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Nickel(II) transport in human blood serum

Studies of nickel(II) binding to human albumin and to native-sequence peptide, and ternary-complex formation with L-histidine

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Detailed studies are reported on the Ni(II)-binding site of human serum albumin (HSA) and the results are compared with those obtained from the *N*-terminal native-sequence peptide, L-aspartyl-L-alanyl-L-histidine *N*-methylamide (Asp-Ala-His-NHMe). Equilibrium dialysis of HSA and Ni(II) in 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.53, demonstrates a specific Ni(II)-binding site on the protein. L-Histidine, the low-molecular-weight Ni(II)-binding constituent of human serum, is shown to have a greater affinity for Ni(II) than does HSA. A small but significant amount of ternary complex HSA–Ni(II)–L-histidine is also present in the equilibrium mixture containing the three components. The log(association constant) values for the binary and ternary Ni(II) complexes are 9.57 and 16.23 respectively. The complex equilibria between Asp-Ala-His-NHMe and Ni(II) have been investigated by analytical potentiometry in aqueous solution (0.15 M-NaCl, 25°C). Several species, including MA, MA_2, $MH_{-2}A$, and $MH_{-1}A_2$ [where M and A represent Ni(II) ion and anionic peptide respectively], were detected in the system, $MH_{-2}A$ being the major complex species. Equilibrium studies involving Asp-Ala-His-NHMe, Ni(II) and L-histidine reveal the presence of a ternary complex $MH_{-1}AB$ (where B represents anionic L-histidine) at physiological pH. Detailed studies of visible-absorption spectra of HSA in the presence of Cu(II) and Ni(II) reveal that the two metal ions bind HSA at the same site. The visible-absorption spectrum of Ni(II)–HSA complex shows a highly absorbing peak at 420nm ($\varepsilon_{max.} = 137$; with shoulder at 450–480nm) characteristic of a square planar or square pyramidal co-ordination arrangement about the metal ion. Similar visible-absorption characteristics were observed for the major species $MH_{-2}A$ in the Asp-Ala-His-NHMe–Ni(II) system ($\lambda_{max.} = 420$nm; $\varepsilon_{max.} = 135$; with shoulder at 450–480nm). The combination of experimental results from the protein studies and the peptide analyses provides strong evidence for the structure of the Ni(II)-binding site of HSA as one that involves the α-amino nitrogen atom, two deprotonated peptide nitrogen atoms, the imidazole nitrogen atom and the side-chain carboxy group of the aspartic acid residue. On the basis of the results obtained from the individual ternary systems involving protein and peptide, a mechanism for the transportation of Ni(II) in the serum is proposed.

At present, there exist two broad categories of investigation into the biological chemistry of Ni(II). It has not as yet been fully established that Ni(II) plays any specific physiological role. That such a physiological role may exist is indicated by such findings as Ni(II) homoeostasis in the blood, Ni(II)-metalloproteins, the pathological alterations in Ni(II) concentration in disease, and Ni(II)-deficiency symptoms provoked in animals by the dietary deprivation of the metal (Nomoto *et al.*, 1971; Sunderman *et al.*, 1972; Nielsen *et al.*, 1975). The second category of research centres on the more pressing environmental nickel health threat and

Abbreviations used: BSA, bovine serum albumin; HSA, human serum albumin; DSA, dog serum albumin; Asp-Ala-His-NHMe, L-aspartyl-L-alanyl-L-histidine *N*-methylamide.

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carcinogenesis (McNeely *et al.*, 1972). The prevalence of cancers of the respiratory tract among workers in nickel refineries is much greater than among the general population (Sunderman, 1977).

The main Ni(II)-binding constituents of human serum have been shown to be the protein albumin and amino acids, with L-histidine exhibiting the greatest affinity by far for Ni(II) (Lucassen & Sarkar, 1979). Some work has been done on the binding of Ni(II) to BSA, specifically in the presence of Cu(II) (Martin, 1961). However, a detailed characterization of the Ni(II)-binding site is lacking. Nickel(II), like Cu(II), blocks the reaction of 1-fluoro-2,4-dinitrobenzene with the α-amino group at the *N*-terminus of BSA; it has also been shown in preliminary tests that increasing amounts of Ni(II) caused the characteristic purple colour of the 1:1 Cu(II)–BSA complex to fade and change to yellow (Peters & Blumenstock, 1967).

The present study was undertaken to investigate the Ni(II)-transport site of HSA in the hope of elucidating the exact position and nature of the Ni(II)-binding to this protein. The work deals with the physicochemical studies of the Ni(II) complexes of HSA and the *N*-terminal native-sequence peptide Asp-Ala-His-NHMe by equilibrium dialysis, potentiometry and spectrophotometry. Studies of the ternary systems HSA–Ni(II)–L-His and Asp-Ala-His-NHMe–Ni(II)–L-His were undertaken to reveal the state of equilibrium between the high-molecular-weight and low-molecular-weight Ni(II)-binding constituents in human serum, especially relevant to the mechanism of transportation of the metal in the blood.

