Bull. Chem. Soc. Jpn., 64, 1814—1820 (1991) [Vol. 64, No. 6

Complex Formation of Gelatin and Chondroitin Sulfate and the Effects on H^+ Ion Binding

Masakatsu Yonese,* Mikio Yano, and Hiroshi Kishimoto
Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467

(Received November 14, 1990)

Soluble and insoluble complex formations of gelatin and chondroitin 6-sulfate (Chs-C) were studied by measuring their viscosities and transmittances; the effects of complex formations on H^+ ion binding were studied by pH titrations. Soluble complexes were formed electrostatically with low ionic strengths; the compositions of the complexes varied with pH from those of solutions. The weight fraction of Chs-C of the saturated soluble complex was found to be 0.294 at pH=5.1. The composition of the insoluble complexes increased in the fraction of Chs-C with decreasing pH. The compositions were found to agree with the theoretical predictions obtained from the condition of electroneutrality. Furthermore, from pH titrations of mixed solutions comprising gelatin and Chs-C, the apparent dissociation constants, $\mathrm{p}K_\mathrm{a}$ of the carboxylic groups of both polymers were obtained. They were found to increase due to soluble complex formation; soluble complex formation depresses the dissociation of H^+ ions due to a shielding of the positively charged amino groups of gelatin.

Chondroitin sulfates (Chs) are important components of mammalian connective tissues found in vivo as proteoglycans that bind with proteins, such as a collagen. They contribute to tissue elasticity and to the control of bone formation, due to their binding properties with Ca^{2+} and other ions.1,2) Several kinds of Chs are found in vivo. Chondroitin 4-sulfate (Chs-A) and 6-sulfate (Chs-C), which are typical Chs, are *N*-acetyl-D-galactosamine 4- and 6-sulfate, together with D-glucuronic acids. Their thermodynamic properties, such as counterion binding properties,3) osmotic coefficients,4) partial molar volumes,5) and dilution enthalpies,6–8) could be explained by the Manning theory based on a line-charge model.9) One of the characteristics resulting from the alternate arrangement of carboxylic and sulfuric groups in Chs contributes to maintaining the activity of Ca^{2+} ions constant when any pH changes.3) Furthermore, no differences in these properties were found between Chs-C and -A.4)

Chs interacts electrostatically with proteins to form complexes. These properties have been studied by light scattering,10) electrophoresis,11) and chromatograph methods.12) In the regions of more acidic pH than the isoionic points of a protein, insoluble complexes were reported to be formed.13) In gel membranes comprising gelatin and Chs, complex formation affects the membrane characteristics: the membranes shrink, and their water contents and permeabilities decrease due to complex formation.14–18)

We studied the complex formation of gelatin and Chs-C in solutions and its effects on H^+ ion binding.

Experimental

Materials. Gelatin (Lot. No. P-1710) was offered by Nitta Gelatin Co., Ltd. By passing the gelatin solution through a mixed ion exchange column comprising cation exchange resins (Amberlite IR-120-B) and anion exchange resins (Amberlite IRA-410) an isoionic gelatin was obtained according to Jenus;19) the isoionic pH was 4.8 (concentration: $C_\mathrm{ge}=1$ w/v%). The isoionic gelatin solution was stored under refrigeration. The amino acid composition is shown in Table 1. Sodium salt of chondroitin sulfate C Chs-C was of commercial origin (Seikagaku Kougyou Co., Ltd., Tokyo, Japan) and was used without any purification. All other reagents were special grade from Katayama Co., Ltd. (Nagoya, Japan). Distilled and deionized water was used for the preparation of all solutions.

Viscosity Measurements. The viscosities of the mixed solutions comprising gelatin and Chs-C were measured at 25.0 °C by an Ostwald-type viscometer in which the falling time of water was 92 s.

pH Titration. The pH values of the solutions were measured by using a digital ion meter (Orion, Type 701A) connected to glass and reference electrodes (Orion, Type 91-01 and 90-02). pH titrations of the mixed solutions comprising isoionic gelatin and Chs-C were carried out at 25.0 °C by adding HCl (1.0 mol dm^{-3}) and NaOH (1.0 mol dm^{-3}) under an atmosphere of nitrogen.

From pH titration curves of the mixed solutions, the binding amounts ν of H^+ and OH^- ions were obtained as follows. Equal volumes of the polymer solution and solvent (V dm^3) were titrated by HCl and NaOH solutions (C mol dm^{-3}). When the titrants are added to the polymer and the solvent by v and v_0 dm^3, respectively, to attain the same pH, the value ν (mol g^{-1}) is obtained by the following equation20)

$$\nu=(v-v_0)\,C/n_\mathrm{p}, \qquad (1)$$

where n_p (g) is the polymer weight in the sample solution. Since the binding amounts of the OH^- ion are equivalent to

Table 1. Amino Acid Composition of Gelatin

	mmol g^{-1}		mmol g^{-1}
Glycine	3.28	Tyrosine	0.0085
Alanine	1.09	Serine	0.296
Valine	0.195	Threonine	0.155
Leucine	0.226	Methionine	0.025
Isoleucine	0.115	Tryptophan	0
Proline	1.04	Cysteine	0
Hydroxyproline	1.21	Arginine	0.459
Phenylalanine	0.115	Histidine	0.046
Aspartic Acid	0.403	Lysine	0.279
Glutamic Acid	0.671	Hydroxylysine	0.050

the dissociated amounts of H^+ ions, they were defined as negative values.[21]

