The Interaction of Ribonuclease with Metal Ions

I. STUDIES OF CUPRIC AND ZINC IONS AND THE EFFECT OF CYTIDYLIC ACID*

(Received for publication, April 25, 1966)

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SUMMARY

The interaction of ribonuclease A with cupric and zinc ions has been studied spectrophotometrically and by potentiometric titration in the presence and absence of cytidylic acid derivatives. In the absence of cytidylic acid, the results suggest that each cupric ion distributes among a set of approximately four spectrally similar sites; each site appears to consist mainly of a single imidazole side chain and its adjacent peptide bond nitrogen atoms at neutral pH. No evidence for the preferential chelation of a single cupric or zinc ion between histidines-12 and -119 has been found.

In the presence of 3'-cytidylate, binding of both cupric and zinc ions is strengthened. In addition, spectrophotometric studies indicate that cupric ion-binding sites are altered in the presence of 3'-cytidylate. These data are compatible with formation of a ternary complex between a cupric or zinc ion, 3'-cytidylate, and ribonuclease. By contrast, 2'-cytidylate does not alter the type of ligand to which cupric ion binds, but appears to diminish very slightly the affinity of ribonuclease for cupric ion; these results suggest that 2'-cytidylate may compete for one of the sites usually available to cupric ion.

Several possible mechanisms for the inhibition of ribonuclease by cupric and zinc ions are suggested. These include blocking of the active site by bound metal ions and conformational changes induced by binding of metal ions elsewhere in the protein. The importance of cooperative interactions between 3'-cytidylate and metal ions in binding to ribonuclease is particularly stressed as a mechanism of inhibition.

Ribonuclease is inhibited by low concentrations of cupric and zinc ions (1), but the mechanism of this inhibition is not understood. Ross, Mathias, and Rabin (2) investigated the interaction of RNase with zinc ions and postulated that formation of a ternary complex between Zn(II), RNase, and 3'-cytidylic acid leads to inhibition of cyclic 2',3'-CMP hydrolysis at low Zn(II) concentrations; however, at higher levels of Zn(II), enzyme inhibition occurred in the absence of 3'-CMP. Crestfield, Stein, and Moore have shown that the rapid alkylation of histidines-12 and -119 by iodoacetic acid at pH 5.5 is inhibited by cupric ions and suggested that a single Cu(II) chelates between these 2 histidines (3). Inasmuch as these residues appear essential to enzyme activity, it might be anticipated that such binding would lead to inactivation.

We were interested in identifying those sites in RNase which bind cupric and zinc ions to learn more about the mechanism of metal ion inhibition. In addition to unique binding sites determined by conformation or special amino acid sequences, studies with other proteins and with model peptides have indicated that several types of nonspecific sites are potentially available to cupric ions at neutral pH (4-10). These are: (a) the α-amino terminus which might bind to Cu(II) through the α-amino and up to 3 adjacent peptide bond nitrogen atoms; (b) histidyl side chains which might alone bind Cu(II) to give simple 1:1 Cu(II)-imidazole complexes or with involvement of adjacent peptide bond nitrogen atoms. The involvement of up to 3 peptide nitrogen atoms together with an adjacent imidazole becomes favored as the pH is raised above 0 (7). The nonspecific sites available to zinc ions at neutral pH appear to be mainly imidazole side chains (11).

We have studied the interaction of cupric and zinc ions with RNase by potentiometric titration and have similarly examined the effect of 2'- and 3'-CMP on this interaction. The sensitivity of the visible spectrum of Cu(II) to the nature of the ligands to which Cu(II) is bound (12, 13) has also been utilized to learn more about the ligands in RNase which bind to Cu(II).

EXPERIMENTAL PROCEDURE

Materials—Bovine pancreatic ribonuclease A was purchased from both Worthington and Mann. RNase A purchased from Worthington was lyophilized, phosphate-free material; the pH of a 1% solution was 9.5 at 25°C, and it was used without further purification. RNase A obtained from Mann was the ethanol-precipitated material; this was deionized by passage through a Dintzis column (14) and subsequently lyophilized. The protein so prepared was identical with that obtained from Worthington in its interactions with hydrogen and metal ions. RNase solutions were standardized either by dry weight or by measurement of absorbance at 278 nm. The calculated molar absorptivity at this wave length was 9900, which is in good agreement with that reported in the literature (15).

Cytidylic acid (a mixture of cytidine 2'- and 3'-phosphates) was obtained from Schwarz BioResearch. The isomers were separated on a Dowex X10 (formate) column (1.2 × 50 cm)

* This research was supported by Grant HE-02739 from the National Institutes of Health.
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Fig. 1. Continuous titration of RNase in the presence of 0, 1, and 2 m eq of CuCl₂. The conditions were: 0.16 ionic strength, 25°, an initial protein concentration of 1.0%. Without CuCl₂: O—O, pH 5.5 to 3; □—□, pH 3 to 11; ■—■, pH 11 to 5.5. Molar ratio of Cu(II) to RNase of 1: O——O, pH 5.5 to 3; □—□, pH 3 to 11; ■—■, pH 11 to 5.5. Molar ratio of Cu(II) to RNase of 2: O——O, pH 5.5 to 3; △—△, pH 3 to 11; ▲, pH 11 to 5.5. Inset: ---, the difference in \( \Delta h \) between the titration curve in the absence of CuCl₂ and the titration curve in the presence of 1 eq of CuCl₂; ----, the difference in \( \Delta h \) between the titration curve in the presence of 1 eq of CuCl₂ and the titration curve in the presence of 2 eq of CuCl₂.

