Differences in the Protein Fluorescence of the Two Iron(III)-Binding Sites of Ovotransferrin

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(*Received* 2 *August* 1974)

1. Changes in the tryptophan fluorescence and the visible absorption spectrum resulting from the combination of apo-ovotransferrin with Fe^{3+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+} and Cd^{2+} were measured. 2. As expected for a radiationless transfer of electronic excitation energy, only the ions Fe^{3+}, Fe^{2+} and Cu^{2+}, which gave complexes with large extinctions between 300 and 370nm, resulted in large decreases in tryptophan fluorescence. 3. The decrease in protein fluorescence was non-linear with increasing occupancy of the Fe^{3+}- and Cu^{2+}-binding sites. The decrease in fluorescence on binding of Fe^{3+} was biphasic and showed that the two metal-binding sites were being occupied sequentially at pH 7.4–8. 4. The first site reacted with Fe^{3+} instantaneously, the second was occupied over a minute. 5. The non-identity of the two sites was also demonstrated by the preparation of a stable hybrid containing both Cu^{2+} and Zn^{2+}.

The transferrins are a family of homologous iron-transporting proteins. Most are single polypeptide chains bearing two iron-binding sites. Other metal ions can bind at these sites with various affinities (Tan & Woodworth, 1969; Luk, 1971). Binding of metal ions leads to increased absorption in the region 242–294nm, which has been attributed to the changed ionization of tyrosine residues (Tan & Woodworth, 1969) and some of the first transition-metal series, Cr^{3+}, Mn^{3+}, Fe^{3+}, Co^{2+} and Cu^{2+}, also confer characteristic colours to the complexes owing to absorption in the region 300–370nm and in the visible region.

The relative affinities of the two metal-binding sites of the transferrins for iron have for some time been a matter of some controversy. Warner & Weber (1953) obtained a value of $1 \times 10^{29} M^{-1}$ for the association constant of Fe^{3+} in ovotransferrin and proposed that the binding of the first iron ion facilitated the binding of the second ion ($K_2 = 100 K_1$) such that both sites on any one molecule were effectively occupied together. By using equilibrium dialysis Davis *et al.* (1962) concluded that $K_1 = 400 K_2$ and that there was sequential occupancy of the two sites, whereas Aasa *et al.* (1963), using the same technique, concluded that the sites were equivalent and independent. On the basis of kinetic studies Woodworth (1966) suggested a positive interaction between the two sites which would result in both sites being occupied together; however, the direct demonstration of three species of serum and ovotransferrin with 0, 1 and 2 atoms of iron per molecule present in the same solution

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(Williams *et al.*, 1970; Aisen *et al.*, 1970) disproves this. The most recent determination of the relative affinities of the two sites by equilibrium dialysis gave $K_1 = 12 K_2$ (Aisen & Leibman, 1968).

Long-range radiationless transfer of electronic excitation energy should occur from the 18 tryptophan residues of transferrin to the two metal-binding sites when the holoprotein has a strong absorption spectrum in the region 300–370nm (Williams, 1962). Even though the local environments of the two metal-binding sites may be the same (giving the same extinction coefficient per site), the decrease in protein fluorescence on occupying the first site will be different from the decrease on occupying the second site. If the 18 tryptophans are randomly distributed throughout the molecular volume then the fluorescence of the apoprotein, one-iron protein and two-iron protein should be in the ratio $x^0 : x^1 : x^2$ (Holbrook, 1972), where x has been called a geometric quenching factor. The differential response of protein fluorescence enables a measurement of the change in protein fluorescence as a function of the degree of saturation of the iron-binding sites to determine whether the two sites are occupied together (a single linear decrease in fluorescence with increased occupancy), sequentially (a linear decrease in fluorescence from that of the apoprotein to that of the one-iron protein, and a second less-steep linear decrease from the fluorescence of the one- to the two-iron protein) or randomly (a smooth non-linear decrease following $F = [1 - \alpha(1 - x)]^2$ where F is the fluorescence relative to the apoprotein, x is the constant geometric quenching factor and α is the degree of occupancy of the binding sites (Holbrook, 1972).

