A Critical Examination of the Interaction between Copper(II) and Glycylglycyl-L-histidine

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The equilibria involved in the copper(II)–glycylglycyl-L-histidine (HL) system have been re-examined by analytical potentiometry in 0.15 mol dm^{-3} NaCl solution at 25 °C. The species found are MH_2L, MHL, ML, $MH_{-1}L$, $MH_{-2}L$, MH_2L_2, MHL_2, ML_2, $MH_{-1}L_2$, and $MH_{-2}L_2$, with log stability constants 16.72, 11.78, 7.55, 2.68, −1.92, 25.81, 20.64, 16.68, 9.73, and 1.43 respectively. The visible absorptions of the total system have been measured at discrete pH values. The spectroscopic characteristics of the predominant species $MH_{-1}L$, $MH_{-2}L$, ML_2, $MH_{-1}L_2$, and $MH_{-2}L_2$ are computed. The combined potentiometric–spectroscopic analysis has helped to clarify the system in greater detail.

HUMAN serum albumin has one specific copper(II) binding site.[1,2] The Cu^{II} bound to albumin which is in equilibrium with Cu^{II} in tissues is considered to be the transport form of Cu^{II} in blood.[1,3] The tripeptide glycylglycyl-L-histidine was shown to simulate the native copper(II) binding characteristics of serum albumin, involving the α-amino-nitrogen, an imidazole nitrogen, and two intervening peptide nitrogens as the ligating sites around the square-planar copper(II) ion.[4,5] It serves as a suitable model for many important studies of the interaction between Cu^{II} and albumin as well as in delineating the mechanism of copper(II) transport, studies which are otherwise not feasible with proteins.[6,7] Therefore, this peptide has attracted a great deal of interest and attention. Although general agreement has been reached on the solution equilibria of the Cu^{II}–glycylglycyl-L-histidine system from several laboratories, there are discrepancies in some of the results.[4,8-14] In order to clarify the uncertainties, a critical re-examination of the Cu^{II}–peptide interaction by analytical potentiometry combined with spectroscopic studies, as a check of the proposed species, was carried out. The results are compared with the literature reports and discussed.

EXPERIMENTAL

The peptide glycylglycyl-L-histidine was synthesized and purified according to the procedures previously described [4] with minor alterations. It was recrystallized from n-propanol–water. The product was dissolved in water and the pH adjusted to 3.0, and then lyophilized. During the process of these treatments, care was taken to avoid any trace-metal contamination. The purity of this peptide was examined both by thin-layer chromatography and by paper electrophoresis. The peptide was always stored in the cold and dark. A stock solution was prepared in water and its concentration determined by quantitative Pauli reaction using L-histidine as a standard as well as by pH titration.

The potentiometric titrations were carried out on a Radiometer automatic titration assembly thermostatted at 25 ± 0.05 °C. The electrodes were calibrated against the National Bureau of Standards buffers. The base used was carbonate-free Na[OH] kept under an argon atmosphere and standardized against primary standard potassium hydrogen phthalate. A stock solution of $CuCl_2$ was prepared in 10^{-3} mol dm^{-3} HCl and standardized complexometrically against ethylenediaminetetra-acetic acid to give the concentration as 0.125 mol dm^{-3}. All the solutions contained 0.15 mol dm^{-3} NaCl and known amounts of HCl to lower the starting pH below that of metal binding and then titrated with 0.098 2 mol dm^{-3} Na[OH] in the presence and absence of Cu^{II} from pH 2.2 to 10.8. All the calculations were performed by a sequential use of three programs [15,16] on a GE 400 computer.

The visible absorptions of the copper(II) complexes were measured on a Beckman Acta model MVI spectrophotometer as a function of pH in 0.15 mol dm^{-3} NaCl solutions at 25 °C.

RESULTS

Proton–Glycylglycyl-L-histidine System.—For the determination of the pK_a values, the concentrations of the peptide used were 5.008×10^{-4}, 7.512×10^{-4}, and 10.016×10^{-4} mol dm^{-3}. Their titration curves are shown in Figure 1. The data were processed [15,16] to yield $\delta H^+/\delta C_L$ as a function of pH. The protonation equilibrium at low pH was well separated from the others and could be treated by the Henderson–Hasselbach equation. The two overlapping equilibria in the region of higher pH were processed as previously described [16] to obtain the protonation constants β_{011} and β_{021} of the species HL and H_2L^+ respectively (where L is the anionic form of glycylglycyl-L-histidine). The refined values of β_{0q1}, given as pK_a values, are compared with literature data in Table 1. They are in good agreement.