Experimental

Materials

HSA (fraction V powder) was obtained as a gift from Connaught Laboratories. Metal-free monomeric protein samples were prepared as previously reported (Sarkar & Wigfield, 1968). The molecular weight for the protein was taken to be 69000. L-Aspartyl-L-alanyl-L-histidine *N*-methylamide (monohydrochloride) was synthesized by the active ester method as previously reported from this laboratory (Iyer *et al.*, 1978) and its purity checked by t.l.c. L-Histidine free base ($[\alpha]_D^{20} = +39.9 \pm 0.5°$) was purchased from ICN Pharmaceuticals. $^{63}NiCl_2$ (specific radioactivity 1–10mCi/mg of Ni) was obtained from the Amersham Corporation, and L-[^{14}C]histidine (specific radioactivity 1.2mCi/mg) from Schwarz Bioresearch. Stock solutions were prepared from distilled and deionized water and stored over pre-purified argon. All glassware used was metal-free. Pre-purified argon was used to maintain an oxygen-free atmosphere throughout the titrations. The base used was carbonate-free 98.3mM-NaOH and standardized against weighed amounts of dried potassium hydrogen phthalate. This base was used to standardize a 0.1M-HCl solution, prepared from a commercial standard acid solution (1.005–0.995M; Fisher Scientific Co.). AnalaR $NiCl_2,6H_2O$ was used to prepare the stock 0.1097M-$NiCl_2$ solution, and a direct titration for the determination of the Ni(II) content was performed by complexometric titration with murexide as indicator.

Potentiometric titrations

Titrations were performed in a Radiometer automatic titration apparatus consisting of a digital PHM64 pH-meter, autoburette ABU13, titrator TTT60 and an automatic recorder REC61 Servograph. The 50ml titration vessel was thermostatically controlled at 25 ± 0.05°C. The electrode pair consisted of a Radiometer G2040C glass electrode and a K4040 reference electrode. Three potentiometric studies were conducted: the two binary systems, Ni(II)–L-His and Ni(II)–Asp-Ala-His-NHMe, and a ternary system, Ni(II)–Asp-Ala-His-NHMe–L-His. The proton–ligand systems were not analysed, as the pK_a values were readily available in the literature (Kruck & Sarkar, 1973; Iyer *et al.*, 1978).

In the metal-concentration-variation titrations for the binary systems, the nickel concentration, C_M, varied from 0.549×10^{-4}M to 1.646×10^{-4}M, in steps of 0.549×10^{-4}M, with C_A (the Asp-Ala-His-NHMe concentration) at 4.474×10^{-4}M and C_B (the L-histidine concentration) at 4.812×10^{-4}M. For the ligand-concentration-variation analysis, $C_A = 4.474 \times 10^{-4}$M, 5.368×10^{-4}M and 7.158×10^{-4}M and $C_B = 3.849 \times 10^{-4}$M, 4.812×10^{-4}M and 7.699×10^{-4}M with C_M constant at 1.097×10^{-4}M.

For the ternary system, the metal-concentration variations were $C_M = 0.439 \times 10^{-4}$M, 1.097×10^{-4}M and 1.755×10^{-4}M at constant $C_A = 8.973 \times 10^{-4}$M and $C_B = 3.843 \times 10^{-4}$M. Ligand-A-concentration-variation samples were 7.178×10^{-4}M, 8.973×10^{-4}M and 10.768×10^{-4}M with $C_B = 3.843 \times 10^{-4}$M and $C_M = 1.097 \times 10^{-4}$M. Ligand-B-concentration-variation samples were 2.883×10^{-4}M, 3.843×10^{-4}M and 5.765×10^{-4}M with $C_A = 8.973 \times 10^{-4}$M and $C_M = 1.097 \times 10^{-4}$M.

High ligand/metal ratios were used to ensure stable titration systems. In the ternary system, at least a 1:2 ratio was maintained for Ni(II) relative to L-histidine; to titrate samples with metal/ligand ratio below the 1:2 results in a strong ratio effect. The ratio effect stems from the stronger and faster binding of the amino acid when compared with the native-sequence tripeptide. Higher concentrations of the peptide relative to L-histidine were used to increase the competitiveness of the native-sequence tripeptide in the reaction equilibria and to follow more

closely the physiological conditions. All the solutions contained known concentrations of HCl to lower the starting pH below that of metal binding. An equilibration period of 5min was allowed at every 0.2 pH reading in the pH range 4.2–5.6 in the Ni(II)–L-histidine titrations. A much slower reaction was observed in the titrations for the binary Ni(II)–Asp-Ala-His-NHMe system in the pH range 5.2–6.8 where, in some cases, more than 1h was needed at the 0.2 pH readings for a steady pH.

