

# Effects of Added Metal Ions on the Interaction of Chitin and Partially Deacetylated Chitin with an Azo Dye Carrying Hydroxyl Groups

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## SYNOPSIS

The binding of chrome violet, which is a monoazo dye and involves two hydroxyl groups in the *o* and *o'* positions to an azo group, to chitin, and partially deacetylated chitin, was examined in the presence of metal ions. Zn<sup>2+</sup> and Cu<sup>2+</sup> ions do not perceptively influence the binding affinity of chrome violet to chitin. In contrast, Co<sup>2+</sup> ion enhances the binding and Ni<sup>2+</sup> ion suppresses it. In the lower free dye concentrations the dye uptake by partially deacetylated chitin was tremendously enhanced by adding Co<sup>2+</sup> ion in the buffer solution of pH 5. The dye uptake by the polymer was considerably increased by the addition of Cu<sup>2+</sup> ion at pH 5 and became much larger at pH 6. The amount corresponded to that in the presence of Co<sup>2+</sup> ion. To investigate further the action of added metal ions, a cobalt- or a chrome-complex dye was prepared, and the binding properties for the polymers were compared with those of chrome violet in the presence of Co<sup>2+</sup> and Cu<sup>2+</sup> ions. Some possible mechanisms for the enhancement of chrome violet binding by the addition of Co<sup>2+</sup> and Cu<sup>2+</sup> ions are described. © 1995 John Wiley & Sons, Inc.

## INTRODUCTION

Many reports concerning chitin and chitosan have been presented because they can be applied to various purposes. These materials show high affinities for various organic compounds and metal ions. Thus, for example, chitosan has been used as coagulant for treatment of waste water and also crosslinked chitin or chitosan as adsorbent.

In a previous article we described that the acid azo dyes carrying two hydroxyl groups in their structures such as chrome violet and 2,4-dihydroxyazobenzene-4'-sulfonate show exceptionally remarkable binding affinity toward polyethylenimine.<sup>1</sup> Moreover, a comparison of the binding of these dyes by polyethylenimine, bovine serum albumin, polyvinylpyrrolidone, and poly-L-lysine indicates that polyethylenimine is markedly superior

to the other polymers in its ability to bind these dyes. It was deduced that the OH groups on the dye interact preferentially with the amine residues on polyethylenimine.

It was revealed that chitin and its derivatives show high affinity for acid and acid mordant dyes.<sup>2-5</sup> Partially deacetylated chitin involves much more amino groups in its structure than chitin. Thus in this article the binding behaviors of chrome violet to chitin and partially deacetylated chitin have been investigated.

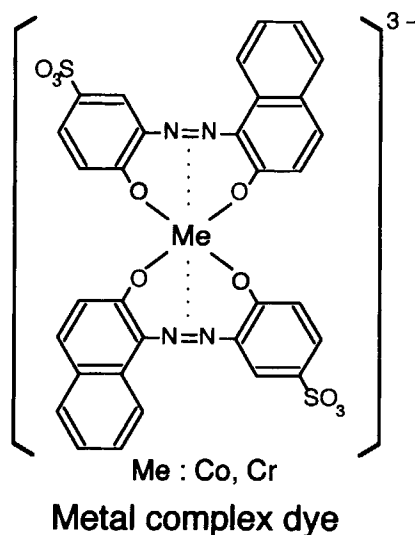
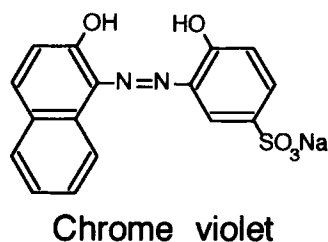
Previously we have studied the binding of a mordant dye, chrome violet with polyethylenimine,<sup>6,7</sup> polyvinylpyrrolidone,<sup>8</sup> polycations,<sup>9</sup> bovine serum albumin,<sup>10</sup> and wool keratin derivatives<sup>11</sup> in the presence of metal ions. In the present investigation the effect of metal ions on the binding of chrome violet by chitin and partially deacetylated chitin and modes of operation of the added metal ions have been examined because the polymers, in particular partially deacetylated chitin, and the dye are ex-

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pected to act as the chelating ligands with metal ions.