Copper(II)–Proton–Glycylglycyl-L-histidine System.—For the metal variation, the concentrations used were: $[L_t] = 7.512 \times 10^{-4}$ mol dm^{-3};† $[Cu^{II}] = 0.501 \times 10^{-4}$, 1.002×10^{-4}, 1.504×10^{-4}, 2.005×10^{-4}, and 2.506×10^{-4} mol dm^{-3}. In the ligand variation, the concentrations used were: $[Cu^{II}] = 1.504 \times 10^{-4}$ mol dm^{-3}; $[L_t] = 2.504 \times 10^{-4}$, 5.008×10^{-4}, 7.521×10^{-4}, 10.016×10^{-4}, and 12.520×10^{-4} mol dm^{-3}. The titration curves are shown in Figures 2 and 3. The data were processed to yield pM, pL, $\delta H^+/\delta C_M$ [Figure 4(*a*)], and $\delta H^+/\delta C_L$ [Figure 4(*b*)] as a function of pH. For the likely species of the complex formed in the metal–ligand system, the following values for p, q, and r were tested: $p = 1$; $q = 2, 1, 0, -1, -2$; $r = 1$ or 2. The calculations indicated that the complex species MH_2L, MHL, ML, $MH_{-1}L$, $MH_{-2}L$, MH_2L_2, MHL_2, ML_2, $MH_{-1}L_2$, and $MH_{-2}L_2$

† $[L_t]$ = Total concentration of L.

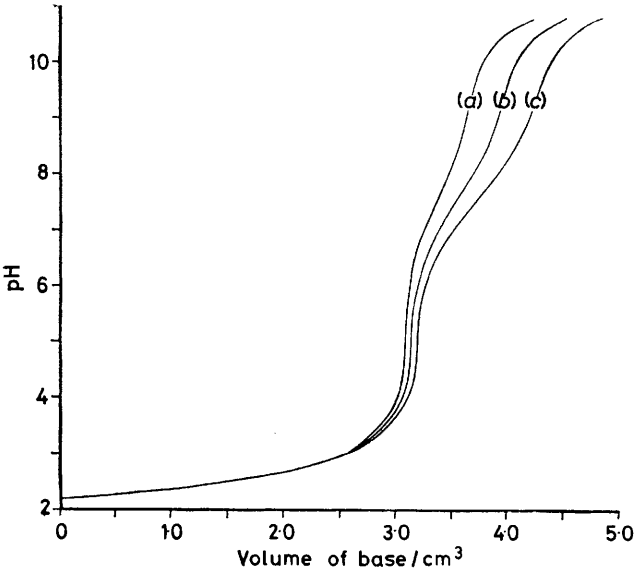

FIGURE 1 Titration curves for the glycylglycyl-L-histidine system. $[L_t] = 5.008 \times 10^{-4}$ (*a*), 7.512×10^{-4} (*b*), and 10.016×10^{-4} mol dm^{-3} (*c*)

were required to give a minimum-error solution, with MH_2L, MHL, and MHL_2 present in very minute amounts (charges are omitted for clarity). The stability constants expressed as log β_{pqr} are included in Table 1, and the species distribution as a function of pH is shown in Figure 5.

Visible Absorption Spectroscopy.—A solution containing 5.12×10^{-3} mol dm^{-3} of Cu^{II} and 5.52×10^{-3} mol dm^{-3} of glycylglycyl-L-histidine was prepared in 0.15 mol dm^{-3} NaCl. The visible absorption spectra as a function of pH were recorded. Representative spectra are shown in Figure 6. The spectral data for various pH values together with the complex species present, more than 10% at the specified pH, were processed by the method previously described [16]