RESULTS AND DISCUSSION

Titration of RNase in Presence of Cupric Ions—RNase was titrated in the absence of added metal ions as well as in the presence of 1 and 2 m eq of CuCl₂ (Fig. 1). In the absence of Cu(II), titration was reversible in the pH region, 3 to 11. In the presence of Cu(II), no precipitate of Cu(OH)_2 was apparent at any pH; however, as noted with myoglobin-Cu(II) complexes (8, 9), some hysteresis is apparent above pH 8. Titration curves of RNase alone are in excellent agreement with those reported by other workers under similar conditions (19).

A convenient way to analyze the titration data is to subtract values of \( \Delta h \) in the presence of a given mole ratio of Cu(II) to RNase from \( \Delta h \) obtained at the same pH in the presence of 1 m eq less of Cu(II). In the absence of Cu(II), titration was reversible in the pH region, 3 to 11. In the presence of Cu(II), no precipitate of Cu(OH)_2 was apparent at any pH; however, as noted with myoglobin-Cu(II) complexes (8, 9), some hysteresis is apparent above pH 8. Titration curves of RNase alone are in excellent agreement with those reported by other workers under similar conditions (19).

The abbreviations used are: pKₐ, the intrinsic dissociation constant of an acid in the absence of metal ion; \( \bar{h} \), the number of H⁺ ions bound per mole of protein.
use of equivalent protein concentrations, and at pH 7 with similar
and 4-fold higher protein concentrations (Table I).  

It can be shown that the titration data are not invalidated by Cu(OH)₂ formation. Consideration of the equilibria between Cu(I) and OH⁻ ions (20, 21) indicates that below pH 6.3 Cu(OH)₂ formation is negligible at the Cu(II) concentrations used. In addition, Sauntry and Stein (22) have reported binding constants for Cu(II) to RNase which indicate that free Cu(II) may be neglected in the pH region 6 to 7. Although preliminary equilibrium dialysis studies in this laboratory give lower binding constants for Cu(II) to RNase than those reported, correction of the data for free Cu(II) and Cu(OH)₂ from these constants also leads to no significant change in the data as plotted. Above pH 8, spectral data given below indicate that Cu(II) binding becomes even stronger; Cu(OH)₂ is apparently negligible above this pH as well.

The rise in $-\Delta \bar{h}/Cu$ between pH 5 and 6.3 indicates that in this pH region each of the first 2 Cu(II) is competing with H⁺ for a multidentate site on RNase; to the nearest integer, two ligands at each Cu(II)-binding site at pH 8 appear to have $p\bar{k}$ values greater than 8 in the absence of Cu(II). No striking differences in RNase affinity for the first 2 Cu(II) are indicated by the titration data, although, as will be seen, there is some suggestion that the second Cu(II) may be bound slightly more strongly than the first.

In view of the suggested bis-imidazole site in RNase (3), it is relevant to compare the titration of RNase-Cu(II) complexes with that of the 1:1 Cu(II) complex of 4,4′(5,5′)bis-imidazolylmethane in which 1 Cu(II) is coordinated with 2 imidazoles (23). The intrinsic stability constant of the 1:1 Cu(II)-4,4′(5,5′)bis-imidazolylmethane complex is $10^{10.41}$. Assuming a similarly strong bis-imidazole-binding site on RNase, cupric ion should effectively compete with H⁺ for 2 RNase imidazoles at relatively low pH. It can, in fact, be calculated that for such a complex, at the Cu(II) concentrations used, the first added Cu(II) should displace at least 1.5 protons from RNase imidazoles between pH 4 and 5, a result not in accord with the experimental data. The titration data, therefore, indicate that no bis-imidazole site of Cu(II)-binding affinity comparable to 4,4′(5,5′)bis-imidazolylmethane is present in RNase.

It may be argued that steric restrictions within RNase weaken a potential bis-imidazole site relative to 4,4′(5,5′)bis-imidazolylmethane. However, it necessarily follows that if 1 of the first 2 Cu(II) bound solely to a site containing 2 imidazoles, then this site must also contain two additional ligands of $p\bar{k}$ greater than 8. This is true since each of the first 2 Cu(II) displaces almost 2 protons at pH 8, whereas the $p\bar{k}$ of histidines-12 and -119 is no higher than 6.5 (19, 24).

The increase in $-\Delta \bar{h}/Cu$ above pH 8 indicates that the binding site of each Cu(II) is altered above pH 8 so that at pH 11 each Cu(II) is bound to four ligands of $p\bar{k}$ greater than 11. This is seen more clearly from the data in Table I; direct pH-stat titration shows that each of the first 8 Cu(II) added to RNase at pH 11 displaces approximately 4 protons. The displacement of 4 protons by each Cu(II) at pH 11 is greater than the value of 3 found in myoglobin derivatives (8, 9) or predicted from model imidazolyl-containing peptides in which binding of Cu(II) occurs at pH 11 to an a- amino or imidazole and 3 adjacent peptide bond nitrogens (7). One interpretation of the titration data at pH 11 is that each Cu(II) is bound to 4 peptide nitrogens in a biuret type complex. As seen below, however, this is only to some extent supported by spectral data.