Composition of Insoluble Complex. The compositions of the insoluble complexes formed under various acidic pH conditions were studied by following two methods: (1) In the transmittance method, mixed solutions comprising gelatin and Chs-C were adjusted at 25 °C to an acidic pH in the region pH=5.6—2.5; after 15 min their transmittances were measured at λ=420 nm. According to A. Nakajima,[22] from compositions showing the transmittance maximum, those of the complexes were determined. (2) In the nitrogen analysis method, from the nitrogen contents of the insoluble complex $(W_N)_{ic}$, gelatin $(W_N)_{ge}$ and Chs-C $(W_N)_{chs}$, the weight fraction of Chs-C of the insoluble complex $(W_{chs})_{ic}$ was obtained by the following relation:

$$(W_{chs})_{ic} = ((W_N)_{ge} - (W_N)_{ic})/((W_N)_{ge} - (W_N)_{chs}). \qquad (2)$$

After adjusting the pH of the mixed solutions to the acidic region, they were allowed to stand for 15 min. Precipitated bulky insoluble complexes were dried in vacuo (at 80 °C), and their nitrogen contents $(W_N)_{ic}$ were analyzed. The nitrogen contents of gelatin, $(W_N)_{ge}$, and Chs-C, $(W_N)_{chs}$, were 0.178 and 0.267, respectively.

Results

Viscosities of Mixed Solutions Composed of Gelatin and Chondroitin Sulfate. The relative viscosities, η_{rel}, of mixed solutions comprising isoionic gelatin and Chs-C are shown in Fig. 1 as a function of the weight fraction of Chs-C W_{chs}. The total polymer concentrations of the mixed solutions were 0.3 g/100 cm³, and the added ionic strengths of NaCl J were 0.0, 0.01, and 0.4 mol dm⁻³. The results of the gelatin solutions did not significantly depend on J, but those of Chs-C solutions did. For J=0.4, the results of the mixed solutions increased linearly with increasing W_{chs}. However, in the cases of J=0.01 and 0, the results deviated from a linear relation; especially in the case of J=0 the deviation became significant. The deviations result from the formation of soluble complexes due to an electrostatic interaction between the gelatin and Chs-C. The ionic strengths resulting from the Na^+ counterion of Chs-C increased from 0 to 0.012 mol dm⁻³ with increasing W_{chs}. Therefore, the total ionic strengths of the mixed solutions varied with W_{chs} and in the region of total ionic strengths smaller than 0.022 mol dm⁻³ soluble complexes were found to be formed.

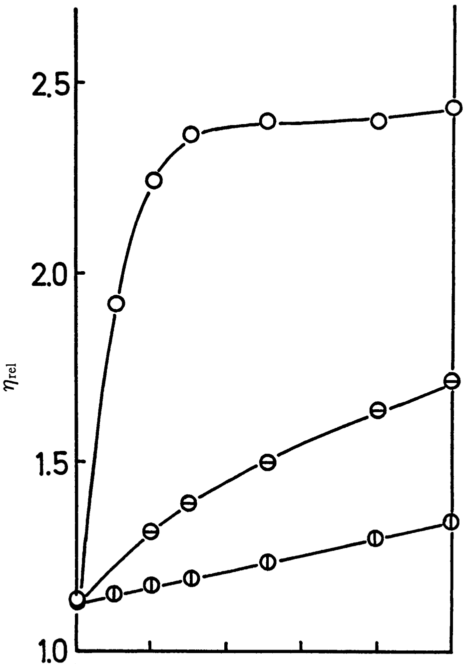

Fig. 1. Relative viscosity of mixed solution composed of gelatin and chondroitin sulfate C in various ionic strengths.
Ionic strength J: O; 0.0, ⊖; 0.01, ⦶; 0.40 mol dm⁻³ Total polymer concentrations of the mixed solutions were 0.3 g/100 cm³, and the ionic strengths were adjusted by adding NaCl.

Chs-C was added to the isoionic gelatin solutions (the concentration C_{ge}=0.3 g/100 cm³) under various conditions. The specific viscosities of mixed solutions, η_{sp}, defined by Eq. 3 are shown in Fig. 2 as a function of the concentration of Chs-C, C_{chs}:

$$\eta_{sp} = (\eta_{rel}/(\eta_{rel})_0 - 1), \qquad (3)$$

where $(\eta_{rel})_0$ is the relative viscosity of the gelatin solution. In the case of J=0.005 mol dm⁻³ and pH=8.0, the results of η_{sp} increased linearly with increasing C_{chs} and were equal to those of Chs-C solutions (C_{ge}=0). In cases satisfying linearity, no complexes were formed. However, in the case of C_{ge}=0.3 g/100 cm³ and J=0.005 mol dm⁻³, the results of η_{sp} showed a steeper increase with C_{chs} and deviated at C_{chs}=0.125 g/100 cm³ (=$(C_{chs})_c$)

