Spectrophotometric Studies of the Copper(II)–D-*o*-Tyrosine Complex. Assignment of the 330-nm Dichroic Band in Copper(II) and Iron(III) Transferrins

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The separation of both enantiomers of racemic *o*-tyrosine by means of binaphthylphosphoric acid has been undertaken. Copper(II) interacts with D-*o*-tyrosine to form two complexes. The first, obtained at pH 6, displays a circular-dichroism (c.d.) spectral pattern characteristic of metal co-ordination through amino-nitrogens and carboxylate oxygens. The second complex starts to form at pH 8 and is fully defined at pH 10.5. Its c.d. spectrum, in the region below 400 nm, is very similar to those of iron(III) and copper(II) transferrins and displays two well defined bands at *ca.* 400 and 330 nm. So far, only the origin of the higher-frequency peak has been well established as arising from a phenolate-oxygen-to-metal charge-transfer transition, whereas that of the lower-frequency peak remains uncertain. Resonance-Raman measurements upon excitation into the envelope of the two absorptions at 400 and 330 nm clearly indicate a common origin to both bands, namely: phenolate oxygen ⟶ Cu^{II} charge-transfer transition.

TRANSFERRINS, the iron transport proteins of biological fluids, have two metal-binding sites which can accommodate a variety of multivalent metal ions.[1] The identification of the metal-binding sites has been the object of numerous studies [2] and it is now well established that in each of them at least one tyrosyl group is co-ordinated to the metal ion. In tyrosyl-to-metal co-ordination absorption spectra as well as in circular dichroism (c.d.) spectra, these proteins exhibit a band in the 400—450 nm region which is attributed to a tyrosyl-to-metal charge-transfer transition.[3] Another band lying at 360 nm has been observed in the c.d. spectra of iron(III) transferrins [4] and has been suggested to arise from a transition of the disulphide bridge of cystine that shifts to lower energies owing to a change in the dihedral angle of at least one disulphide bridge upon iron binding.[5]

The present study was undertaken essentially to determine the origin of this band; we first show that the higher-energy band is present at 330 nm in the c.d. spectra of copper(II) transferrin. Secondly, using a very simple model, bis(D-*o*-tyrosinato)copper(II), in which the carboxylate oxygen, amino-nitrogen, and phenolate oxygen are the only potential ligating atoms, we demonstrate that the binding of the latter to Cu^{II} is characterized by the appearance of two c.d. bands at 330 and 390 nm. Moreover, c.d. and resonance-Raman (r.R.) studies enable us to attribute these two bands to tyrosyl-to-copper charge-transfer transitions. To perform these experiments we have chosen the *ortho*-isomer of tyrosine since it is well known that *m*- and *p*-tyrosine are unable to bind to metals *via* phenolate oxygen even at high pH.[6,7] This type of co-ordination is only observed in the case of the *para*-isomer when its α-amino- and carboxyl groups are included in a long polypeptide chain.[8]

Resolution of racemic *o*-tyrosine by crystallization of its diastereoisomeric salts with binaphthylphosphoric acid is also reported, the D-*o*-tyrosine diastereoisomer being used throughout.

RESULTS AND DISCUSSION

Racemic *o*-tyrosine was resolved by Hooker and Schellman [9] by means of the L-amino-acid oxidase method of Parikh *et al.*,[10] in which the L enantiomer is destroyed. We found it more convenient to resolve this compound by crystallization of its diastereoisomeric salts with binaphthylphosphoric acid (bnp), a resolving agent which is readily accessible in both (+) and (−) forms.[11] The compounds (+)-bnp (pK_a 2.50) [12] and (±)-*o*-tyrosine rapidly formed a mixture of crystalline diastereoisomers, from which the less soluble [(+)-bnp, (+)-*o*-tyrosine] salt was easily separated, owing to a large solubility difference between the two salts. By means of (−)-bnp the [(−)-bnp, (−)-*o*-tyrosine] salt was subsequently isolated from the mother liquor.

