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FLUORESCENCE POLARIZATION STUDY ON THE INCREASE OF MEMBRANE FLUIDITY OF HUMAN ERYTHROCYTE GHOSTS INDUCED BY SYNTHETIC WATER-SOLUBLE POLYMERS

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The effect of water-soluble polymers on the membrane fluidity of human erythrocyte ghosts was investigated and was compared with that of concanavalin A by means of the fluorescence polarization technique. 8-Anilino-1-naphthalene sulfonic acid sodium salt and 1,6-diphenyl-1,3,5-hexatriene were used as probe molecules. The membrane fluidity was increased by the addition of polycations with concentrations of less than $2 \cdot 10^{-3}$ wt% 60 min after mixing. The fluidity changes were affected by the chemical structure (hydrophobicity, charge density, etc.) of polycations. Thus, the membrane fluidity increased markedly with increasing charge density on the chain backbone of polycations. On the other hand, nonionic polymers such as poly(ethylene glycol) and poly(*N*-vinyl-2-pyrrolidone) changed the membrane fluidity in a biphasic manner. That is, the fluidity of human erythrocyte ghost was temporarily increased and then decrease. For example, 20 wt.% of poly(ethylene glycol) gave a maximum fluidity 15 min after mixing with erythrocyte ghosts. A similar fluidity change was observed by adding concanavalin A. Such fluidity changes were not observed when lipid bilayer vesicles were used instead of cell membranes. These results suggested that the increase of membrane fluidity resulted from the intramembraneous aggregation of membrane-bound proteins which was induced by the added polymers. Cell agglutination was also induced by the addition of a large amount of polymers. This agglutination was considered to be due to the intermembraneous aggregation of membrane-bound proteins.

Introduction

Some kinds of synthetic polymer have been used as biochemical reagent in the fields of cell engineering and related technology. For example, dextran is used to protect cell membranes [1,2] and poly(ethylene glycol) has been used as fusogen for various cell lines [3–5]. The mechanism of the interaction between synthetic polymers and cell membranes has not hitherto been clarified in spite of wide usages of synthetic polymers. The authors have already studied the interaction of synthetic polymers with vesicle membranes by means of ^{1}H-NMR [6,7] and fluorescence spectrometry [8]. Synthetic polymers decrease the molecular motion of lipid molecules monotonically and the interaction deeply depends on chemical structure of the applied polymers.

Recently, the aggregation of intramembraneous particles on the cell membrane surface was observed when an excess of poly(ethylene glycol) was added by means of the freeze-fracture technique [9]. There have been few studies on the changes of membrane fluidity caused by adding several polymers [10,11]. The study on the interaction of cell membranes and synthetic polymers was required for the development of new biochemical active reagents such as polymeric

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Abbreviations: ANS, 8-anilino-1-naphthalene sulfonic acid sodium salt; DPH, 1,6-diphenyl-1,3,5-hexatriene; PVI, poly(*N*-vinylimidazole), PEG, poly(ethylene glycol), PVPo, poly(*N*-vinyl-2-pyrrolidone)

fusogens. In the present paper, the interaction of synthetic polymers with human erythrocyte membranes has been studied by means of the fluorescence polarization technique.

Materials and Methods

Materials

Human erythrocyte ghosts were obtained from adult donors. After removing plasma by centrifugation (800 × *g* for 5 min at 4°C), erythrocytes were washed and centrifuged (800 × *g* for 10 min at 4°C) three times with 10 vol. 0.9 wt% NaCl solution to remove the buffy coats by aspiration. The erythrocytes were lysed with hypotonic Tris buffer solution (pH 7.4, 30 mosM) according to the method reported by Dodge et al. [12].

8-Anilino-1-naphthalene sulfonic acid sodium salt (ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Tokyo Kasei Co. Ltd. Erythrocyte ghosts were labeled with ANS or DPH by incubation (at 37°C for 60 min) with gentle shaking. The labeled erythrocyte ghosts were washed once with Tris buffer solution. Centrifuged ghosts were resuspended in the same buffer to a concentration of 1.0 vol.% and were used for fluorescence measurements.