The main objective of this series of research<sup>6-11</sup> is to clarify the general mechanisms of added metal ions in macromolecule–small molecule binding and the role of coordinate interactions in the binding. For this purpose, we have searched many kinds of polymers and found that the added  $\text{Cu}^{2+}$  and  $\text{Co}^{2+}$  ions contribute differently to the enhancement of the chrome violet binding for nitrogen-containing polymers, in particular polyethylenimine. In addition to the strong binding ability of chitin and its derivatives for small organic substrates and ions, when chitin and partially deacetylated chitin are employed, many kinds of polymers with different

amino group contents are available. Subsequently it can be expected to clarify the difference between the added  $\text{Cu}^{2+}$  and  $\text{Co}^{2+}$  ions in the chrome violet binding in more detail and to establish the general mechanisms for the macromolecule–small molecule interaction in the presence of the metal ions. The mechanisms that we proposed in this series of work<sup>6-11</sup> are very important in macromolecule–small molecule interactions, which are related to many fields, and also might contribute to the molecular design and preparation of polymers with increased affinity for small substrates. Thus in this investigation we have chosen chitin and partially deacetylated chitin as nitrogen-containing polymers and the  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ , and their related divalent metal ions as added metal ions.



## EXPERIMENTAL

### Materials

Chitin (48–145 mesh) purchased from Nakarai Tesque Co. was used without further purification. Partially deacetylated chitin was prepared as follows: chitin was refluxed in an aqueous solution of sodium hydroxide (30%) for 1 h, neutralized by hydrochloric acid, washed, dried, and sieved from 48- to 145-mesh size. The content of primary amino group in chitin and partially deacetylated chitin was determined by ninhydrin method. The values of chitin and partially deacetylated chitin were  $8.17 \times 10^{-5}$  and  $7.04 \times 10^{-4}$  mol/10 g, respectively. The degree of deacetylation of partially deacetylated chitin determined by infrared (IR) method was found to be about 65%.

The dye used in this experiment was chrome violet (C. I. Mordant Violet 5, Tokyo Kasei Co.) in-

volving two phenolic hydroxy groups. It was recrystallized from 50% ethanol solution. Divalent metal salts  $\text{CuCl}_2$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , and  $\text{ZnCl}_2$  are the same as those used in the earlier work.<sup>6</sup> A 2 : 1 cobalt–complex dye and a 2:1 chrome–complex dye prepared from the corresponding metal ions and chrome violet were employed to compare the modes of action of the added metal ions.<sup>8</sup>

### Equilibrium Binding Measurements

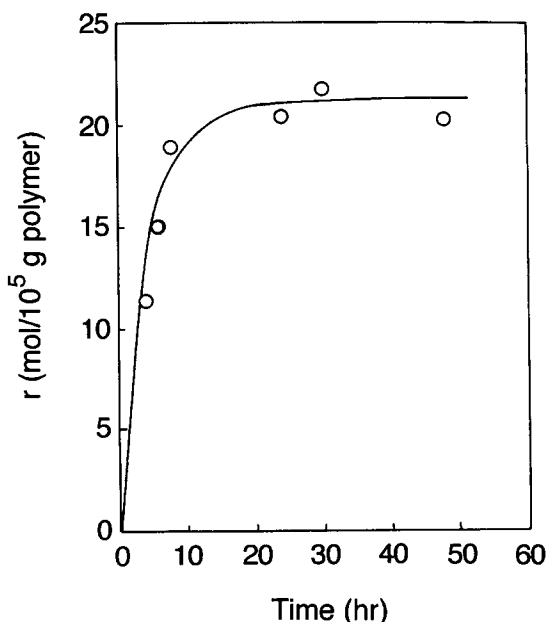
The procedure for the dye binding experiments in the presence or the absence of metal ion is as follows: 0.015 g of chitin or partially deacetylated chitin, varying amounts of dye, metal ion, and buffer solution were placed in Erlenmeyer flasks with reflux condenser. The mixed solution was continuously

stirred at  $30.0 \pm 0.1^\circ\text{C}$  for 48 h. The concentration of added metal ion was  $1 \times 10^{-4}$  mol/L. The measurements were carried out in 0.01 or 0.1M acetate buffer at pH 5 and 6. After equilibrium a part of the solution was centrifuged. The amount of free dye was determined spectrophotometrically by measuring the dye concentration in the supernatant solution. The amount of dye bound to the polymer matrix was obtained by subtracting its amount from the initial dye amount. As it was found that chitin and partially deacetylated chitin dissolve slightly in the buffer solution, an appropriate correction was made on the bound amounts. Also the metal ions were subjected to binding with chitin and partially deacetylated chitin. The concentration of metal ion was determined by using an atomic absorption spectrophotometer (Nippon Jarrell-Ash model AA-880MK-2).