The enantiomeric purity of the above samples was checked by high-performance liquid chromatography (h.p.l.c.) on a macroporous polyacrylamide gel grafted with L-piperidine-2-carboxylic acid and subsequently complexed with Cu^{II}, as described by Lefebvre *et al.*[13]

Chromatograms for (±)-*o*-tyrosine and for the resolved sample having α (1 mol dm^{-3} HCl, 303 K, 589 nm) = +25.0° are shown in Figure 1. It may be concluded from these data that (*i*) (+)-*o*-tyrosine should belong to the D series, in agreement with c.d. results (see below), and (*ii*) the (+) sample studied, if not pure, certainly contains less than 2—3% of (−) isomer (limit of detection) which corresponds to an enantiomeric excess in the range of 95—100%. A similar conclusion may be drawn for the L-(−)-*o*-tyrosine described above, which exhibits the same optical rotation, in absolute terms, as the D-(+) sample.

This straightforward resolution of *o*-tyrosine suggests

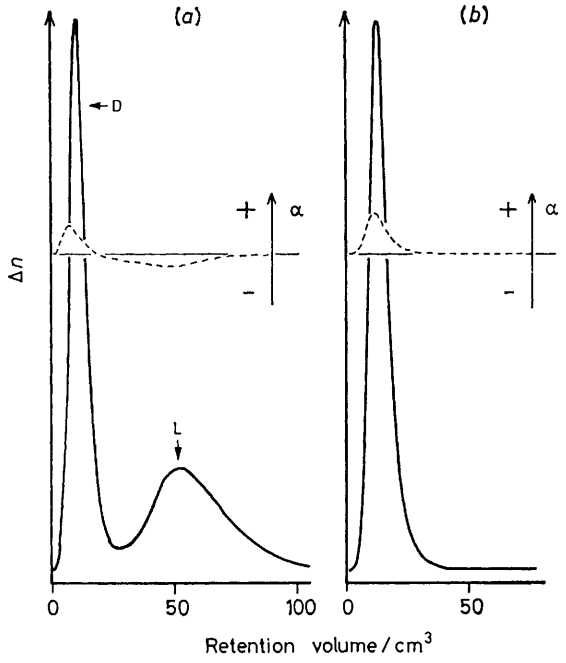

FIGURE 1 Chromatogram (h.p.l.c.) of *o*-tyrosine on a polyacrylamide gel grafted with L-piperidine-2-carboxylic acid and complexed with Cu^{II}. (*a*) Racemate; (*b*) (+) sample having α(1 mol dm^{-3} HCl, 303 K, 589 nm) + 25.0°. Detection: refractometric (——); polarimetric (— — —)

that (+)- and (−)-bnp could be effective for the resolution of other underivatized amino-acids as well, both L and D enantiomers of which might be accessible in this way.

Chromatographic results indicating that (+)-*o*-tyrosine belongs to the D series are confirmed by c.d. data: Figure 2 exhibits the c.d. spectrum of an aqueous

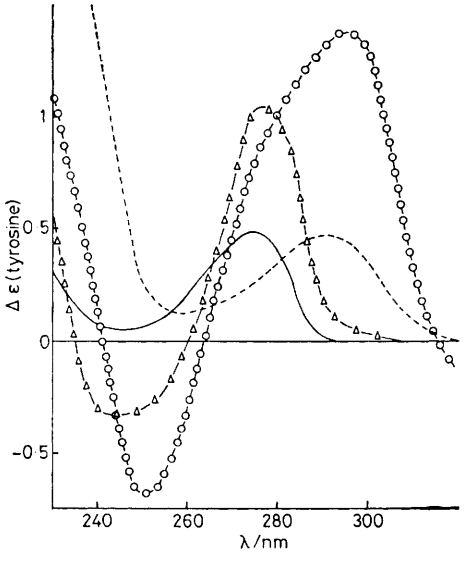

FIGURE 2 Circular dichroism spectra of D-*o*-tyrosine (2×10^{-4} mol dm^{-3}): in the absence of copper at pH 7 (——) and pH 11 (— — —); in the presence of copper at 2:1 D-*o*-tyrosine to metal molar ratio, at pH 7 (△) and pH 11 (○)