Distilled *N*-vinylimidazole was dissolved in dehydrated methanol. The solution was added with azobis(isobutyronitrile) as an initiator of radical polymerization, then this solution was packed in a vacuum tube. This was degassed three times and then the tube was sealed. The polymerization of *N*-vinylimidazole was performed by gentle shaking in a thermost-controlled bath at 50°C for 6 h. The polymer solution obtained was poured into an excess of diethylether. Poly(*N*-vinylimidazole), obtained as white precipitate, was washed with dehydrated diethylether and was dried in vacuo.

Poly(*N*-vinylimidazole) (PVI) was quaternized by alkylbromides such as ethylbromide, hexylbromide or laurylbromide. Dimethylsulfoxide solutions of PVI were mixed with alkylbromides in vacuum tubes. Degassed solutions were sealed under reduced pressure and were kept at 40°C (for the ethylbromide-added system) or 80°C for 20–48 h. As partially quaternized PVI by laurylbromide was less soluble in an aqueous medium, this polycation was requaternized by ethylbromide in ethanol at 40°C for 20 h

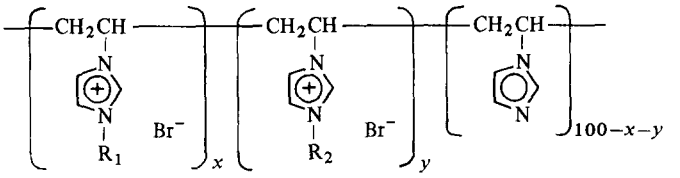
The structure of the applied polycations is summarized in Table I.

Poly(ethylene glycol)s (PEG) with average molecular weights of 7500 and 20 000 were purchased from Wako Pure Chem. Co. Ltd. A chloroform solution of PEG was poured into an excess of dehydrated diethylether to precipitate the purified PEG. White precipitate was washed several times by dehydrated diethylether on glass filter to remove the contaminants of low molecular weight and was dried in vacuo Poly(*N*-vinyl-2-pyrrolidone) (PVPo) was obtained by radical polymerization of *N*-vinyl-2-pyrrolidone in degassed methanol at 50°C for 6 h with azobis(isobutyronitrile) as an initiator. The reaction mixture was poured into an excess of diethylether. Then the washed polymer was dried in vacuo. Its average molecular weight was 40 000, which was calculated from viscosity measurement.

Highly purified, salt-free concanavalin A was purchased from Sigma, and was used without further purification.

TABLE I

LIST OF THE APPLIED POLYCATIONS



Polycation	R_1	R_2	*x* (%)	*y* (%)
PVI [a]	–	–	0	0
PIE_{16} [b]	$-C_2H_5$	–	16	0
PIE_{35}			35	0
PIE_{62}			62	0
PIE_{93}			93	0
PIH_3 [c]	–	$-C_6H_{13}$	0	3
PIL_{10} [d]	$-C_2H_5$	$-C_{12}H_{25}$	19	10

[a] Poly(*N*-vinylimidazole), $[\eta]^{30°C}_{EtOH} = 0.28$ (dl/g), systematic name poly[1-(1-imidazolyl)ethylene] [b] Systematic name poly[1-(1-imidazolyl) ethylene-co-1-(3-ethylimidazolium-1-yl)ethylene bromide] [c] Systematic name. poly[1-(1-imidazolyl)ethylene-co-1-(3-hexylimidazolium-1-yl)ethylene bromide]. [d] Systematic name· poly[1-(1-imidazolyl)ethylene-co-1-(3-ethylimidazolium-1-yl)ethylene bromide-co-1-(3-laurylimidazolium-1-yl)ethylene bromide].

