Hoffmann, L., Blumenfeld, O. O., Mondshine, R. B., and Park, S. S. (1972), *J. Appl. Physiol. 33,* 42.
Johanson, W. G., and Pierce, A. K. (1972), *J. Clin. Invest. 51,* 288.
Johanson, W. G., and Pierce, A. K. (1973), *J. Clin. Invest. 52,* 2921.
John, R., and Thomas, J. (1971), *Int. J. Biochem. 2,* 529.
John, R., and Thomas, J. (1972), *Biochem. J. 127,* 261.
Lansing, A. I., Rosenthal, T. B., Alex, M., and Dempsey, E. W. (1952), *Anat. Rec. 114,* 555.
Oderr, C. (1964), *Am. Rev. Respir. Dis. 90,* 410.
Partridge, S. M., Davis, H. F., and Adair, G. S. (1955), *Biochem. J. 61,* 21.
Paz, M. A., Gallop, P. M., Blumenfeld, O. O., Henson, E., and Seifter, S. (1971), *Biochem. Biophys. Res. Commun. 43,* 289.
Pereyra, B., Blumenfeld, O. O., Paz, M. A., Henson, E., and Gallop, P. M. (1974), *J. Biol. Chem. 249,* 2212.
Pereyra, B., Paz, M. A., Gallop, P. M., and Blumenfeld, O. O. (1973), *Biochem. Biophys. Res. Commun. 55,* 96.
Pierce, J. A., and Ebert, R. V. (1965), *Thorax 20,* 469.
Pierce, J. A., Eisen, H. K., and Lingra, D. (1969), *Trans. Assoc. Am. Physicians 57,* 87.
Pierce, J. A., and Hocott, J. B. (1960), *J. Clin. Invest. 39,* 8.
Pump, K. K. (1974), *Chest 65,* 431.
Richmond, V. (1974), *Biochim. Biophys. Acta 351,* 173.
Serafini-Fracassini, A., Field, J. M., Rodger, G. W., and Spina, M. (1975), *Biochim. Biophys. Acta 386,* 80.
Serafini-Fracassini, A., and Smith, J. W. (1974), in The Structure and Biochemistry of Cartilage, Serafini-Fracassini, A., and Smith, J. W., Ed, Churchill Livingstone, Edinburgh, United Kingdom, p 7.
Snider, G. L., Hayes, J. A., Franzblau, C., Kagan, H. M., Stone, P. S., and Karthy, A. L. (1974), *Am. Rev. Respir. Dis. 110,* 254.
Spina, M., Garbin, G., Field, J. M., and Serafini-Fracassini, A. (1975), *Biochim. Biophys. Acta 400,* 162.
Tanzer, M. L., Mechanic, G., and Gallop, P. M. (1970), *Biochim. Biophys. Acta 207,* 548.
Verhoeff, F. H. (1908), *J. Am. Med. Assoc. 50,* 876.
Wright, R. R. (1961), *Am. J. Pathol. 39,* 355.

Resonance Raman Spectra of Iron(III)–, Copper(II)–, Cobalt(III)–, and Manganese(III)–Transferrins and of Bis(2,4,6-trichlorophenolato)diimidazolecopper(II) Monohydrate, a Possible Model for Copper(II) Binding to Transferrins†

Yoshio Tomimatsu,* Saima Kint, and James R. Scherer

ABSTRACT: Fe(III), Cu(II), Co(III), and Mn(III) complexes of ovo- and human serum transferrins show resonance enhanced Raman bands near 1600, 1500, 1270, and 1170 cm^{-1} upon excitation with laser frequencies which fall within the visible absorption bands of those metalloproteins. Comparison of the visible absorption and resonance Raman spectra of the Cu(II)–transferrin complexes with those for the Cu(II) model compound, bis(2,4,6-trichlorophenolato)diimidazolecopper(II) monohydrate, indicates that the resonance Raman bands are due to enhancement of phenolic vibrational modes. For the model Cu(II) compound, a normal coordinate analysis was used to aid our assignment of the observed resonance bands at 1562, 1463, 1311, and 1122 cm^{-1} to A_1 vibrational modes of the 2,4,6-trichlorophenolato moiety. These assignments are consistent with those made for Cu(II)–transferrins. The latter assignments were based upon calculated A_1 frequencies for *p*-methylphenol (Cummings, D. L., and Wood, J. L. (1974), *J. Mol. Struct. 20,* 1). The wavelength shifts in the resonance bands for the model compound from those for Cu(II)–transferrins are due to the influence of the chloro substituents on the planar vibrations of phenol. These results clearly identify tyrosine as a ligand in copper binding to transferrins.

Unlike that for heme proteins, the physical structure of the two metal binding sites of transferrins has not been resolved. From studies of proton displacement upon introduction of the metal, Warner and Weber (1953) suggested that the phenolic group of tyrosine residues acts as ligands in Fe(III) and Cu(II) binding to ovotransferrin. This suggestion was supported by difference spectra (Wishnia et al., 1961; Tan and Woodworth, 1969; Lehrer, 1969), NMR[1] (Woodworth et al., 1970) and chemical modification (Komatsu and Feeney, 1967) studies. Fluorescence measurements (Luk, 1971) also pointed to tyrosine as ligand in the binding of trivalent lanthanide ions to transferrins. ESR measurements, which showed extra hyperfine splitting of the $g_\perp$ peak (Windle et al., 1963; Aasa et al., 1963; Aasa and Aisen, 1968), provided evidence for participation of nitrogen-containing ligands in Cu(II) binding to transferrins. The imidazole group of histidine residue has been proposed as the nitrogen-containing ligand (Aasa et al., 1963), although other nitrogen-containing groups, such as amide (Feeney and Komatsu, 1966) and guanidyl (Windle et al., 1963), have also been suggested. Chemical modification studies indicate the possible participation of histidyl groups (Line et al., 1967; Bezkorovainy and Grohlich, 1971) but not amino

† From the Western Regional Research Center, Agricultural Research Service, United States Department of Agriculture, Berkeley, California 94710. *Received May 24, 1976.*

[1] Abbreviations used: NMR, nuclear magnetic resonance; TCPI, bis(2,4,6-trichlorophenolato)diimidazolecopper(II) monohydrate; ESR, electron spin resonance.

groups (Zschocke et al., 1972) in Fe(III) binding to human serum transferrin.