Spectrophotometry

Absorbance measurements over the range 340–800nm were made in 1cm cells at 25°C with a Beckman ACTA MVI spectrophotometer. Similar metal/ligand ratios were used to those in the titration studies. All solutions for the spectral measurements were prepared in 0.15M-NaCl. For the Ni(II)–Asp-Ala-His-NHMe solutions, considerable equilibration time was allowed during the 'slow' pH range. Where manual calculation of individual spectra was not possible, the spectral data for various pH values were processed by the method described previously (Kruck & Sarkar, 1975). Considerable equilibration time was allowed for the slower reaction of Ni(II) with HSA both in the presence and in the absence of Cu(II). For the very slow reactions, samples were kept at 6°C between scans to avoid protein denaturation resulting from prolonged sitting at room temperature.

Determination of stability constants

The equilibrium reactions occurring between metal ion M, proton H, anionic peptide A and L-histidine B can be represented by general reaction equation:

$$pM + qH + rA + sB \rightleftharpoons M_pH_qA_rB_s \qquad (1)$$

where p, q, r and s are the stoicheiometric quantities of M, H, A and B respectively. The stabilities of the species formed are represented by the stoicheiometric equilibrium constant β_{pqrs} expressed in terms of concentrations at constant ionic strength, temperature, and pressure:

$$\beta_{pqrs} = \frac{[M_pH_qA_rB_s]}{m^p h^q a^r b^s} \qquad (2)$$

where m, h, a and b are the concentrations of free metal ion, hydrogen ion, anionic peptide and L-histidine respectively. The relations to obtain values for the unbound portions of metal ion and ligands A and B at any specified pH values have been described (Sarkar & Kruck, 1973; Sarkar, 1977). The experimental results and titration curves [$-\log h$ = f(base)] were obtained from the solutions containing different concentrations of C_M, C_H, C_A and C_B as outlined above. This set of data was fed to the program PLOT-3 to give values for the unbound portions of metal and the proton-liberation term $\delta H^+/\delta C_M$ together with $\delta H^+/\delta C_A$, and $\delta H^+/\delta C_B$, and the free ligand concentrations. By using the data from PLOT-3, the program GUESS-3 set up a matrix of the terms $m^p h^q a^r b^s$ for each proposed species at each selected pH value. This matrix then served as the input to the program LEASK-4, which uses an iterative least-squares minimization procedure to calculate the stability constants, β_{pqrs}. All calculations were performed by a sequential use of these three programs on a GE-400 computer. A detailed account of the data processing has been previously reported (Sarkar & Kruck, 1973; Sarkar, 1977).

Equilibrium dialysis

Nickel(II)-binding to HSA was determined by an equilibrium-dialysis procedure (Lau & Sarkar, 1971; Lau *et al.*, 1974). The dialysis buffer was 0.1M-*N*-ethylmorpholine/HCl buffer, pH7.53 and I0.16. Each half-unit of the dialysis cells contained a total of 4ml of buffer with one half-unit containing a protein concentration of 0.1mM, in which the Ni(II)/HSA ratio was increased from 0.1 to 3.0. After 6 days of dialysis with gentle shaking at 6°C, equal samples from each pair of compartments were counted for ^{63}Ni(II) radioactivity in a liquid-scintillation counter (Nuclear–Chicago mark 1) (Appleton & Sarkar, 1971). In the second series of experiments, the total HSA and Ni(II) concentrations were kept constant in one half-unit at 0.10mM and 0.098mM respectively, whereas in the other the concentration of L-[^{14}C]histidine was varied. Double-isotope analyses of ^{63}Ni(II) and ^{14}C were performed with a pre-programmed Beckman LS 8100 liquid-scintillation counter. Radioactivity was measured both before and after dialysis.

Results

Equilibrium dialysis

Equilibrium dialysis of purified HSA against Ni(II) at pH7.53 reveals the presence of a first specific binding site on HSA. This is clearly shown in Fig. 1, where moles of unbound Ni(II) per mole of HSA are plotted against an increasing value of the Ni(II)/HSA ratio. The second binding site on HSA displays a lower affinity for Ni(II).

The results of the equilibrium-dialysis study of the ternary system HSA–Ni(II)–L-His are shown in Table 1. The calculations were made from the ^{63}Ni and L-[^{14}C]histidine double-labelling radioactivity counts before and after dialysis (Lau & Sarkar, 1971). The results show that even low concentrations of the amino acid decrease the concentration of protein-bound Ni(II) considerably owing to the avidity of L-histidine for Ni(II). There is a

Table 1. *Calculated equilibrium-dialysis data of the ternary system HSA–Ni(II)–L-His*

The equilibrium dialysis was performed in 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.5 and *I* 0.16, at 6°C. Total HSA and Ni(II) concentrations were constant at 0.10 mM and 0.098 mM respectively, whereas the total concentration of L-histidine $[L\text{-His}]_t$ was varied. For full details see the text.