Experimental

Preparation of ovotransferrin

A modification of the method of Williams (1968) was used. One volume of saturated (at room temperature) $(NH_4)_2SO_4$ was added to the whites of 36 hen's eggs with stirring at room temperature over 2h. A precipitate was centrifuged down at 700g for 15min and discarded. The supernatant was adjusted to pH4.7 with $0.5M$-H_2SO_4 and the precipitate that formed was removed by centrifugation at 6000g for 20min and discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant at the rate of 8g/100ml and the precipitate that formed was recovered by centrifugation at 6000g for 20min and redissolved in the minimum volume of distilled water (about 100ml). The solution was desalted by passing it through a column (6.5cm×44cm) of Sephadex G-50 (coarse grade) equilibrated with distilled water. The protein-containing eluate was adjusted to pH6 with aq. NH_3, cooled to 4°C and two-thirds of its volume of $0.02M$-NaCl in 50% (v/v) ethanol was added with stirring. Stirring was continued overnight at 4°C. A precipitate was recovered by centrifugation at 6000g for 10min at −2°C and was redissolved in a minimum volume of distilled water (about 100ml) and was mixed with $0.1M$-Fe^{3+}–nitrilotriacetate until no more pink colour formed. The protein solution was clarified by centrifugation at 6000g for 10min and then passed through a column (6.5cm× 44cm) of Sephadex G-50 (coarse grade) equilibrated with distilled water adjusted to pH9.5 with aq. NH_3. To the protein eluate was added one-ninth of its volume of $1M$-sodium acetate, pH5.5, and the solution was then applied to a column (4cm× 8cm) of CM-Sephadex (C-50) equilibrated in $0.1M$-sodium acetate, pH5.5. The transferrin was retained and the column was washed until the eluate was free of absorption at 280nm. Transferrin (a pink protein) was eluted with $0.5M$-sodium acetate, pH5.5, desalted by passage through a Sephadex G-50 column equilibrated with distilled water and then freeze-dried. About 4g of protein was obtained from 36 eggs. Protein was determined by using $E^{1\%}_{280} = 11.4$ (Warner & Weber, 1953). The transferrin migrated as a single protein-staining band during polyacrylamide-gel electrophoresis in sodium dodecyl sulphate by using the conditions of Tanner & Boxer (1972). The migration was as expected for a polypeptide chain of relative molecular mass 80000. Additional chromatography on DEAE-Sephadex was not necessary.

Iron-free ovotransferrin

Iron was removed by a modification of the method of Warner & Weber (1951). A solution of 80mg of ovotransferrin in 2ml of water was adjusted to pH4.7 with $1M$-citric acid and made $0.01M$ in citrate

by addition of $0.1M$-sodium citrate, pH4.7. Amberlite IRA-400 (Cl^- form) was added at a rate of 1g/ml of protein solution and the suspension was filtered through a Millipore filter (RAWP01300, 1.2μm) and the residue washed with 1ml of $0.1M$-sodium citrate, pH3.7. The combined filtrates were desalted by passage through a column of Sephadex G-50 (coarse grade) that had been equilibrated with the buffer required in the subsequent titration experiment.

Iron-saturated transferrin

The preparation does not yield the fully iron-saturated protein. A fourfold molar excess of Fe^{3+}–nitrilotriacetate was added to a solution of ovotransferrin in $0.01M$-Tris–$0.01M$-NaHCO$_3$ at pH8 with 11M-HCl. The solution was incubated at 4°C for 12h and then desalted by passage through Sephadex G-50 (coarse grade) equilibrated with the buffer required in the subsequent experiments.

Metal-ion solutions

The 1:1 complex of Fe^{3+} and nitrilotriacetic acid was prepared as described by Woodworth (1966). The 1:1 complex of Cu^{2+} and nitrilotriacetic acid was prepared in an analogous manner. The complex of Fe^{3+} with citrate with $[Fe^{3+}]/[citrate] = 0.1$ at pH8 was prepared by the method of Bates et al. (1967a).

Recombination of metal-free ovotransferrin with metal ions

The conclusions in this paper stem from a knowledge of the protein fluorescence of solutions of known degree of saturation with metal ion. The degree of saturation of the metal-binding sites was equated to $\Delta E/\Delta E_{max.}$ at a given wavelength where ΔE is the change in absorbance on addition of metal ion and $\Delta E_{max.}$ is the maximum change observed in the presence of excess of metal ion. Fe^{3+} was monitored at 330nm or more usually 470nm. Cu^{2+} binding was monitored at 330 or 440nm (Lehrer, 1969). Binding of other metal ions was monitored at 245nm (Tan & Woodworth, 1969). When sufficient time was allowed for equilibrium to be reached ΔE increased linearly with metal ion added up to at least 95% saturation. The absorbance measurements were made either with a Hilger–Gilford spectrophotometer or with a split-beam spectrophotometer (Stinson & Holbrook, 1973). Protein fluorescence at about 335nm was measured through a Kodak–Wratten 18A filter from solutions in 1cm^2 cuvettes excited at right angles with radiation at 295nm from a 250W xenon arc at 21°. The fluorimeter described by Stinson & Holbrook (1973) was used. Fluorescence is expressed as the fraction of the fluorescence of apo-ovotransferrin and was corrected for dilution and for inner-filter effects at the