The resonance Raman technique (Behringer, 1967) has been recently applied (Tomimatsu et al., 1973; Carey and Young, 1974; Gaber et al., 1974) to further elucidate the structure of the metal binding sites of transferrins. Resonance enhanced Raman spectra were observed upon excitation of Fe(III)-transferrins with laser frequencies which fell within the visible absorption band of the metalloproteins. The resonance Raman bands were assigned to phenolate vibrational modes by comparison with an Fe(III) complex of ethylenediaminebis(*o*-hydroxyphenylacetate) (Gaber et al., 1974). The transferrins bind other metal ions, e.g., Cu(II), Co(III), and Mn(III), to form stable complexes which show intense visible absorption bands (Inman, 1956; Aasa et al., 1963; Ulmer and Vallee, 1963; Aisen et al., 1969). Based on the published work which implicates the phenolic group of tyrosine residue as the oxygen-containing ligand and the imidazole group of histidine residue as the nitrogen containing ligand, we prepared bis(2,4,6-trichlorophenolato)diimidazolecopper(II) monohydrate (TCPI) as a possible model for Cu(II) binding to transferrins. The crystal and molecular structure of the model compound has been published (Wong et al., 1976). We report a comparison of the visible absorption and resonance Raman spectra of Fe(III), Cu(II), Co(III), and Mn(III) complexes of ovo- and human serum transferrins. The spectral properties of Cu(II)--transferrins are compared with those of the model Cu(II) compound. Results of normal coordinate analysis are used to assign the observed resonance Raman bands to specific vibrational modes of the phenolate moiety of the model compound.

Materials and Methods[2]

TCPI was prepared by reacting imidazole (0.2 mol) dissolved in 25 ml of 0.4 M $CuSO_4 \cdot 5H_2O$ with 2,4,6-trichlorophenol (0.2 mol) dissolved in 25 ml of 0.8 M NaOH (Wong et al., 1976). Imidazole (EK 4733) and 2,4,6-trichlorophenol (EK 1469) were obtained from Eastman Kodak Company. Chicken ovotransferrin and human serum transferrin were purchased from Sigma Chemical Company. Human serum transferrin, essentially free of iron contamination ($E_{1\%}^{1cm} <$ 0.01 at 460 nm), was used without further purification. Chicken ovotransferrin was purified on a cellulose exchange column (Woodworth and Schade, 1959). The Fe(III), Cu(II), Co(III), and Mn(III) chelates of the transferrins were prepared by modifications of published procedures (Warner and Weber, 1951; Aisen et al., 1969). For the Fe(III) chelates, a weighed amount of solid apoprotein was added to a known volume of 0.1 M sodium citrate solution which was 0.025 M in $NaHCO_3$ and contained a 50% excess of Fe(III) (3 mol of $Fe(NO_3)_3$ per mol of transferrin). The solution was adjusted to pH 9 with 1 N NaOH and allowed to stand at 4 °C until maximum color developed. After exhaustive dialysis against glass-distilled water (pH 9), lyophilization yielded Fe(III)-transferrin which was used to prepare aqueous solutions for Raman measurements. For the Cu(II), Co(III), and Mn(III) chelates, stoichiometric amounts of the apoproteins were added to known volumes of acidified (10^{-3} N HCl) solutions of the appropriate metal ($CuSO_4$, $CoCl_2$, and $MnCl_2$). Sufficient concentrated $NaHCO_3$ solution was added to give (metal)/(HCO_3^-) = 0.5. With efficient stirring, at 4 °C, small increments of 0.1 N NaOH were slowly added to a final pH of 9. For the Co(II) and Mn(II) chelates, small increments of 0.05 M H_2O_2 were added to promote oxidation to the colored Co(III) and Mn(III) chelates (Aisen et al., 1969). After maximum color development, the solutions were filtered through an ultrafine sintered glass filter and used directly for Raman measurements.

A Cary 15 spectrophotometer was used to monitor color development during preparation of the transferrin metal chelates and to obtain their visible absorption spectra. A Coherent Radiation 52 B argon ion laser was used for 488.0- and 514.5-nm excitation. Raman scattering was analyzed by a Spex 1401 double monochromator and detected with a cooled RCA C31034A gallium arsenide photomultiplier. Details of the data acquisition system and the procedure for correcting photomultiplier and optical efficiency have been reported (Scherer and Kint, 1970). For solids, Raman scattering at 90° to the incident beam was measured on samples contained in open-ended capillary (0.8 mm i.d.) cells (imidazole and 2,4,6-trichlorophenol) or in a grooved, metal rotating cell (model Cu(II) compound) similar to that described by Kiefer and Bernstein (1971a). For aqueous solutions of the transferrin metal chelates, 90° Raman scattering was measured in aluminized capillary (0.5 mm i.d.) cells (Bailey et al., 1967). The optical arrangement (Bailey et al., 1967) and the techniques for filling with degassed solution and sealing the cell (Scherer et al., 1973) have been described. A 180° back-scattering geometry (Scherer et al., 1971) was used to measure the Raman spectrum of an acetone solution of the model Cu(II) compound contained in a quartz rotating liquid sample holder (Kiefer and Bernstein, 1971b). A laminar air flow apparatus (Scherer et al., 1973) was used to adjust and control sample temperature (±0.5 °C at 0 °C).