$[L\text{-His}]_t$ (μM)	[Ni(II)] (nM)	[Ni(II)–(L-His)$_2$] (μM)	[L-His] (μM)	[Ni(II)–HSA] (μM)	[HSA] (μM)	[HSA–Ni(II)–L-His] (μM)	log K_a Ni(II)–HSA	log K_a HSA–Ni(II)–L-His
10	12.96	3.5	1.4	91.8	7.4	1.58	9.62	16.09
20	2.15	8.0	1.9	86.4	11.5	2.00	9.54	16.31
40	1.76	16.8	3.2	78.5	18.2	3.24	9.38	16.15
60	1.19	25.4	4.7	67.8	27.6	4.54	9.32	16.10
80	0.18	30.6	13.4	60.9	33.7	5.44	10.01	16.51

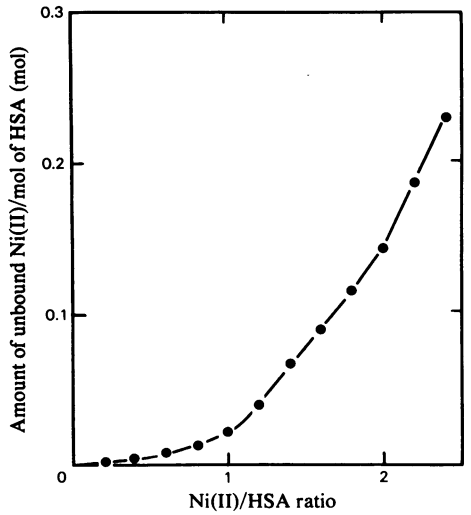

Fig. 1. *Equilibrium dialysis at pH 7.5 of HSA against increasing molar equivalents of Ni(II)*

The HSA concentration was 0.1 mM in 0.1 M-*N*-ethylmorpholine buffer containing 0.06 M-NaCl. For full details see the text.

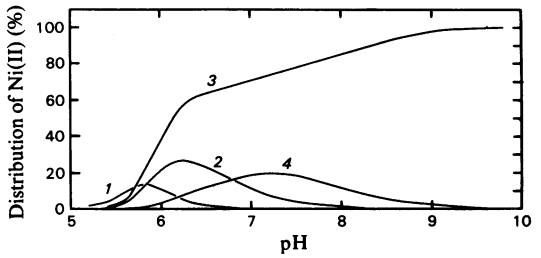

Fig. 2. *Species distribution for the Ni(II)–Asp-Ala-His-NHMe system as a function of pH*

$C_M = 1.097 \times 10^{-4}$ M, $C_A = 4.474 \times 10^{-4}$ M; curve *1*, MA ($\log\beta_{101} = 5.50$); curve *2*, MA_2 ($\log\beta_{102} = 11.56$); curve *3*, $MH_{-2}A$ ($\log\beta_{1-21} = -5.94$); curve *4*, $MH_{-1}A_2$ ($\log\beta_{1-12} = 4.75$). For full details see the text.

small but significant amount of a ternary complex formed. The average value of the log(association constant) for HSA–Ni(II)–L-His is 16.23 and that for Ni(II)–HSA is 9.57.

Complex species in Ni(II)–Asp-Ala-His-NHMe binary system

In order to confirm further the species and the nature of the Ni(II)-binding site of HSA, detailed studies were undertaken with the synthetic native-sequence tripeptide. The proton-liberation reaction begins at a pH slightly above 5 and a rapid increase in $\delta H^+/\delta C_M$ occurs to a maximum of almost 3.5 at pH 6.4. This rapid displacement is attributed to the formation of the major species $MH_{-2}A$ (Fig. 2). At pH 9.5–10.0, $\delta H^+/\delta C_M$ is steady at a value of 2, where the species $MH_{-2}A$ is present exclusively. Since in this pH range the β-carboxy, imidazole and amino groups are already titrated, the protons liberated can be attributed to complex-formation by the two peptide amide groups. From the species distribution, it can be seen that the initial complex is an MA species, which at its maximum at pH 5.8 represents 13% of the total Ni(II). By pH 5.8, this complex is overtaken by the species $MH_{-2}A$, which is the major complex species up to pH 9.5, where it is present exclusively. Other important complexes present are the MA_2 and the $MH_{-1}A_2$ complexes. The log(stability constant) values of the species are given in the legend to Fig. 2.

Complex species in Ni(II)–L-His and Ni(II)–Asp-Ala-His-NHMe–L-His systems

Because of the importance of L-histidine as the major low-molecular-weight Ni(II)-binding constituent in human serum, the complex equilibria between Ni(II) and the amino acid were examined for the purpose of investigating the ternary-complex equilibria. Although previously studied by other workers (Leberman & Rabin, 1959; Andrews &

Zebolsky, 1965; Perrin & Sharma, 1967; Sóvágó *et al.*, 1978), no clear mapping of the species as a function of pH at high ligand/metal ratios was available. Two major complexes, MB and MB_2, dominate the distribution, with $\log\beta_{101}$ and $\log\beta_{102}$ equal to 8.57 and 15.57 respectively (Fig. 3*a*). The species $MH_{-1}B_2$ ($\log\beta_{1-12} \simeq 4.8$) probably involves co-ordination by the deprotonated (anionic) imidazole moiety (Carlson & Brown, 1966).

