Ultraviolet Difference Spectral Studies of Conalbumin Complexes with Transition Metal Ions*

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ABSTRACT: Evidence was sought for the binding of various transition metal ions to hen's egg conalbumin using ultraviolet difference spectroscopy. Difference spectra of metal–conalbumin *vs.* conalbumin were recorded in the 230–320-mμ region at pH 8.5. In addition to the known binding of Fe^{3+}, Cu^{2+}, and Zn^{2+} to conalbumin, Cr^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+} were found to react with the two specific binding sites of the protein.

The difference spectra were characterized by two maxima near 245 and 295 mμ. The λ_{max} and ϵ_{max} depended upon the specific metal bound. These spectral shifts may be attributed to perturbations caused by the binding of different metal ions to the tyrosyl residues of the binding sites and by the involvement of ligands other than tyrosine in the metal–conalbumin binding. The increase in absorbance at 245 and 295 mμ on binding of metal ions by conalbumin revealed that two tyrosyl residues are coordinated to each trivalent metal ion whereas one tyrosyl residue is coordinated to each divalent metal ion. Displacement of bound metal by other metal ions showed that the relative stability of the transition metal conalbumin complexes is in the following order: $Fe^{3+} > Cr^{3+}$ $Cu^{2+} > Mn^{2+}$, Co^{2+}, $Cd^{2+} > Zn^{2+} > Ni^{2+}$.

Conalbumin from hen's egg white binds 2 moles of Fe^{3+}/mole of protein to form a stable salmon-colored complex characterized by a broad visible absorption band with a maximum at about 465 mμ (Schade *et al.*, 1949; Fraenkel-Conrat and Feeney, 1950). The interaction of conalbumin with the following metals has also been studied by various groups during the last two decades: Cu^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, and Mn^{2+}. A mole of conalbumin can bind 2 moles of Cu^{2+} with the formation of a yellow complex with a maximum absorption at 440 mμ (Schade *et al.*, 1949; Frankel-Conrat and Feeney, 1950; Warner and Weber, 1953) and forms a loose colorless complex with 2 moles of Zn^{2+} (Warner and Weber, 1953). From the results of their metal displacement studies Warner and Weber concluded that conalbumin shows only a small combining ability for Co^{2+} and Cd^{2+} ions, but none for Ni^{2+}. It is generally accepted that conalbumin combines with these various metal ions at specific binding sites on the protein molecule (Buttkus *et al.*, 1965).

There have been few reports concerning the interaction of conalbumin with metal ions other than Fe^{3+} and Cu^{2+}. Recently, Michaud and Woodworth (Michaud and Woodworth, 1966; Michaud, 1967) reported the observation of two maxima at 242 and 294 mμ in the ultraviolet difference spectrum of iron-saturated *vs.* iron-free conalbumin. This observation was explained in terms of the binding of Fe^{3+} to conalbumin resulting in the dissociation of tyrosyl residues in the specific binding sites. These findings suggest that if conalbumin combines with metals other than iron at the same specific sites one should be able to detect the resulting complexes using the difference spectrophotometric method. This report presents our findings regarding the possible interaction of conalbumin with the first transition series ions Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} and the second transition series ion Cd^{2+}.

Materials and Methods

Conalbumin was prepared as previously reported (Woodworth and Schade, 1959) with the modification that the protein was chromatographed on a column of CM-Sephadex C-50 rather than CM-cellulose. The preparation was found to be pure by spectrophotometric and metal-binding criteria and to be homogeneous on electrophoresis in polyacrylamide gel (Woodworth and Clark, 1967).

Chemicals were reagent grade and were used without further purification. CM-Sephadex C-50 was obtained from Pharmacia Fine Chemicals, New Market, N. J. Glass-distilled water was used for making all solutions and dilutions.

Stock aqueous metal ion solutions were 0.02 M in $CuCl_2$, $Zn(Ac)_2$, $MnCl_2$, $NiSO_4$, $CdCl_2$, $CoCl_2$, $CrCl_3$, and $Fe(NH_4)_2(SO_4)_2$.

All glassware was soaked in 8 N HNO_3 for several hours, rinsed several times with distilled water and two to three times with glass-distilled water, and dried to ensure that it was free of traces of heavy metals.