Methods

Fluorescence intensities of ANS or DPH which were incorporated into the ghost membranes were measured by fluorescence spectrometer JASCO FP-550. Fluorescence intensities were corrected for light-scattering background by subtracting a probe-free blank solution which contained ghost membranes and polymers. The wavelengths of 360 and 366 nm were used for excitation of membrane-incorporated ANS and DPH, respectively. The emission intensities from ANS and DPH were detected at 476 and 433 nm, respectively. The slit widths for both excitation and emission were 5 nm. The fluorescence polarization and intensities were obtained by measuring the fluorescence intensities polarized parallel ($I_{//}$) and perpendicular ($I_{\perp}$) to the direction of the polarized excitation beam. Degree of polarization (P) is defined by the following equation.

$$P = (I_{//} - I_{\perp})/(I_{//} + I_{\perp}) \quad (1)$$

Results

Polycation-induced increase of membrane fluidity of human erythrocyte ghosts

The interaction of poly(L-lysine) or poly(L-glutamic acid) with phospholipid bilayer vesicles were studied by some workers [7,13,14]. These polypeptides can interact with lipid membranes through some secondary binding forces such as electrostatic force, hydrogen bond, hydrophobic interaction force, and so on. It is known that they decrease the fluidity of phospholipid vesicle membranes monotonically [8,14]. On the other hand, polycations increased the membrane fluidity of human erythrocyte ghosts immediately after mixing with relatively concentrated polycation solutions (more than $(1-2) \cdot 10^{-2}$ wt%). So the effect of polycations on the fluidity of ghost membrane was checked in a dilute polycation solution. The degree of polarization (P), defined by Eqn. 1, was used as one of the fluidity parameters of cell membranes. Each solution of polycation and ghosts was mixed with equivolume at 37°C. It was observed that the changes of membrane fluidity induced by a dilute polymer solution were relatively slow. So the polarization (P) was calculated by means of fluorescence measurement 60 min after mixing (incubated at 37°C). The increase of membrane fluidity of human erythrocyte ghosts was remarkable when polycations with higher cation density were added (see Fig. 1). Further addition of these polycations caused the agglutination of ghost membranes.

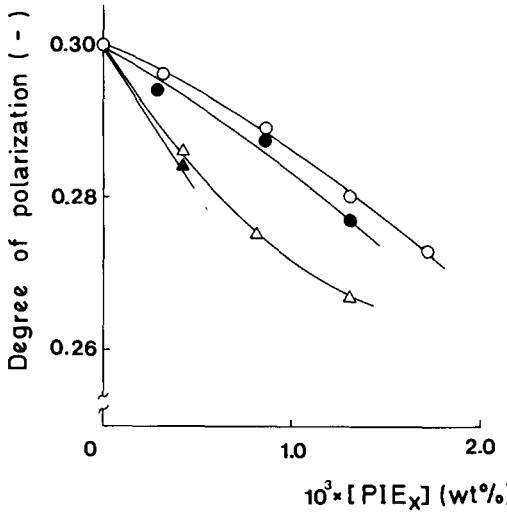


Fig. 1. Effect of polycations (PIE_X) on the fluidity of human erythrocyte ghosts 60 min after mixing at 37°C. DPH was incorporated into ghost membrane by 60-min incubation Labeled ghosts were washed once and buffered solution of the ghosts (2 wt%) was added to solutions of polycations with different concentrations by equivolume mixing at 37°C Every solution was incubated at 37°C for 60 min, then fluidity was measured by means of the fluorescence polarization method. Degree of polarization was calculated from Eqn. 1 (see Methods) [DPH] = 1 0 10^{-6} M ○ PIE_{16}, ● PIE_{35}, △ PIE_{62}, ▲: PIE_{93}

PIH_3, quaternized PVI with hexylbromide at a repeating unit mol% of 3, showed strong interaction with ghost membranes. The polarization trace varied with successive addition of PIH_3 in a bell-shaped manner when ANS was used as fluorescent probe. The concentration of PIH_3 at which the polarization reached minimum after 60 min incubation was low ($2.5 \cdot 10^{-5}$ wt%) in spite of quite low cation density. This concentration was about 100-fold smaller than those of PIEs (PVI quaternized with ethylbromide). On the other hand, when DPH was used as probe molecule, the polarization increased a little, reaching a constant value.

PIL_{10}, quaternized PVI with laurylbromide and ethylbromide at 10% and 19%, respectively, showed much stronger interaction with ghost membranes. The polarization (P) reached constant (about 0.42)

TABLE II

Final polymer concentration (C_{min}) at which the minimum polarization of human erythrocyte ghosts was obtained after 60-min incubation at 37°C.