The analysis of the ternary system with the native-sequence tripeptide shows the presence of a small but significant amount of a ternary complex $MH_{-1}AB$ at physiological pH (Fig. 3*b*). A very similar distribution to that of the binary Ni(II)–L-His system exists up to neutral pH, with the complexes MB and MB_2 predominating. At high pH, the species $MH_{-1}AB$ gave a better fit than an $MH_{-1}B_2$ or $MH_{-2}A$ species. However, it is evident that the Ni(II)–(L-His)$_2$ bis-complex is still a very stable species as the pH is increased. A comparison of log(stability constant) values for the ternary complex ($\log\beta_{1-111} = 4.84$) with the binary peptide complex $MH_{-1}A_2$ ($\log\beta_{1-12} = 4.75$) indicates an enhancement of stability with the ternary co-ordination complex. The higher avidity of L-histidine for Ni(II) than that of the peptide is shown by a comparison of log(stability constant) values, $\log\beta_{pq}$ for the mono- and bis-complexes: MB, 8.57; MA, 5.50; MB_2, 15.57; MA_2, 11.56. It should be noted that it is probable that small amounts of other complexes are also present in the species distribution (up to about 5%), for example, the species MHB and $MH_{-2}A$. Indeed, the distribution is very much ratio-dependent; analysis of the system at a 1:1:1 value of M:A:B would undoubtedly result in the formation of large amounts of the species $MH_{-2}A$.

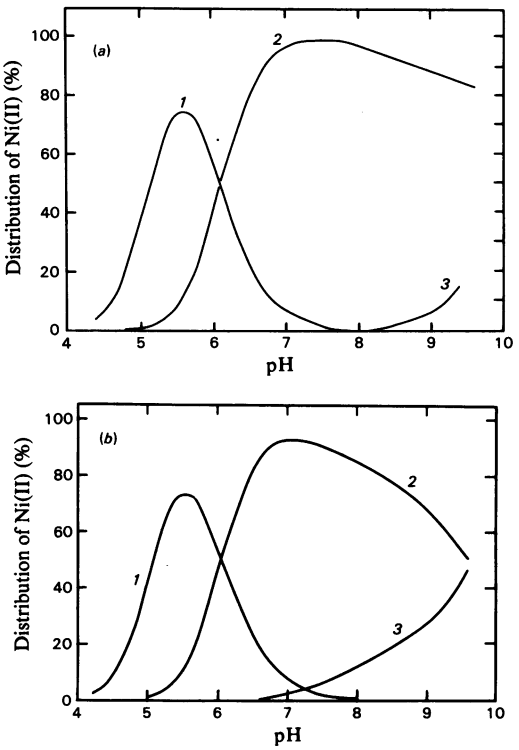

Fig. 3. *Species distributions for (a) the Ni(II)–L-His system and (b) the Ni(II)–Asp-Ala-His-NHMe–L-His ternary system*

(*a*) $C_M = 1.097 \times 10^{-4}$M; $C_B = 4.812 \times 10^{-4}$M; curve *1*, MB; curve *2*, MB_2; curve *3*, $MH_{-1}B_2$. (*b*) $C_M = 1.097 \times 10^{-4}$M; $C_A = 8.973 \times 10^{-4}$M; $C_B = 3.843 \times 10^{-4}$M; curve *1*, MB; curve *2*, MB_2; curve *3*, $MH_{-1}AB$. For full details see the text.

Absorption spectra of Ni(II) complexes of human serum albumin

Under physiological conditions, only the first specific Ni(II)-binding site of HSA is expected to be occupied owing to the low concentration of Ni(II) in human serum, relative to the concentration of HSA [HSA concentration of 0.6mM, normal nickel mean concentration 0.04μM (Callan & Sunderman, 1973)]. Analysis of the spectra as a function of pH, in the presence of 1 equiv. of Ni(II), shows some interesting results (Fig. 4*a*). The spectrum of hexaquo-Ni(II) with absorption maxima at 395 and at 650–720nm (broad doublet) changes rapidly with increasing pH to a highly absorbing peak at 420nm, indicative of a square planar or square pyramidal geometry about the metal ion (Lever, 1968). By pH6.9, the peak is well developed and a shoulder in the region of 450–480nm is evident. At higher pH values, the increases in absorption are less and a smaller peak develops at 340nm. No exact assignment of peaks is being made here. The same high-pH requirement for the full development of the yellow colour was previously observed in equimolar mixtures of BSA and Ni(II) (Martin, 1961).

Absorption spectra of Ni(II)–Asp-Ala-His-NHMe system

The visible-absorption spectra are shown in Fig. 4(*b*) from 340 to 800nm. At pH3.5, a spectrum of hexaquo-Ni(II) is observed. When the pH is increased to the starting pH approx. 5.1, the spectrum is shifted to a $\lambda_{max.}$ of 385nm and a broad peak at about 640nm. Some initial carboxylate interaction with Ni(II) before proton liberation may be taking place, yielding a complex of octahedral symmetry. By pH5.4, the peak maximum has shifted to 415nm and the solution is a mixture of octahedral Ni(II) and mainly the highly absorbing MA complex ($\lambda_{max.} = 428$nm, $\varepsilon_{max.} = 202$). [The largest error in the assigned $\lambda_{max.}$ values occurs for the species MA