The difference spectra were recorded with a Cary 15 spectrophotometer. A Zeiss PMQII spectrophotometer was used to determine precisely the absorption maxima and their corresponding absorbances.

Protein concentrations were chosen to give an A_{440} of 0.3–0.8 for Cu–conalbumin. Protein concentrations were determined by titrating aliquots of stock conalbumin solutions photometrically at 440 mμ against 0.02 M $CuCl_2$ solution or by measuring A_{280}. $\epsilon_{280}^{1\%}$ 12.0 was used for calculating concentrations of metal-free conalbumin (Woodworth and Schade, 1959).

Recording of pH Difference Spectra. Both the sample and

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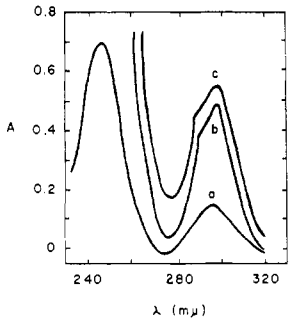

FIGURE 1: Conalbumin pH difference spectra. (a) pH 10.8 *vs.* pH 7.0; (b) pH 12.5 *vs.* pH 7.0, recorded 5 min after the sample solution was adjusted to pH 12.5; and (c) pH 12.5 *vs.* pH 7.0 recorded 2.5 hr after the sample solution was adjusted to pH 12.5. Conalbumin concentration = 0.18 mM; 0.2 N KCl. Sample solution unbuffered, reference solution 0.01 M in Tris-HCl (pH 7.0). Optical path length = 0.5 mm.

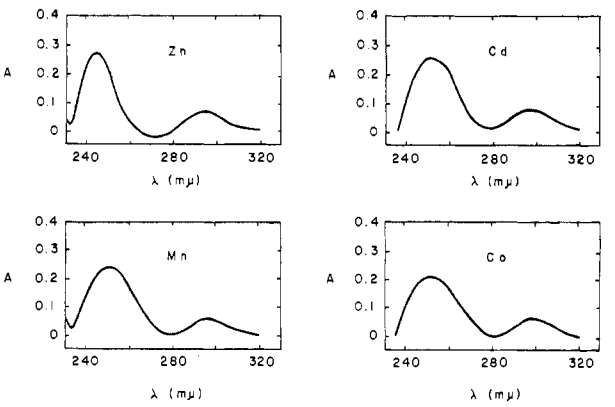

FIGURE 2: Zn-, Cd-, Mn-, and Co-saturated conalbumin *vs.* metal-free conalbumin ultraviolet difference spectra. Conalbumin concentration = 0.18 mM, in 0.01 M Tris-HCl–0.005 M $NaHCO_3$ (pH 8.5). Optical path length = 0.5 mm.

reference solution had a conalbumin concentration of 0.18 mM in 0.2 N KCl. The unbuffered sample solution was adjusted to the desired pH by the addition of 10 N KOH, whereas the pH of the reference solution was kept constant in 0.01 M Tris-HCl buffer (pH 7.0). The difference spectra were recorded 5 min after the pH of the sample solution was adjusted, unless otherwise stated. The pH of the sample solution was also measured after the spectrum was recorded. The changes in pH before and after recording were less than 0.01 pH unit. The change in volume owing to the addition of 10 N KOH was less than 1%. No correction was made to account for this small volume change.

Metal Binding Studies. Solutions of conalbumin were prepared by mixing 2.0 ml of 0.18 mM conalbumin, 20 μl of 1 M Tris-HCl buffer (pH 8.5), and 10 μl of 1 M $NaHCO_3$. Metal-saturated conalbumin solutions were prepared by adding 4 moles of aqueous metal ion/mole of conalbumin. The complexing of Mn, Co, Ni, Cu, Zn, and Cd with conalbumin appeared to be completed within less than 3 min as judged from the fact that no changes in absorbance in the 230–320-mμ region were observed when the metal–conalbumin samples stood for 10 min as compared with those for 3 min. The Fe– and Cr–conalbumin complexing were somewhat time dependent, but the ultraviolet spectra of these metal–conalbumin complexes were constant after the samples had stood for more than 40 hr. To ensure complete reaction, Fe^{3+}– and Cr^{3+}–conalbumin were allowed to stand for 2 days, and other metal conalbumin complexes for more than 10 min, before difference spectra were recorded. The difference spectra were obtained by placing the metal-saturated conalbumin in a 1.0-cm silica absorption cell in the sample beam and the metal-free conalbumin in a matching cell in the reference beam. The path length in each cell was reduced to 0.5 mm with a 9.5-mm silica spacer.