Polymer	Probe	C_{min} in wt.%
PEG	ANS, DPH	$2.3 \cdot 10^{-2}$
PVPo	ANS, DPH	$2.2 \cdot 10^{-2}$
PVI	ANS	$1.9 \cdot 10^{-2}$
PIE_x	ANS, DPH	more than $2.0 \cdot 10^{-3}$ [a]
PIH_3	ANS	$2.5 \cdot 10^{-5}$
PIL_{10}	ANS, DPH	less than 10^{-10} [b]

[a] Further addition of polymer made the aggregation of ghost membranes instantaneous.

[b] Ghost membranes were partially lysed, see text.

60 min after mixing with PIL_{10} in the concentration range from 10^{-10} to 10^{-6} wt% when DPH was used as probe molecule. The same tendency was observed using ANS instead of DPH. The minimum point would be obtained if more dilute PIL_{10} solutions were added. The destruction of ghost membranes was also observed with the addition of a relatively concentrated PIL_{10} solution which was detected by electron microscopy. Polycations, having longer alkyl chains, were found to induce extreme agglutination and hemolysis of human erythrocytes, as previously predicted [15]. Table II shows the concentrations of several polymers at which maximum fluidity was observed 60 min after mixing (incubated at 37°C).

Nonionic polymer-induced increase of membrane fluidity of human erythrocyte ghosts

Fig. 2 shows the degree of polarization (P) of human erythrocyte ghosts after 60-min incubation with poly(*N*-vinyl-2-pyrrolidone). It can be seen in Fig. 2 that membrane fluidity reached maximum when PVPo at a concentration of approx. 0.02 wt% was added. This value (approx. 0.02 wt%) was not dependent on the species of the fluorescent probe. Thus, the polarization changes of ANS were quite similar to those of DPH. On the other hand, in the case of PEG, the changes in degree of polarization caused by ANS were larger than those caused by DPH, as shown in Fig. 3. The concentrations of polymers at which the polarization reached minimum 60 min after mixing (C_{min}) are listed in Table II. C_{min} is the polymer concentration at which the

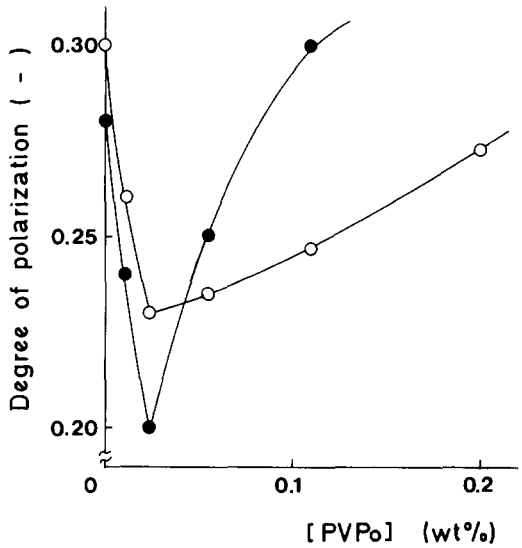


Fig 2. Effect of poly(*N*-vinyl-2-pyrrolidone) on the fluidity of human erythrocyte ghosts 60 min after mixing at 37°C DPH (○) and ANS (●) were used as probe molecules. The procedure was as noted in Fig. 1 [DPH] = [ANS] = $1.0 \cdot 10^{-6}$ M.