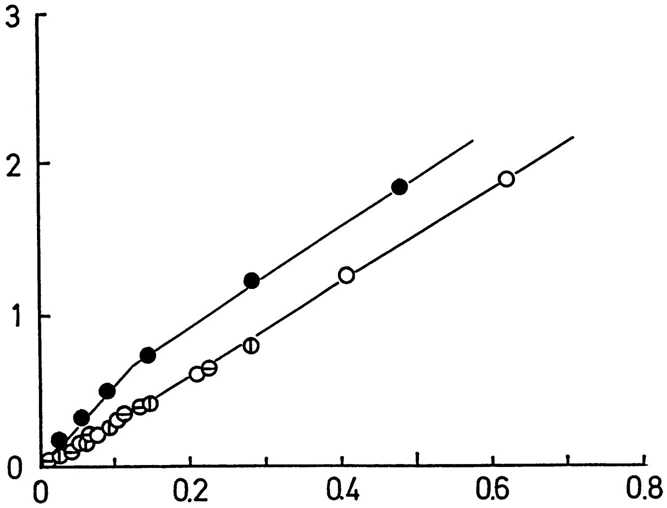

Fig. 2. Effects of adding chondroitin sulfate C to gelatin solutions on specific viscosities.
O: C_{ge}=0 g/100 cm³, J=0.005 mol dm⁻³, pH=4.8—5.6, ●: C_{ge}=0.3, J=0.005, pH=4.8—5.4, ⊖; C_{ge}=0.15, J=0.1, pH=4.8—5.4, ⦶; C_{ge}=0.3, J=0.005, pH=8.0.

from linearity. This results from the formation of the soluble complexes. In the region $C_{chs}>(C_{chs})_c$ complexes were formed, and their compositions seemed to vary with C_{chs}, i.e. the fraction of Chs-C in the complexes increased with increasing C_{chs}. At $C_{chs}=(C_{chs})_c$, saturated complexes were formed. In the region $C_{chs}>(C_{chs})_c$, the saturated complex and the free Chs-C present in the solution and the latter increased with increasing C_{chs}. Nakagaki et al. have reported that for soluble complexes formed by a bovine serum albumin (BSA) and Chs-C, BSA molecules bind strongly with Chs-C in a manner like the Langmuir type.[10] By assuming that all of the gelatin and Chs-C contribute to form complexes at $(C_{chs})_c$, the weight fraction of Chs-C in the saturated soluble complex $(W_{chs})_{ssc}$ was estimated to be 0.294.

pH Titrations of Mixed Solutions. The pH values of mixed solutions comprising isoionic gelatin and Chs-C solutions are shown in Fig. 3 as a function of W_{chs} for $J=0$ and 0.1 mol dm^{-3}. Their total concentrations were 1 g/100 cm^3 and the added ionic strengths J were adjusted by NaCl. The solid curves indicate the pH values calculated by assuming an ideal mixture. In the case of $J=0.1$ mol dm^{-3}, the results agreed with the calculated values. However, in the case of $J=0$, the experimental results were more alkaline than the calculated ones in the region $W_{chs}<0.3$; upon increasing W_{chs}, both of the results approached each other. The increments of pH in the region $W_{chs}<0.3$ are considered to result from the formation of soluble complexes.

The binding amounts of H$^+$ ion on isoionic gelatin and Chs-C, ν mol g^{-1}, were obtained from their pH titration curves by using Eq. 1. The values of ν are shown in Fig. 4 as a function of pH. The saturated binding amount of the isoionic gelatin, ν_{ge}^{∞}, was 0.85X10^{-3}, mol g^{-1}. This is equivalent to the total amount of the basic amino acids of the gelatin,[21] which was 0.83X10^{-3} mol g^{-1} (Table 1). The saturated binding amount of Chs-C, ν_{chs}^{∞}, was 1.60X10^{-3} mol g^{-1}, equal to the concentration of glucuronic acid in Chs-C (1.67X10^{-3} mol g^{-1}), as determined by the carbazole-sulfuric acid method.[23] Figure 4 also shows the results for ν using mixed solutions (W_{chs}=0.177, 0.334, and

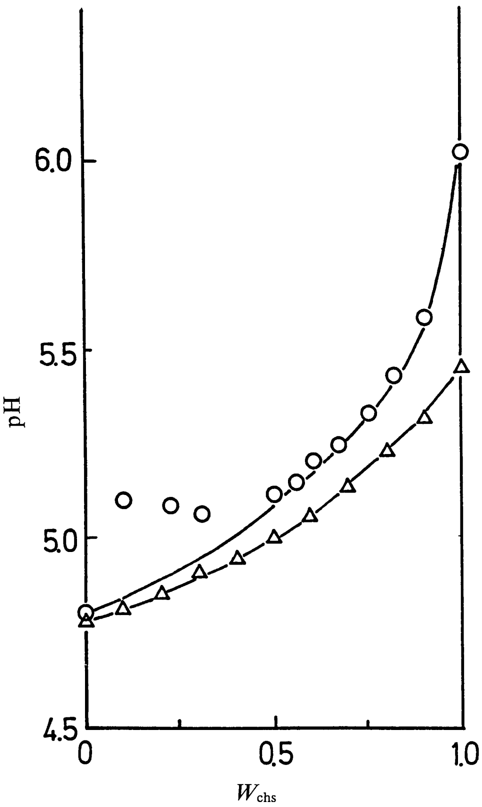

Fig. 3. pH of mixed solutions vs. weight fraction of chondroitin sulfate C.
J: ○; 0.0, △; 0.10 mol dm^{-3}. Solid curves are the calculated values obtained by assuming an ideal mixtures.