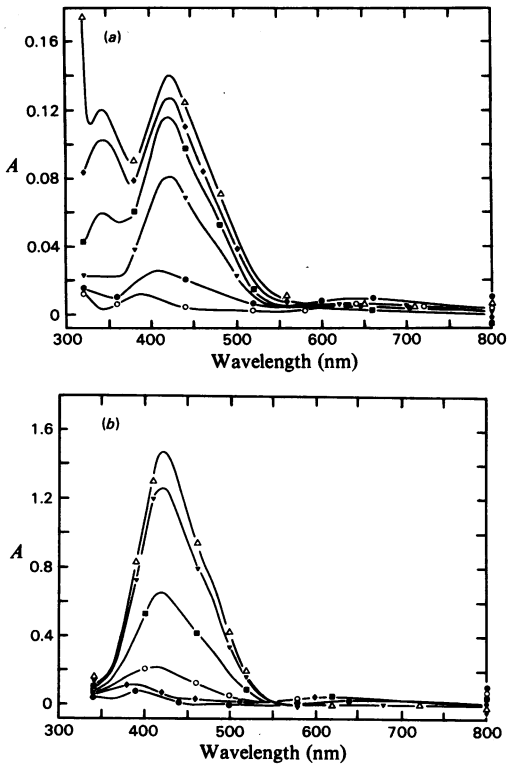

Fig. 4. *Visible-absorption spectra as a function of pH for (a) the Ni(II)–HSA system and (b) the Ni(II)–Asp-Ala-His-NHMe system*

(*a*) [Ni(II)–HSA] = 1.01 mM in 0.15 M-NaCl, 1 cm cell path, with protein as reference at 25°C. ○, pH 5.34; ●, pH 6.36; ▼, pH 6.94; ■, pH 8.19; ♦, pH 9.19; △, pH 10.14. (*b*) [Ni(II)] = 10.97 mM; [Asp-Ala-His-NHMe] = 50 mM in 0.15 M-NaCl at 25°C. ●, pH 3.49; ♦, pH 5.12; ○, pH 5.41; ■, pH 5.77; ▼, pH 6.27; △, pH 7.43–10.0.

and MA_2, owing to their lower concentration and the likely presence before 100% Ni(II) complex-formation of weakly absorbing protonated complexes that fail to be picked up by proton-displacement analyses.] With increasing pH, the absorption at 420 nm increases sharply, coinciding with the increase in the species $MH_{-2}A$ ($\lambda_{max.} = 420$, $\varepsilon_{max.} = 135$) as shown in the species distribution. A shoulder in the region 450–480 nm is present on the high-wavelength side of the 420 nm peak. At physiological pH, significant amounts of the complexes MA_2 ($\lambda_{max.} = 410$, $\varepsilon_{max.} = 47$) and $MH_{-1}A_2$ ($\lambda_{max.} = 420$, $\varepsilon_{max.} = 167$) are present.

Absorption spectra of Ni(II)–Asp-Ala-His-NHMe–L-His system

In the analysis of the visible-absorption spectra as a function of increasing pH, the colour changed

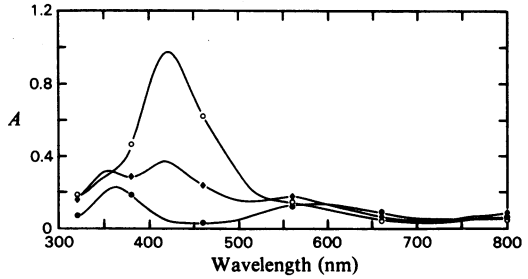

Fig. 5. *Visible spectra for the ternary system Ni(II)–Asp-Ala-His-NHMe–L-His as a function of pH*

[Ni(II)] = 21.94 mM; [Asp-Ala-His-NHMe] = 175.5 mM; [L-His] = 87.76 mM. ●, pH 4.83; ♦, pH 7.00; ○, pH 9.02.

from purple to yellow, indicating a change from octahedral to planar co-ordination about the Ni(II) ion (Fig. 5). At pH 4.8, the spectrum corresponds to a mixture of octahedral species, $Ni(H_2O)_6$ and MB. At pH 7.0, the peaks at 355 nm and 560 nm represent the predominant MB_2 complex while a highly absorbing peak at 420 nm is developing. Indeed, the ternary complex $MH_{-1}AB$ ($\lambda_{max.} = 420$ nm; $\varepsilon_{max.} = 159$; with shoulder at approx. 480 nm) because of its much greater molar absorption coefficient, dominates the spectrum at pH 9.0, despite representing only 27% of the complexed Ni(II). This complex has a similar absorption profile to the $MH_{-1}A_2$ complex ($\lambda_{max.} = 420$ nm; $\varepsilon_{max.} = 167$).

