

## Complex Formation of Hyaluronic Acid or Chondroitin Sulfate with Glycol Chitosan

Katsuro SHINODA and Akio NAKAJIMA\*

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The interaction between a basic polysaccharide, glycol chitosan, and an acidic polysaccharide with relatively low charge density, chondroitin sulfate A, chondroitin sulfate C or hyaluronic acid, was studied by turbidimetric, potentiometric and metachromasy measurements. The results suggest that a 1:1 ladder form or a form of pyranose-pairing aggregate may be the most favorable structure for these systems.

### INTRODUCTION

In the previous paper,<sup>1)</sup> we investigated the interactions between glycol chitosan and anionic polysaccharides with high charge density, heparin and sulfated cellulose. The result indicated that the backbone chain conformations of component polymers together with the location of ionizable groups are important factors for those systems.

In this paper, we report the interactions between glycol chitosan (GC) with anionic polysaccharides with relatively low charge density, chondroitin sulfate A (CSA), chondroitin sulfate C (CSC) and hyaluronic acid (HA) in order to investigate further the effect of the conformations and the chemical structure of the polysaccharides on the interactions.

HA is an alternating copolymer of N-acetyl-D-glucosamine and D-glucuronic acid. CSA is an alternating copolymer of N-acetyl-D-galactosamine-4-sulfate and D-glucuronic acid. CSC is 6-sulfate instead of 4-sulfate in CSA. Recently the crystalline chain conformations of HA, CSA, and CSC were elucidated by X-ray diffraction investigation.<sup>2)</sup> Helical conformations were indicated for these polysaccharides. GC is a copolymer of N-acetyl-D-glucosamine-6-glycol and D-glucosamine-6-glycol. Both HA and CS are widely distributed in different connective tissues. Chitin, the parent polymer of GC, is an important component of the skeletal material of the crustasia.

### EXPERIMENTAL

#### Materials

Glycol chitosan (Lot: LM9931) was purchased from Wako Junyaku Co., Kyoto, and was used after dialysis followed by deionization through an ion exchange resin, Amberlyte MB-1. Chondroitin sulfate A-Na (Lot: V3P8089), chondroitin sulfate

\* 篠田勝郎, 中島章夫: Department of Polymer Chemistry, Faculty of Engineering, Kyoto University, Kyoto.

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C-Na (Lot: V3P8090) and hyaluronic acid-K (Lot: V3K5808) were purchased from Nakarai Chemicals Co., Kyoto, and were used after dialysis.

#### Characterization of Polymer Samples

The molecular weights  $M$  of these polymer samples were estimated from the limiting viscosity numbers  $[\eta]$  in specified solvent systems.<sup>3-5)</sup> Contents of ionizable groups of these polymers were measured by conductometric titrations with a Yanagimoto Conductivity Outfit Model MY-8. The experimental results were shown in Table I.

Table I. Characterization of Polysaccharides

Polymer	(dl/g)	$M$	Remarks on ionizable groups	Purity (%)
GC	0.612 (in 0.1 N NaOH)	105,000 <sup>3)</sup>	0.62 $\text{NH}_2$ /pyranose	
HA	1.405 (in 0.2 M NaCl)	850,000 <sup>4)</sup>	1 COOH/2.6 pyranose	78
CSA	0.120 (in 0.15 M sodium phosphate buffer contg. 0.2 M NaCl)	43,000 <sup>5)</sup>	$\text{HSO}_3$ : COOH=1.0: 1.0	81
CSC	0.121 (ditto)	45,000 <sup>5)</sup>	$\text{HSO}_3$ : COOH=1.0: 1.0	80

The  $\text{NH}_2$  content in GC was 0.62 per pyranose ring. The ratio of the numbers of two ionizable groups, *i.e.*, the number of sulfonic groups to that of carboxyl groups, in CSA and CSC was determined to confirm the theoretical relation,  $\text{HSO}_3$ : COOH=1:1. The purity cited in the fifth column designates the % of COOH observed to the theoretical amount of COOH. The concentrations of ionizable groups mentioned hereafter are based on these purities.