exciting wavelength by comparing the protein fluor-escence with the fluorescence of a $1\mu M$ solution of tryptophan to which an identical concentration of metal ion was added. No change in the fluorescence emission spectrum of tryptophan nor in its absorption spectrum was detected in the presence of the concen-trations of metal ions used in these experiments. In some experiments the metal ion and protein solutions were incubated together for a long time before measurements of absorption and fluorescence were made. In other experiments the metal ion was con-tinuously titrated into the stirred contents of a cuvette at rates between 0.5 and $100\mu l\cdot min^{-1}$.

Results

The spectra of the Fe^{3+} and Cu^{2+} complexes of ovo-transferrin were measured and showed the character-istic pink and yellow colours associated respectively with visible absorption bands at 470 and 440nm. Both showed a strong shoulder of absorption at 330nm (Lehrer, 1969) suitable to overlap the tryptophan fluorescence of ovotransferrin. Taking $E_{470}^{1\%} = 0.62$ for Fe^{3+}-transferrin (Warner & Weber, 1953) then $E_{280}^{1\%} = 14$. Similarly taking $E_{440}^{1\%} = 0.57$ for Cu^{2+}-transferrin (Fraenkel-Conrat & Feeney, 1950) gives $E_{280}^{1\%} = 12$.

Titrations of ovotransferrin with copper

Titrations of solutions of ovotransferrin that had been incubated for 12h at pH7.9 with $CuSO_4$ are shown in Fig. 1. The linear increase in absorbance at
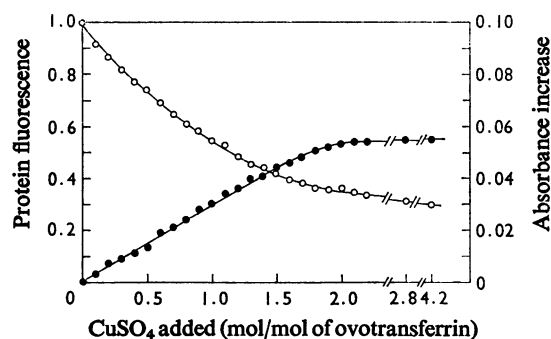
440nm is accompanied by a non-linear decrease in the protein fluorescence. Continuous titrations of transferrin with either $CuSO_4$ or Cu^{2+}-nitrilotri-acetate showed that the binding of the hydrated metal was much more rapid than that of the chelate. The final protein fluorescence was approx. 0.32 irrespec-tive of the form in which the copper had been added.

Titrations of ovotransferrin with iron

Aisen *et al.* (1973) claimed that the two iron-binding sites of ovotransferrin have unequal extinction co-efficients at 330nm but are indistinguishable at 470nm. Fig. 2 shows titration at both of these wave-lengths. Both in this experiment and in continuous-titration experiments the extinction at both wave-lengths increased linearly to the end-point. Although it is not contested that both sites are spectrophoto-metrically equivalent at 470nm, we were also unable to detect any large difference between them at 330nm. The protein fluorescence of solutions used for Fig. 2 was also measured, and as shown in Fig. 3 this de-creased non-linearly as the two sites were occupied. The precision of the measurements made on the large number of different solutions used to construct Fig. 3 is not high. More reproduceable results were obtained when the ovotransferrin solution was maintained in a cuvette and the Fe^{3+} solution was added from a syringe in portions of about 7% of the sites. After each addition the fluorescence was recorded until no further change occurred. The biphasic decrease in fluorescence is clear from the results in Fig. 4. Identical results were obtained in an experiment when the bicarbonate was omitted. In both series of experi-ments the time required to achieve a stable signal was fast [within the mixing time (3s) of the apparatus] during the addition of the first equivalent of iron, but
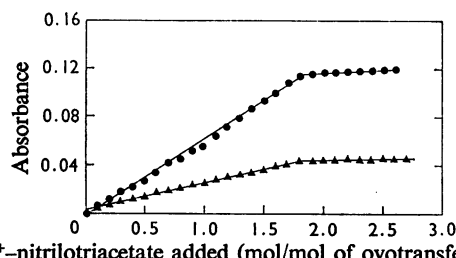