solution of D-*o*-tyrosine in the region of the L_b transition (using Platt notation). When D-*o*-tyrosine is in the protonated form, the band due to the L_b transition occurs at 275 nm with Δε(tyrosine) = +0.5. The sign of this band is in agreement with that reported by Hooker and Schellman [9] for D-*o*-tyrosine but its amplitude is somewhat lower than the value obtained by these authors. Upon deprotonation, the band moves to 292 nm; it is still positive with Δε(tyrosine) = +0.5. As can be seen in Figure 4, where Δε(292 nm) is plotted as a function of pH, deprotonated D-*o*-tyrosine is formed in the range pH 8—10.5.

On addition of Cu^{II} to a D-*o*-tyrosine solution at a 2 : 1 amino-acid-to-metal molar ratio, two spectroscopically well defined complexes are obtained, the formation of which is dependent on pH. This is illustrated by the plot of Δε and/or ε of the different bands as a function of pH which exhibits a two-step behaviour. At pH 6 the first step is reached and from pH 6 to 8 no further variations of spectra are observed. These results are comparable with those obtained by Letter and Bauman [6] using potentiometric and calorimetric measurements which indicate that *o*-tyrosine forms a 2 : 1 complex with Cu^{II} at *ca.* pH 7 ($K_1K_2 = 3 \times 10^5$, $I = 0.16$ mol dm^{-3}). Since in our system the experimental conditions are slightly different, it is reasonable to conclude that the species formed at pH 6, hereafter called (I), is also a 2 : 1 complex.

Absorption and c.d. spectra of complex (I) are shown in Figures 2 and 3. The c.d. spectrum exhibits bands at

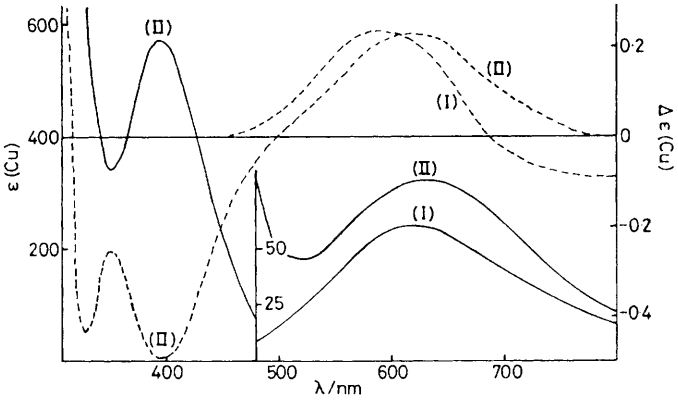

FIGURE 3 Absorption (——) and c.d. (— — —) spectra of complexes (I) and (II)

800 (−0.09), 590 (+0.23), 277 [Δε(tyrosine) = +1.05], and 245 (−0.66) nm. If one ignores the band at 277 nm due to the L_b transition, this spectrum is typical of a bis-(amino-acid) complex bound to Cu^{II} as a substituted glycinate.[14] The two bands in the visible spectrum originate from *d–d* transitions although it should be noted that their amplitudes are lower than those of bis(D-*p*-tyrosinato)copper(II);[14b] the band at 250 nm can be attributed to amino-nitrogen and/or carboxylato-oxygen-to-copper charge-transfer transitions.[15] In this complex the tyrosyl is in its phenolic form as evidenced

by the L_b band remaining unshifted at 275 nm. Its amplitude is, however, twice as strong in the spectra of the complex than in that of the free ligand (Figure 2).

This enhancement may be interpreted by assuming that a weak interaction exists between the phenolic ring and the copper(II) bis chelate. The crystal structures of bis(L-tyrosinato)copper(II) and of the dimeric copper(II) chelate of glycyl-L-leucyl-L-tyrosine show that one of the phenolic rings is located beneath the metal co-ordination plane at a distance slightly greater than 3 Å indicating that such interaction really does take place in these complexes.[16,17] A similar, but closer, approach most probably occurs in complex (I) and the following data confirm this assumption.