The appropriate solvent spectrum was subtracted from Raman spectra of transferrin metal chelates in water and Cu(II) model compound in acetone. The intense Rayleigh scattering of the incident laser beam by acetone solutions of the Cu(II) model compound led to excitation of the Raman spectrum of the quartz sample holder. Therefore, a spectrum of quartz was used to eliminate this scattering from the solution Raman spectrum of the Cu(II) model compound. All of these adjustments were carried out using existing computer routines for Raman data reduction (Kint et al., 1976).

A normal coordinate calculation was carried out for the 2,4,6-trichlorophenolato group of the model Cu(II) compound.[3]

Results

The visible absorption spectra of Fe(III), Cu(II), Co(III), and Mn(III) chelates of ovo- and human serum transferrins in water are shown in Figures 1A and 1B, respectively. Peak positions and absorptivities are similar to those reported by others and summarized by Feeney and Komatsu (1966). The resonance Raman spectra of Fe(III), Cu(II), Co(III), and Mn(III) chelates of ovo- and human serum transferrins in water are shown in Figures 2A and 2B, respectively. The spectra of the Fe(III)- and Cu(II)-transferrin chelates are similar to those already reported (Tomimatsu et al., 1973; Carey and Young, 1974; Gaber et al., 1974; Siiman et al., 1974). In these spectra, the intensity of the 1002-1004-cm^{-1} band, which is assigned to a phenyl ring breathing mode of phenylalanine residues (Lord and Yu, 1970), served as refer-

[2] Reference to a company or product name does not imply approval or recommendation of that product by the United States Department of Agriculture to the exclusion of others that also may be suitable.

[3] Details of this calculation are available upon request.

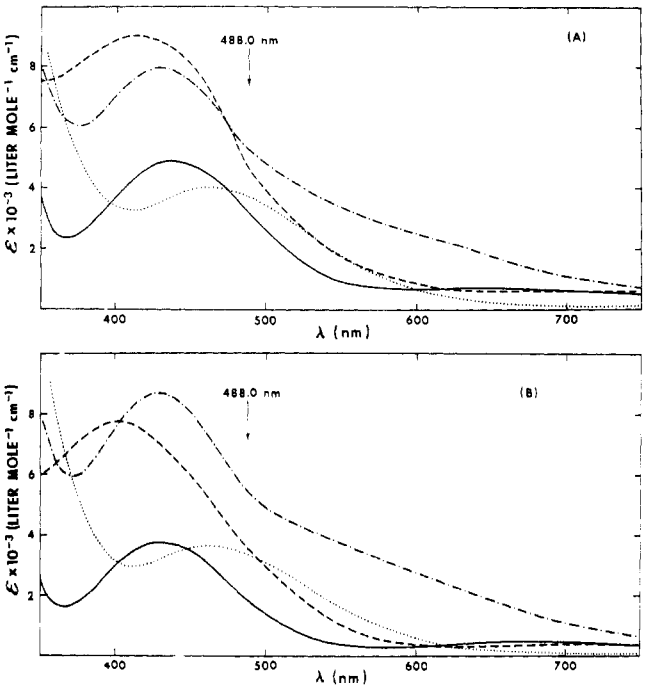

FIGURE 1: (A) Absorption spectra of aqueous solutions of Fe(III)- (. . .), Cu(II)- (—), Co(III)- (- - -), and Mn(III)- (—·—·) ovotransferrin. Protein concentration and pH's are: Fe(III), 1.31×10^{-4} M, pH 7.0; Cu(II), 9.35×10^{-5} M, pH 8.4; Co(III), 8.81×10^{-5} M pH 8.8; Mn(III), 1.16×10^{-4} M, pH 8.9. (B) Absorption spectra of Fe(III)- (. . .), Cu(II)- (—), Co(III)- (- - -), and Mn(III)- (—·—·) human serum transferrin. Protein concentrations and pH's are: Fe(III), 1.23×10^{-4} M, pH. 7.0; Cu(II), 8.57×10^{-5} M, pH 8.5; Co(III), 8.65×10^{-5} M, pH 8.5; Mn(III), 1.10×10^{-4} M, pH 8.8.

ence for intensity comparisons. Observed phenylalanine intensities were adjusted to the same conditions of protein concentration, laser power, and slit width. The adjusted phenylalanine intensities for all spectra agreed to ±15%. Therefore, for each spectrum, the intensities of all of the bands were normalized to a phenylalanine band intensity of 10. No adjustments were made for the influence of absorption on band intensities (Behringer, 1967), nor for the difference in phenylalanine content between ovotransferrin and human serum transferrin (Feeney and Komatsu, 1966), since calculations showed these effects to be small compared with the estimated reliability of ±15% indicated above. The frequencies and normalized relative intensities of the resonance Raman bands of the different metal chelates of ovo- and human serum transferrins are compared in Table I.

The Raman spectra of solid samples of the Cu(II) model compound and its component parts, 2,4,6-trichlorophenol and imidazole are shown in Figure 3. The model compound spectrum shows Raman bands which are readily associated with intense bands in the spectra of the components. The bands at 376 and 873 cm^{-1} in the model compound spectrum correspond to the intense bands at 380 and 870 cm^{-1} in the trichlorophenol spectrum. The two intense imidazole bands at 1263 and 1150 cm^{-1} correspond to the relatively weak band at 1263 cm^{-1} and to the shoulder on the high frequency side of the band at 1140 cm^{-1}, respectively, in the Cu(II) complex spectrum. The sharp band at 1087 cm^{-1} in the model compound spectrum is a $CaCO_3$ Raman band excited in the polarization scrambler plate at the entrance slit to the spectrometer. This leaves the relatively intense bands at 1569, 1460, 1304, 1140, and 750 cm^{-1} unaccounted for in the model Cu(II) compound spectrum.