Fig. 1. *Titration of apo-ovotransferrin with copper*

$CuSO_4$ (1.97mM) was mixed with 3ml samples of apo-ovo-transferrin (26.3μM in metal-binding sites) in 0.01 M-Tris adjusted to pH8 with 11M-HCl and incubated at 4°C for 8h. Measurements were then made of the increase in absorbance at 440nm (●) and the protein fluorescence (○) relative to the unliganded protein (excited at 295nm, emis-sion via Kodak–Wratten filter 18A with a maximum at about 335nm).

Fig. 2. *Comparison of the extinction coefficients of Fe^{3+}–transferrin at 330 and 470nm*

Portions of Fe^{3+}-nitrilotriacetate were mixed with samples of apo-ovotransferrin (0.95μM in metal-binding sites) in 0.01 M-Tris–0.01 M-NaHCO$_3$ adjusted to pH8 with 11M-HCl. After incubation for 12h at 4°C measurements were made of the increase in absorbance at 330 (●) and 470nm (▲).
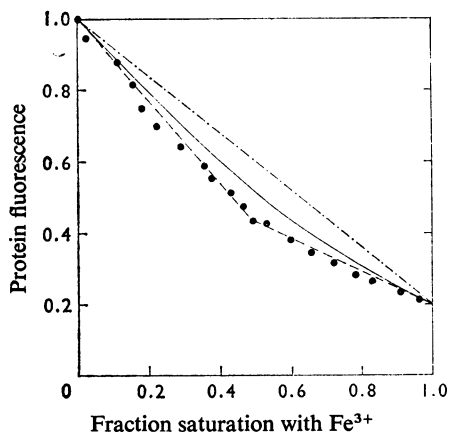
Fig. 3. *Variation in the protein fluorescence of Fe^{3+}–ovo-transferrin with increasing occupancy of the iron-binding sites*

The procedure was as described in the legend to Fig. 2 except that the residual protein fluorescence of each sample was measured. The fractional saturation (α) was equated to the absorbance increase divided by the absorbance increase at the break-point of the titration. Protein fluorescence (F) is expressed relative to the protein fluorescence of the apo-ovotransferrin. ---- are the two straight lines expected for sequential binding. —— is the curve $F = [1 - \alpha(1 - x)]^2$ for random binding with $x = 0.1936^{1/2}$ and –·–·–· is the single straight line expected for both sites being occupied together. ●, Experimental points.
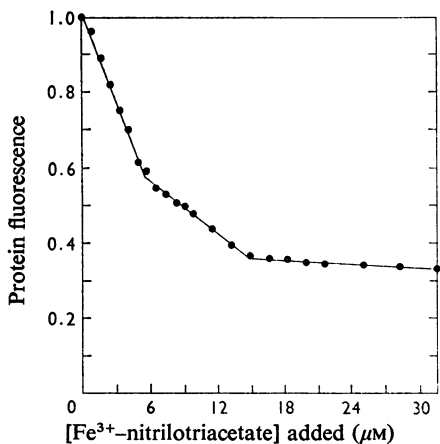

Fig. 4. *Protein fluorescence during the titration of a single sample of apo-ovotransferrin with Fe^{3+}–nitrilotriacetate*

A sample of apo-ovotransferrin (6.8 μM in metal-binding sites) was placed in a fluorimeter and slowly stirred in a buffer of 0.01 M-Tris–0.01 M-NaHCO₃ adjusted to pH 8 with 11 M-HCl. Portions of 5 mM-Fe³⁺–nitrilotriacetate were added from a micrometer syringe to give the final concentrations shown above. The protein fluorescence was recorded after each addition until no further change was observed.
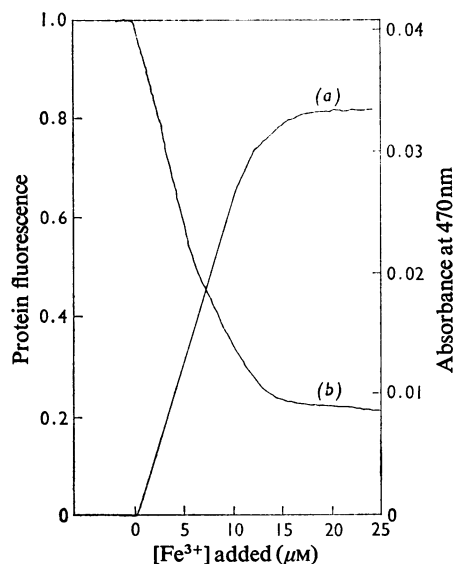