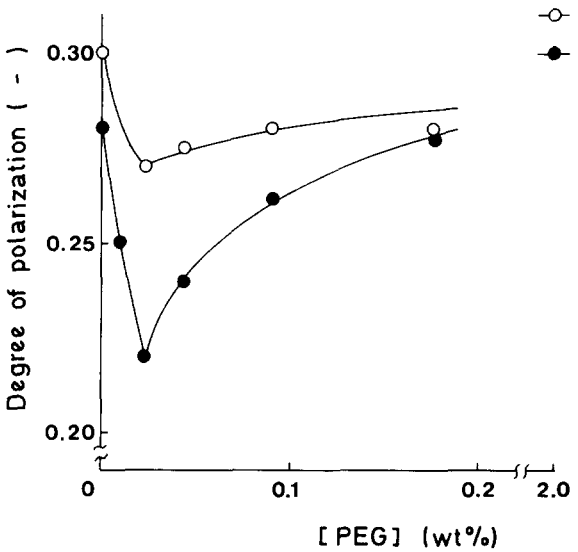


Fig. 3. Effect of poly(ethylene glycol) on the fluidity of human erythrocyte ghosts 60 min after mixing at 37°C DPH (○) and ANS (●) were used as probe molecules. The procedure was as noted in Fig. 1 [DPH] = [ANS] = $1.0 \cdot 10^{-6}$ M.

membrane fluidity reached maximum 60 min after mixing (incubated at 37°C).

Time course and polymer concentration dependence of the increase of membrane fluidity induced by poly(ethylene glycol)

It was suggested from the results described above that there should exist a kind of relationship between the membrane fluidity and polymer concentration or incubation time. The most-fluid state of ghost membrane could be expected after a shorter period of incubation if more concentrated polymer solutions were applied. Fig. 4 shows the time-course of polarization changes induced by PEG with a molecular weight of 7500. There appeared an obvious dependence of PEG concentration on the time at which the polarization reached minimum (T_{min}). Thus, the membrane fluidity was increased immediately by the addition of a concentrated PEG solution. The minimum polarization also depended on PEG concentration. The minimum P values was 0.220 and T_{min} was 60 min when PEG with a final concentration of $2.3 \cdot 10^{-2}$ wt% was added. On the other hand, the P value was reduced a little (0.245) but T_{min} fell to about 15 min when PEG with a concentration of 20 wt% was added. The effect of PEG concentration on the (temporary) increase of membrane fluidity is summarized in Fig. 5.

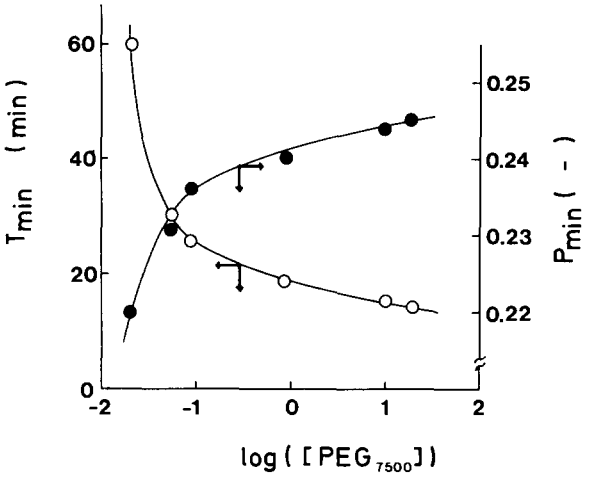


Fig. 5. Effect of PEG concentration (in wt%) on the increase of membrane fluidity of human erythrocyte ghosts at 37°C. A labeled ghost dispersion was mixed with PEG solution at 37°C and fluorescence intensities were measured immediately. Intensities were successively recorded for 60 min at 37°C. Average molecular weight of PEG is 7500. T_{min}, time at the degree of polarization reached minimum (most-fluid state), P_{min}, the minimum degree of polarization.

The same experiments were done using PEG with a molecular weight of 20000 to clarify the effect of

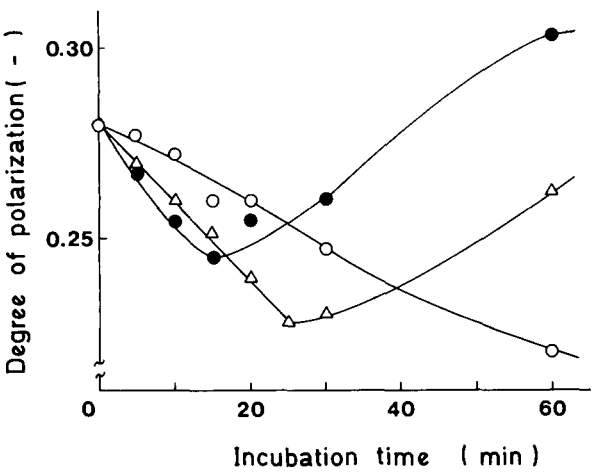


Fig 4. Time-dependence of the fluidity changes of human erythrocyte ghosts induced by the addition of poly(ethylene glycol)s (M_r = 7500) with different concentrations at 37°C. Final concentrations of PEG were 20 (•), 0 9 (△) and $2.3 \cdot 10^{-2}$ (○) in wt%, respectively. ANS was used as probe molecules.