Determination of Moles of Metal Ion Bound per Mole of Conalbumin. Aliquots of standard 0.02 M metal ion solution were added to a solution of conalbumin of known concentration in 0.02 M Tris-HCl buffer (pH 8.4) with a Hamilton syringe attached to a Hamilton PB-600-1 repeating dispenser. The changes in absorbance at 245 and 295 mμ in the cases of Zn, Cd, Mn, Co, or Ni and at 465, 440, or 435 mμ in the Fe, Cu, or Cr binding studies, respectively, were recorded 10–15 min after each addition of an aliquot of metal ion. The moles of metal ion bound per mole of conalbumin were calculated from the end point of the titration curve.

Metal Displacement Studies. A solution of competing metal ion, in twofold molar excess over the bound metal, was added to the metal–conalbumin complex, and let stand for 10 min in the case of competing metals other than Fe or Cr, and for 2 days in the case of Fe or Cr. This newly equilibrated sample was compared with samples of conalbumin saturated with initially bound metal and with competing metal ion by recording the respective difference spectra. These difference spectra were then compared with the difference spectrum of conalbumin saturated with competing metal *vs.* conalbumin saturated with initially bound metal. In another set of displacement experiments, a tenfold molar excess of competing metal ion was used. In all cases equilibrium was ensured on the basis of the constancy of the ultraviolet absorption spectra of the metal–conalbumin complexes, before difference spectra were recorded.

Results

pH-Dependent Difference Spectra of Conalbumin. The ultraviolet difference spectra of conalbumin at alkaline *vs.* neutral pH showed maxima at 245 and 295 mμ with an absorbance ratio of 4.6:1 and a negative absorbance in the 275-mμ region. Figure 1a shows a difference spectrum for pH 10.8 *vs.* pH 7.0. The close similarity of this spectrum to that for the ionized *vs.* un-ionized phenol group of *N*-acetyl-L-tyrosine (Tachibana and Murachi, 1966) assures us that this pH-dependent difference spectrum of conalbumin represents the dissociation of tyrosyl residues only.

The difference spectra of conalbumin at pH > 11.2 *vs.* pH 7.0 showed that although the two tyrosyl dissociation maxima were still observed, A_{245}/A_{295} was greater than 4.6 and increased with increasing pH. Moreover, a shoulder at 290–292 mμ appeared as did positive absorbances in the 275-mμ region. These time-dependent spectral changes (Figure 1b,c) have been attributed to transfer of buried tryptophyl residues into an aqueous environment (Tan and Woodworth, 1968a).

Ultraviolet Difference Spectra of Metal–Conalbumin vs. Conalbumin. The difference spectra of metal–conalbumin *vs.* conalbumin are shown in Figures 2 and 3. Two sharp maxima

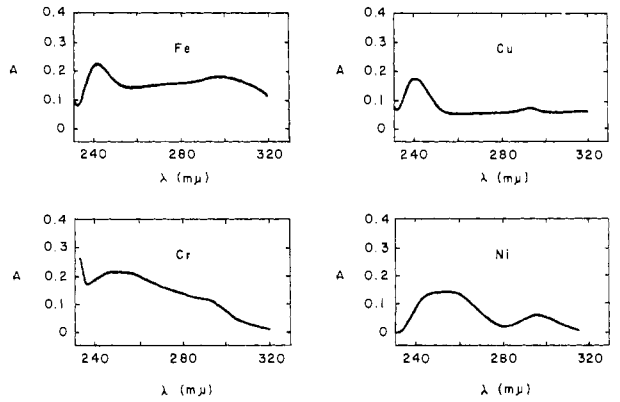

FIGURE 3: Fe-, Cu-, Cr-, and Ni-saturated conalbumin *vs.* metal-free conalbumin ultraviolet difference spectra. Conalbumin concentration = 0.18 mM, in 0.01 M Tris-HCl–0.005 M $NaHCO_3$ (pH 8.5). Optical path length = 0.5 mm.