Fig. 5. *Continuous titration of apo-ovotransferrin with Fe^{3+}*

A solution (3 ml) of apo-ovotransferrin (12.5 μM in binding sites) in the same buffer used for Fig. 4 was slowly stirred in a spectrophotometer/spectrofluorimeter and a solution of 6 mM-Fe³⁺–nitrilotriacetate was slowly added at a rate of 5 μl·min⁻¹. In one experiment (curve a) the increase in absorbance at 470 nm was continuously recorded. In a second experiment a few minutes later the decrease in protein fluorescence was recorded (curve b). The two traces are superimposed.

much slower (half-time about 20 s) during the addition of the second equivalent of iron.

The biphasic decrease in protein fluorescence (F) was also observed during continuous-titration experiments in which the rate of addition of iron was varied 200-fold. An experiment in which iron was added at 10 μM·min⁻¹ is shown as Fig. 5. In this experiment and in seven others at rates of addition between 1 and 200 μM·min⁻¹ there was a linear decrease in fluorescence to a value of 0.53 followed by a non-linear decrease to an end-point at $F = 0.22$. The amount of iron required for the initial decrease to $F = 0.53$ was independent of the rate of addition and was equivalent to one-half the total iron-binding sites (as judged from the spectrophotometric titration). The binding of iron during the second phase was slow and only at the slowest rates of addition did the recombination process approach stoicheiometric binding. If the continuous addition was stopped when $F = 0.7$ the fluorescence only decreased by a further 1 % over 45 s. If the addition was stopped when $F = 0.55$ a further decrease to $F = 0.51$ took place over the next 85 s. Several unsuccessful attempts were made to monitor the binding of ferric citrate to apo-ovotransferrin.

Binding was very slow and conditions for stoicheiometric binding were not found, presumably because of the complex, polymeric nature of ferric citrate solutions at high pH values (Bates *et al.*, 1967*a,b*, 1973; Spiro *et al.*, 1967*a,b*).

Metal-displacement studies

Only metal ions that form complexes with ovotransferrin with a large absorption spectrum between 300 and 370 nm should give major decreases in protein fluorescence owing to radiationless transfer of electronic excitation energy. We confirmed the results of Tan & Woodworth (1969) and Lehrer (1969) that Cd^{2+}, Mn^{2+} and Zn^{2+} form complexes with ovotransferrin with a difference spectrum at 245 nm but with no absorption spectrum above 300 nm. The protein fluorescence of these complexes was, at 0.5 mg and 0.1 mg of protein/ml, 0.82 and 0.87 for Mn^{2+}, 0.81 and 0.92 for Cd^{2+} and 0.82 and 0.98 for Zn^{2+} (uncorrected values). The decrease in fluorescence was not dependent on the wavelength of the exciting radiation in the range 275–295 nm even though there is a difference between the spectrum of the apoprotein and the complex in this region (Tan & Woodworth, 1969). The very small changes in protein fluorescence when transferrin forms complexes with no colour above 300 nm is consistent with the suggestion that the large decreases with Fe^{3+} and Cu^{2+} are a result of energy transfer to oscillators with wavelengths 300–370 nm. The small decreases in protein fluorescence when colourless (above 300 nm) complexes form have not been fully investigated. The protein concentration-dependence might indicate trivial reabsorption although the spectra would not support this suggestion. There are known to be small conformational changes when ions bind to transferrin (Fuller & Briggs, 1956; Williams *et al.*, 1970) and with a protein containing 18 tryptophans this could easily give an average decrease of 10% in the protein fluorescence. Equally a change of 6 tyrosines to 6 tyrosinates due to the metal binding might be sufficient to give changed energy transfer from tryptophan at about 300 nm. Whatever the mechanism, such small changes, even if due to energy transfer, will be approximately linear with the fraction of the binding sites occupied (Holbrook, 1972), and the formation of hybrids may be detected.