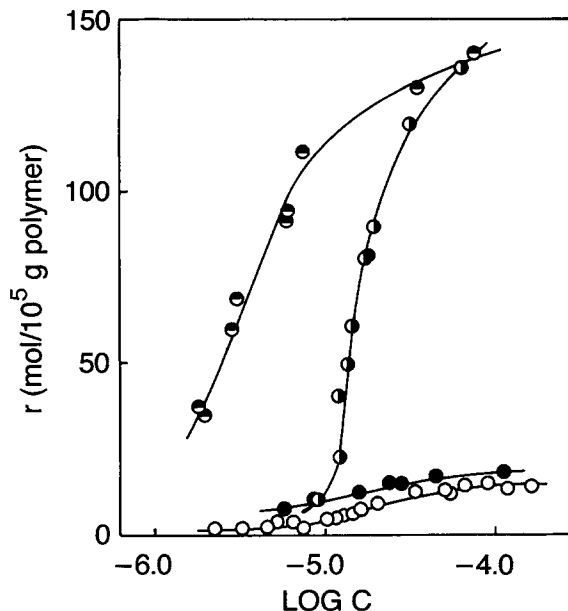
## RESULTS AND DISCUSSION

The rate of binding of chrome violet to chitin at  $30^\circ\text{C}$  is given in Figure 1, in which  $r$ , average number of bound dye per a common unit of weight— $10^5$  g of polymer—is plotted against time. As is apparent in Figure 1, the equilibrium is reached in the period of 24 h. Thus all the binding experiments were carried out for 48 h.

The ability of chitin and partially deacetylated chitin to bind chrome violet has been examined. The



**Figure 1** Rate of the binding of chrome violet by chitin in 0.1M acetate buffer, pH 5.0 at  $30^\circ\text{C}$ .



**Figure 2** Extent of the binding of chrome violet by chitin and partially deacetylated chitin in 0.01 or 0.1M acetate buffer, pH 5.0 at  $30^\circ\text{C}$ : (O) chitin, 0.1M buffer; (●) chitin, 0.01M buffer; (◐) partially deacetylated chitin, 0.1M buffer; (◑) partially deacetylated chitin, 0.01M buffer.

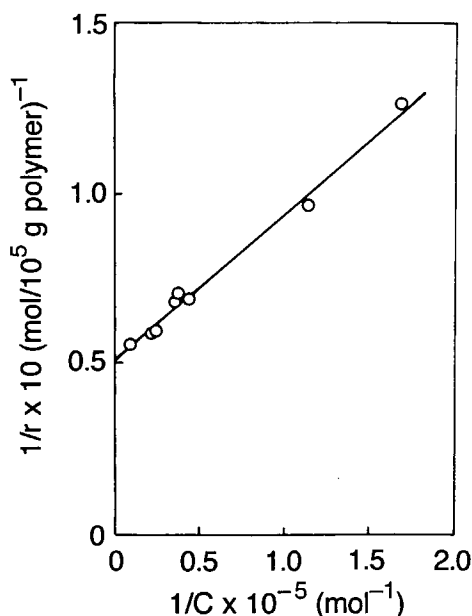
variation of bound dye with the concentration of free dye is shown in Figure 2, in which the binding isotherm is expressed as  $r$  as a function of  $C$ , the free cosolute concentration in the solution of equilibrium to facilitate a comparison of different polymers or different ionic strengths of the buffer used.

An interesting trend becomes apparent in Figure 2. A tremendously greater extent of binding of dye by partially deacetylated chitin can be seen as compared to chitin. Deacetylation of chitin induces the formation of primary amino groups in its structure. Therefore partially deacetylated chitin involves more primary amino groups than chitin. The content of amino groups in partially deacetylated chitin increases by a factor of 8.6 compared to that in chitin.