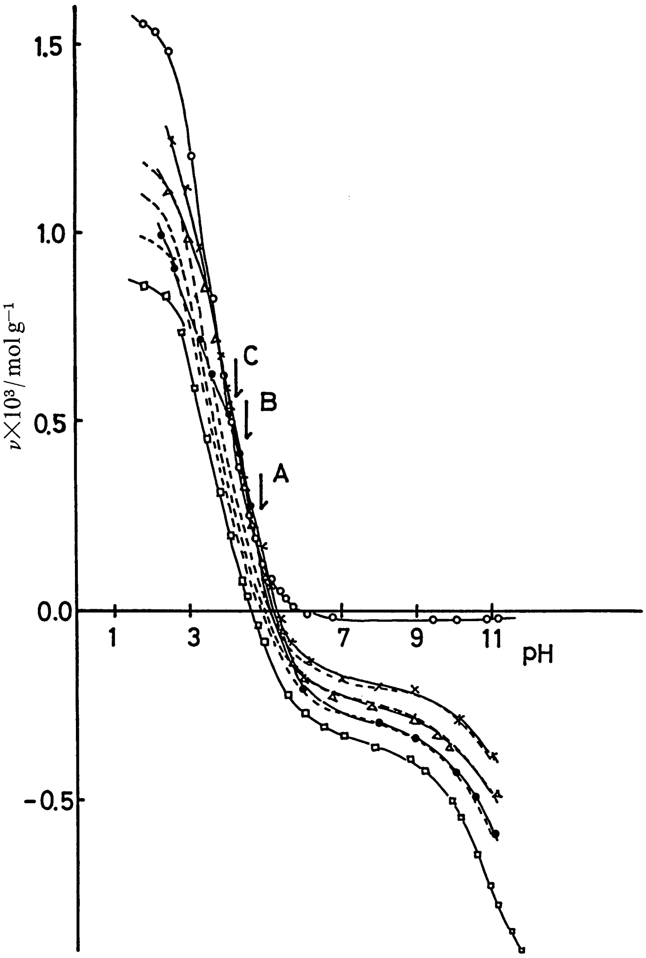

Fig. 4. H$^+$ and OH$^-$ ion binding amounts of mixed solutions.
W_{chs}: ○; 1.0, ×; 0.461, △; 0.334, ●; 0.177, □; 0.
Arrows show the value of pH at which the insoluble complexes start to be formed, (pH)$_{ic}$, and capital letters by the arrows show W_{chs}, A; 0.177, B; 0.334, C: 0.461. Broken lines are calculated values from Eq. 4 by assuming no effects of interactions between gelatin and Chs-C.

0461); these are the binding amounts of H^+ ions per 1 g of polymers. The results were found to be close to that for a Chs-C solution in the pH region 4.5—6. Upon decreasing the pH of mixed solutions, they became opaque due to the formation of insoluble complexes. The arrows in Fig .4 indicate the pH values at which insoluble complexes start to be formed ($=(\mathrm{pH})_{\mathrm{ic}}$). Since the insoluble complexes were deposited onto the glass electrode in the region $\mathrm{pH}<(\mathrm{pH})_{\mathrm{ic}}$, the experimental results might not be very exact in these regions.

When there was no interaction between the gelatin and Chs-C, the ideal binding amounts of H^+ ions, ν^{ideal}, could be expressed by summing up the binding amounts of the gelatin and Chs-C, ν_{ge} and ν_{chs},

$$\nu^{\mathrm{ideal}}=(1-W_{\mathrm{chs}})\,\nu_{\mathrm{ge}}+W_{\mathrm{chs}}\,\nu_{\mathrm{chs}}. \tag{4}$$

The calculated values are shown in Fig. 4 with broken lines. In the region of pH greater than about 6, the experimental and calculated results were almost equal. However, in the region of smaller pH, the former were more than the latter. An enhancement of H^+ ion binding is expected from the results shown in Fig. 3, and is considered to result from the formation of complexes.

Compositions of Insoluble Complexes. Upon decreasing the pH of mixed solutions comprising gelatin and Chs-C, a phase separation occurred. The transmittances of the mixed solutions of various compositions were measured at various times after adjusting to acidic pH in the range pH=2.5—5.0. During the initial stage a milky turbidity appeared, but with an elapse of time the bulky insoluble complexes precipitated, even under stirring conditions. The results mesured 15 min after adjusting the pH showed the clearest peaks as a function of W_{chs} (Fig. 5). The values of W_{chs} showing the transmittance maximum at arbitrary acidic pHs, $(W_{\mathrm{chs}})_{\mathrm{ic}}$, are considered to represent the compositions of insoluble complexes.[22] The $(W_{\mathrm{chs}})_{\mathrm{ic}}$ results are shown in Fig. 6 as a function of the pH of the solutions. $(W_{\mathrm{chs}})_{\mathrm{ic}}$ decreased with increasing pH of the solutions.