The degrees of dissociation of  $\text{NH}_2$  group in GC, and of COOH group in CSA, CSC, and HA were determined by potentiometric titrations. The initial concentration of polyelectrolyte was about 0.003 mol ionizable groups per liter. Titrations

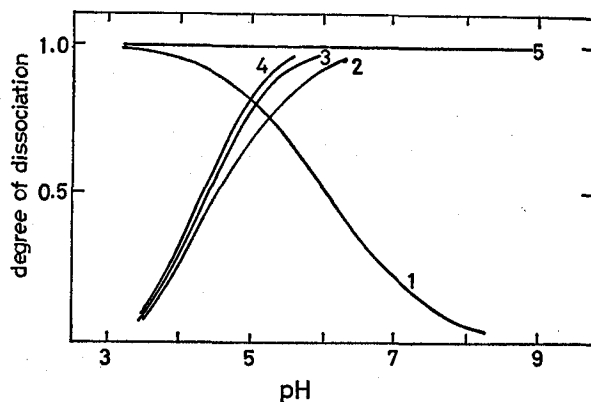


Fig. 1. Degrees of dissociation plotted against pH for  $\text{NH}_2$  groups of GC (1), for COOH groups of CSA (2), CSC (3), and HA (4), and for  $\text{SO}_3\text{H}$  groups (5).

were performed in the presence of about 0.005 M NaCl under N<sub>2</sub> atmosphere with a Hitachi-Horiba pH-Meter Model F-7<sub>ss</sub>. In Fig. 1, the degree of dissociation was plotted against the pH for NH<sub>2</sub> group of GC (curve 1), and COOH group of CSA (curve 2), CSC (curve 3), and HA (curve 4). With respect to SO<sub>3</sub>H group, a strong electrolyte, degree of dissociation is unity independent of pH, as a matter of course. The apparent dissociation constants pK<sub>a</sub> were listed in Table II.

Table II. Apparent Dissociation Constants pK<sub>a</sub> of Ionizable Groups in Polysaccharides

Polymer	Ionizable group	pK <sub>a</sub>
	<i>Polycation</i>	
GC	-NH <sub>2</sub>	6.0
	<i>Polyanion</i>	
HA	-COOH	4.3
CSA	-COOH	4.5
CSC	-COOH	4.4

### Formation of Polyelectrolyte Complexes

The polysaccharides mentioned above were, unless otherwise noted, dissolved at ca. 0.015 g/dl concentration in various buffer solutions of different pH values of ionic strength of ca. 0.005. The polyanion solution at a fixed pH was then added with stirring in different ratios to the polycation solution having the same pH. The pH differences between before and after the addition of the polyanion were within 0.02. Complex formation was followed by the measurements of turbidity and metachromasy of the mixtures. Turbidity measurements were carried out at a wave length of 430 nm with a Shimadzu-Kotaki Photoelectric Nephelotitrator Type NT 3H. Metachromasy measurements were carried out as follows. After the polyanion solution was added to the polycation solution, the precipitates if any were removed by centrifuge, and Toluidine Blue was added to the supernatant solution. Thus the color change of Toluidine Blue was measured with the visible spectrum with a Hitachi Spectrophotometer Model EPS-3T. The turbidity  $\tau$  of the mixtures and the absorption metachromasy were plotted against the mixing ratio of polyanion to polycation.

### RESULTS AND DISCUSSION

In Fig. 2 and Fig. 3, the turbidity  $\tau$  was plotted against the mixing ratio  $R$  for GC-CSA and GC-HA systems. The mixing ratio  $R$  was defined as

$$R = \frac{PC_{py}}{PC_{py} + PA_{py}} \quad (1)$$

where PC<sub>py</sub> and PA<sub>py</sub> are the numbers of ionizable groups represented by the numbers of pyranose rings for polycation and polyanion, respectively. In these systems, the value of  $\tau$  changed with increasing time of standing. Figure 2 shows the effect of time standing after the mixing. At near the maximum point, the complex formed precipitates with increase of time and thus the turbidity of the supernatant solution

### Complex Formation of Hyaluronic Acid

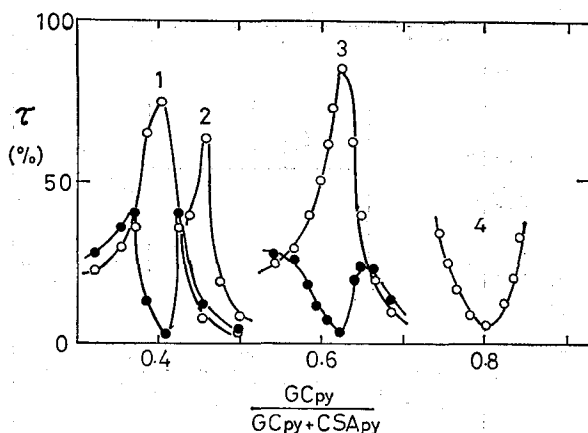


Fig. 2. Turbidity vs. mixing ratio for GC-CSA system at pH=2.75 (1), 4.50 (2), 6.86 (3), and 7.95 (4). ○: at 15 min after mixing, ●: at 24 hr after mixing.