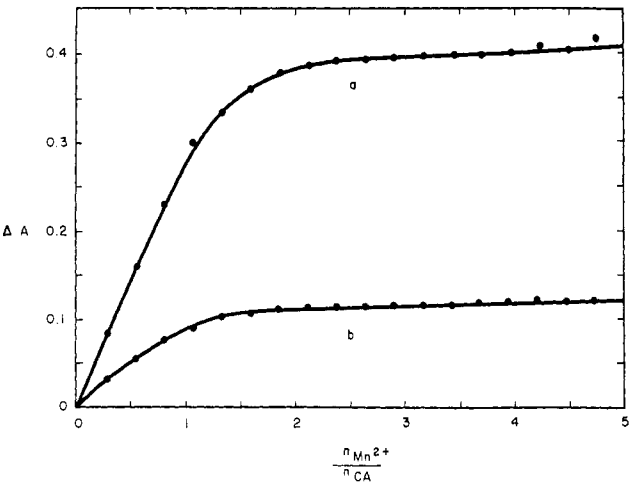

FIGURE 4: Titration of conalbumin with Mn^{2+}. To 2 ml of conalbumin (0.019 mM in 0.02 M Tris-HCl, pH 8.4) increments of 0.02 M $MnCl_2$ were added. (a) Changes observed at 245 mμ; (b) changes observed at 295 mμ. Optical path length = 1.0 cm.

appear in the region of 245 and 295 mμ in some of these spectra. The exact location of the maxima and their corresponding absorbances depend upon the particular metal bound. The Zn-binding difference spectrum has two maxima at 245 and 295 mμ, and a negative absorption at 270 mμ. This spectrum is identical with Figure 1a and with that for the ionized *vs.* unionized phenol group in *N*-acetyl-L-tyrosine (Tachibana and Murachi, 1966). Although the Ni-binding difference spectrum has two maxima at 245 and 295 mμ, identical in position with those for Zn, A_{245}/A_{295} is smaller than in the case of Zn. Furthermore, instead of negative absorption in the 270-mμ region, appreciable absorption in the 260-mμ region is observed. The difference spectra for Co, Cd, and Mn binding show a shift in maxima to 250 and 297 mμ and also appreciable absorption in the 260-mμ region.

The difference spectra of the colored complexes, *i.e.*, Fe^{3+}–, Cr^{3+}–, and Cu^{2+}–conalbumin *vs.* conalbumin, are more complex. Although maxima in the region of 245 and 295 mμ are still observed, there are also strong absorptions in other regions. The shapes of these spectra are not the same as the tyrosine dissociation curve.

There were no changes in the shapes and only small increases in ϵ_{max} of the difference spectra on addition of metal ions in excess of 2 moles of metal ion/mole of conalbumin.

Metal Titration Studies. The spectroscopic titration curves for Fe^{3+} or Cu^{2+} binding to conalbumin showed a linear increase in absorbance at 465 or 440 mμ, on addition of aliquots of standard metal ion solutions but leveled off when the ratio of 2 moles of metal ions/mole conalbumin was reached.

The Cr^{3+}–conalbumin complex has a light blue-green color. Its visible spectrum has two maxima at 435 and 610 mμ. A titration study based on the increase of absorbance at these wavelengths showed that on the addition of excess Cr^{3+} the absorbance continued to increase in a linear manner rather than level off. The slope of this line was less than the slope of the linear increase in absorption from 0 to 2 moles of Cr^{3+} per mole of conalbumin, indicating that the nature of the binding of excess Cr^{3+} ions was different from that of the first two Cr^{3+} ions.

For titration of conalbumin with metal ions yielding no colored complexes, the increases in absorbance at 245 and 295 mμ were recorded, as sequential aliquots of metal ion were added. The shapes of all these titration curves were similar to that for Mn^{2+} and conalbumin shown in Figure 4. These titration curves demonstrated that 2 moles of each transition metal ion investigated can bind to the specific binding sites per mole of conalbumin.