**Table I**

<table>
<thead>
<tr>
<th>Molar addition of CuCl</th>
<th>Number of protons displaced*</th>
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<tbody>
<tr>
<td></td>
<td>At pH 5.3</td>
</tr>
<tr>
<td></td>
<td>No CMP</td>
</tr>
<tr>
<td>1st</td>
<td>0.55</td>
</tr>
<tr>
<td>2nd</td>
<td>0.55</td>
</tr>
<tr>
<td>3rd</td>
<td>0.45</td>
</tr>
<tr>
<td>4th</td>
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<td>5th</td>
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<tr>
<td>6th</td>
<td>0.27</td>
</tr>
<tr>
<td>7th</td>
<td>3.7</td>
</tr>
<tr>
<td>8th</td>
<td>3.7</td>
</tr>
</tbody>
</table>

* This number has the same significance as the term $-\Delta \bar{h}/Cu$ calculated from continuous titration data.

protons by each Cu(II) at pH 11 is greater than the value of 3 found in myoglobin derivatives (8, 9) or predicted from model imidazolyl-containing peptides in which binding of Cu(II) occurs at pH 11 to an a- amino or imidazole and 3 adjacent peptide bond nitrogens (7). One interpretation of the titration data at pH 11 is that each Cu(II) is bound to 4 peptide nitrogens in a biuret type complex. As seen below, however, this is only to some extent supported by spectral data.

**Spectra of RNase in Presence of Cu(II) Ions**—The visible absorption spectra of RNase-Cu(II) complexes were determined with a 3 to 4% solution of RNase in 0.16 M KCl sequentially adjusted to the desired pH in the presence of a known molar ratio of CuCl₂. In Fig. 2A, the effect of pH upon the spectrum of RNase in the presence of 1 added mole of CuCl₂ is shown. It is immediately apparent that, in the pH region 5 to 6.3, an important change in spectrum occurs which parallels the rise in $-\Delta \bar{h}/Cu$ by the first Cu(II) from 0 to 1.7 in the same pH region. It is also apparent, however, that in the pH interval 6.3 to 8.1, over which $-\Delta \bar{h}/Cu$ remains relatively constant (Figs. 1 and 3), the spectrum is not constant but exhibits progressive shifts of the main absorption peak to lower wave lengths with increasing pH. Above pH 8, another more marked change in spectrum occurs which parallels the dissociation of 2 additional protons by Cu(II) in this region. The effect of pH upon absorbance between 700 and 750 nm is of particular interest; absorbance in this region increases between pH 5 and 6.3 and decreases progressively above pH 6.3. Such data indicate that the spectral changes between pH 5 and 8 result from the formation of at least two different species as the pH is increased above 5.

Some protein precipitation may attend the addition of Cu(II) to RNase. In the spectra shown, this was minimal. There is some evidence that the degree of precipitation and the sharpness of the spectra depend on the particular sample of RNase used and the conditions under which Cu(II) is added to the protein.

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2 The extent to which Cu(II) is bound is dependent on the concentration of the Cu(II)-RNase mixture. Below pH 5.5, binding is incomplete at concentrations of 1%. Therefore, within this pH region, values of $-\Delta \bar{h}/Cu$ will increase as the concentration is increased above 1%.

3 A. W. Girotti and E. Breslow, unpublished observations.
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FIG. 2. Spectra of RNase in the presence of CuCl₂. The conditions were: protein concentration of 3 to 4%: ionic strength, 0.16.
A, molar ratio of CuCl₂ to RNase of 1. B, molar ratio of CuCl₂ to RNase of 3. All data are corrected for a small amount of scattering estimated from the optical density at 450 mμ. Results are reported as the extinction per mole of RNase. The ordinate in B is 3 times the ordinate in A.

The relationship between ligands bound to Cu(II) ions and Cu(II) spectra has been studied in many simple systems (12, 25, 26). In general, such studies show that, in complexes in which Cu(II) is bound to 1 or more oxygen atoms only, the visible absorption bands lie above 700 mμ. As the number of nitrogen atoms bound to Cu(II) increases, the absorption spectrum shifts to lower wave lengths (12, 27). In Table II, the position of the main absorption bands in the visible wave length region for a variety of Cu(II)-complexes is shown and may be compared with the spectra of Cu(II)-RNase at different pH values. Such comparisons indicate that the decrease in λₘₐₓ from 700 to 640 mμ between pH 5 and 6.3 is compatible with binding of Cu(II) to a site which contains two nitrogen ligands at pH 6.35, 6. At pH 8, such comparisons suggest that the first Cu(II) may be coordinated with 3 nitrogen atoms.6 Above pH 8, as many as 4 nitrogen atoms may be bound to Cu(II).

5 For simplicity, the spectrum of the Cu(II)-RNase complex at pH 5.5 was omitted from Fig. 2A but is seen in Fig. 4. The λₘₐₓ at pH 5.5 is at 675 mμ. These results indicate that the species formed at pH 6.3 does not develop in an all or none manner and may be assigned as pKₐ and pKₐ', subsequently assigned.

6 Strict comparison of the 640 mμ λₘₐₓ in Cu(II)-RNase complexes at pH 6.3 with models in Table II indicates that such a λₘₐₓ could arise from bonding of Cu(II) to 1 nitrogen and 2 COO⁻ as well as from the postulated bonding to 2 nitrogen ligands. Such bonding would not be due to the observed proton displacement at pH 6.3, however, and this alternative can be ruled out. The 600 mμ λₘₐₓ at pH 8 is not grossly incompatible with bonding to two nitrogen ligands and 2 COO⁻, but such a site would necessarily be determined by a special sequence or conformation. As each of 4 Cu(II) seem to give the same spectra at pH 8, such special sites seem less likely than binding of each Cu to three nitrogen-containing ligands which involves no such special assumptions.