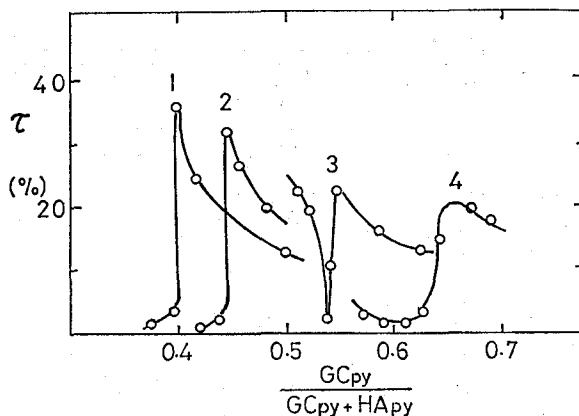


Fig. 3. Turbidity vs. mixing ratio for GC-HA system at pH=3.50 (1), 5.02 (2), 6.03 (3), and 6.75 (4).

decreases. However, the mixing ratio of the maximum turbidity appeared at earlier stage of standing coincides with that of minimum point at later stage in a similar manner as GC-heparin system.<sup>1)</sup> At any rate, it is evident that yield of polyelectrolyte complex is maximum at the mixing ratio where the turbidity is maximum or minimum.

Complex formation was pursued also with metachromasy, parallel to the turbidity measurements. The metachromatic complexes of acidic polysaccharides with cationic dyes have been the basis of observation and identification for long years. Recently, the conformation of some mucopolysaccharides in aqueous solution has been studied using this phenomena.<sup>6)</sup> In Fig. 4, the ratio of absorptions at 644 nm to 544 nm, *i.e.*, absorption of the original color of Toluidine Blue to that of the metachromatic complex, was plotted against the mixing ratio (at pH 7.95). The mixing ratio  $R$  at the point of color change, *i.e.*,  $E_{644}/E_{544}=1.0$ , coincides with that of the minimum point of turbidity. (see curve 4 in Fig. 2) This result may afford a basis for that the mixing ratio of maximum or minimum turbidity is regarded as the composition of the poly-

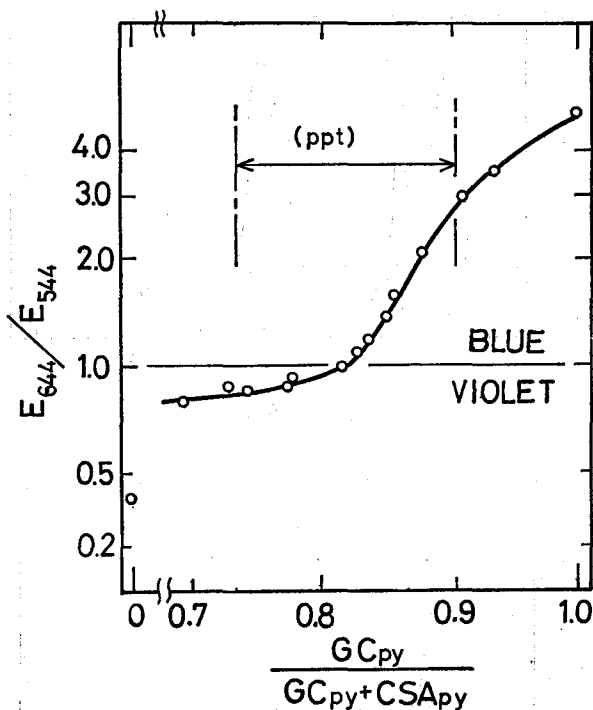


Fig. 4. Absorption ratio at 644 nm to 544 nm plotted against mixing ratio for GC-CSA system at pH=7.95. Initial GC concentration: 0.0004 mol/l. Toluidine Blue concentration: 0.0005 mol/l.

electrolyte complex. With respect to HA, metachromatic reaction was not observed under similar condition to GC-CSA system.