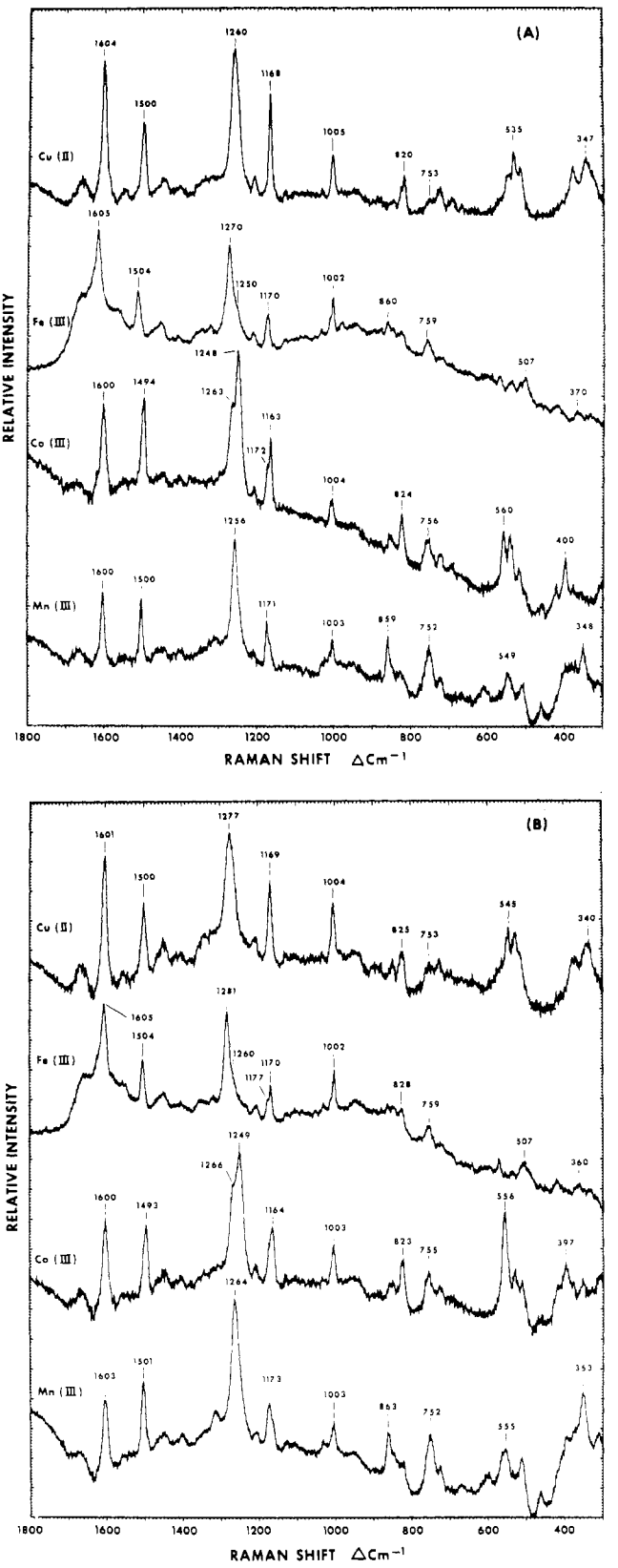

FIGURE 2: (A) Raman spectra of aqueous solutions of ovotransferrin-metal chelates obtained using 488.0-nm laser excitation and 4.0-cm^{-1} slit width. Protein concentration and laser power were as follows: Fe(III), 1.01×10^{-4} M, 150 mW; Cu(II), 0.91×10^{-4} M, 200 mW; Co(III), 0.88×10^{-4} M, 100 mW; and Mn(III), 1.16×10^{-4} M, 100 mW. (B) Raman spectra of aqueous solutions of human serum transferrin-metal chelates obtained using 488.0-nm laser excitation and 4.0-cm^{-1} slit width. Protein concentrations and laser power were as follows: Fe(III), 1.23×10^{-4} M, 200 mW; Cu(II), 0.86×10^{-4} M, 170 mW; Co(III), 0.87×10^{-4} M, 230 mW; and Mn(III), 1.11×10^{-4} M, 160 mW.

TABLE I. Comparison of Resonance Band Frequencies and Relative Intensities[a] of the Different Metal Chelates of Ovotransferrin and Serum Transferrin.

Fe(III)		Cu(II)		Co(III)		Mn(III)	
cm^{-1}	Int	cm^{-1}	Int	cm^{-1}	Int	cm^{-1}	Int
		Ovotransferrin[b]					
1605	19	1604	32	1600	36	1600	23
1504	12	1500	17	1494	33	1500	21
1270	24	1260	33	1263 (sh)	36	1256	40
1250 (sh)	9			1248	57		
				1172 (sh)	16		
1170	9	1168	23	1163	26	1171	14
759	5	753	4	756	12	752	17
		Human Serum Transferrin[b]					
1605	18	1601	23	1600	21	1603	18
1504	11	1500	12	1493	16	1501	18
1281	23	1277	20	1266 (sh)	21	1264	36
1260 (sh)	7			1249	29		
1177 (sh)	5						
1170	8	1169	13	1164	12	1173	10
759	5	753	5	755	10	752	14

[a] All intensities normalized to an intensity of 10 for the phenylalanine band near 1002 cm^{-1}. [b] sh denotes shoulder.