Previously it was found that dyes with two OH groups are bound to polyethylenimine much more strongly than dyes with one OH group.<sup>1</sup> Also polyethylenimine is far superior to any other polymers, including bovine serum albumin, polyvinylpyrrolidone, and poly-L-lysine, in its ability to form complexes with the dye carrying two OH groups such as chrome violet. The preferential and specific binding of chrome violet to polyethylenimine was explained on the basis of the formation of hydrogen bonding between the hydroxyl groups or the oxygen atoms on the quinonoid rings in the hydrazone form of the dye and the primary, secondary, or tertiary amine

on polyethylenimine. Accordingly, it is likely that the primary amino residues on deacetylated chitin participate preferentially in complex formation with two OH groups involved in chrome violet. The outstanding difference in the binding affinities between chitin and partially deacetylated chitin shown in Figure 2 is due to the content of amino groups in the polymers. In other words, the results obtained for chitin and partially deacetylated chitin also clarify strongly the anomalous binding behaviors of chrome violet.

To facilitate a quantitative comparison of binding ability, we evaluated the first binding constants  $k_1$  (equal to  $nk$ , where  $k$  and  $n$  represent the intrinsic binding constant and the number of binding sites per  $10^5$  g of the polymer, respectively) accompanying the binding from a linear transformation (Klotz plot,  $1/r$  vs.  $1/C$ ) of the fundamental binding equation, which is most convenient for graphic representation of the data, in the usual way. A typical example is shown in Figure 3. The resultant values of  $k_1$  at  $30^\circ\text{C}$  are listed in Table I. As is evident in Figure 2 and Table I, partially deacetylated chitin shows stronger binding for chrome violet than the unmodified parent chitin at pH 5. The amount of binding of partially deacetylated chitin increases by a factor of ca. 20 compared to that of chitin in buffered solutions of any ionic strengths. An increase in binding affinity can be created in chitin upon introduction of amino



**Figure 3** Relationship between  $1/r$  and  $1/C$  for the binding of chrome violet by chitin in  $0.01\text{ M}$  acetate buffer, pH 5.0 at  $30^\circ\text{C}$ .

**Table I** First Binding Constants  $k_1$  for the Binding of Chrome Violet by Chitin and Partially Deacetylated Chitin

Polymer	Buffer <sup>a</sup> (mol/L)	$k_1 \times 10^{-6b}$
Chitin	0.01	22.9
	0.1	4.10
Partially deacetylated chitin	0.01	423
	0.1	78.4

<sup>a</sup> Acetate buffer, pH 5.0.

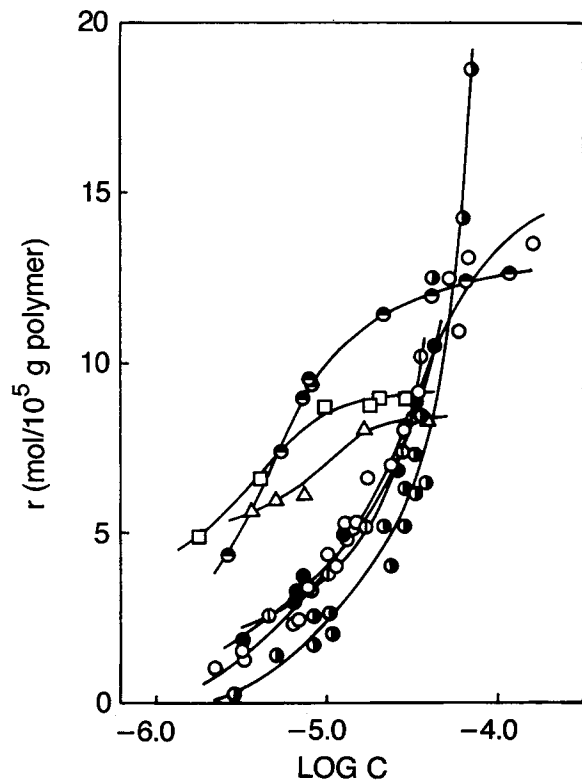
<sup>b</sup> Measurements at  $30^\circ\text{C}$ . Calculated from  $r$  values computed for  $10^5$  g of the polymer.

groups. Also the binding affinity measured in  $0.01\text{ M}$  acetate buffer is approximately five times that in  $0.1\text{ M}$  acetate buffer. These results reveal that the protonated amino groups in partially deacetylated chitin contribute greatly to the binding of chrome violet with a sulfonate anion and the binding interaction is suppressed by the competition between the dye and the acetate anions. Also, evidently, a greater extent of binding by partially deacetylated chitin can be seen at pH 5 than pH 6 (the data are shown below). This is due to the increase of protonated nitrogen atoms on the polymer at lower pH. Therefore it is clear that electrostatic interactions are involved in the binding of chrome violet to the polymer.