The second step is reached at pH 10.5, as illustrated by Figure 4 where values of $\Delta\varepsilon$ for various bands are plotted against pH. Boggess and Martin [7] have already shown that a solution containing DL-*o*-tyrosine and Cu^{II} at 2 : 1 molar ratio yields two types of complexes, the second being formed, as observed by us, between pH 8 and 10.5, after the addition of two equivalents of base. The visible absorption spectrum of their solution at pH 10.5 is similar to that of our complex, hereafter called (II), exhibiting bands at 630 nm ($\varepsilon = 80$ dm^3 mol^{-1} cm^{-1}) and 390 nm ($\varepsilon = 575$).

Three interesting points should be noted at this stage: (*i*) complex (II) is formed in the same pH range at which deprotonation of the phenol moiety of free tyrosine occurs (Figure 4); (*ii*) complex (II) exhibits an absorption band at 390 nm; and (*iii*) c.d. spectra of bis(D-*o*-tyrosinato)copper(II) solutions from pH 8 to 10.5 exhibit isodichroic points at 608, 280, and 244 nm. In addition, each intermediary spectrum comprises a linear combination of the spectra of complexes (I) and (II) indicating that only two well defined species are present. From these observations, the first conclusion to be drawn is that phenolate–metal binding is involved in complex (II) formation. Similarly, as inferred by the presence of the isodichroic point at 280 nm, between pH 8 and 10.5 the phenol moiety of tyrosine must be present only in two forms: the protonated form interacting slightly with the copper chelate, and the deprotonated form bound to the metal. The presence of a third entity in variable concentrations, such as unbound deprotonated tyrosine, should therefore be neglected.

The foregoing statements imply that two phenolate oxygens must be co-ordinated to each Cu^{II}. Hence, for complex (II) we propose a structure like that of the complex (I) bis chelate with two phenolate oxygens co-ordinated in apical positions.

Figures 2 and 3 show the absorption and c.d. spectra of complex (II). There is only one positive band in the visible region due to *d–d* transitions lying at 620 nm (+0.22). The negative band at 250 nm (−1.40) has the same origin as in complex (I). Here again the amplitude of the positive band at 295 nm [$\Delta\varepsilon$(tyrosine) = +1.36], assigned to the L_b transition of phenolate, is higher in the complex than in the free-ligand spectra. Hence, a similar interaction to that observed in the case of complex (I) exists between the deprotonated phenolic ring and the metal bis chelate, strongly suggesting that apical co-ordination to phenolate is favoured by this interaction.

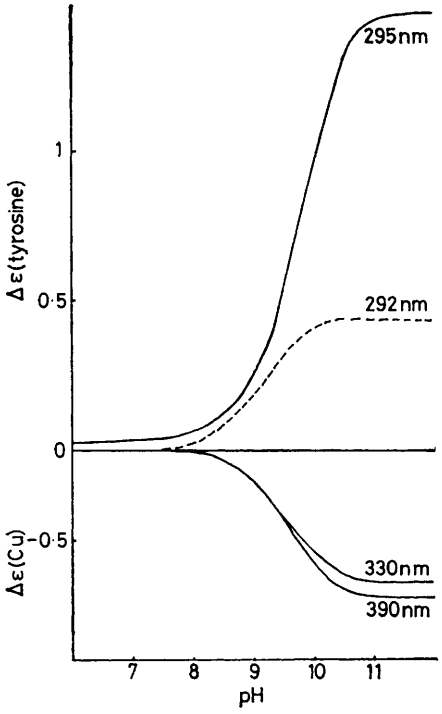

FIGURE 4 Variation of molar c.d. coefficients of different bands as a function of pH; (— — —) [D-*o*-tyrosine] = 2×10^{-4}, [Cu^{2+}] = 0; (——) [D-*o*-tyrosine] = 2×10^{-4}, [Cu^{2+}] = 10^{-4} mol dm^{-3}. $\Delta\varepsilon$ is expressed per mole of tyrosine for the bands at 292 and 295 nm and per mole of copper for the bands at 330 and 390 nm