From the nitrogen contents, $(W_{\mathrm{N}})_{\mathrm{ic}}$, of the insoluble complexes at pH=3, 3.5, and 4.4, the results of $(W_{\mathrm{chs}})_{\mathrm{ic}}$ were obtained using Eq. 2. They did not depend on the compositions of the mixed solutions, but on the pHs. The mean values of the results are shown in Fig. 6. Taking the errors of the nitrogen analysis into consideration, the results were found to agree fairly well with those from the transmittance method.

The compositions of saturated soluble complexes can vary with the pH of mixed solutions. However, the composition of saturated soluble complexes at only pH=5.1 was estimated to be $(W_{\mathrm{chs}})_{\mathrm{ssc}}=0.294$ from viscosity measurements. As shown in Fig. 6, $(W_{\mathrm{chs}})_{\mathrm{ic}}$ and pH.

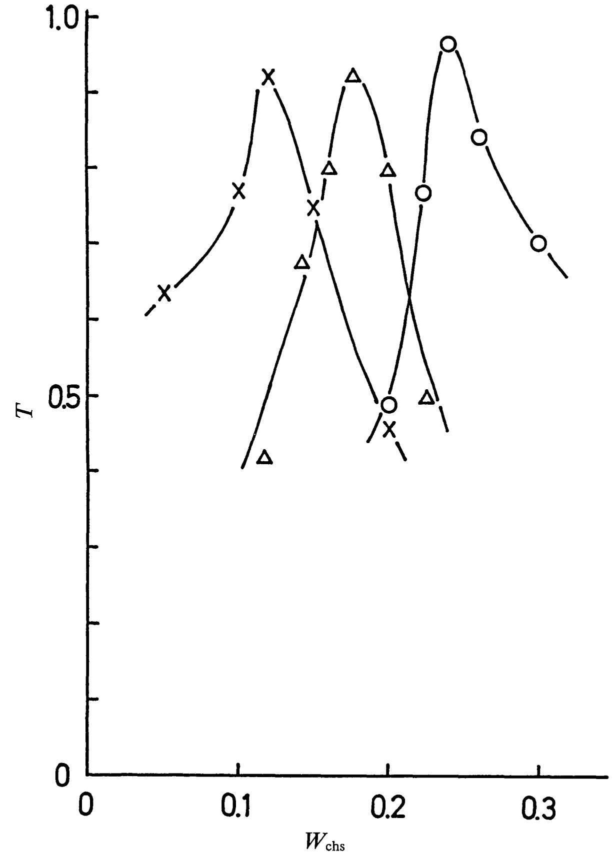

Fig. 5. Transmittance T of mixed solutions at various pH.
pH of the mixed solution: O; 3.0, Δ; 3.5, ×; 4.0.

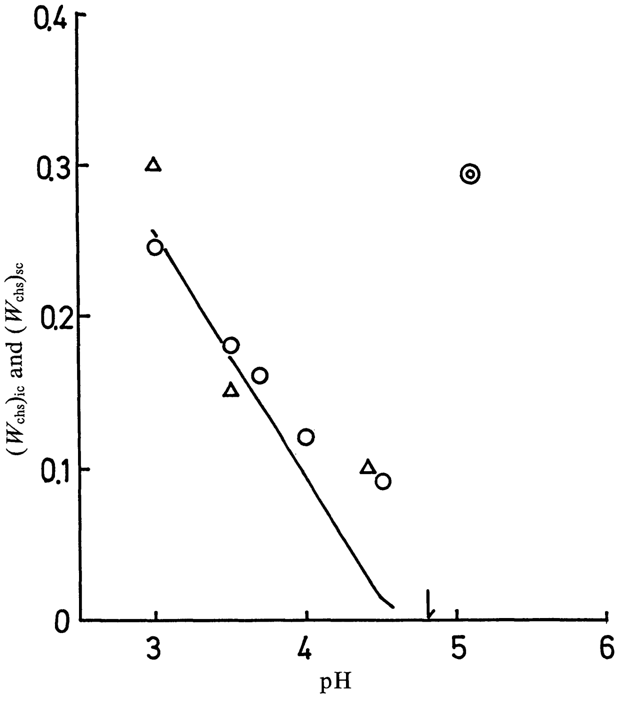

Fig. 6. Fraction of Chs-C of insoluble complexes formed in acidic pH region and saturated soluble complex.
$(W_{\mathrm{chs}})_{\mathrm{ic}}$: O; Transmittance method, Δ; Nitrogen analysis method, $(W_{\mathrm{chs}})_{\mathrm{ssc}}$: ◎; exprimental result obtained from viscosity measurement. Solid curve shows the calculated values $(W_{\mathrm{chs}})_{\mathrm{co}}$ from Eq. 10 under the condition of electroneutrality between Z_{chs} and Z_{ge}. An arrow shows the isoionic point of the gelatin.