Estimation of $\Delta\epsilon_{245}$ *and* $\Delta\epsilon_{295}$. Increases in molar absorbance at 245 and 295 mμ, $\Delta\epsilon_{245}$ and $\Delta\epsilon_{295}$, on addition of Cu^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+} to conalbumin at pH 8.4 were calculated from the absorbance increases following addition of twice the amount of metal ion necessary to saturate the conalbumin. The data for Cu^{2+} and Co^{2+} were corrected for absorbance caused by complexes of these metal ions with the Tris buffer. Control solutions of Cd, Mn, Ni, or Zn ions in the Tris buffer used did not give measurable absorption in the 240–320-mμ region. The values of $\Delta\epsilon_{245}$ and $\Delta\epsilon_{295}$ were constant over the pH range 8.0–9.0, but fell off sharply outside of this range, and are listed in Table I.

The values of $\Delta\epsilon_{245}$ and $\Delta\epsilon_{295}$ for Fe^{3+}– and Cr^{3+}–conalbumin were constant over the pH range 7.0–9.0. The values listed in Table I for these two complexes were obtained by subtracting the values for $\Delta\epsilon_{245}$ and $\Delta\epsilon_{295}$ at pH 11.0 from those at pH 7.0. At pH 11.0 all exposed tyrosyl residues in Fe^{3+}–conalbumin and conalbumin are ionized, so that the difference spectrum for these two species represents the non-pH-dependent portion of the spectrum arising from ligands other than tyrosine (Michaud, 1967). Subtraction of this residual spectrum from the difference spectrum at pH 7.0 results in a spectrum identical with that in Figure 1a. Similar considerations hold for the Cr^{3+}–conalbumin complex. Therefore the values listed in Table I for these complexes represent ionization of tyrosine owing to binding of the metal ions.

Metal Displacement Studies. Comparison of the various difference spectra yielded the results of metal displacement studies, on a 1:1 basis, which are summarized in Table II. Because of the inherent error in comparing small differences in difference spectra, quantitative determination of metal substitution studies is difficult, and the errors of the magnitudes listed in Table II were expected to be large. However, since the results were reproducible, it is not unreasonable to say that

TABLE I: The Effect of Metals on $\Delta\epsilon_{245}$ and $\Delta\epsilon_{295}$ of Conalbumin (CA).[a]

Metals	$\Delta\epsilon_{295}$	No. of Tyr/Mole of CA	$\Delta\epsilon_{245}$	No. of Tyr/Mole of CA	No. of Expt
Fe^{3+}	9,500 ± 95	4.04 ± 0.04	36,333 ± 1,530	3.63 ± 0.15	3
Cr^{3+}	8,326 ± 651	3.54 ± 0.28	38,743 ± 2,806	3.87 ± 0.28	3
Cu^{2+}	4,850 ± 401	2.06 ± 0.17	19,906 ± 1,756	1.99 ± 0.18	4
Mn^{2+}	4,875 ± 506	2.07 ± 0.22	20,337 ± 1,996	2.03 ± 0.34	8
Co^{2+}	4,845 ± 518	2.06 ± 0.22	20,495 ± 2,916	2.04 ± 0.29	8
Cd^{2+}	5,262 ± 797	2.24 ± 0.34	22,432 ± 3,600	2.24 ± 0.36	14
Zn^{2+}	4,938 ± 670	2.10 ± 0.29	24,961 ± 4,149	2.45 ± 0.41	12
Ni^{2+}	6,032 ± 790	2.57 ± 0.34	19,238 ± 2,210	1.92 ± 0.22	8

[a] $\Delta\epsilon_{295}$ 2350 and $\Delta\epsilon_{245}$ 10,000 were used to calculate the number of tyrosines ionized per mole of conalbumin bound to 2 moles of metal ions. The $\Delta\epsilon_{295}$ and $\Delta\epsilon_{245}$ due to the binding of Co and Cu reported here were corrected for the absorption of excess metal and Tris buffer. The errors in $\Delta\epsilon$ and the number of tyrosines per mole of conalbumin are the standard deviations.

TABLE II: Results of Metal Substitution Reaction of Conalbumin.[a]

	Displacing Metal Ion							
Bound Metal Ion	Zn^{2+}	Cd^{2+}	Mn^{2+}	Co^{2+}	Fe^{3+}	Cu^{2+}	Cr^{3+}	Ni^{2+}
Zn^{2+}		1	1	1	2	1	>1	0
Cd^{2+}	<1		1	1	<2	<2	1	0
Mn^{2+}	1	<1		<1	<2	<2	1	0
Co^{2+}	<1	1	1		>1	>1	<1	0
Fe^{3+}	0	0	0	0		0	0	0
Cu^{2+}	1	0	1	1	2		0	0
Cr^{3+}	1	1	<1		>1	1		0
Ni^{2+}	2	2	2	2	2	2	2	

[a] For explanation, see text.

the quantities listed in Table II are of the right order of magnitude.