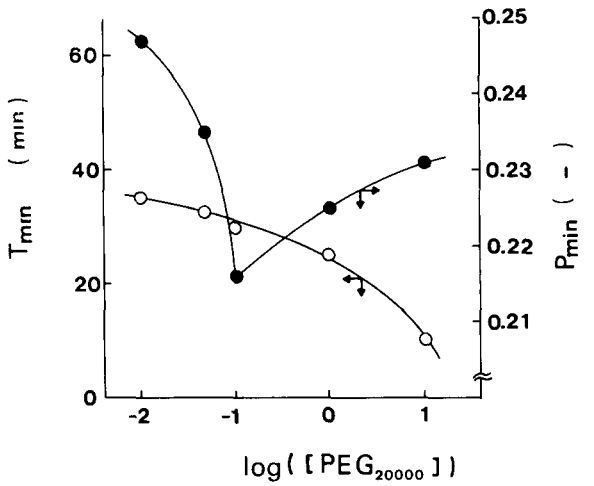


Fig. 6. Effect of PEG concentration (in wt%) on the increase of membrane fluidity of human erythrocyte ghosts at 37°C. The average molecular weight of PEG was 20000. The experimental procedure was as noted in Fig. 5 [ANS] = $1.0 \cdot 10^{-5}$ M

the molecular weight of applied polymers. Though PEG with molecular weight 7500 shows a normal relationship, as shown in Fig. 5, PEG with molecular weight 20000 shows an unusual relationship. Results of the $PEG_{20\,000}$-added system are shown in Fig. 6. T_{min} decreased monotonically with the increase in PEG concentration, as shown by the open circles. However, P_{min} varied, with a sharp bend at a PEG concentration of about 0.1 wt%. The extraordinary behaviour was explained by the difference of chain length of PEG added, in other words, by the difference in their interaction force.

Discussion

It is well-known that several kinds of polymers can interact with each-other through electrostatic interaction force, hydrogen bonding and/or hydrophobic interaction force, and form interpolymer complexes in solution [16–19]. Amorphous polyelectrolyte complexes are formed by rapid desolvation upon mixing two solutions of oppositely charged polyelectrolytes. An extended polyelectrolyte chain shrinks and the intrapolymer mobility is reduced by the complex formation [17,19]. The surface of the cell membrane is surrounded by oligosaccharide chains which are covalently bound to membrane proteins such as sialoglycoproteins. The polymer complex formation can also be observed on the cell surface when water-soluble polymers are added. In general, water-soluble polymers can cause cell agglutination, and in some cases, membrane-bound proteins are forced to form clusters on the membrane [9].

Polycations such as PIE_x can interact with not only acidic residues of glycoproteins such as sialic acid but also with phospholipids through electrostatic interaction force. When polycations can be expected to interact with oligosaccharide chains initially on the surface of cell membranes, this might induce the cluster formation of glycoproteins. This step might correspond to the increase of membrane fluidity shown in Fig. 1. Then the lipid layers were exposed to the water phase by cluster formation. As polycations can interact strongly with saccharides, proteins or lipids through intracellular and/or intercellular interaction force, almost all polycations show remarkable ability to agglutinate the cell membranes.