Discussion

Apparent Dissociation Constant of Carboxylic Groups in Mixed Solutions. The degree of dissociation, α, of the carboxylic group of weak acidic polymers is related to the pH of the solutions by Eq. 5 in the range where α is not close to 0 or 1:[24)]

$$\mathrm{pH} = \mathrm{p}K_\mathrm{a} + n \log(\alpha/(1-\alpha)), \tag{5}$$

where pK_a is an apparent dissociation constant and n is a constant. Since the sulfuric groups of Chs-C can be approximated to be fully dissociated in mixed solutions of gelatin and Chs-C,[25,26)] their carboxylic groups are responsible for the binding of H^+ ions. Eq. 5 can, thus, be applied to mixed solutions. In mixed solutions the mean value of the degree of dissociation of their carboxylic groups is obtained by

$$\alpha = \frac{(1-W_\mathrm{chs})\nu_\mathrm{ge}^\infty + W_\mathrm{chs}\,\nu_\mathrm{chs}^\infty - \nu}{(1-W_\mathrm{chs})(n_\mathrm{car})_\mathrm{ge} + W_\mathrm{chs}(n_\mathrm{car})_\mathrm{chs}}, \tag{6}$$

where $(n_\mathrm{car})_\mathrm{ge}$ and $(n_\mathrm{car})_\mathrm{chs}$ mol g^{-1} are the contents of the carboxylic groups of the gelatin and Chs-C. $(n_\mathrm{car})_\mathrm{ge}$ is 1.074×10^{-3} mol g^{-1}, which is the total content of the acidic amino groups obtained from Table 1; $(n_\mathrm{car})_\mathrm{chs}$ is 1.67×10^{-3} mol g^{-1}, which is the content of the glucuronic acid of Chs-C. The relations between pH and $\log(\alpha/(1-\alpha))$ were found to be linear, even for mixed solutions (Fig. 7). The values of pK_a and n obtained from these linearities are the mean values of the carboxylic groups of both polymers. Although the n values were almost constant (the mean value=1.24), the values of pK_a were found to have a maximum at about W_chs=0.25 (Fig. 8).

By using the mean value of n, the values of pK_a of the mixed solutions over the full range of W_chs can be obtained from the pH results for the mixed solutions shown in Fig. 3. The pK_a values, thus obtained, are

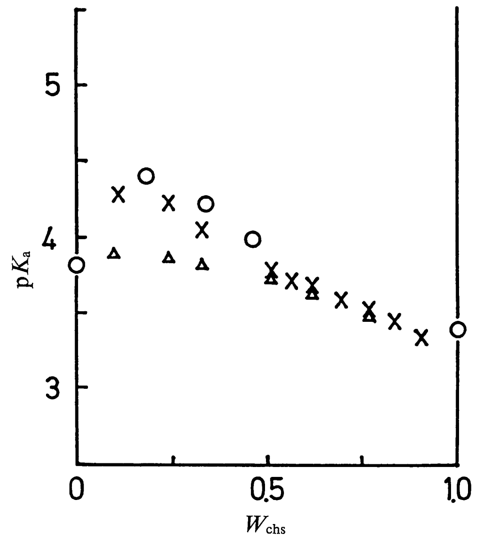

Fig. 8. pK_a values of carboxylic groups in mixed solutions.
○: Results obtained from the linear relations in Fig. 7. ×: Results obtained from Eq. 5 using the mean values of n and α obtained from pH titration curves. △: Calculated values assuming the ideal mixtures.

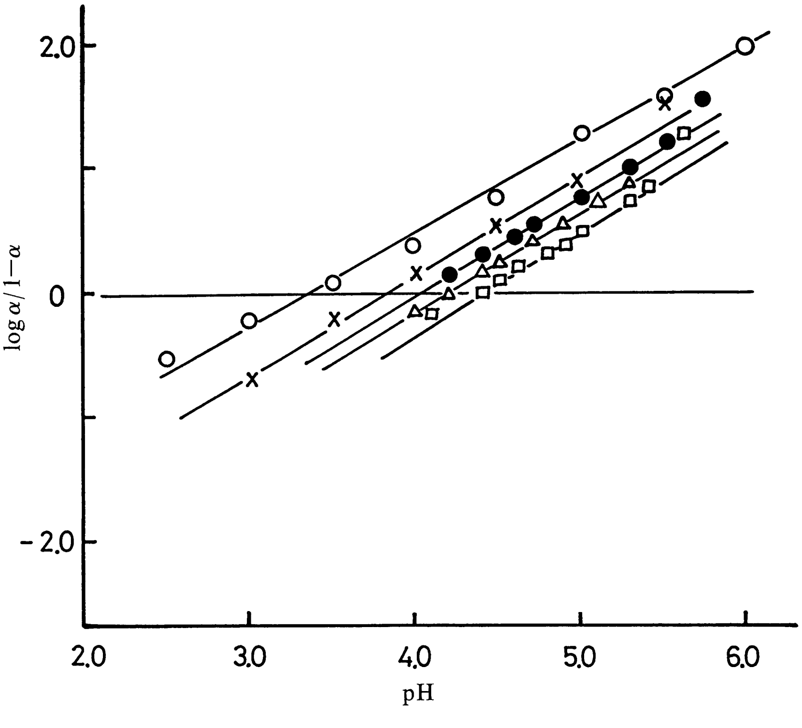

Fig. 7. $\log(\alpha/(1-\alpha))$ vs. pH.
W_chs: ×; 0, □; 0.177, △; 0.334, ●; 0.461 , ○; 1.0.

