrease in viscosity $[\eta]$ with increasing shear stress f (or Δp). In contrast to this, at higher salt solutions, where PLP molecules may assume the nearly disordered structures, *i.e.* the flexible, random coil conformation, the deformation effect will play a more important role in reducing the viscosity with increasing shear stress. That is the random coil form of the PLP molecule may change its shape from a sphere at $\Delta p = 0$ to an ellipsoid with the major axis parallel to the flow direction at higher Δp. Accordinly, the PLP molecules may flow easier with this shear deformation, or equivalently, $[\eta]$ decreases with increasing Δp. From the change of the non-Newtonian viscosity behavior of PLP in aqueous solution caused by the addition of salt as displayed in Figure 6, we can also see that the orientation effect for a rod-like molecule seems to be stronger than the deformation effect for a random coil molecule in the non-Newtonian flow of PLP. Thus, we may conclude that in case of pure water, the non-Newtonian effect is strong, while for highly concentrated aqueous salt solutions the effect is weak, which is probably equivalent to saying that the larger the value of v (or $\langle r^2 \rangle^{3/2}$), the stronger the non-Newtonian effect for the PLP-aq. $CaCl_2$ system.

In order to understand more easily the effect of salt concentration on the non-Newtonian viscosity behavior of PLP in aqueous solution, the data presented in Figure 6 are re-
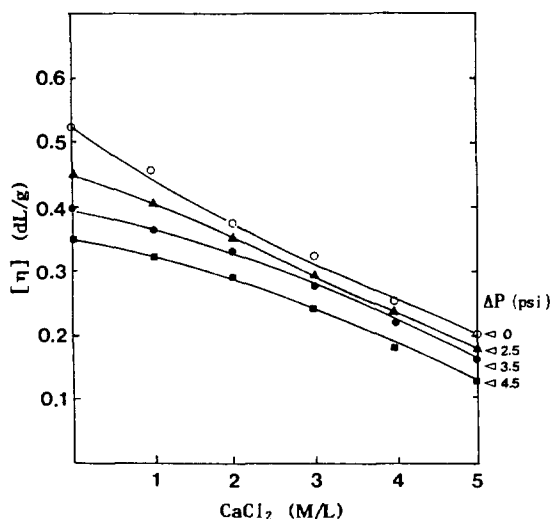
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Figure 8. The same interpretation as in Figure 6 except for the PLP molecular weight of $M_v = 19,000$.
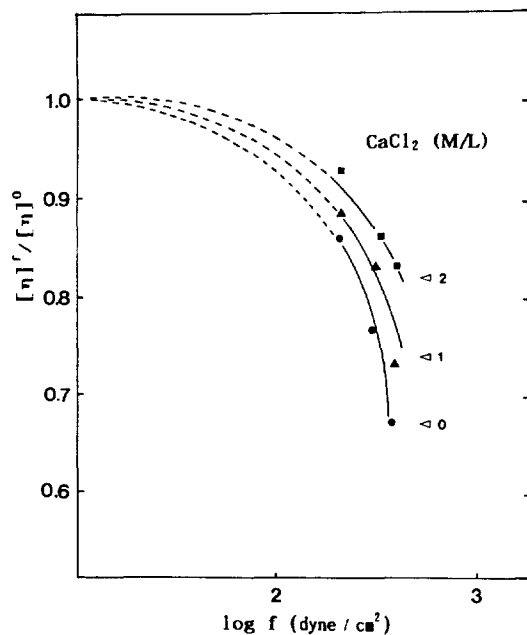

Figure 9. The same interpretation as in Figure 7 except for the PLP molecular weight of $M_v = 19,000$.

plotted as $[\eta]^r/[\eta]^o$ *vs.* $\log f$ in Figure 7, where $[\eta]^r$ and $[\eta]^o$ are the intrinsic viscosities at shear stress $f=f$ and $f=0$, respectively. The (relative) flow curves in Figure 7 clearly demonstrate that the degree of the non-Newtonian viscosity effect decreases with increasing salt concentration in aqueous solution of PLP, which may be attributable to the disordering effect of salt, *i.e.*, the decrease in molecular asymmetry (or in ν) of PLP caused by the presence of salt in large quantities, as mentioned above.

In order to investigate the effect of polymer molecular weight on the non-Newtonian viscosity effect of PLP in aqueous salt solutions, the same plots as those in Figures 6 and 7 except for a PLP molecular weight of $M_v = 19,000$

are represented in Figures 8 and 9, respectively.

As expected, these figures exhibit similar results to those for Figures 6 and 7. However, the non-Newtonian viscosity effect appears to be somewhat less pronounced as compared to the case for $M_v = 32,000$ at the corresponding experimental conditions, indicating that the non-Netwonian effect for the PLP-aq. CaCl$_2$ system will be stronger for larger molecular weights of PLP samples.

From the results obtained above, we may conclude that the non-Newtonian viscosity behavior of PLP in aqueous salt solution is largely affected by molecular conformation (in turn, dependant on salt concentration) and molecular weight of PLP. In addition, these findings could be accounted for, though qualitatively, in therms of conformational changes of PLP during the reverse mutarotation (helix-helix transition) or in highly concentrated aqueous salt solutions (helix-coil transition) through use of the *M-H* exponent ν or the hydrodynamic volume $\langle r^2 \rangle^{3/2}$.

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