The appearance of two new negative bands at 395 (−0.5) and 330 nm (−0.44) is the most striking feature of the c.d. spectrum of complex (II). It should be noted that the band at 330 nm is partially masked by the positive band at 295 nm and should therefore lie at somewhat higher energy. In that region of the absorption spectrum only one band at 400 nm ($\varepsilon = 575$ dm^3 mol^{-1} cm^{-1}) is actually detectable, that at 330 nm appearing as a weak shoulder on the edge of the band at 295 nm. The origin of the band at 400 nm is well known: it has been assigned to a ligand-to-metal charge-transfer transition; it is observed in the absorption spectrum of copper(II)–poly(α-amino-acid) complexes containing tyrosine and especially in that of copper(II) transferrin.[3] This band around 400 nm is even used as evidence of phenolate-to-metal co-ordination in the absence of more direct proof. In our complex, in addition, the origin of the band at 330 nm is easy to elucidate and may safely be attributed to phenolate–copper(II) co-ordination.

We can go further by comparing the energy difference of 6 400 cm^{-1} between the L_b and L_a transitions of phenolate with that of 5 300 cm^{-1} between the bands at 400 and 330 nm and tentatively assign the latter to charge-

transfer transitions from π_a and π_b phenolate orbitals to Cu^{II} respectively.

Figure 5 shows the c.d. spectrum of copper(II) transferrin which exhibits, in addition to the visible bands, two

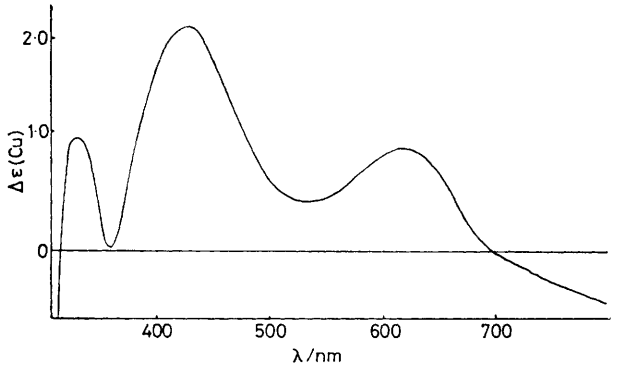

FIGURE 5 Circular dichroism spectrum of copper(II) transferrin (0.9×10^{-4} mol dm^{-3}) in 0.01 mol dm^{-3} $Na[HCO_3]$, 0.1 mol dm^{-3} NaCl, and 0.01 mol dm^{-3} hepes buffer (pH 8)

positive well defined bands at 430 and 330 nm. This spectrum is similar to that of complex (II) of bis(D-*o*-tyrosinato)copper(II) and we can assign these two bands to charge-transfer transitions from π_b and π_a phenolate orbitals to the $d_{x^2-y^2}$ Cu^{II} orbital respectively. The second band (330 nm) has also been observed in the iron(III) transferrin c.d. spectrum,[4] so it can now be attributed unambiguously to a phenolate-to-metal charge-transfer transition. Based on the spectrum of our model compound, one can rule out the interpretation of this band as arising from a red shift of the S–S transition owing to a change in the dihedral angle of at least one disulphide bridge upon iron binding.

The r.R. studies of complex (II) confirm our assignments. A typical r.R. spectrum of complex (II) in D_2O upon excitation into the absorption at *ca.* 390 nm is illustrated in Figure 6. A similar pattern is obtained using the two u.v. exciting lines of an Ar^+ laser at 334.5 and 363.8 nm. The resonance-enhanced bands are all phenolic ring fundamentals: two doublets at 1 482—1 457 and 1 599—1 570 cm^{-1} assigned to the C–C stretches of the phenolic ring correspond to the ν_{13} and ν_{16} doubly degenerate modes of benzene respectively,[18] the band at 1 272 cm^{-1} is due to the C–O stretching vibration of phenolate,[19] and the bands at 1 044 and 1 164 cm^{-1} are attributed to CH deformation modes (ν_{14} and ν_{17} respectively in benzene [18a]). Excluding the peak at 1 044 cm^{-1}, a similar pattern is observed in the r.R. spectra of metal transferrins and in those of copper(II)–poly(α-amino-acid) complexes containing tyrosine residues when metal–phenolate co-ordination takes place.[8] As the excitation profiles of Figure 7 demonstrate, these bands show characteristic selective enhancement. The CO stretching vibration and one of the two C–C ring stretches (1 599—1 570 cm^{-1}) are coupled with both electronic transitions, whereas the C–C stretch at 1 482—1 457 cm^{-1} is only in resonance with the low-energy transition and both C–H bending modes (1 164 and 1 044 cm^{-1}) with the high-energy one. This difference in enhancement pattern most probably reflects the aforementioned difference in the tyrosine molecular orbitals (π_a and π_b) involved in the electronic transitions at 330 and 390 cm^{-1}.