The spectrum of the first Cu(II) added to RNase is not unique. As higher levels of Cu(II) are added at each pH, the absorbance at all wave lengths increases regularly with no discernible change in wave length of the absorption maximum. For instance, in Fig. 2B, the spectrum of RNase in the presence of 3 Cu(II) is shown at the same pH values as in the presence of 1 Cu(II); the two sets of curves are almost identical except for a 3-fold difference in molar absorptivity. A similar effect is noted on addition of the fourth molar equivalent of Cu(II). In fact, it can be shown that the increase in ε at the wave length maximum at any pH is linearly related to the Cu(II) to RNase ratio up to the fourth equivalent of Cu(II) added. At the fifth mole of added Cu(II), precipitation of Cu(OH)₂ is apparent at neutral pH. Such data suggest that RNase contains a number of Cu(II)-binding sites strong enough to compete with OH⁻ ion for Cu(II). That the number of such sites is probably between three and five is indicated by the formation of Cu(OH)₂ when the fifth Cu(II) is added and by preliminary equilibrium dialysis studies3 which show that at pH 7, at protein concentrations equivalent to those used for spectra, approximately 2.7 moles of Cu(II) are bound to RNase when 3 moles of Cu(II) have been added. Furthermore, the observation that the spectral changes accompanying each added Cu(II) are almost identical indicates either that: (a) the binding constants for all sites are of similar magnitude so that each bound Cu(II) is distributed among a set of sites that overlap, or (b) the sites differ in their binding affinities but their spectral properties are indistinguishable. A distinction between these two possibilities is clearly relevant to the question of whether there is a special site on RNase, upon which activity depends, which has a particularly high affinity for cupric ion. In the absence of binding constants, a final answer...
cannot be given, but the essentially identical pH dependence of
the spectrum at each level of Cu(II) in the pH region 5 to 8,
together with comparable proton displacements by each Cu(II),
suggests that the sites must be very similar in their intrinsic
affinity for Cu(II). It is therefore most likely that each bound
Cu(II) is distributed among a set of sites.

Possible Identity of Cu(II)-binding Sites—Between pH 8 and
11, titration studies have suggested that each of the first 8 Cu(II)
may coordinate with 4 peptide nitrogens. This would seem to
be substantiated by the finding that at least 29 Cu(II) can be
added to RNase at pH 11 with almost no diminution in the molar
absorptivity of each added Cu(II) (Table III). However, as
shown in Table III, the wave length maximum of the complex
changes between the 5th and 29th Cu(II) bound and is, in any
event, higher than that of the potentially analogous Cu(II)-
biuret complex (12). Such data suggest a somewhat more com-
plicated binding pattern of each Cu(II) than to four peptide bonds
alone between pH 8 and 11.

It is in the pH region between 5 and 8, however, that identifica-

Fig. 3 Proton displacement by Cu(II) from RNase in the
presence and absence of CMP. The conditions were: protein con-
centration, 1%; 0.16 ionic strength. Experimental: ●, the difference
in $\Delta h$ between the titration curve of RNase alone and in the
presence of 1 eq of Cu(II); O—O, the difference in $\Delta h$ between
the titration curve of RNase + 1 eq of 3'-CMP and the titration
curve of RNase + 1 eq of 3'-CMP + 1 eq of Cu(II); □—□, the
differences in $\Delta h$ between the titration curve of RNase + 1 eq of
2'-CMP and the titration curve of RNase + 1 eq of 2'-CMP + 1
eq of Cu(II). ---, theoretical curve for the interaction of Cu(II)
with RNase in the absence of CMP; see text for details.

<table>
<thead>
<tr>
<th>Ratios</th>
<th>$\lambda_{max}$</th>
<th>$\Delta$R/HKase</th>
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<tr>
<td>1</td>
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<td>170</td>
</tr>
<tr>
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</tr>
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<tr>
<td>7</td>
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<td>4600</td>
<td>154</td>
</tr>
</tbody>
</table>

* The ligands cited represent the groups other than H$_2$O which
are bound to Cu(II). Except where specifically stated, these
are assumed to be among the four closest ligands to Cu(II)
in their respective complexes and hence the main contributors to
the Cu(II) ligand field and visible absorption spectrum (12).
* These data are taken from the structure of the Cu(II)-glu-
tamate complex. The 2 C=O are not among the four closest
ligands to Cu(II) (12, 28).
peptides (Table II). Moreover, since approximately four of the sites are spectrally similar, the four imidazoles of RNase appear uniquely attractive as the ligands in question. The possibility that one of these sites contains both His-12 and His-119 appears uniquely attractive as the ligands in question. The possibility of binding are considered. Contribution of a potential site at the α-amino terminus may be present, but cannot be assessed from the present data.

It is undoubtedly naive to assume that the 4 imidazoles of RNase behave identically toward Cu(II) ions as they do in simple peptides in which the backbone conformations are necessarily less constrained. Nevertheless, it is of interest to see to what extent the titration and spectrophotometric behavior of Cu(II)-RNase complexes are compatible with such a simplification. Deviations from ideality are then ultimately interpretable in terms of the structure of the protein. It was therefore decided to see whether the titration behavior of RNase in the presence of 1 Cu(II) could be fit with the simple assumption that each Cu(II) distributes over a set of 4 independent imidazoles the interaction of which with Cu(II) is similar to that of imidazoles in smaller peptides (7).