When tenfold excess competing metal ion was used, bound metals other than Fe^{3+}, Cr^{3+}, and Cu^{2+} were displaced almost completely by the competing ions. Ten times excess Fe^{3+} displaced almost all of other bound metals but only traces of bound Fe^{3+} were displaced by tenfold excess of competing metals such as Cu^{2+} and Cr^{3+}.

Discussion

The role of tyrosyl residues as ligands in metal binding by conalbumin was first proposed by Warner and coworkers (Warner and Weber, 1953; Wishnia *et al.*, 1961) on the basis of potentiometric and spectrophotometric titration results. This proposal was supported by chemical modification investigations (Komatsu and Feeney, 1967). Difference spectral studies (235–310 mμ) and difference iodination rate studies of iron–conalbumin *vs.* conalbumin have substantiated the involvement of tyrosine in Fe binding to conalbumin (Michaud, 1967). The results of the present studies extend these findings to the specific binding of other transition metal ions.

The difference spectrum for the ionization of the phenol group of tyrosine is characterized by two maxima in the region of 242 and 294 mμ and a negative absorption with a minimum near 275 mμ, *e.g.*, two maxima for free tyrosine occur at 242 and 295 mμ with an absorbance ratio of 5.1:1 (Hermans, 1962). For poly-L-tyrosine with an average degree of polymerization of 30 these two maxima occur at 242 and 293 mμ, with an absorbance ratio of 4.6:1 (Katchalski and Sela, 1953). In *N*-acetyltyrosine, they occur at 242 and 294 mμ with an absorbance ratio of 4.8:1 (Tachibana and Murachi, 1966; Donovan, 1964). A negative absorption with a minimum between 267 and 277 mμ was also observed in all these difference spectra. The tyrosine dissociation spectra in proteins are similar in shape to those of free tyrosine and tyrosyl peptides with only a slight shift to longer wavelengths in the two maxima. Thus, a maximum at 295 mμ was observed in ribonuclease (Gorbunoff, 1967), papain (Glazer and Smith, 1961), stem bromelan (Tachibana and Murachi, 1966), hemoglobins and myoglobins (Hermans, 1962), and aldolase (Donovan, 1964); 297.5 mμ for β-lactoglobin and 299 mμ for α-lactalbumin (Gorbunoff, 1967). The maximum in the lower wavelength region is also shifted to a longer wavelength in proteins, *e.g.*,

243 mμ in aldolase (Donovan, 1964), 244 mμ in cytochromes C from various species (Hamaguchi *et al.*, 1967), and 245 mμ in myoglobins and hemoglobins (Hermans, 1962). In all these studies the absorbance ratio of these two maxima was about 4.5:1. The tyrosine dissociation spectrum in conalbumin (Figure 1a) is identical with that in other proteins and has two maxima at 245 and 295 mμ with an absorbance ratio of 4.6:1 and a minimum in the 275-mμ region. The Zn–conalbumin *vs.* conalbumin difference spectrum shown in Figure 2 has two maxima at 245 and 295 mμ with an absorbance ratio of 4:7.1, and a negative absorption in the 275-mμ region. The close similarity between this spectrum and those of tyrosine and tyrosyl peptides as well as the tyrosyl dissociation curves in proteins assures us that we are dealing with a tyrosine ionization difference spectrum. As a consequence, the involvement of tyrosyl residues in Zn^{2+} binding to conalbumin is beyond doubt.

In the difference spectra of Cd^{2+}–, Co^{2+}–, and Mn^{2+}–conalbumin *vs.* conalbumin, however, although the shapes are similar to that of the tyrosine dissociation difference spectrum, the maximum in the 242-mμ region has shifted to about 250 mμ. The shift may be due to a perturbation brought about by the binding of tyrosyl residues in conalbumin to these metal ions, although the possibility of the contribution by the binding of ligands other than tyrosine to conalbumin also exists.