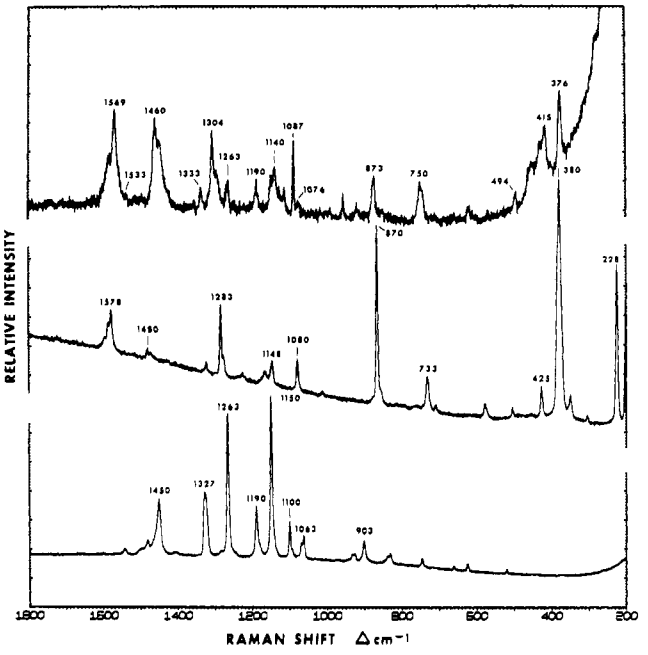

FIGURE 3: Raman spectra of solid samples of bis(2,4,6-trichlorophenolato)diimidazolecopper(II) monohydrate (top curve), 2,4,6-trichlorophenol (middle curve), and imidazole (bottom curve). Experimental conditions: 514.5-nm laser excitation, 4-cm^{-1} slit width, and laser power of 600, 360, and 140 mW for top, middle, and bottom curves, respectively.

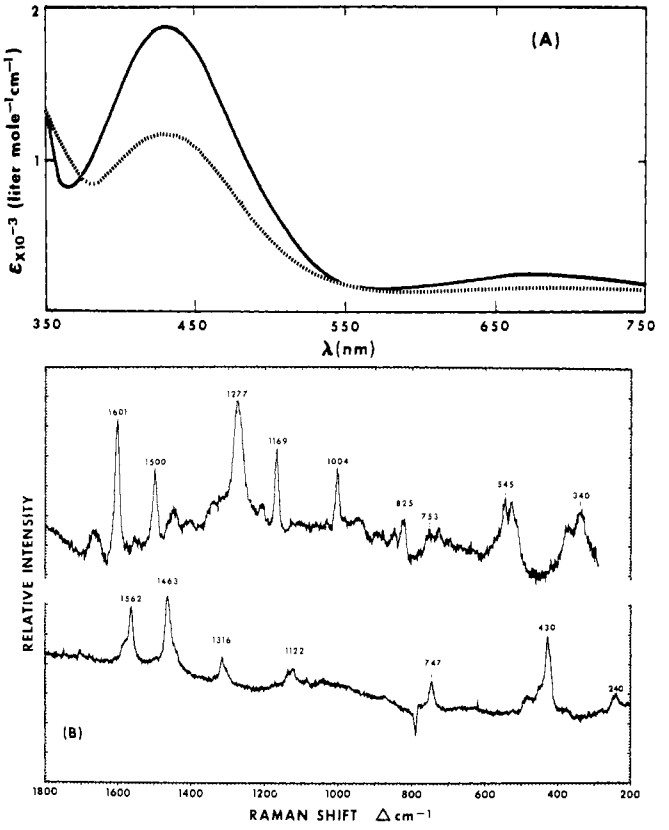

FIGURE 4: (A) Absorption spectra of bis(2,4,6-trichlorophenolato)diimidazolecopper(II) monohydrate, 1.594×10^{-3} M in acetone (dashed curve) and Cu(II)-human serum transferrin, 8.57×10^{-5} M in water (solid curve). Absorptivity (ϵ) is per mole of Cu(II). (B) Raman spectra of bis(2,4,6-trichlorophenolato)diimidazolecopper(II) monohydrate in acetone (lower curve) and Cu(II)-human serum transferrin in water (upper curve). Experimental conditions: 488.0-nm laser excitation, 4-cm^{-1} slit width, laser power of 180 and 170 mW and concentrations of 8.11×10^{-3} and 0.86×10^{-4} M for lower and upper curves, respectively.

The visible absorption and resonance Raman spectra of TCPI in acetone are shown in Figures 4A and 4B, respectively. The corresponding spectra of Cu(II)-serum transferrin are also shown for comparison. The absorption curves are similar, both showing strong bands centered at 430 nm and weak, broad bands at higher wavelengths. Since the model Cu(II) compound contains no bicarbonate, this similarity of the absorption spectra suggests that coordination of bicarbonate specifically (Aisen, 1973) may not be necessary for color development in solutions of Cu(II)-transferrins. In the case of Fe(III)-serum transferrin, Young and Perkins (1968) observed similar absorptivities at 465 nm upon replacement of bicarbonate with other anions, e.g., cyanide, cyanate, oxalate, or phosphate. For TCPI, the *o*-chloro axial ligands may function as the required anions, and perturb the Cu(II) d orbitals sufficiently to allow

observation of the 430-nm electronic transition. The absorptivities per mole of copper are smaller for the Cu(II) model compound, 1200 and 150 l. mol^{-1} cm^{-1} at 430 and 710 nm, respectively, compared with 1890 and 235 l. mol^{-1} cm^{-1} at 430 and 680 nm, respectively, than for Cu(II)-human serum transferrin. The resonance Raman bands (Figure 4B) of the model Cu(II) compound appear at frequencies different from those for Cu(II)-human serum transferrin. The wavelength shifts are shown (see below) to be due to the influence of the chloro substituents on the planar vibrations of phenol.

Discussion

The similarity of the resonance Raman spectra of the various metal chelates of ovo- and human serum transferrins (Figures 2A and 2B) provide evidence that Fe(III), Cu(II), Co(III), and Mn(III) all occupy the same binding sites. This is in agreement with the results of metal displacement studies by ultraviolet difference spectroscopy (Tan and Woodworth, 1969). However, there are differences in resonance band frequency and intensity between the various metal chelates and between the two transferrins (Table I). Enhancement of Raman modes by excitation with laser frequencies within the absorption band is expected of those modes which couple to the electronic transition (Behringer, 1967). Consideration of the quantum mechanical expressions for induced dipole moment and for scattering intensities (Woodward, 1967) leads to the expectation that, for metalloproteins with similar absorption band frequencies, resonance Raman band intensities will be stronger for the metalloproteins with the larger molar absorptivity. Resonance band intensities will decrease as the incident laser frequency becomes further removed from the absorption maximum. These relationships between resonance band intensity, laser frequency, and absorptivity were observed for permanganate and chromate ions (Kiefer and Bernstein, 1972). The resonance band intensities are comparable for Fe(III) and Mn(III) chelates of the two transferrins, but, for the Cu(II) and Co(III) chelates, the intensities are considerably larger for ovotransferrin. These observations are in accord with the similarities and differences in molar absorptivity (Figures 1A and 1B) for the metal chelates at 488 nm, the wavelength of the incident laser light.