In the absence of the α-amino terminus, the binding of Cu(II) in a 1:1 ratio to such peptides below pH 8 is described by the following equations (7).

\[
\begin{align*}
Cu^{++} + L^- & \rightleftharpoons CuL^+ \quad K_1 = \frac{(CuL^+)}{(Cu^{++})(L^-)} \quad (1) \\
CuL^+ & \rightleftharpoons CuL^0 + H^+ \quad K_e = \frac{(CuL^0)(H^+)}{(CuL^+)} \quad (2) \\
CuL^0 & \rightleftharpoons CuL^- + H^+ \quad K_{e'} = \frac{(CuL^-)(H^+)}{(CuL^0)} \quad (3)
\end{align*}
\]

where \( L^- \) is a histidine-containing peptide with the imidazole in the unprotonated state. \( K_1 \) therefore represents the intrinsic association constant between Cu(II) and the unprotonated imidazole. \( K_e \) and \( K_{e'} \) describe the dissociation of the first and second peptide bond protons which accompanies interaction of Cu(II) with sequential peptide bond nitrogen atoms in the Cu(II) complexes. In a series of peptides, values of log \( K_1 \) were found to vary from 3 to 4.4; values of \( pK_e \) and \( pK_{e'} \) ranged from 5.95 to 7.35.

Also to be considered along with Equations 1 to 3 is the equilibrium between imidazoles and \( H^+ \) ions. In RNase, in the presence of metal, this is described at 25°C by the equation

\[
K_h = \frac{\rho e^{+\epsilon Z}}{(n - \rho_h - \rho_e)(H^+)} = 10^{e - a}
\]

where \( n \), the number of imidazoles per mole, is equal to 4; \( \rho_h \) and \( \rho_e \) are the number of imidazoles carrying protons or metal ions, respectively; \( \epsilon Z \) is the net protein charge; and \( a \) is 0.06 at 0.16 ionic strength (19).

Assuming that Cu(II) binds to RNase as it does to imidazole-containing peptides, then Equations 1 to 3 indicate that

\[
\rho_m = \frac{(CuL^+)}{(CuL^0) + (CuL^-) + (CuL^+)}
\]

An apparent pH-dependent constant, \( K_m \), can be defined as

\[
K_m = \frac{e^{+\epsilon Z}}{(Cu^{++})(n - \rho_m - \rho_e)}
\]

where \( (Cu^{++}) \) is the free Cu(II) concentration, \( e^{+\epsilon Z} \) is the electrostatic interaction term for Cu(II) ion, and \( K_m \) is related to \( K_1, K_e, \) and \( K_{e'} \) by

\[
K_m = K_1 \left( \frac{1 + K_e e^{+\epsilon Z}}{(H^+)} + \frac{K_{e'} e^{+\epsilon Z}}{(H^+)^2} \right)
\]

Substituting for \( (n - \rho_m - \rho_e) \) in Equations 6 and 4, it follows that at any pH

\[
\frac{\rho_m}{\rho_a} = \frac{K_m(Cu^{++}) e^{+\epsilon Z}}{K_1(H^+)}
\]

At a constant pH, the number of protons displaced \( -\Delta n / Cu \) in the presence of a given Cu(II) concentration is the difference between \( \rho_a \) in the absence and presence of Cu(II) plus the number displaced from peptide bond nitrogens. The latter can be estimated from Equations 2 and 3 substituting \( K_e e^{+\epsilon Z} \) and \( K_{e'} e^{+\epsilon Z} \) for \( K_e \) and \( K_{e'} \), respectively, to allow for electrostatic interactions in the protein.

To fit the titration curve of RNase in the presence of 1 Cu(II) with the above assumptions, values of log \( K_1, pK_e \), and \( pK_{e'} \) for RNase should be chosen so as to be comparable to peptide imidazoles of similar basicity (7). From model data, values of \( pK_e \) and \( pK_{e'} \) of approximately 5.9 and 6.9, respectively, are predicted. With these constants and Equations 4 to 8, the titration data were analyzed by successive approximations to obtain self-consistent values of \( -\Delta n / Cu \) for Cu(II) and RNase with the use of the relation

\[
(Cu^{++})_{total} = (Cu^{++})_{free} + (RNase)\rho_m
\]

The value of \( K_h \) necessary to fit the data was \( 10^{e - a} \), somewhat higher than the value of \( 10^{e - a} \) interpolated from the data of Bryce, Roeseke, and Gurd (7).

A theoretical plot of \( -\Delta n / Cu \) with respect to pH (calculated for the same Cu(II) and RNase concentrations used in titration) is shown in Fig. 3 along with the experimental curve. Since the Cu(II)-binding sites change above pH 8, no attempt was made to fit the data above this pH. The fit of experimental and theoretical curves is rather good, particularly when ambiguities surrounding the basicity of RNase imidazoles are considered (24, 29). This suggests that the main Cu(II)-binding sites in RNase below pH 8 are the 4 imidazoles and their adjacent peptide bond nitrogens. In addition, the relatively good agreement between theoretical and experimental titration curves is paralleled by good agreement between the observed Cu(II)-RNase spectra and that predicted by theory. The ascribed values of \( pK_e \) and \( pK_{e'} \), together with the different wave length maxima of absorption peaks associated with the species CuL^+, CuL, and CuL^0, predict a progressive decrease in wave length maximum between pH 5 and 8 from about 700 nm to about 600 nm, in good agreement with that found.