In the previous article,<sup>11</sup> we investigated the interaction of wool keratin derivatives such as *S*-carboxymethylated, *S*-cyanoethylated, and regenerated keratin derivatives with chrome violet in  $0.1\text{ M}$  acetate buffer, pH 4.0, at  $25^\circ\text{C}$ . The values of first binding constant were  $1.09 \times 10^6$  for *S*-cyanoethylated keratin,  $9.2 \times 10^5$  for regenerated keratin, and  $2.43 \times 10^5$  for *S*-carboxymethylated keratin.

The  $k_1$  value obtained for chitin is similar to that of the keratin derivatives, and the value for partially deacetylated chitin is considerably larger than that of the keratin derivatives, although the ionic strength of acetate buffer is the same but the pH value and the binding temperature are slightly different.

The influence of added metal ions,  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Zn}^{2+}$  ions, on the binding of chrome violet by chitin and partially deacetylated chitin has been studied. The results for chitin are shown in Figure 4 in which the concentration of added metal ions was fixed at  $1 \times 10^{-4}$  mol/L. As can be seen in Figure 4, the extent of binding of chrome violet by chitin is markedly enhanced in the presence of  $\text{Co}^{2+}$



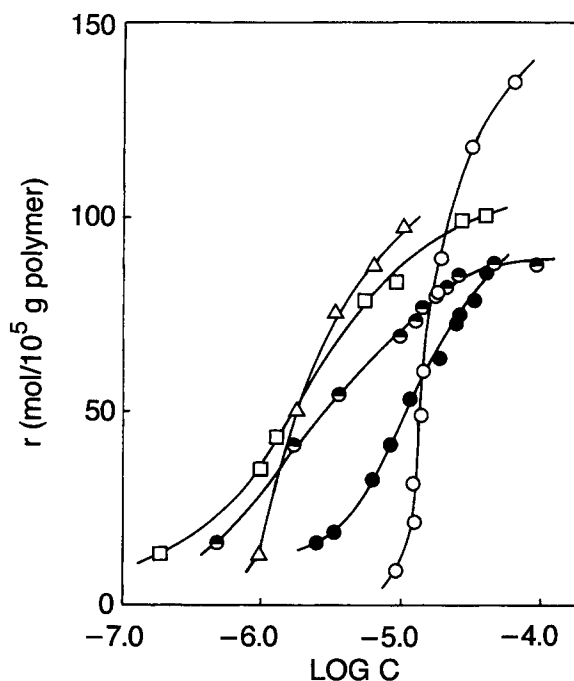
**Figure 4** Extent of the binding of chrome violet by chitin in the presence of metal ions ( $1 \times 10^{-4}$  mol/L) in 0.1M acetate buffer, pH 5.0 at 30°C: (○) none, (●)  $\text{Cu}^{2+}$ , (◐)  $\text{Co}^{2+}$ , (◑)  $\text{Ni}^{2+}$ , (⊕)  $\text{Zn}^{2+}$ , (△) cobalt-complex dye, (□) chrome-complex dye.

ion, in particular at low concentrations of free dye. In contrast,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  ions do not perceptibly influence the binding affinity of the dye.  $\text{Ni}^{2+}$  ion slightly suppresses the binding. The 2:1 chrome- or cobalt-complex dye also exhibits a strong affinity for the polymer similar to that shown in the presence of  $\text{Co}^{2+}$  ion. The results for partially deacetylated chitin are given in Figure 5. With chitin and partially deacetylated chitin, the values of first binding constant in the presence of  $\text{Co}^{2+}$  ion were  $2.58 \times 10^6$  and  $4.04 \times 10^7$ , respectively. The binding affinity of chrome violet in the presence of  $\text{Co}^{2+}$  ion is approximately five times that in the absence of the metal ion for chitin and partially deacetylated chitin. The binding behaviors of partially deacetylated chitin are also very similar to those of chitin except for added  $\text{Cu}^{2+}$  ion. With  $\text{Cu}^{2+}$  ion at pH 5, the addition of  $\text{Cu}^{2+}$  ion to the binding environment increases considerably the binding of chrome violet to partially deacetylated chitin, in particular at low concentrations of free dye. As is readily apparent in a comparison of the extent of binding by partially deacetylated chitin at pH 5 (Fig. 5) and 6 (Fig. 6), the