It is reported that HA is a poor chromotrope in dilute solution owing to the low charge density and high tendency to form a multimolecular structure.<sup>7)</sup> Therefore, direct measurement would be difficult under these conditions. However, indirect method, in which a small amount of strong anionic polysaccharide is added, indicated that  $R$  of maximum turbidity was in agreement with  $R$  where the color change occurred.

When a polyelectrolyte reacts with an oppositely charged polyelectrolyte stoichiometrically, a neutral complex is formed. In such a case, the following equations should hold for GC-CS and GC-HA systems, respectively.

$$C_{(\text{HSO}_3)} + \alpha C_{(\text{COOH})} = \beta C_{(\text{NH}_2)} \quad \text{for GC-CS} \quad (2)$$

$$\alpha C_{(\text{COOH})} = \beta C_{(\text{NH}_2)} \quad \text{for GC-HA} \quad (3)$$

where  $C_{(\text{HSO}_3)}$ ,  $C_{(\text{COOH})}$  and  $C_{(\text{NH}_2)}$  are the molar concentrations of the ionizable groups designated in the parentheses, and  $\alpha$  and  $\beta$  are the degrees of dissociation of COOH and  $\text{NH}_2$ , respectively. If we assume that the coexisting oppositely charged polyions have no influence on the value of  $\alpha$  and  $\beta$ , the stoichiometric composition at given pH can be calculated by using the results given in Fig. 1 without any corrections. Such stoichiometric composition as well as the experimental plots obtained from Fig. 2 and Fig. 3 was shown in Fig. 5 and Fig. 6.

With respect to experimental result of CSC, the turbidity curves was similar to

Complex Formation of Hyaluronic Acid

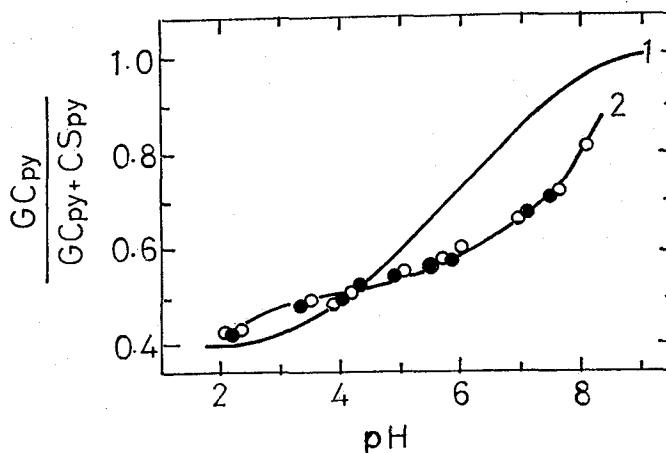


Fig. 5. Composition of complex plotted against pH for GC-CSA (○) and GC-CSC (●) systems.

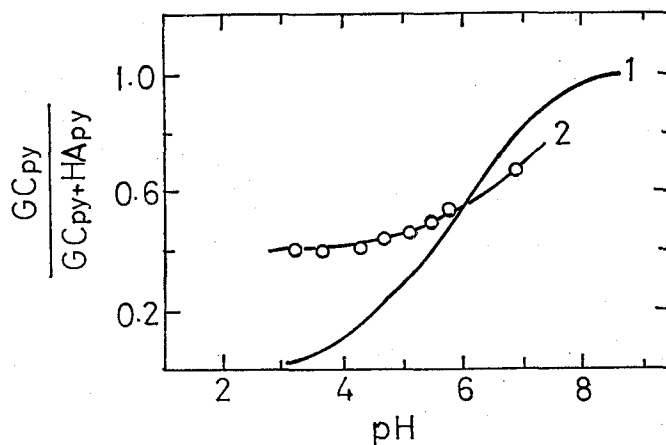
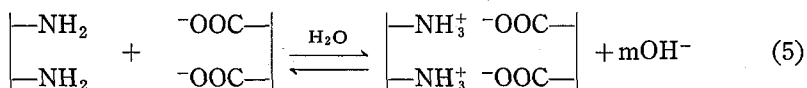
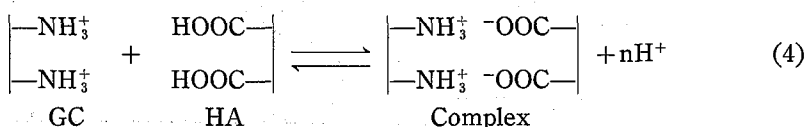


Fig. 6. Composition of complex plotted against pH for GC-HA system.