While the above, therefore, implies that RNase behaves similarly toward Cu(II) as do less complex imidazole-containing pep-
It is apparent that deviations between theoretical and experimental titration curves do occur below pH 5.3 and between pH 7 and 8. Below pH 5.3 these may represent Cu(II)-carboxylate interactions; above pH 7 deviations are potentially interpretable as a difference among the sites in values of $pK_a$ and $pK_{eq}$. However, it is particularly probable that conformational restraints in RNase affect Cu(II) RNase interactions in an unpredicted manner. Relevant to this is the fact that the titration of RNase in the presence of 2 Cu(II) can only be fit with a higher intrinsic Cu(II)-binding constant than used to fit the titration in the presence of 1 Cu(II). This can be seen in Fig. 1 and Table I where it is evident that between pH 5 and 6 the second Cu(II) displaces a somewhat greater number of protons from RNase than the first Cu(II), whereas simple statistical assumptions demand that it should displace fewer protons if $K_1$ were unchanged. These data suggest that the second Cu(II) is bound more strongly than the first and that cooperative interactions, probably arising from conformational changes, occur in the binding of Cu(II) to RNase.

Interaction of Cytidic Acids with RNase—The effect of 2'-CMP and 3'-CMP on Cu(II)-binding was studied initially by titration. Interpretation of the data, however, demanded that the titration of RNase and CMP derivatives together, in the absence of Cu(II), first be determined. For this, 2'-CMP and 3'-CMP were first each titrated alone and then in the presence of 1 eq of RNase. The titration curves of the nucleotides alone were in good agreement with literature data (17). The titration curves of the nucleotides in the presence of RNase indicated that the binding of either 2'-CMP or 3'-CMP to RNase is accompanied by marked changes in $H^+$ ion equilibria. With 2'-CMP, the results were in essentially complete agreement with the data of Hummel and Witzel (30). With 3'-CMP, changes in $H^+$ ion equilibria which accompanied binding to RNase were qualitatively identical with those occurring with 2'-CMP, but of lesser magnitude at the same concentration. This quantitative difference is a probable reflection of the weaker binding of the 3' derivative to RNase (31); a fundamental difference in the nature of the apparent pK shifts is not indicated. Of relevance to the present study is that binding of either CMP derivative to RNase is accompanied by no net change in proton equilibria at pH 5.5. Above pH 5.5, binding is accompanied by an uptake of protons, and, below pH 5.5, binding is accompanied by proton release.

Effect of 3'-CMP on Cu(II) Binding—RNase plus 1 eq of 3'-CMP was titrated in the presence of 1 eq of CuCl$_2$ in the pH region 3 to 11; no precipitation was observed during the titration. The difference in $h$ at a given pH ($-\Delta h/Cu$) between this curve and that of RNase plus 3'-CMP is plotted in Fig. 3 along with the equivalent data for RNase alone and in the presence of 2'-CMP. It is readily seen that, in the presence of 3'-CMP, Cu(II) displaces a much larger number of protons in the pH region 4.5 to 5.8 than in the absence of 3'-CMP. This effect was verified at pH 5.3 by direct pH-stat titrations of H$^+$ ion released by Cu(II) in the absence of CMP and in the presence of 2'-CMP and 3'-CMP (Table I); the increased proton liberation in the presence of 3'-CMP is clearly seen.

In the presence of CMP, values of $-\Delta h/Cu$ were calculated from the continuous titration curves by subtraction of $h$ at a given pH in the presence of Cu(II) and CMP from the parent titration curve of RNase + CMP in the absence of Cu(II).

The increase in $-\Delta h/Cu$ at and below pH 5.5 in the presence of 3'-CMP indicates that binding of 3'-CMP facilitates the interaction of Cu(II) with RNase; it is also possible that part of the change in $-\Delta h/Cu$ originates from a change in ligands to which Cu(II) is bound. (The increase in $-\Delta h/Cu$ cannot be ascribed to competition between 3'-CMP and Cu(II) for the same set of sites such that 3'-CMP is displaced by Cu(II).) This follows since the increase is particularly marked at pH 5.5 and displacement of 3'-CMP from RNase leads to no net change in $h$ at this pH. Other thermodynamic considerations as well indicate that the increase in $-\Delta h/Cu$ can only reflect a cooperative interaction among 3'-CMP, Cu(II), and RNase.

Equilibrium dialysis studies, to be reported in full in a subsequent communication, do indicate that binding of Cu(II) to RNase is considerably strengthened by 3'-CMP. That, in fact, a change in ligands bound to the first Cu(II) also occurs in the presence of 3'-CMP is indicated by the spectrum of RNase (Fig. 4). It is apparent that 3'-CMP significantly lowers the absorbance of the Cu(II)-RNase complex at pH 7.2, and, at all pH values, the $\lambda_{max}$ of the Cu(II)-visible absorption band is somewhat higher in the presence than in the absence of 3'-CMP.

The direction of the spectral change in the presence of 3'-CMP indicates that the bound Cu(II) is in a less intense ligand field and suggests that perhaps an oxygen has been substituted for a nitrogen ligand to Cu(II) (12). One explanation of these data is that, in the presence of 3'-CMP, a new site becomes available in which Cu(II) is bound to the phosphate oxygen of 3'-CMP and at least one other ligand, probably a nitrogen. A similar thesis has been advanced for the enhancement of Zn(II)-inhibition of RNase in the presence of 3'-CMP (2). However, study of the effect of 3'-CMP upon the second Cu(II) added to RNase indicates that more than 1 Cu(II) is affected by 3'-CMP.
Fig. 4B, it can be seen that the spectrum of the second Cu(II) added to the 3'-CMP-RNase complex is even more displaced to longer wave lengths than that of the first. Moreover, pH-stat titrations at pH 5.5 also indicate that at least the first 2 Cu(II) added in the presence of 3'-CMP bind differently than in the absence of 3'-CMP (Table I).