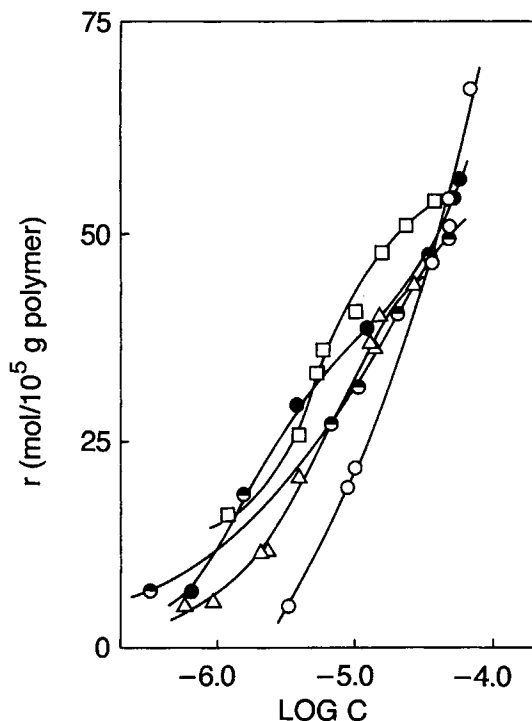
effect of added  $\text{Cu}^{2+}$  ion increases significantly at pH 6 and is similar to that of added  $\text{Co}^{2+}$  ion. The metal complex dyes also exhibit similarly strong affinity toward the polymer to that shown in the presence of  $\text{Co}^{2+}$  ion.

To further clarify the influence of added metal ions on the binding of chrome violet, the extent of complexation of metal ions with chitin and partially deacetylated chitin was measured. The amounts of metal ion bound on  $10^5$  g of polymer at pH 5 and 6 were evaluated. The results are shown in Table II, in which the initial concentration of added metal ions was  $1 \times 10^{-4}$  mol/L and the other conditions were the same as those of the dye binding. As Table II indicates, only  $\text{Cu}^{2+}$  ion interacts with partially deacetylated chitin at higher pH. However, there is no indication that  $\text{Co}^{2+}$  ion is markedly bound to chitin and partially deacetylated chitin. Thus the coordination between the cobalt atom and the polymers is very weak. Nevertheless, the addition of  $\text{Co}^{2+}$  ion to the dye-polymer binding system markedly increases the binding.

In the previous articles,<sup>6-11</sup> the influences of added  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Zn}^{2+}$  ions on the binding of chrome violet by various kinds of polymers such as polyethylenimine, polyvinylpyrrolidone, polycations, bovine serum albumin, and wool keratin de-



**Figure 5** Extent of the binding of chrome violet by partially deacetylated chitin in the presence of metal ions ( $1 \times 10^{-4}$  mol/L) in 0.1M acetate buffer, pH 5.0 at 30°C. Symbols have the same meaning as those in Figure 4.



**Figure 6** Extent of the binding of chrome violet by partially deacetylated chitin in the presence of metal ions ( $1 \times 10^{-4}$  mol/L) in 0.1M acetate buffer, pH 6.0 at 30°C. Symbols have the same meaning as those in Figure 4.

derivatives have been investigated to clarify the formation of the polymer-metal atom-dye coordination linkage. With  $\text{Co}^{2+}$  ion the tendencies observed in the present study are very similar to those of polyvinylpyrrolidone, the polycations, bovine serum albumin, and the wool keratin derivatives. Namely, in spite of little or no binding between the metal and the polymers, the addition of  $\text{Co}^{2+}$  ion significantly improved the chrome violet binding. In this case, there exists no possibility that polymer-metal-dye bonds are formed. To explain these situations, we deduced that in the presence of  $\text{Co}^{2+}$  ion, a 2 : 1 type metal-complex dye with a trivalent negative charge is formed in the binding medium; the resultant species interacts preferentially with the positive charge on the polymers through electrostatic forces.<sup>8,9</sup>