It was reported in the studies of fluorescence polarization [10] or ESR [11,20] measurements that the increase in fluidity of human peripheral lymphocytes or hen erythrocytes was induced by some lectins. ANS and DPH were usually used as fluorescent probes to evaluate the fluidity of cell membranes [21–25]. It has been reported in these studies that the aggregation of intramembraneous particles (cluster formation) was observed after about 30-min incubation with several lectins. It should be noted that the fluorescence polarization was deeply affected by the fluorescence lifetime of the probe molecules. The lifetimes of ANS and DPH were 6.8 ns and 10.8 ns, respectively. These values were not affected by the addition of some synthetic polymers. The lifetimes were almost the same as those which have already reported [24,25]. So the changes of fluorescence polarization induced by the addition of polymers were considered to be due to the changes of molecular motion. Fig. 7 shows the fluidity change of human erythrocyte ghost with the addition of concanavalin A. As concanavalin A has a strong affinity for a specific site of saccharide chains, a small amount of concanavalin A can bind with saccharide chains to form clusters of glycoproteins on cell membranes. As shown in Figs. 2, 3 and 4, the membrane fluidity

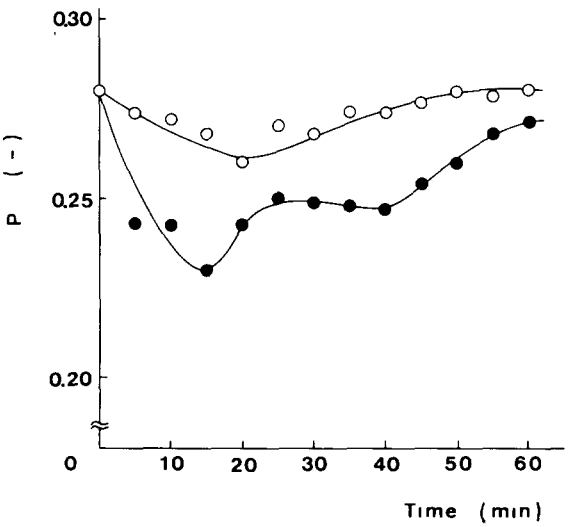


Fig. 7. Effect of concanavalin A concentration on the changes of membrane fluidity of human erythrocyte ghost at 37°C. [ANS] = $1.0 \cdot 10^{-5}$ M.

changes induced by water-soluble polymers were very similar to those induced by concanavalin A. Recently, the aggregation of intramembraneous particles has been observed by adding a large amount of PEG using the freeze-fracture technique [9]. PEG or PVPo can interact with proton-donating polymers to form polymer complexes through hydrogen bond and/or hydrophobic interaction force [18,19,26,27]. They also bind with hydroxy (alcoholic) groups of the oligosaccharide chains of glycoproteins [28]. Such polymer complexes should also be formed on the surface of ghost membranes. As the result, clusters of proteins or glycoproteins could be formed to exposure lipid layers to the water phase. As the result of cluster formation, the lipid layers are considered to be exposed to the water phase and molecular motion of lipids is increased. An excess of polymer then attack the exposed lipid layers. This step was observed as the decrease of membrane fluidity. The membrane fluidity was decreased when an excess of polymers interacted with the exposed lipid layers. This interaction has already been clarified by the analysis of the interaction between several polymers and phospholipid vesicle membranes [7,8].

The interaction between soluble polymers was strengthened with increasing chain lengths of polymers; that is one of the characteristic of polymer complex formation [26]. It is clear from the results shown in Figs. 5 and 6 that the change of membrane fluidity induced by PEG with molecular weight 20 000 was more dynamic than that induced by PEG with shorter chain length. PEG with molecular weight 20 000 interacted more strongly with saccharide chains due to its high molecular weight. The dependence of molecular weight of PEG on the membrane fluidity was observed especially when PEG solution with relatively high concentrations (more than 0.1 wt%) were added, as shown in Fig. 6. A considerable bend in the [PEG] vs. P_{min} curve may be observed (as seen in Fig. 6). Although the exact reason for the appearance of this bend has not been clarified yet, it might be explained by the higher molecular weight of PEG. In dilute PEG solution, it takes longer than 30 min for the cluster formation of glycoprotein. A part of PEG chains which have already attached to saccharide chains on cell membranes may simultaneously attach to naked phospholipid layers during a longer period of incubation (more than 30 min). Thus the clustering of glycoproteins may not be observed sufficiently.

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