These data suggest that either 2 Cu(II) interact directly with 3'-CMP bound to RNase or that formation of a ternary complex between the first Cu(II), 3'-CMP, and RNase leads to a conformational change which alters the binding pattern of subsequent Cu(II) ions. A less likely interpretation is that a conformational change in RNase induced by 3'-CMP itself alters the binding pattern of Cu(II) without direct interaction between bound Cu(II) and bound 3'-CMP. This is improbable since 2'-CMP has a very different effect from 3'-CMP upon the equilibria between Cu(II) and RNase (Fig. 3), while both 2'-CMP and 3'-CMP have at least qualitatively similar effects upon the conformation of RNase (32).

Effect of 2'-CMP on Cu(II) Binding—In Fig. 3, the pattern of proton displacement by Cu(II) from RNase in the presence of 2'-CMP is also shown. Comparison of the data with that for RNase alone indicates that a slight but significant decrease in the number of protons released by Cu(II) below pH 6 occurs in the presence of 2'-CMP. This effect can be seen more clearly from pH-stat titrations at pH 5.5 as shown in Table I; it suggests that, in contrast to the effect of 3'-CMP, the effect of 2'-CMP is to diminish slightly the affinity of RNase for Cu(II). Moreover, spectral studies show that at pH 7 the spectrum of RNase in the presence of 1 eq of Cu(II) is unaltered by 2'-CMP, again in contrast to the effect of 3'-CMP. These data suggest that 2'-CMP does not alter the ligands to which the first Cu(II) is bound at pH 7.

It can also be shown that the first Cu(II) does not significantly displace 2'-CMP at pH 7. Our data and those of Hummel and Witzel (30) indicate that any release of 2'-CMP would be accompanied by an appreciable proton release above pH 5.5. It is not possible to reconcile the identity of Cu(II)-RNase spectra in the presence and absence of 2'-CMP with the identity of values of $\Delta H$; Cu near pH 7 if a significant fraction of bound 2'-CMP is displaced by the first Cu(II). Thus, although 2'-CMP somewhat diminishes the affinity of RNase for Cu(II), these data indicate that RNase can accommodate simultaneously 1 2'-CMP and 1 Cu(II) without apparent alteration of the ligands to which each is bound.

The diminished affinity of RNase for Cu(II) in the presence of 2'-CMP could result either from the effect of 2'-CMP upon the conformation of RNase or from direct competition by 2'-CMP for one of the several sites usually available to Cu(II).9 Histidines-12 and -119 appear necessary for the binding of 2'-CMP (cf. Reference 31). Since binding of Cu(II) in the absence of 2'-CMP also appears to involve these residues, direct competition between 2'-CMP and Cu(II) is particularly probable. The net effect of such competition would be to reduce the number of sites over which Cu(II) distributed in the presence of 2'-CMP.

It is of interest that, near pH 7, 2'-CMP affects neither the proton displacement nor the spectrum of the first bound Cu(II). Such data are not readily compatible with an important contribution of a bis-imidazole site composed of His-12 and His-119 to the usual set of Cu(II)-binding sites. Bound 2'-CMP would necessarily alter (and probably remove) the availability of such a site to Cu(II). It is unlikely that such effects upon a unique member of a set of Cu(II)-binding sites would not have discernible repercussions either on the spectrum or the number of protons displaced by Cu(II) at pH 7. The preferred postulate of the essential identity of all the Cu(II)-binding sites allows removal of either His-12 or His-119 from availability to Cu(II) with no change in those properties.

Interaction of RNase with Zinc Ions—The effects of 1 mole of added ZnCl2 on RNase, RNase plus 3'-CMP, and RNase plus 2'-CMP were determined by continuous titration in identical manner with the studies of Cu(II) interactions. All titrations were freely reversible between pH 3 and 11 and, as with Cu(II), no precipitation was observed at any pH. Values of $\Delta H$/Zn at a given pH were computed from the titration data in the usual manner and are shown in Fig. 5. In the absence of CMP, no effect of Zn(II) upon the H+ ion equilibria of RNase is seen below pH 5.5. Between pH 5.5 and 7.3, slight H+ ion displacements are observed which suggest competition between Zn(II) and H+ for the protein in this pH region. Above pH 7.5, 2 additional protons are titrated in the presence of Zn(II); it is

9 The effect of 2'-CMP upon the Cu(II)-RNase interaction is too great to be ascribed to competition between free 2'-CMP and free RNase for Cu(II). Control titrations indicate that binding of Cu(II) by 2'-CMP and 3'-CMP is relatively very weak compared with the Cu(II)-RNase interaction. Similarly, no important differences between the two free nucleotides in their interaction with Zn(II) and Cu(II) have been found which could explain their different effects on metal binding by RNase.
not clear whether this represents formation of Zn(OH)_2 or binding
of Zn(II) to protonated side chains, although no precipitation
occurs. As in the case of Cu(II), however, the number of protons
displaced by Zn(II) below pH 6 is too low to allow for the pres-
ence of a bis-imidazole site of comparable affinity for Zn(II) as
4,4'(5,5')bis-imidazolylmethane (23). Although a weaker
binding of Zn(II) to a bis-imidazole site is not ruled out by the
present data, the simplest explanation of the data lies in assum-
ing 1:1 complex formation between Zn(II) and imidazole side
chains.

In Fig. 5, the effect of 1 Zn(II) on the titration of RNase in
the presence of CMP is also shown. In the presence of 3'-CMP,
the interaction pattern of Zn(II) with RNase is strikingly
changed; Zn(II) becomes a much stronger competitor with H^+
ion for binding sites on the protein. At pH 6.5, Zn(II) appears
bound to at least one site whose pK_a is usually above 6; the
existence of a second ligand to Zn(II) with pK_a between 6 and
7.5 is also suggested by the values of ΔH/Zn in this pH region.
As in the absence of 3'-CMP, it is not possible to interpret the
titration of 2 additional protons above pH 7. The effect of 2'
CMP clearly differs from the effect of 3'-CMP, but no interpreta-
tion can be made at this time.