those of CSA, therefore, only the experimental points were shown in Fig. 5. As shown in Fig. 5, both plots of CSA (open circles) and CSC (filled circles) fall on a same curve (curve 2). In this case, the calculated curve (curve 1) crosses curve 2 at a complex composition  $R$  of 0.5. Since  $GC_{py}$  and  $CS_{py}$  are given by the numbers of pyranose ring,  $R = GC_{py} / (GC_{py} + CS_{py}) = 0.5$  means that the formed polyelectrolyte complex is composed of equal numbers of pyranose rings of polycation and polyanion. In other words, the complex GC-CS may be in a 1:1 ladder form or in a form of pyranose-pairing aggregates. Furthermore, the slope of curve 2 is small near the point of  $R=0.5$ . This may indicate that such pyranose-pairing form is the most favorable structure for the GC-CS system.

The GC-HA system is a combination of a weak base and a weak acid. As is shown in Fig. 6, curve 1 crosses curve 2 at  $R$  of ca. 0.5. The  $R$  value of all experimental points ranging in pH from 3 to 7 are nearly 0.5. This may also indicate that the 1:1 ladder structure is the most favorable structure for the GC-HA system. Discrepancy between both curves in Fig. 6 is large at pH 3.50 where GC is fully but HA is slightly

charged. This discrepancy may be caused by the following two factors: (1) geometrical hindrance may let charged groups free and (2) the dissociation of uncharged dissociable groups is induced by the coexisting oppositely charged groups. In the former case, the complex should have the net charge calculated from the composition and the values of  $\alpha$  and  $\beta$  obtained from Fig. 1 as a function of pH. So the discrepancy may be an indicative of the ionic nature of the complex. In the latter case, the following reactions should occur.



pH measurement may be used to examine this factor. The pH difference between before and after the mixing of GC with HA solution having the same pH and not including buffer should relate to the number of hydrogen or hydroxyl ion originated along complex formation. From Equations (4) and (5), the apparent increase in hydrogen ion concentration and in hydroxyl ion concentration is estimated from:

$$\Delta[\text{H}^+] = n - m \quad (6)$$

$$\Delta[\text{OH}^-] = m - n \quad (7)$$

Where  $n$  and  $m$  are the numbers of induced dissociated COOH and  $\text{NH}_2$  groups, respectively. If no mutual interaction exists, then no pH change will be observed. In Fig. 7, the amount of increased hydrogen ion is plotted against the mixing ratio for the mixture of HA and GC solutions both having the same pH ( $\text{pH}=3.51$ ), and the same concentration (0.00033 mol/l). At pH 3.51, GC is almost fully charged but HA is slightly charged. (see Fig. 1) So, the  $\Delta[\text{H}^+]$  may depend mostly upon the induced dissociation of COOH groups. We can find that about 70% of  $\text{NH}_3^+$  groups in GC-HA complex formed at pH 3.5 is neutralized, by adopting the result of Fig. 7

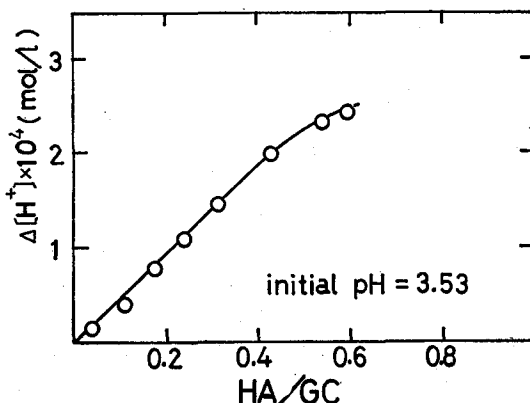


Fig. 7. Amount of the increased hydrogen ion plotted against mixing ratio for GC-HA system. GC concentration: 0.0004 mol/l (based on ionizable groups). Initial pH=3.53.

### Complex Formation of Hyaluronic Acid

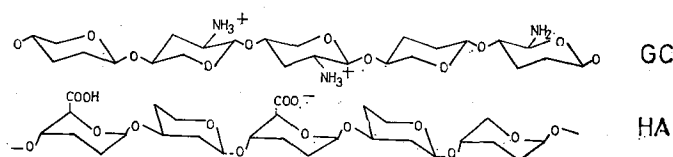


Fig. 8. Schematic model for a ladder complex composed of 1 HA pyranose and 1 GC pyranose.