In 0.01 M-Tris adjusted to pH 8 with 11 M-HCl the fluorescence of 12.8 μM (in binding sites) transferrin was decreased to 0.82 by addition of 2 equivalents of $ZnSO_4$. Addition of 2 equivalents of $CuSO_4$ decreased the fluorescence of another sample of transferrin to 0.36. However, if excess of $CuSO_4$ (60 μM) was added to a solution of the transferrin that had previously been saturated with $ZnSO_4$, the fluorescence only decreased from 0.82 to 0.49. Further additions of $CuSO_4$ up to a final concentration of 310 μM caused no

further change. However, addition of 128 μM-Fe^{3+}–nitrilotriacetate to either the Zn^{2+}–transferrin, the Cu^{2+}–transferrin or the mixed hybrid caused a drop in the fluorescence to $F = 0.2$ characteristic of the two-iron ferric–transferrin.

Discussion

It has been emphasized by Holbrook (1972) that protein-fluorescence changes caused by resonance transfer over long distances can be used to distinguish classes of protein molecules bearing different numbers of ligands at otherwise indistinguishable and identical sites. In his primary study of the protein fluorescence of transferrins Lehrer (1969) calculated that energy transfer occurred over critical distances of 2.1 and 1.8 nm for the Fe^{3+} and Cu^{2+} complexes. The considerations noted by Holbrook (1972) predict that any decrease in protein fluorescence due to tryptophan–ligand energy transfer will be non-linear with the increase in occupancy of the ligand-binding sites of a polydentate protein. Such non-linear decrease had been observed by Lehrer (1969) for Fe^{3+} but not for Cu^{2+}. The result for Cu^{2+} will be discussed below. Such non-linearity can provide information as to whether the two iron-binding sites of the protein are occupied together ($K_2 \gg K_1$), sequentially ($K_1 \gg K_2$), or statistically ($K_1 = 4K_2$), and the different patterns of protein fluorescence changes expected are noted in the introduction. Such a distinction is usually impossible from measurements of absorption-spectra changes since they usually only respond to the local environment of the bound ligand. The changes in the absorption spectra of transferrin on binding metal ions are linearly related to the concentration of bound ion.

Although Lehrer (1969) observed there was a non-linear decrease in protein fluorescence on binding of Fe^{3+} the more precise results in the present paper (Fig. 3 and especially Fig. 4) show that the decrease takes place in two linear portions. As noted in the introduction this is only found if the two iron-binding sites are occupied sequentially. The differences between the sites are also seen in that the first site binds iron more rapidly than the second. Although the pattern of protein-fluorescence change leaves no doubt that the two sites are occupied sequentially it is more difficult to quantify the association constants of the two sites. By comparison with model curves it is estimated that K_1/K_2 must be greater than 30, although even small experimental errors would mask K_1/K_2 ratios that were much greater than 100.

The sequential binding we observed above is at first sight at variance with the random binding that was suggested by the electrophoretic separations of Aisen *et al.* (1966) and Williams *et al.* (1970). However, careful examination of the distributions of the

three forms of protein in partially liganded transferrins shown in these works shows that the distributions are indeed characteristic of sequential binding as we show above. Of course the two ligand-binding sites of a single polypeptide chain must be intrinsically non-equivalent if observation is extended over the whole protein. However, judging by the identity of the electronic spectra of the two bound ligands, the local environment of both iron ions must be very similar. Aisen *et al.* (1973) have claimed that there was a slight difference in the extinction coefficients at 330nm but we were unable to confirm this. The protein fluorescences of transferrins with 0, 1 and 2 iron ions are as 1:0.47:0.22. The different fluorescence increments (0.53 and 0.25) on occupation of each site do not need to arise from different distances, or orientations of the ligands to the tryptophan residues of the protein. The protein fluorescence values are very close to the series $1:x:x^2$ as predicted by Holbrook (1972) for identical sites. The different increments then arise simply because of long-distance energy transfer from locally identical sites.

The sequential binding of iron at pH7.4–8 must be contrasted with the random distributions of iron observed by Wenn & Williams (1968) in isoelectric focusing experiments at pH5–7. A direct comparison is difficult because, with the possibility of three changed ionizations per ion bound, the affinity of the protein for iron will show a high power-dependence on pH value. One of us (R. W. Evans, unpublished experiments) has observed a slow decrease in the extinction at 470nm when Fe^{3+}–transferrin was incubated at pH6 in phosphate buffer such that only one-third of the extinction remained after 4h. Thus isoelectric-focusing experiments in the acid pH range may be difficult to interpret simply.