One might argue, however, that some vibrations could be in resonance with the $\pi_b(PhO^-) \rightarrow Cu^{II}$ and/or the

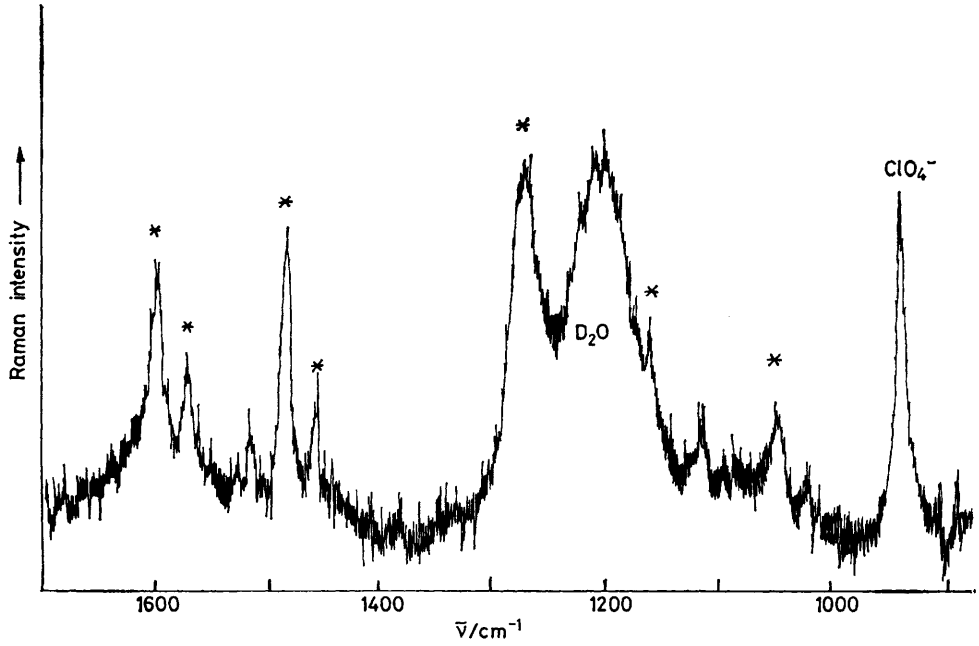

FIGURE 6 Resonance-Raman spectrum of complex (II) in D_2O using the 454.5-nm exciting line, 80 mW, and [Cu] = 5×10^{-3} mol dm^{-3}. Other conditions: slit width 5 cm^{-1}, scanning speed 25 cm min^{-1}, time constant 1 s. Asterisks indicate the resonance-enhanced bands