The two transferrins have similar resonance band frequencies, except for the band near 1270 cm^{-1}. This band appears at higher frequencies for human serum transferrin, the difference amounting to 17 cm^{-1} for (Cu(II), 11 cm^{-1} for Fe(III), 8 cm^{-1} for Mn(III), and essentially no difference for Co(III). Except for Co(III), there appears to be a rough correlation with atomic radius of the uncomplexed metals. The atomic configurations are d^9, d^6, d^5, and d^4 for Cu(II), Co(III), Fe(III), and Mn(III), respectively. In Co(III)-serum transferrin, cobalt has a spin of 0, compared with spins of ½, 5/2, and 2 for the metals in Cu(II)-, Fe(III)-, and Mn(III)-transferrins (Aisen et al., 1969), respectively. All six d electrons in cobalt are paired in Co(III)-transferrin and probably occupy the lowest lying 3d orbitals. This is consistent with a cobalt atomic radius which appears to be the smallest among the four metals and which may minimize steric effects. However, steric effects might account for the difference in Raman shifts of the band near 1270 cm^{-1} between the two transferrins when complexed with the other metals. Steric effects have been observed in the binding of trivalent lanthanide ions to transferrins (Luk, 1971) and in iron binding to hemoglobin (Brunner et al., 1972; Brunner and Sussner, 1973; Spiro and Strekas, 1974). However, this steric explanation does not account for the frequency difference among the metal chelates for the same transferrin. Except for a reversal between Cu(II) and Fe(III), the higher frequencies correspond to the larger metal ions. Clearly, in the case of the transferrin-metal chelates, a knowledge of the effect of the metal ion on the force constants and of the coupling among the various coordinates associated with the resonance band near 1270 cm^{-1} is required to account for the observed differences in frequency. This aspect will be discussed further below.

Studies of the model Cu(II) compound provide some insight into the structure of the metal binding sites of transferrins. By comparison with the electronic spectrum of the Fe(III) complex of ethylenediaminebis(*o*-hydroxyphenylacetate), Gaber et al. (1974) assigned the visible absorption band of Fe(III)-human serum transferrin to a charge-transfer transition from a $p\pi$ orbital on the phenolate oxygen atom of tyrosine residue to a $d\pi^*$ orbital of Fe(III). A similar assignment was tentatively made by those authors for the visible absorption band of Cu(II)-human serum transferrin, in this case a transition from a phenolate $p\pi$ orbital to a $d\sigma^*$ orbital of Cu(II). The similarity of the visible absorption spectrum of our model Cu(II) compound to that of Cu(II)-transferrins (Figure 4A) supports this assignment. An alternate assignment is a Cu(II) to phenolate charge transfer, as suggested by Harrod (1969) for the intense absorption band observed near 440 nm for phenoxo complexes of Cu(II) containing halophenoxo and amine ligands. This suggests that, like the Fe(III)-transferrins (Gaber et al., 1974), all of the Cu(II)-transferrin resonance bands should be assignable to vibrational modes of the phenolate moiety of tyrosine and that the vibrational modes of the imidazole moiety of histidine are not resonance enhanced. Resonance enhancement of imidazole vibrational modes was not observed for Cu(II)- (Colyvas et al., 1973; Siiman et al., 1974) or Co(II)- (Yoshida et al., 1975) imidazole complexes. In fact, reasonable assignments of the observed Cu(II)-transferrin resonance bands, based upon calculated A_1 vibrational frequencies (Cummings and Wood, 1974) for *p*-methylphenol, can be made. The normal coordinate analysis of the 2,4,6-trichlorophenolato group of the model Cu(II) compound provided frequency data for a similar assignment of the observed resonance bands of TCPI (Figure 4B). The frequencies of the observed resonance bands and their assignments for Cu(II)-human serum transferrin and the Cu(II) model compound are listed in Table II.

Upon laser excitation of the Cu(II) model compound and Cu(II)-serum transferrin within their absorption bands, the resonance enhanced vibrational modes are those which are coupled to the electronic transition. The phenolate vibrational modes most likely to be enhanced are those involving the C-O and phenyl ring stretching coordinates. A band which is largely due to a nonring vibrational coordinate could be resonance enhanced, provided a C-O or ring stretching coordinate contributes to the potential energy distribution of the band. Such contributions are evident for all of the model compound resonance bands listed in Table II. The observed model compound resonance band at 1562 cm^{-1}, which corresponds to the 1601 cm^{-1} band in Cu(II)-serum transferrin, is primarily a C-C ring stretching vibration. The band at 1463 cm^{-1} in the model compound, which corresponds to the transferrin band at 1500 cm^{-1}, is also largely due to a C-C ring stretching coordinate, but the calculations show considerable contribution to the potential energy distribution from a C-O stretching coordinate. For Cu(II)-transferrin, the band at 1169 cm^{-1} is assigned to a CH bending coordinate which is typical of benzene, monosubstituted benzenes, and para-disubstituted benzenes (Scherer, 1963). The model Cu(II) compound has no coun-

TABLE II: Calculated and Observed Resonance Band Frequencies of the Model Cu(II) Compound and Cu(II)-Serum Transferrin and Their Assignments.