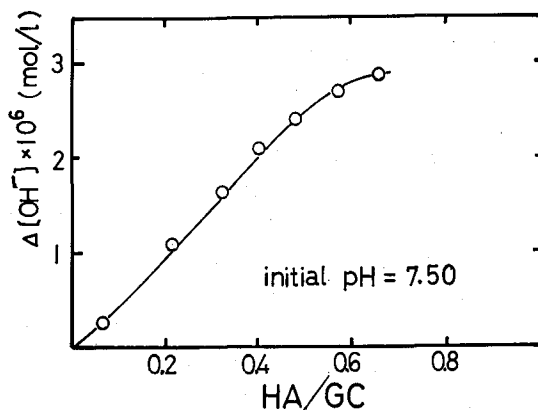


Fig. 9. Amount of the increased hydroxyl ion plotted against mixing ratio for GC-HA system. GC concentration: 0.0004 mol/l. Initial pH=7.50.

to that of Fig. 5. Katchalsky, *et al.*<sup>8)</sup> and Nagasawa, *et al.*<sup>9)</sup> suggested that the nearest-neighbor charges play very important role on the dissociation equilibrium of a polyelectrolyte. Therefore, such a large number of induced dissociation of HA indicates that the polyanion and polycation may form a ladder-like complex as shown in Fig. 8 which is regarded as the most approachable structure for the oppositely charged polyelectrolytes in question. Similar structure was proposed for  $\alpha$ -chitin by Carlstrom<sup>10)</sup> from the X-ray data.

Similar plot in a weak basic pH region was indicated in Fig. 9. In this pH region, HA is almost but GC is slightly charged, contrary to the condition of Fig. 7. In this case, the amount of increased hydroxyl ions is very small, that is, induced dissociation on the GC molecules is rarely achieved. These results may be caused by the following reasons. Since polyanion solution was added to polycation solution throughout these experiments, the initial conformation of GC should be important. In the low pH region, GC would adopt stretched conformation by its high charge density, therefore, added HA can be easily ionized and form a ladder-like complex. On the other hand, in the basic pH region, GC would adopt coiled conformation, therefore, added HA may randomly react with GC and induces scarce dissociation on account of geometric hindrance. As a result, the formed complex may be in a form of scramble salt or random aggregate.

To confirm such presumption, scanning electron micrographs of the complex were shown in Fig. 10. A and B in Fig. 10 are the GC-HA complexes formed at pH 3.4 and pH 7.5, respectively. In A, fibrous network structures were clearly observed. On the other hand, the complex formed at pH 7.5 is amorphous. These structures



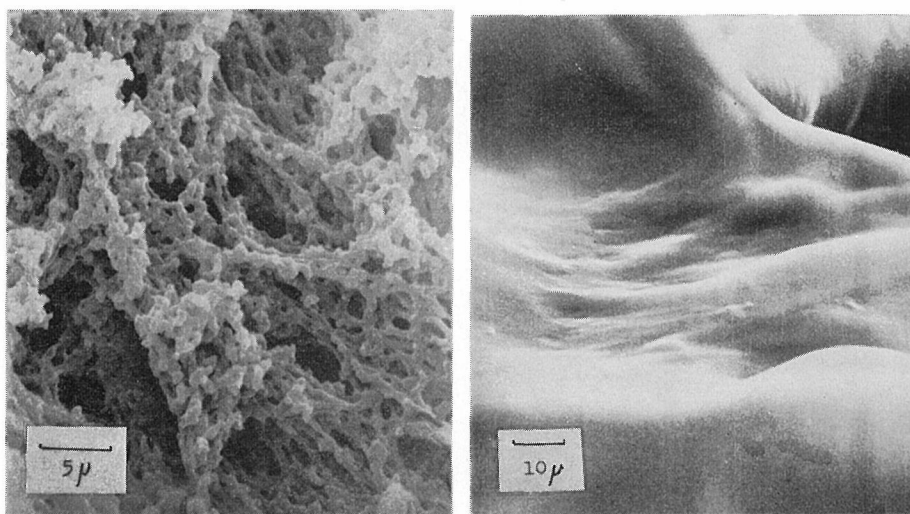


Fig. 10. Scanning electron micrographs of GC-HA complex. A, formed at pH 3.45; B, formed at pH 7.50.

may support our proposed mechanism for complex formation.

Our conclusion for the present systems is that the backbone chain conformation of component polymers together with the kind and location of ionizable groups is important factor to discuss the formation and structure of polyelectrolyte complexes.

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