also shown in Fig. 8, and are found to be in good agreements with the results mentioned above. The theoretical values of pK_a obtained by assuming ideal mixtures of Chs-C and gelatin are also shown in Fig. 8. The experimental results of pK_a were found to be greater than the theoretical values in the region $0<W_{\mathrm{chs}}<0.4$, and the maximum to be around $W_{\mathrm{chs}}=0.25$, which is very near to $(W_{\mathrm{chs}})_{\mathrm{ssc}}$ (=0.29). The carboxylic groups of the gelatin and Chs-C are responsible for the association of H^+ ions. The latter can be considered to be depressed in the association of H^+ ions for the following reasons: (1) competitive binding with H^+ ions and the amino groups of the gelatin, and (2) electrostatic shielding of the neighboring sulfuric groups due to interaction with the gelatin. The latter reason is expected from the fact that pK_a of chondroitin is less than that of Chs-C.[27] Chondroitin is the same structure as Chs-C except for the lack of sulfuric groups. Therefore, the enhancement of the H^+ ion association due to soluble complex formation is ascribed to that of the carboxylic groups of the gelatin due to a shielding of neighboring positively charged amino groups.

Complex Formations and Charge Densities of Gelatin and Chs-C. The saturated binding amounts of H^+ ions on isoionic gelatin molecules $\nu_{\mathrm{ge}}^{\infty}$ was 0.85×10^{-3} mol g^{-1}, as shown in Fig. 4. The charge amounts of the gelatin at the isoionic pH, $(Z_{\mathrm{ge}})_{\mathrm{iip}}$, is obtained by

$$(Z_{\mathrm{ge}})_{\mathrm{iip}}=(C_{\mathrm{OH}}-C_{\mathrm{H}})/C_{\mathrm{ge}}, \tag{7}$$

where C_{ge} is the concentration of gelatin (g dm^{-3}); C_{OH} and C_{H} are those of OH^- and H^+ ions (mol dm^{-3}) at the isoionic pH. From the isoionic pH of the gelatin (=4.8), $(Z_{\mathrm{ge}})_{\mathrm{iip}}$ was determined to be -1.585×10^{-6} mol g^{-1}, which can be negligible. The isoionic pH can thus be approximated to be equal to the isoelectric point, and the maximum charge amount of the gelatin Z_{ge}^{∞} to be $\nu_{\mathrm{ge}}^{\infty}$. The charge amounts, Z_{ge}, vary with pH, due to a dissociation of H^+ ions, and can be obtained by subtracting the dissociated amounts of H^+ ions, r_{ge}, from Z_{ge}^{∞},

$$Z_{\mathrm{ge}}=Z_{\mathrm{ge}}^{\infty}-r_{\mathrm{ge}}. \tag{8}$$

Cl^- or Na^+ ions come from the HCl or NaOH present in the solutions. However, the concentrations of these ions were so small that their binding amounts might be negligible. The charge of Chs-C Z_{chs} is the summation of the contributions of the sulfuric and the carboxylic groups ($(Z_{\mathrm{sul}})_{\mathrm{chs}}$ and $(Z_{\mathrm{car}})_{\mathrm{chs}}$),

$$Z_{\mathrm{chs}}=(Z_{\mathrm{sul}})_{\mathrm{chs}}+(Z_{\mathrm{car}})_{\mathrm{chs}}=(Z_{\mathrm{sul}})_{\mathrm{chs}}-r_{\mathrm{chs}}, \tag{9}$$

where r_{chs} is the dissociated amounts of H^+ ions from chondroitin sulfuric acid. The values of Z_{ge} and Z_{chs} in the solutions can be related to the formation of both soluble and insoluble complexes. The pHs which start to form the soluble complexes were almost pH=6, at which Z_{ge} was negative (=−0.026 mol dm^{-3}). Soluble complex formation was, thus, found to occur even between both the negatively charged gelatin and Chs-C, being considered to result from an electrostatic interaction between the positively charged amino groups of the gelatin and the charged groups of Chs-C. As the pH decreases, Z_{ge} increases and the insoluble complexes come to be formed; a phase separation thus occurs.

Composition of Complexes. When the complexes formed by electrostatic interactions satisfy electroneutrality, the weight fraction of the Chs-C of the complex $(W_{\mathrm{chs}})_{\mathrm{co}}$ are related to Z_{chs} and Z_{ge} by the following equation:

$$(W_{\mathrm{chs}})_{\mathrm{co}}=-Z_{\mathrm{ge}}/(Z_{\mathrm{chs}}-Z_{\mathrm{ge}}). \tag{10}$$

Using Z_{ge} and Z_{chs}, the values of $(W_{\mathrm{chs}})_{\mathrm{co}}$ were calculated. They are shown in Fig. 6 together with the experimental results for insoluble complexes $(W_{\mathrm{chs}})_{\mathrm{ic}}$, and a saturated soluble complex at pH=5.1 $(W_{\mathrm{chs}})_{\mathrm{ssc}}$. The results for the insoluble complexes $(W_{\mathrm{chs}})_{\mathrm{ic}}$ agreed with the calculated values. Therefore, the formation of insoluble complexes is found to be due to electrostatic interactions between the negative charge of Chs-C and the effective positive charge of the gelatin. However, when the pH value approaches the isoionic point of the gelatin, the experimental results, $(W_{\mathrm{chs}})_{\mathrm{ic}}$, became greater than the calculated values.