Model Cu(II) Compound			Cu(II)-Serum Transferrin		
Calcd[a] (cm^{-1})	Obsd (cm^{-1})	Assignment[a]	*p*-Methylphenol[b] (cm^{-1})	Obsd (cm^{-1})	Assignment[c]
1572.2	1562	CC str (87), CH bend (20)	1608	1601	CC str
1465.1	1463	CC str (49), CO str (37), CH bend (23)	1519	1500	CC str
1301.0	1316	CO str (43), CC str (26), CH bend (21)	1263	1277	CO str
			1167	1169	CH bend
1139.4	1122	CCl str (48), CC str (32), CC ring def (30)			
741.2	747	CCl str (56), CC ring def (14), CC str (11)			
			721	753	CO str + CCH_3 str

[a] 2,4,6-Trichlorophenolato group (this work). Numerical values in parentheses are the percent contribution to the potential energy distribution. Abbreviations: str, stretch; def, deformation. [b] Calculated for *p*-methylphenol (Cummings and Wood, 1974). [c] Based on assignments for *p*-methylphenol.

terpart to this mode and no resonance band is seen near 1170 cm^{-1}. On the other hand, the model compound resonance band at 1122 cm^{-1}, which is largely due to a C-Cl stretching coordinate, is absent for Cu(II)-transferrin. Gaber et al. (1974) assigned the resonance band at 1174 cm^{-1} in both Fe(III)- and Cu(II)-serum transferrin to an in-plane C-O bending mode with substantial ring character, based on correlation with phenol modes (Green et al., 1971; Pinchas et al., 1965). However, in those references and others (Evans, 1960; Jakobsen, 1965; Cummings and Wood, 1974), the two phenol and para-substituted phenol bands appearing near 1170-1180 cm^{-1} are assigned as follows: (a) a band near 1170 cm^{-1} to a CH bending mode which is sensitive to deuteration of the ring hydrogens but not the hydroxyl hydrogen, and (b) a band near 1180 cm^{-1} to a C-O-H (not C-O) bending mode which is sensitive to deuteration of the hydroxyl hydrogen but not the ring hydrogens. However, the C-O-H bending mode is not present in Cu(II)-transferrins because the hydroxyl proton is displaced by a coordination bond to the metal. Further, the calculated and the observed C-O bending mode for phenol appears at 404 cm^{-1} (Evans, 1960; Cummings and Wood, 1974).

The 747-cm^{-1} model compound band and the 753-cm^{-1} Cu(II)-transferrin band may or may not be corresponding resonance bands. The transferrin band is assigned to C-O and C-CH_3 stretching modes. In the model compound the band is largely due to C-Cl stretching modes, primarily that of the chlorine located para to the phenolic oxygen, i.e., similar to the methyl group in tyrosine. The C-O stretching coordinate contributes 7% to the potential energy distribution of the band in the model compound.

A C-O stretching coordinate contributes appreciably to the potential energy distribution of the bands at 1316 and 1277 cm^{-1}, respectively, for the model compound and Cu(II)-serum transferrin. As expected, the 1316-cm^{-1} model compound band and, to a lesser extent, the band at 1463 cm^{-1} are sensitive to changes in the C-O force constant. Increasing the force constant increases the frequencies of the bands. This is consistent with the observed difference in C-O stretch frequencies, 1249 and 1281 cm^{-1}, between phenol and phenolate ion, respectively (Pinchas, 1972). Ionization increases the double-bond character, shortens the bond length, and increases the C-O force constant. The C-O stretch band is at 1283 and 1304 cm^{-1}, respectively, for 2,4,6-trichlorophenol and the model compound (Figure 3). The frequency difference of 21 cm^{-1}, compared with 32 cm^{-1} for the phenol to phenolate ion change, indicates that coordination of the oxygen atom to Cu(II) results in a C-O bond whose double-bond character is nearer to that for phenolate ion than that for phenol. The C-O bond distance of 1.32 Å for the model compound (Wong et al., 1976) is significantly shorter than the 1.37 Å reported for phenol (Forest and Dailey, 1966; Quade, 1968; Pedersen et al., 1969). The effect of metal coordination on the C-O stretch constant is expected to depend upon the electronegativity of the metal ions. Since the electronegativities are in the order Cu > Co > Fe > Mn (Cotton and Wilkinson, 1962) and the respective C-O stretch frequencies for ovotransferrin are 1260, 1248, 1270, and 1256 cm^{-1} (Table I), there is no correlation. Although the metal binding ligands of transferrin apparently display sufficient flexibility to accommodate metal ions of various sizes, ESR studies (Windle et al., 1963; Aasa et al., 1963) indicate considerable difference in environmental symmetry between Cu(II) and Fe(III). The environment about Cu(II) shows only a small departure from axial symmetry, while Fe(III) is in a rhombically distorted octahedral environment. This suggests that the metal-oxygen-carbon bond angle differs for the various metals so that metal coordination affects the C-O stretch force constant differently than expected from their relative electronegativities. For the Fe(III), Cu(II), and Mn(III) chelates, the serum transferrin C-O stretch frequencies are larger than those for ovotransferrin (Table I), so that binding of those three metals is weaker for serum transferrin. For the Fe(III) chelates, this is consistent with the larger stability constants observed for ovotransferrin (Aasa et al., 1963; Aisen and Leibman, 1968).

Correspondence of the first three Raman bands (Table II) of the model Cu(II) compound to those of the Cu(II)-transferrin and the consistent assignments of the other two bands clearly identify tyrosine as a ligand in Cu(II) binding to transferrins. Resonance of the C-O stretch band demonstrates, for the first time, direct coordination of the phenolic oxygen function to the bound copper. The present study does not provide information on whether imidazole is a ligand. Resolution of the low frequency bands between 200 and 650 cm^{-1} (Figures 2A, 2B, and 4B), which are probably metal-O or metal-N stretch bands (Nakamoto, 1968), could provide evidence for the participation of N-containing ligands in metal binding to transferrins.

Acknowledgment

We thank G. Secor for the nitrogen analyses.

References

Aasa, R., and Aisen, P. (1968), *J. Biol. Chem. 243,* 2399.