Absorption spectra of human serum albumin in the presence of Cu(II) and Ni(II)

The Cu(II)-transport site of the HSA involves co-ordination of the α-amino nitrogen atom of the *N*-terminal aspartic acid residue, two intervening peptide nitrogen atoms and the imidazole nitrogen atom of the histidine residue in the third position (Neumann & Sass-Kortsak, 1967; Peters & Blumenstock, 1967; Sarkar & Wigfield, 1968; Bradshaw & Peters, 1969; Iyer *et al.*, 1978). Furthermore, the carboxy group of the aspartic acid residue has been implicated in the co-ordination about the Cu(II) ion, yielding a penta-co-ordinate structure (Laussac & Sarkar, 1980*a*). To examine the position and the relative strength of binding of both Cu(II) and Ni(II) to HSA, the spectral characteristics of the mixtures Ni(II)–HSA with $CuCl_2$ and Cu(II)–HSA with $NiCl_2$ at pH 7.5 were examined at various time intervals (Figs. 6*a* and 6*b*). As shown in Fig. 6(*a*), the addition of 1 equiv. of Cu(II) to the yellow Ni(II)–HSA complex initially produces a broad peak at 680 nm, characteristic of the second binding site of Cu(II) on the protein. However, with time, the absorptions at 420 nm [Ni(II)–HSA complex] and

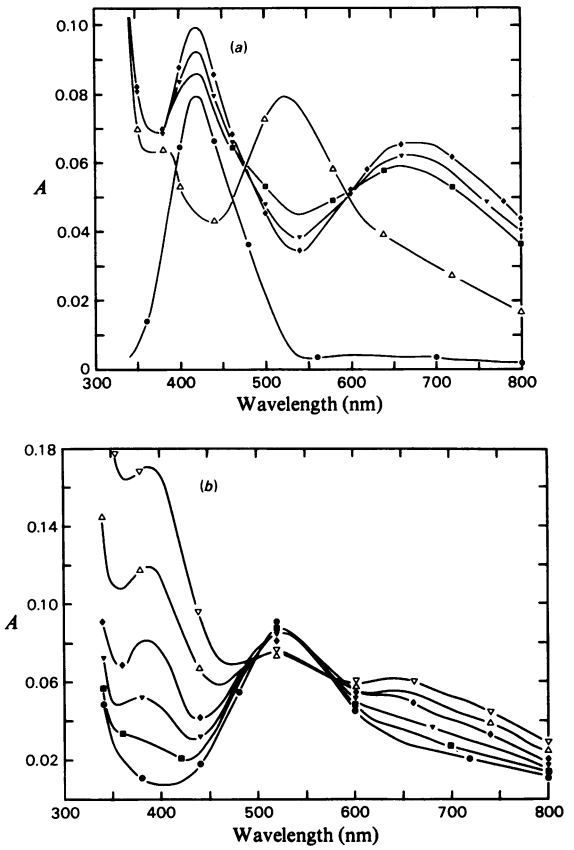

Fig. 6. *Visible-absorption spectra of the reaction mixtures (a) Ni(II)–HSA + $CuCl_2$ and (b) Cu(II)–HSA + $NiCl_2$ at pH 7.5 in 0.1 M-N-ethylmorpholine buffer containing 0.06 M-NaCl (25°C) as a function of time*
(*a*) [Ni(II)–HSA] = 1.0 mM. ●, Ni(II)–HSA; ♦, Ni(II)–HSA + 1 equiv. of Cu(II), initial spectrum; ▼, after 1 h; ■, after 2.5 h; △, final spectrum. (*b*) [Cu(II)–HSA] = 1.0 mM. Initial spectra; ●, Cu(II)–HSA; ■, Cu(II)–HSA + 1 equiv. of Ni(II); ▼, Cu(II)–HSA + 2 equiv. of Ni(II); ♦, Cu(II)–HSA + 4 equiv. of Ni(II). Cu(II)–HSA + 4 equiv. of Ni(II); △, after 24 h; ▽, after 96 h.

at 680 nm [Cu(II)–HSA second-site complex] decrease while the absorption at 525 nm increases. The Cu(II), as expected, exchanges with the Ni(II) ion. The final spectrum shows a distinct peak maximum at 525 nm, reflecting the specific binding of Cu(II) to HSA; the less intense peaks evident at 385 nm and in the region of 600–750 nm reflect octahedral co-ordination of the replaced Ni(II) by HSA, presumably at its second Ni(II)-binding site.

Similarly, the spectra of Cu(II)–HSA ($\lambda_{max.}$ = 525 nm) were recorded before and after the addition of Ni(II) (Fig. 6*b*). The initial spectra, recorded after the addition of 1, 2 and 4 equiv. of Ni(II), show the development of two extra peaks, one at 385 nm and the other in the range 600–750 nm. The Ni(II) appears to have entered an octahedral environment, while the Cu(II) ion remains at its *N*-terminal specific binding site. There is no development of a peak at 420 nm, characteristic of the first Ni(II)-binding site. With time, the peaks representing octahedral co-ordination by Ni(II) increased in intensity, probably reflecting the slow reaction of Ni(II) with its other binding site on HSA.