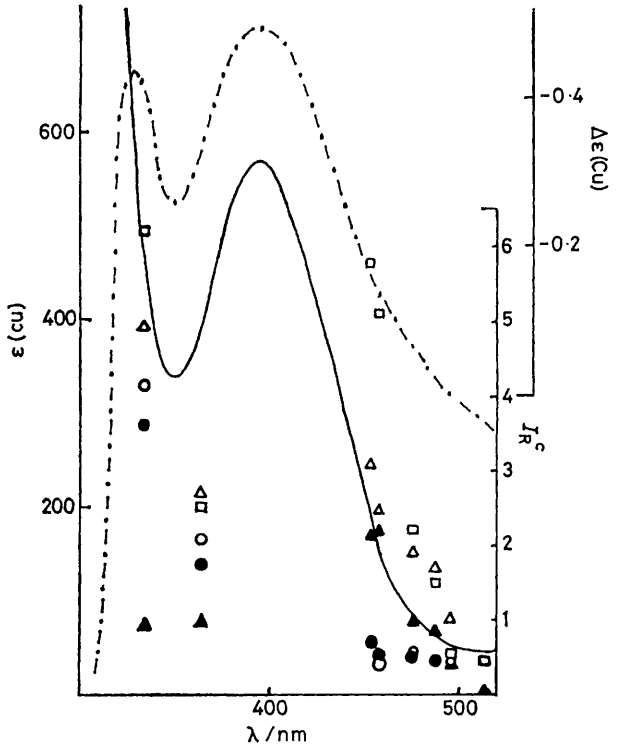

FIGURE 7 Excitation profiles of the Raman bands of complex (II). I_R^c is the molar Raman intensity measured as indicated in the text. (——), Absorption spectrum; (— · — · —), c.d. spectrum; (●), 1 044 cm^{-1}; (○), 1 164 cm^{-1}; (□), 1 272 cm^{-1}; (▲), 1 482—1 457 cm^{-1}; (△), 1 599—1 570 cm^{-1}. For the sake of clarity only the high-energy band instensity of the doublets has been plotted

$\pi_a(PhO^-) \rightarrow Cu^{II}$ ligand-to-metal charge transfer (l.m.c.t.) transitions and the $\pi_a \rightarrow \pi^*$ and/or $\pi_b \rightarrow \pi^*$ transitions. In such circumstances the Raman enhancement observed in the u.v. region could be ascribed to a pre-resonance Raman spectrum of the chromophore absorbing at 275 nm, namely the $\pi_b \rightarrow \pi^*$ transition. In fact, the Raman spectra of tyrosine at pH 11, using the u.v. exciting lines, show the resonance enhancement of several bands, in particular those at 1 270 and 1 600 cm^{-1}. However, the intensity increase is at most only one fifth of that observed by us in the r.R. spectra of complex (II).[20]

EXPERIMENTAL

Human apotransferrin (Behringwerke) from Hoechst Pharmaceuticals was used. The molar ratio of iron to transferrin was less than 0.03 : 1 as estimated from the visible spectra. Solutions of copper(II) transferrin (0.92×10^{-4} mol dm^{-3}) in 0.01 mol dm^{-3} $Na[HCO_3]$, 0.1 mol dm^{-3} NaCl, and 0.01 mol dm^{-3} *N*-(2-hydroxyethyl)piperazine-*N'*-ethane-2-sulphonic acid (hepes) buffer, pH 8, were used.

Resolution of o-*Tyrosine*.—DL-*o*-Tyrosine was purchased from Sigma Laboratories. (*a*) (+)-o-*Tyrosine*. Racemic *o*-tyrosine (1.8 g, 10 mmol) and (+)-binaphthylphosphoric acid (3.5 g, 10 mmol) were mixed in hot ethanol (200 cm^3); in most experiments, crystallization of the salts occurred before complete dissolution of the reactants was achieved. After the mixture was allowed to stand at room temperature for *ca.* 1 h, the salt formed (A) was filtered off; yield 2 g (3.8 mmol). Recrystallization of this salt from ethanol (200 cm^3) afforded 1.2 g (2.3 mmol, 46%) of essentially pure [(+)-bnp, (+)-*o*-tyrosine] salt (B).

To a stirred suspension of (B) (1.19 g, 2.25 mmol) in absolute ethanol (20 cm^3) was added aqueous 6 mol dm^{-3} Na[OH] (0.37 cm^3, 2.22 mmol), which resulted in the dissolution of the salt, followed by the crystallization of free *o*-tyrosine; 0.34 g (1.9 mmol) was collected. This amino-acid, which was contaminated with traces of (+)-bnp, was purified by dissolution in a mixture of water (7 cm^3) and HCl (1.9 cm^3, mol dm^{-3}). The opaqueness which resulted (due to bnp) was eliminated by filtration through a sintered glass filter; neutralization of the filtrate (with ammonia) afforded a precipitate of pure (+)-*o*-tyrosine (0.2 g): α(aqueous 1 mol dm^{-3} HCl, *c* 0.84, 303 K): + 25.0 (589), +26.5 (578), +30.6 (546), and +55.7° (436 nm).