The shapes as well as the ratios of the two absorption maxima of difference spectra of Fe^{3+}–, Cr^{3+}–, Ni^{2+}–, and Cu^{2+}–conalbumin *vs.* conalbumin varied appreciably from that of the tyrosine dissociation difference spectrum. Clearly, perturbations from binding of these metals to tyrosyl residues in conalbumin was not solely responsible for these discrepancies.

Each mole of conalbumin was found to bind specifically two moles of all the metal ions investigated as revealed by the spectrophotometric titration curves. The fact that the absorbances of the uv difference spectra at all wavelengths increased with the amount of metal ion added until the equivalent amount of 2 moles of metal ion/mole of conalbumin was reached and that only very small increases and no change in shape of the spectra occurred upon further addition of metal ions is a good indication that these two metal ions bound conalbumin at the specific binding sites. These results do not rule out the possibility of nonspecific metal binding to conalbumin after saturation of the two specific sites. For example, in the case of Cr^{3+} there was almost no change in the Cr–conalbumin *vs.* conalbumin ultraviolet difference spectrum beyond the 2:1 equivalence point, however, a marked increase in absorbance at the two maxima, 435 and 610 mμ, beyond the 2:1 equivalence point, was still observed, suggesting the occurrence of nonspecific binding.

The value of $\Delta\epsilon_{295}$ for Fe–conalbumin is in good agreement with those reported before (Wishnia *et al.*, 1961; Michaud, 1967). The values of $\Delta\epsilon_{245}$ and $\Delta\epsilon_{295}$ are frequently used to estimate the number of tyrosyl residues undergoing ionization in a protein as the environment changes (Wetlaufer, 1962). The $\Delta\epsilon_{295}$ per tyrosyl ionizing ranges from 2130 for poly-L-tyrosine (Katchalski and Sela, 1953) to 2630 for ribonuclease (Tanford *et al.*, 1956), whereas $\Delta\epsilon_{245}$ ranges from 9700 in sperm whale myoglobin to 12,000 in hemoglobins (Hermans, 1962). For conalbumin, $\Delta\epsilon_{295}$ was estimated to be 2350 (Wishnia *et al.*, 1961). If this value is used, the number of tyrosyl residues in conalbumin involved in binding each transition metal ion is two for trivalent ions and one for divalent ions. This conclusion agrees with that reached from consideration of the values of $\Delta\epsilon_{245}$.

These conclusions disagree with those drawn from potentiometric titration studies, wherein three hydrogen ions were found to be released for each Fe^{3+} ion bound (Warner and Weber, 1953), and earlier spectrophotometric titration studies (Wishnia *et al.*, 1961). The spectrophotometric data of this latter study do not disagree with ours or with Michaud's (Michaud, 1967), but interpretation does differ. Wishnia *et al.* stated that their data were best fit by *assuming* titration of 11 accessible tyrosyl residues in native conalbumin, prior to its denaturation, and 5 such residues in iron–conalbumin, giving a difference of 6 tyrosyl residues coordinated to the two bound ferric ions. Perhaps assumption of these numbers of normally ionizing tyrosyl residues is necessary for the best theoretical fit of their data. However, Michaud has shown that the tyrosyl residues involved in iron binding are distinctly not normal, two having pK_a' of 8.5 and two having pK_a' less than 10 (Michaud, 1967). The statement that the $\Delta\epsilon_{295}$ between conalbumin and iron–conalbumin at normal pH is consistent with the ionization of 6 tyrosyl residues (Wishnia *et al.*, 1961), fails to take into account the large contribution from other ligands and a charge-transfer band of iron for which correction is made in the present study. Detailed analysis of this correction and the residual difference spectrum of iron–conalbumin *vs.* conalbumin at high pH appear in Michaud's thesis (Michaud, 1967).

In the earlier studies it was assumed that the three hydrogen ions necessarily all originate from the ionization of tyrosyl residues (Warner and Weber, 1953; Wishnia *et al.*, 1961). This need not be the case, however. An alternative possibility is that two hydrogen ions arise from ionization of tyrosyl residues whereas the third arises on binding of a tryptophanyl residue as has been suggested for the colored metal–conalbumin complexes (Tan and Woodworth, 1968b).