The effect of Zn(II) upon 3'-CMP-RNase titrations is not due
to competition between 3'-CMP and Zn(II) for sites on RNase.
Above pH 5.5, dissociation of 3'-CMP from RNase is accom-
panied by H^+ ion release. However, in the pH region 6.5 to 8,
the data indicate that more protons are displaced by Zn(II)
from the 3'-CMP-RNase complex than would be allowed by
even complete dissociation of 3'-CMP from RNase and combina-
tion of Zn(II) with its usual sites on free RNase. This is seen
in Fig. 6, where a theoretical curve representing the titration of
Zn(II)-RNase in the presence of free 3'-CMP is compared with
the experimental titration curve of Zn(II) and RNase in the
presence of 3'-CMP.

By arguments previously advanced for Cu(II), the data there-
fore indicate that the affinity of RNase for Zn(II) is increased
by 3'-CMP. This effect has been confirmed by equilibrium
dialysis and is in accord with the studies of Ross et al. (2). Al-
though it has not particularly been shown here, it is also possible
that the ligands to which Zn(II) is bound are altered in the
presence of 3'-CMP.

Possible Mechanisms of RNase Inhibition by Zinc and Cupric
Ions. It is not known how many zinc or cupric ions must be
bound to RNase in order that catalysis be completely inhibited.
The data presented here suggest that several types of inhibition
may occur.

For Cu(II), in the absence of nucleotides, there are several
potential binding sites, each of which most likely contains 1
imidazole and 2 peptide nitrogens at the usual pH of RNase
assay. Although we find no evidence for coordination of a single
Cu(II) between both His-12 and His-119, Cu(II) may well in-
teract with these residues individually. The importance of
His-12 and -119 to enzymic activity has been well documented.
Binding of a single Cu(II) to either His-12 or His-119 individually
should therefore reduce activity, particularly if such binding
involved reorientation of the imidazole ring to allow formation
of a chelate involving the adjacent peptide bond nitrogens.
Binding of Cu(II) to imidazoles other than those of His-12 and
His-119, however, might also lead to inactivation. Binding of

\[ \text{Ternary 3'CMP-Cu(II) Complex} \]

\[ \text{2'CMP Complex + Cu(II)} \]

\[ \text{Scheme 1} \]

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**Fig. 6.** Titration of RNase in the presence of 1 eq of ZnCl_2
and 3'-CMP. **Experimental:** □, pH 5.5 to 3; ○, pH 3 to 11; △,
pH 11 to 5.5. **Theoretical** curve calculated from the sum
of the titration curve of RNase in the presence of 1 eq of ZnCl_2
and the titration curve of free 3'-CMP.
Cu(II) to myoglobin and apomyoglobin disrupts the native structure presumably because helical segments are broken by formation of imidazole Cu(II) peptide N chelates (33). Moreover, it has recently been reported that binding of Cu(II) to a histidine-containing peptide derived from residues 38 to 61 of RNase disrupts its helical conformation in trifluoroethanol (34). Therefore, binding of Cu(II) to imidazoles other than those at the active site itself may well lead to disruption of the conformation necessary for activity.

In the presence of 3'-CMP, it is clear that the binding of both Zn(II) and Cu(II) to RNase is altered. Not only do the Cu(II)-binding sites differ in the presence and absence of 3'-CMP, but Zn(II) and Cu(II) binding to RNase are strengthened by 3'-CMP. Thermodynamically, it follows then that the binding of 3'-CMP is strengthened by Zn(II) and Cu(II) ions. Since 3'-CMP is an inhibitor, an increase in its affinity for RNase could explain some of the inhibition of cyclic 2',3'-CMP hydrolysis found in the presence of Zn(II) and Cu(II) ions. In the particular case that Cu(II) and Zn(II) might interact directly with 3'-CMP in the complex, then increased inhibition by these metals in the presence of 3'-CMP, as shown for Zn(II) (2), might be due to relocation of a bound metal ion from a set of sites among which it is distributed in the absence of 3'-CMP to the active site itself.

The different effects of 2'- and 3'-CMP upon metal ion-interaction seen here have also in part been observed by Ross et al. for Zn(II) interaction (2). The possibility that the conformation of RNase differs in the presence of the two analogues cannot completely be ruled out. The explanation which we prefer for this difference, however, is that the phosphate of only bound 3'-CMP is located within Cu(II)- or Zn(II)-bonding distance of another potential ligand (N-1) to these metals, whereas the phosphate of the 2' derivative sterically interferes with binding to a usually available Cu(II)-binding site (N-2). Such a situation may be depicted as shown in Scheme 1. In the light of existing information, ligands N-1 and N-2 could be His-12 and His-119, but other ligands are not precluded.

It is apparent that further studies are necessary to ascertain the exact relationship between Cu(II)- and Zn(II)-binding sites and the active site of RNase. It is also clearly of interest to learn whether the effects of 3'-CMP upon Cu(II)- and Zn(II)-bonding are peculiar to 3'-CMP or whether they are common to all products (or substrates) of RNase. The data presented here, however, do indicate the potential relevance of these effects to the mapping of the active site of RNase.

Acknowledgment—The authors wish to express their appreciation to Dr. A. Crestfield for many invaluable discussions.

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