(*b*) (−)-o-*Tyrosine*. The mother-liquors of salt (A) (preceding experiment) were evaporated to dryness to give *ca.* 3.3 g of a mixture of diastereoisomeric salts. Recrystallization from ethanol (50 cm^3) afforded another crop (0.7 g) enriched in the less soluble salt (C). The new mother-liquors, enriched in the more soluble [(+)-bnp, (−)-*o*-tyrosine] salt, were decomposed by 6 mol dm^{-3} Na[OH] (*ca.* 0.8 cm^3), yielding a precipitate of enantiomerically impure (−)-*o*-tyrosine (0.66 g, 3.6 mmol), α(1 mol dm^{-3} HCl, *c* 0.82, 303 K, 589 nm) — 15°. When this sample was combined with (−)-bnp (1.26 g, 3.6 mmol) in ethanol (70 cm^3), crystallization of the salt [(−)-bnp, (−)-*o*-tyrosine] occurred instantly. This salt (1.17 g, 2.2 mmol) did not need further purification and was decomposed by 6 mol dm^{-3} Na[OH] (0.3 cm^3) in absolute ethanol (20 cm^3) as described in (*a*) above. The sample of (−)-*o*-tyrosine obtained in this way (0.34 g) was submitted to the same purification procedure as for the (+) enantiomer, yielding 0.16 g of the pure amino-acid: α(aqueous 1 mol dm^{-3} HCl, *c* 0.81, 303 K) — 25.2 (589), — 25.6 (578), —30.2 (546), and —55.2° (436 nm).

Enantiomeric Purity Determination.—Preparation of the macroporous polyacrylamide gel has been described.[13a] The polymer (pearls of size 10—20 μm) was grafted by reaction with L-piperidine-2-carboxylic acid and formaldehyde; the amount of amino-acid linked was 1.5 milli-equivalents g^{-1} of dry resin. Copper was incorporated by shaking the resin in a formate buffer containing copper(II) ions. The stationary phase so obtained was packed into a stainless-steel column (length 5 cm, internal diameter 4.8 mm). A Waters liquid chromatograph equipped with refractometric and polarimetric detectors was used. The samples of *o*-tyrosine were injected as aqueous solutions, and eluted with water at room temperature, at a flow rate of 1 cm^3 min^{-1}. Under these conditions the retention volume of the D isomer was *ca.* 11 cm^3 and that of the L isomer *ca.* 50 cm^3.

Instrumental Details.—Absorption spectra in the range 185—200 nm were recorded on a Cary 14 spectrometer. Circular-dichroism spectra in the range 185—800 nm were obtained by means of a Jobin–Yvon Dochrograph model mark III, calibrated with a solution of epiandrosterone in dioxan as indicated by the manufacturers. The solutions for these measurements were prepared such that they gave values of optical density not exceeding 1.5. Results are reported in terms of ε (molar absorption coefficient) and $\Delta\varepsilon = \varepsilon_l - \varepsilon_r$ (molar c.d. coefficient), in dm^3 mol^{-1} cm^{-1}.

Raman spectra were measured on a Coderg D 800 and a

Jobin–Yvon Ramanor spectrophotometer for the visible and u.v. regions respectively, using the exciting lines of Ar^+ and Kr^+ Spectra Physics lasers; the turning-cell technique and 90° scattering were used throughout.

Raman intensity measurements were determined relative to the intensity of the ClO_4^- ν_1 band (936 cm^{-1}) and are given as the ratio of the area under a band (measured by the product: peak height × half-band width) to the area under the ClO_4^- band. The ratios were corrected for sample absorption, instrumental response, and ν dependence.

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