The weight fraction of Chs-C of the saturated soluble complex $(W_{\mathrm{chs}})_{\mathrm{ssc}}$ was 0.294 at pH=5.1. However, it can not be estimated from Eq. 10 since Z_{ge} (=−0.05 mol dm^{-3})<0. The soluble complexes are thus considered to be formed by an interaction between Chs-C and the positively charged amino groups of the gelatin. Although the theoretical value of $(W_{\mathrm{chs}})_{\mathrm{ssc}}$ is difficult to estimate from our results, as an extreame estimation, $(W_{\mathrm{chs}})_{\mathrm{ssc}}$ was calculated from the electroneutrality between Chs-C and all of the positively charged amino groups at pH=5.1: 0.217, and less than the experimental result. This difference might be explained as follows. Near the isoionic point of gelatin, since it is in a shrinking random coil state, parts of the positively charged amino groups are considered to take part in complex formation. Furthermore, parts of the negatively charged groups of Chs-C are also considered to take part in it, and the residual parts are free states in solutions.

Conclusion

(1) The apparent dissociation constants of carboxylic groups of Chs-C and gelatin decrease due to complex formation; thus, the dissociation of H^+ ions of the carboxylic groups of gelatin was depressed by a shielding of the positively charged amino groups due to an interaction with Chs-C.

(2) Along with decreasing pH of the solutions, insoluble complexes were formed due to the electrostatical interactions between Chs-C and the effective positive charges of gelatin; the composition agrees with the values calculated according to electroneutrality.

References

1) J. H. Fessler, *Biochem. J.*, **76**, 124 (1960).

2) A. Courts and B. G. Giles, "Struc. Function Connective Skeletal Tissue," Proc. St. Andrews, Scotland (1965), p. 235.

3) M. Yonese, H. Tsuge, and H. Kishimoto, *J. Phys. Chem.*, **91**, 1971 (1987).

4) M. Yonese, H. Tsuge, and H. Kishimoto, *Nippon Kagaku Kaishi,* **1978**, 108.

5) H. Tsuge, M. Yonese, and H. Kishimoto, *Nippon Kagaku Kaishi,* **1978**, 609.

6) H. Tsuge, M. Yonese, and H. Kishimoto, *Bull. Chem. Soc. Jpn.*, **52**, 2846 (1979).

7) M. Yonese and H. Kishimoto, *Bull. Chem. Soc. Jpn.*, **54**, 20 (1981).

8) H. Tsuge and M. Yonese, *Nippon Kagaku Kaishi,* **1982**, 1583.

9) G. S. Manning, *Annu. Rev. Phys. Chem.*, **23**, 117 (1972).

10) M. Nakagaki and Y. Sano, *Bull. Chem. Soc. Jpn.*, **45**, 1011 (1972).

11) M. B. Mathews, *Biochem. J.*, **96**, 710 (1965).

12) B. Obrink and A. Wasteson, *Biochem. J.*, **121**, 227 (1971).

13) E. A. MacMullan and F. R. Eirich, *J. Colloid Sci.*, **18**, 526 (1963).

14) M. Yonese and M. Nakagaki, *Yakugaku Zasshi,* **95**, 75 (1975).

15) M. Yonese and M. Nakagaki, *Yakugaku Zasshi,* **95**, 641 (1975).

16) M. Yonese and M. Nakagaki, *Yakugaku Zasshi,* **95**, 665 (1975).

17) M. Yonese and M. Nakagaki, *Yakugaku Zasshi,* **96**, 299 (1976).

18) M. Yonese and M. Nakagaki, *Yakugaku Zasshi,* **101**, 493 (1981).

19) J. W. Janus, A. W. Kenchington, and A. G. Ward, *Research,* **4**, 247 (1951).

20) M. Nakagaki, N. Koga, and H. Terada, *Yakugaku Zasshi,* **86**, 849 (1966).

21) C. Tanford, "Physical Chemistry of Macromolecules," University of Tokyo Press, Tokyo (1969), p. 548.

22) A. Nakajima and K. Shinoda, *J. Colloid Interface Sci.*, **55**, 126 (1976).

23) T. Bitter and H. M. Muir, *Anal. Biochem.*, **4**, 330 (1962).

24) A. Katchalski, N. Shavit, and H. Eisenberg, *Polym. Sci.*, **13**, 69 (1954).

25) M. B. Mathews, *Biochim. Biophys. Acta,* **35**, 9 (1959).

26) I. G. F. Gilbert and N. A. Myers, *Biochim. Biophys. Acta,* **42**, 469 (1960).

27) M. Yonese, unpublished data.