Aasa, R., Malmström, B. G., Saltman, P., and Vänngård, T. (1963), *Biochim. Biophys. Acta 75,* 203.

Aisen, P. (1973), in Inorganic Biochemistry, Vol. 1, Eichhorn, G. L., Ed., Amsterdam, Elsevier, Chapter 9.

Aisen, P., Aasa, R., and Redfield, A. G. (1969), *J. Biol. Chem. 244,* 4628.

Aisen, P., and Leibman, A. (1968), *Biochem. Biophys. Res. Commun. 30,* 407.

Bailey, G. F., Kint, S., and Scherer, J. R. (1967), *Anal. Chem. 39,* 1040.

Behringer, J. (1967), in Raman Spectroscopy, Szymanski, H. A., Ed., New York, N.Y., Plenum Press, p 168.

Bezkorovainy, A., and Grohlich, D. (1971), *Biochem. J. 123,* 125.

Brunner, H., Mayer, A., and Sussner, H. (1972), *J. Mol. Biol. 70,* 153.

Brunner, H., and Sussner, H. (1973), *Biochim. Biophys. Acta 310,* 20.

Carey, P. R., and Young, N. M. (1974), *Can. J. Biochem. 52,* 273.

Colyvas, K., Cooney, R. P., and Walker, W. R. (1973), *Aust. J. Chem. 26,* 2059.

Cotton, F. A., and Wilkinson, G. W. (1962), in Advanced Inorganic Chemistry, New York, N.Y., Interscience, p 93.

Cummings, D. L., and Wood, J. L. (1974), *J. Mol. Struct. 20,* 1.

Evans, J. C. (1960), *Spectrochim. Acta 16,* 1382.

Feeney, R. E., and Komatsu, S. K. (1966), *Struct. Bonding (Berlin) 1,* 149.

Forest, H., and Dailey, B. P. (1966), *J. Chem. Phys. 45,* 1736.

Gaber, B. P., Miskowski, V., and Spiro, T. G. (1974), *J. Am. Chem. Soc. 96,* 6868.

Green, J. H. S., Harrison, D. J., and Kynaston, W. (1971), *Spectrochim. Acta, Part A 27,* 2199.

Harrod, J. F. (1969), *Can. J. Chem. 47,* 637.

Inman, J. K. (1956), Ph.D. Dissertation, Harvard University.

Jakobsen, R. J. (1965), *Spectrochim. Acta 21,* 433.

Kiefer, W., and Bernstein, H. J. (1971a), *Appl. Spectrosc. 25,* 609.

Kiefer, W., and Bernstein, H. J. (1971b), *Appl. Spectrosc. 25,* 500.

Kiefer, W., and Bernstein, H. J. (1972), *Mol. Phys. 23,* 835.

Kint, S., Elsken, R. H., and Scherer, J. R. (1976), *Appl. Spectrosc. 30,* 281.

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

Lehrer, S. S. (1969), *J. Biol. Chem. 244,* 3613.

Line, W. F., Grohlich, D., and Bezkorovainy, A. (1967), *Biochemistry 6,* 3393.

Lord, R. C., and Yu, N. T. (1970), *J. Mol. Biol. 50,* 509.

Luk, C. K. (1971), *Biochemistry 10,* 2838.

Nakamoto, K. (1968), in Spectroscopy and Structure of Metal Chelate Compounds, Nakamoto, K., and McCarthy, P. J., Ed., New York, N.Y., Wiley, p 216.

Pedersen, T., Larsen, N. W., and Nygaard, L. (1969), *J. Mol. Struct. 4,* 59.

Pinchas, S. (1972), *Spectrochim. Acta, Part A 28,* 801.

Pinchas, S., Sadek, D., and Samuel, D. (1965), *J. Phys. Chem. 69,* 2259.

Quade, C. R. (1968), *J. Chem. Phys. 48,* 5490.

Scherer, J. R. (1963), in Planar Vibrations of Chlorinated Benzenes, Midland, Mich., The Dow Chemical Company.

Scherer, J. R., Bailey, G. F., and Kint, S. (1971), *Anal. Chem. 43,* 1917.

Scherer, J. R., Go, M. K., and Kint, S. (1973), *J. Phys. Chem. 77,* 2108.

Scherer, J. R., and Kint, S. (1970), *Appl. Opt. 9,* 1615.

Siiman, O., Young, N. M., and Carey, P. R. (1974), *J. Am. Chem. Soc. 96,* 5583.

Spiro, T. G., and Strekas, T. C. (1974), *J. Am. Chem. Soc. 96,* 338.

Tan, A. T., and Woodworth, R. C. (1969), *Biochemistry 8,* 3711.

Tomimatsu, Y., Kint, S., and Scherer, J. R. (1973), *Biochem. Biophys. Res. Commun. 54,* 1067.

Ulmer, D. D., and Vallee, B. L. (1963), *Biochemistry 2,* 1335.

Warner, R. C., and Weber, I. (1951), *J. Biol. Chem. 191,* 173.

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

Windle, J. J., Wiersema, A. K., Clark, J. R., and Feeney, R. E. (1963), *Biochemistry 2,* 1341.

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

Wong, R. Y., Palmer, K. J., and Tomimatsu, Y. (1976), *Acta Crystallogr., Sect. B. 32,* 567.

Woodward, L. A. (1967), in Raman Spectroscopy, Szymanski, H. A., Ed., New York, N.Y., Plenum Press, p 1.

Woodworth, R. C., Morallee, K. G., and Williams, R. J. P. (1970), *Biochemistry 9,* 839.

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

Yoshida, C. M., Freidman, T. B., and Loehr, T. M. (1975), *J. Am. Chem. Soc. 97,* 1028.

Young, J. W., and Perkins, D. J. (1968), *Eur. J. Biochem. 4,* 385.

Zschocke, R. H., Chiao, M. T., and Bezkorovainy, A. (1972), *Eur. J. Biochem. 27,* 145.