The appearance of the characteristic spectrum of the competing metal–conalbumin complex with the concomitant disappearance of the characteristic spectrum of the original bound metal–conalbumin complex after the competing metal ion was added suggested that the added metal ion was competing with the bound metal for specific binding sites in conalbumin. As mentioned previously, the inherent error in the comparison of small differences in the difference spectra prevents us from giving a quantitative conclusion concerning metal displacement studies. However, the results do indicate that Fe^{3+} is the most tightly and Ni^{2+} the least tightly bound metal ion and that other transition metal ions range between these two in binding strength.

The appearance of color when transition metal ions interact with proteins is a good indication of complex formation; the absence of color, however, does not necessarily mean no complex is formed. Although transition metal complexes often show absorption in the visible region, colorless complexes are not uncommon. The present study indicates that transition metal ions such as Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} combine with the tyrosyl residues in the specific metal binding sites of conalbumin despite the fact that color did not appear.

The present investigation demonstrates that owing to the changes of spectral properties of tyrosyl residues upon binding metal ions and with the advent of the modern spectrophotometer, the difference spectra recorded in the uv region not only can detect, but also allow estimation of the number of tyrosyl residues actually involved in binding. Furthermore, with care-

ful experimentation and intelligent interpretation the ultraviolet difference spectroscopic method may be used to demonstrate the involvement of other amino acid residues in binding, *e.g.*, the thiol group in thionein with Cd and Zn (Kägi and Vallee, 1961), the histidyl residues with iron in hemoproteins (Brill and Sandberg, 1968), and tryptophanyl residues in conalbumin with certain transition metals to form colored complexes (Tan and Woodworth, 1968b). In addition, the difference spectrophotometric method is rapid, sensitive, and capable of detecting the presence of weak complexes.

References

Brill, A. S., and Sandberg, H. E. (1968), *Biophys. J. 8*, 669.

Buttkus, H., Clark, J. R., and Feeney, R. E. (1965), *Biochemistry 4*, 998.

Donovan, J. W. (1964), *Biochemistry 3*, 67.

Fraenkel-Conrat, H., and Feeney, R. E. (1950), *Arch. Biochem. 29*, 101.

Glazer, A. N., and Smith, E. L. (1961), *J. Biol. Chem. 236*, 2948.

Gorbunoff, M. J. (1967), *Biochemistry 6*, 1606.

Hamaguchi, K., Ikeda, K., Sakai, H., Sugeno, K., and Narita, K. (1967), *J. Biochem. (Tokyo) 62*, 99.

Hermans, J., Jr. (1962), *Biochemistry 1*, 193.

Kägi, J. H. R., and Vallee, B. L. (1961), *J. Biol. Chem. 236*, 2435.

Katchalski, E., and Sela, M. (1953), *J. Am. Chem. Soc. 75*, 5284.

Komatsu, S. K., and Feeney, R. E. (1967), *Biochemistry 6*, 1136.

Michaud, R. L. (1967), Ph.D. Thesis, University of Vermont, Burlington, Vt.

Michaud, R. L., and Woodworth, R. C. (1966), 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept, 220.

Schade, A. L., Reinhart, R. W., and Levy, H. (1949), *Arch. Biochem. 20*, 170.

Tachibana, A., and Murachi, T. (1966), *Biochemistry 5*, 2756.

Tan, A. T., and Woodworth, R. C. (1967), *Fed. Proc. 26*, 778.

Tan, A. T., and Woodworth, R. C. (1968a), *Biochem. Biophys. Res. Commun. 32*, 739.

Tan, A. T., and Woodworth, R. C. (1968b), IUPAC International Symposium on Macromolecular Chemistry, Sept., Toronto, Preprints of Scientific Papers (Structure and Function of Biopolymers), BI.

Tanford, C., Hauenstein, J. D., and Rands, D. G. (1956), *J. Am. Chem. Soc. 77*, 6409.

Warner, R. C., and Weber, I. (1953), *J. Am. Chem. Soc. 75*, 5094.

Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.

Wishnia, A., Weber, I., and Warner, R. C. (1961), *J. Am. Chem. Soc. 83*, 2071.

Woodworth, R. C., and Clark, L. G. (1967), *Anal. Biochem. 18*, 295.

Woodworth, R. C., and Schade, A. L. (1959), *Arch. Biochem. Biophys. 82*, 78.