The metal-complex dye consisting of chrome violet and chromium is very popular, and its chemical structure has been extensively examined and identified. The complex dye bears one additional, negative charge (the difference between four ionized hydroxyl groups and the  $\text{Cr}^{3+}$  ion). The negative charge is distributed over the four metallizable hydroxyl groups or situated at the metal atom. Sub-

sequently, the metal-complex dye contains the net negative charges of three, two from sulfonates and the other from hydroxyl groups. For the cobalt-complex dye Kimura et al. proposed a similar structural formula.<sup>12,13</sup>

We also demonstrated in the earlier publication that chrome violet coordinates with the  $\text{Co}^{2+}$  ion in aqueous solution to give a 2 : 1 kind of complex as the results of the continuous variation method applied to the  $\text{Co}^{2+}$ -chrome violet system indicate.<sup>8</sup>

Accordingly, it is reasonable to assume that the significant effect of added  $\text{Co}^{2+}$  ion is not due to the formation of chitin derivative-Co-chrome violet coordination linkage but to the increase in electrostatic interactions between the polymer and the metal-complex species like the formula of the cobalt-complex dye formed in the binding medium. As has been already discussed in the previous articles in detail,<sup>6-11</sup> a 2 : 1 type metal-complex dye with a trivalent negative charge is formed in the binding medium in the presence of  $\text{Co}^{2+}$  ion. The resultant species interacts preferentially and energetically with the protonated amino groups of chitin or partially deacetylated chitin, in particular the latter polymer. The facts that the binding behaviors of the cobalt- or chrome-complex dye are very similar to those of chrome violet in the presence of  $\text{Co}^{2+}$  ion strongly suggest the above argument.

As Table II shows, partially deacetylated chitin chelates  $\text{Cu}^{2+}$  ion at higher pH but chitin does not. This is a manifestation of the many nitrogen ligands in partially deacetylated chitin. The polymer provides frameworks to which the metal ion can be affixed. Thus it would be expected that such polymer-bound metal ions can offer favorable binding sites

**Table II** Binding of Metal Ion by Chitin and Partially Deacetylated Chitin

Polymer	Metal Ion	pH <sup>a</sup>	Amount of Bound Metal Ion <sup>b</sup>
Chitin	$\text{Cu}^{2+}$	5.0	0
	$\text{Co}^{2+}$	5.0	0
	$\text{Ni}^{2+}$	5.0	0
Partially deacetylated chitin	$\text{Cu}^{2+}$	5.0	4.42
	$\text{Cu}^{2+}$	6.0	14.5
	$\text{Co}^{2+}$	5.0	0
	$\text{Co}^{2+}$	6.0	0.375

<sup>a</sup> 0.1M acetate buffer.

<sup>b</sup> Mol/10<sup>5</sup> g polymer at 30°C.

for the dye with two hydroxyl group ligands through chelate formation. In fact, as Figures 4 and 5 demonstrate, the effect of added  $\text{Cu}^{2+}$  ion on the chrome violet binding is more pronounced for partially deacetylated chitin than for chitin. Also as is apparent in Figures 5 and 6, with partially deacetylated chitin the effect is more significant at pH 6 than pH 5. Namely, partially deacetylated chitin shows stronger binding for chrome violet in the presence of  $\text{Cu}^{2+}$  ion at pH 6: added  $\text{Cu}^{2+}$  ion increases the degree of dye binding. This influence of  $\text{Cu}^{2+}$  ion might be ascribed to the formation of the polymer-copper atom-dye coordination linkage. In the case of added  $\text{Cu}^{2+}$  ion, a 1 : 1  $\text{Cu}^{2+}$ -dye complex is formed in aqueous solution as measured by the continuous variation method.<sup>8</sup> Residual coordination positions in the cupric ion are still available for occupation by groups in chitin and partially deacetylated chitin, in particular the latter polymer, and also acetate ions of the buffer used.

It is made apparent in Figures 4, 5, and 6 that a steep rise in binding with increasing concentration of dye can be seen in the absence of metal ion. The steepness is a clear indication of cooperative interactions in this binding. This phenomenon might be interpreted in terms of cooperativity in binding, similar to that shown previously in systems of the polycations with chrome violet.<sup>9</sup>

The authors wish to thank Mr. H. Hasegawa for his experimental assistance.

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Received December 17, 1993

Accepted June 30, 1994