Under aerobic conditions we confirmed the observation by Bates *et al.* (1973) that Fe^{2+} binds to apotransferrin to give a product with the spectral and protein fluorescence characteristics of Fe^{3+}–transferrin. In particular the binding of both iron ions was rapid and the protein fluorescence in one preliminary experiment decreased linearly with the iron added. This would indicate a positive interaction between the two sites and merits further study. We did not determine whether the agent which oxidizes Fe^{2+} to Fe^{3+} on the protein was O_2, O_2 through the agency of a protein side-chain, or a protein side-chain.

Copper(II) either as $CuSO_4$ or as a complex with nitrilotriacetate reacts with ovotransferrin to give a complex with a decreased protein fluorescence. Although the rate of reaction was faster with Cu^{2+}_{aq}. neither the absorption spectrum nor protein fluorescence of the product depended on the form in which the copper was added. In contrast with Lehrer (1969) we find (Fig. 6) that the decrease in protein fluorescence is non-linear. In comparison with Fe^{3+}, the non-linearity is much less and could easily be over
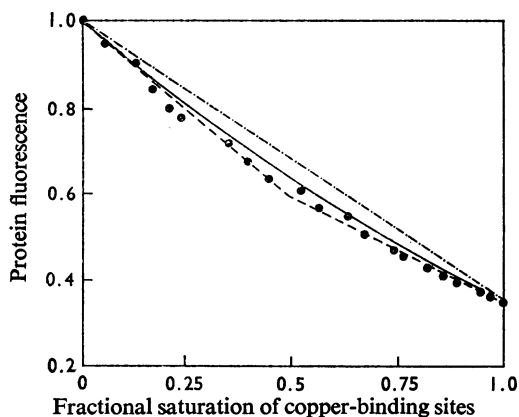

Fig. 6. *Variation in the protein fluorescence of transferrin with increasing occupancy of the copper-binding sites*

Results are from a stepwise titration similar to that in Fig. 1. Symbols have the same meaning as defined in the legend to Fig. 3. ●, Experimental points; —·—·—·—, line calculated for both sites occupied together; ————, two straight lines calculated for sequential occupancy; ———, line calculated from random binding, taking $x = 0.36^{1/2}$.

looked and this might account for the discrepancy with the earlier work. However, as explained by Holbrook (1972), the non-linearity will become more difficult to detect experimentally the less the overall decrease in fluorescence. The higher protein fluorescence of Cu_2^{2+}–transferrin compared with Fe_2^{3+}–transferrin is fully consistent with the lower extinction coefficient of the copper complex in the region 300–370nm when compared with the ferric complex. Although the result in Fig. 6 is clearly non-linear it is not sufficiently precise to distinguish unequivocally between random and sequential occupancy of the two copper-binding sites, although it more closely resembles the two straight lines predicted for sequential binding than the curve calculated for geometric quenching and random binding.

Metal interchange reactions of the type:

$$A_2\text{–protein} + B = AB\text{–protein} + B = B_2\text{–protein}$$

can be detected by protein fluorescence measurements if there is a significant difference between the protein fluorescence in the A_2 and B_2 complexes. Zn_2^{2+}–transferrin has a high fluorescence (0.82) and Cu_2^{2+}–transferrin has a much lower fluorescence because of the possibility of energy transfer (0.36). As noted above, addition of Cu^{2+} to Zn_2^{2+}–transferrin did not decrease the protein fluorescence to the value of 0.36 characteristic of Cu_2^{2+}–transferrin but gave a complex with protein fluorescence 0.49 which was stable over a wide range of added excess of copper. The predicted fluorescence of Cu^{2+}–Zn^{2+}–transferrin

is $(1.0-0.09) \cdot (0.36)^{1/2} = 0.54$ in reasonable agreement with the value observed. The formation of this hybrid is unexpected. Tan & Woodworth (1969) have determined the relative stability of the metallotransferrins to be: $Fe^{3+} > Cr^{3+}$, $Cu^{2+} > Mn^{2+}$, Co^{2+}, $Cd^{2+} > Zn^{2+} > Ni^{2+}$ and on this basis excess of copper should displace both zinc atoms. Although the precise molecular mechanism of this interesting effect remains obscure it is yet one more demonstration of the difference between the two metal-ion-binding sites of ovotransferrin.

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