Discussion

One important result arising from the series of spectral studies is that both Cu(II) and Ni(II) have specific binding sites at the same position on HSA. Although HSA can bind both metal ions at the same time, only one can bind at its specific binding site at equimolar ratios. It has been suggested that Ni(II) would be expected to be less tightly bound than Cu(II) owing to its slightly greater size than the Cu(II) ion (Peters, 1970). The stronger binding of Cu(II) is shown not only by the observed replacement of Ni(II) by Cu(II) but also by a comparison of log(association constant) values for the two protein complexes: Cu(II)–HSA, 16.1; Ni(II)–HSA, 9.5. Indeed, in a previous dialysis study (Westerik & Sarkar, 1975), the specific Ni(II)-binding was shown to be absent in the presence of Cu(II).

The combination of experimental results from the protein studies and the native-sequence peptide analysis gives a clear picture of the specific Ni(II)-binding site of HSA. A comparison of the proton displacement with the percentages of species present at the pH of maximum proton liberation is informative; it is clear that the Ni(II) co-ordination in the $MH_{-2}A$ species involves both the α-amino nitrogen atom of the *N*-terminal aspartic acid residue and the imidazole nitrogen atom of the histidine residue, as well as the involvement of the two peptide nitrogen atoms. The lack of nickel-binding specificity of DSA reinforces the involvement of the third-position histidine residue, since DSA has a tyrosine residue in the same position (Dixon & Sarkar, 1974). [Nickel(II)-binding to DSA was performed at pH 7.5 with 0.1 M-*N*-ethylmorpholine/HCl buffer, *I* 0.16. The results show the lack of a first specific binding site on DSA. The spectral results as a function of pH concur with this contention (J. D. Glennon & B. Sarkar, unpublished work).] The slow reaction of Ni(II) with peptides, specifically on the ionization of amide hydrogen atoms, is a well-documented phenomenon (Martin, 1961). Similar properties were exhibited in the binding of Ni(II) to Asp-Ala-His-NHMe.

A comparison of the spectral characteristics of the Ni(II)–peptide complex $MH_{-2}A$, the predominant

complex at physiological pH, with those of Ni(II)–HSA strengthens the suggestion of a similar binding site in both cases. The same rapid increase in absorption at 420nm [Ni(II)–HSA $\varepsilon_{max.} = 138$; Ni(II)–Asp-Ala-His-NHMe $\varepsilon_{max.} = 135$] between pH 5 and 7 is seen in the Ni(II) spectra of both the native-sequence tripeptide and the protein, as is the shoulder in the region of 450–480nm on the 420nm peak. Furthermore, the stronger binding of Cu(II) is observed in the peptide system as was observed with HSA: $\log\beta_{1-21} = -0.55$ ($CuH_{-2}A$) and -5.94 ($NiH_{-2}A$). Recent ^{13}C- and ^{1}H-n.m.r.-spectroscopic studies in this laboratory have implicated the carboxy group of the aspartic acid residue in the co-ordination sphere of the Ni(II)–Asp-Ala-His-NHMe complex besides the above-mentioned four nitrogen-atom ligands (Laussac & Sarkar, 1980*b*). So the final picture of the specific Ni(II)-binding site on HSA emerges as that of a penta-co-ordinated structure involving the α-amino nitrogen atom, two deprotonated peptide nitrogen atoms, the imidazole nitrogen atom and the side-chain carboxy group of the aspartic acid residue. A similar structure has also been proposed for the Cu(II)-binding site of human serum albumin (Laussac & Sarkar, 1980*a*).

From the standpoint of the transport of Ni(II) in the serum, the detection of the ternary complex HSA–Ni(II)–L-His is very significant. An intermediate of this nature may make it possible for Ni(II)–HSA to transfer Ni(II) to the low-molecular-weight constituents of human serum, which in turn could transport the metal ion across the biological membrane. It has been shown in the ternary system of HSA, Ni(II) and L-histidine that relatively low concentrations of the amino acid lower the concentration of protein-bound Ni(II) considerably at physiological pH. Under physiological conditions, Ni(II) is in equilibrium with L-histidine and HSA, the relative amount bound to each constituent being dependent on the exact concentration of Ni(II). The potentiometric results from the ternary analysis show that the peptide, like the protein, is unable to compete with L-histidine for the metal ion. The proposed model for the transportation of Ni(II) centres on the ability of the amino acid to remove the metal from the transport protein HSA via a ternary complex and on the ability of the low-molecular-weight L-histidine complex to transverse the biological membrane.

Despite the similarities in the position and symmetry of the specific Cu(II)- and Ni(II)-binding to HSA, the equilibria involved in the metal ion ternary systems are dissimilar. The important difference is due to the lower affinity of Ni(II) for HSA and the avidity of L-histidine for the metal ion. Low concentrations of L-histidine, similar to the physiological state, remove Ni(II) from HSA, since L-histidine has a greater affinity for Ni(II) than does HSA. These results confirm the findings of previous studies in this laboratory (Lucassen & Sarkar, 1979). The equilibrium in favour of the low-molecular-weight Ni(II)-binding constituent may explain the observed rapid urinary excretion of Ni(II) from animals injected with Ni(II) (Onkelinx *et al.*, 1973; Sarkar, 1980, 1981).

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