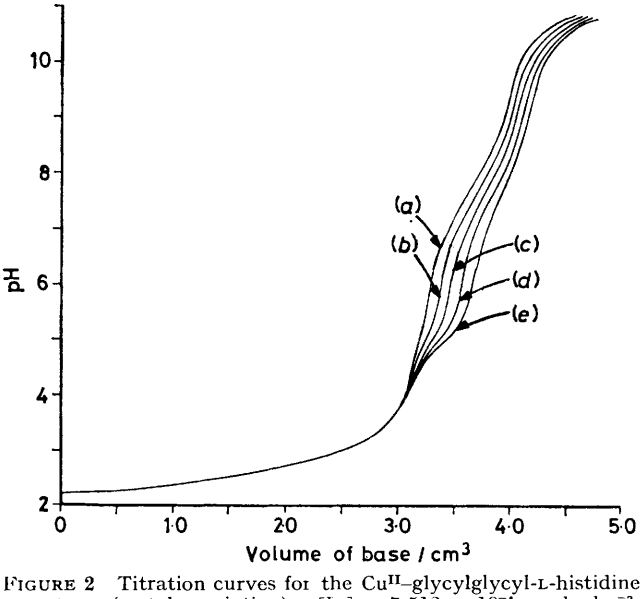

FIGURE 2 Titration curves for the Cu^{II}–glycylglycyl-L-histidine system (metal variation). $[L_t] = 7.512 \times 10^{-4}$ mol dm^{-3}. $[Cu^{II}] = 0.501 \times 10^{-4}$ (*a*), 1.002×10^{-4} (*b*), 1.504×10^{-4} (*c*), 2.005×10^{-4} (*d*), and 2.506×10^{-4} mol dm^{-3} (*e*)

to obtain the calculated spectrum for individual species. The results are shown in Table 2.

DISCUSSION

Among the species detected in the Cu^{II}–glycylglycyl-L-histidine system, MH_2L, MHL, ML, MH_2L_2, and MHL_2

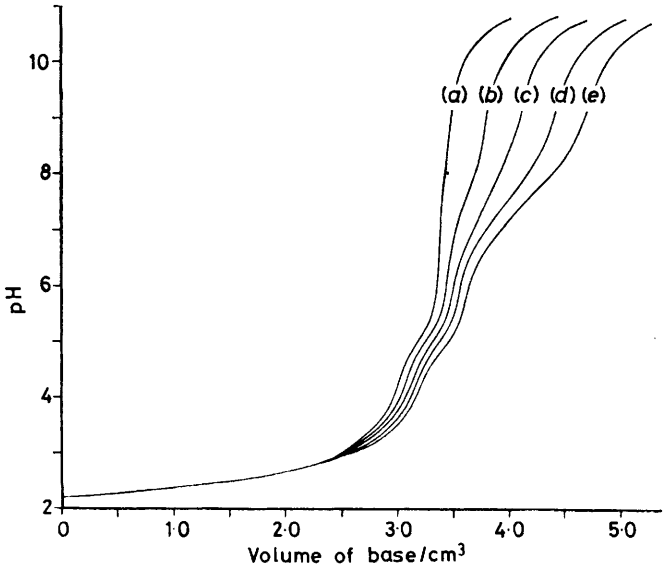

FIGURE 3 Titration curves for the Cu^{II}–glycylglycyl-L-histidine system (ligand variation). $[Cu^{II}] = 1.504 \times 10^{-4}$ mol dm^{-3}. $[L_t] = 2.504 \times 10^{-4}$ (*a*), 5.008×10^{-4} (*b*), 7.512×10^{-4} (*c*), 10.016×10^{-4} (*d*), and 12.520×10^{-4} mol dm^{-3} (*e*)

are never present in appreciable amounts, only as the deprotonated forms $MH_{-1}L$, $MH_{-2}L$, ML_2, $MH_{-1}L_2$, and $MH_{-2}L_2$. However, it is noted that the stepwise pK_a values for the successive deprotonation of MH_2L to

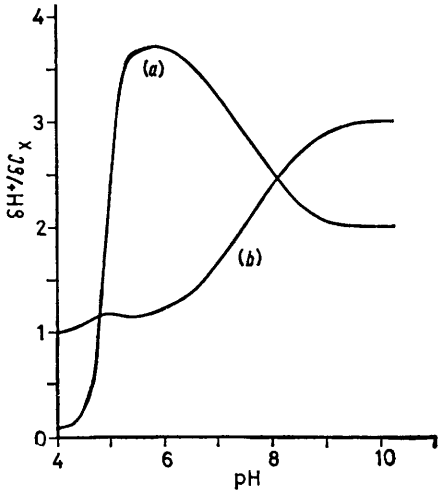

FIGURE 4 Proton displacement $\delta H^+/\delta C_X$ as a function of pH for Cu^{II}–glycylglycyl-L-histidine: X = Cu^{II} (*a*) and L (*b*). $[Cu^{II}] = 1.504 \times 10^{-4}$ mol dm^{-3}, $[L_t] = 7.512 \times 10^{-4}$ mol dm^{-3}

$MH_{-2}L$ are 4.94, 4.23, 4.87, and 4.60. Similar results were reported by Bryce *et al.*[8] for the last three steps of deprotonation. Such co-operative action has been ascribed to the copper(II) ion-promoted ionizations of

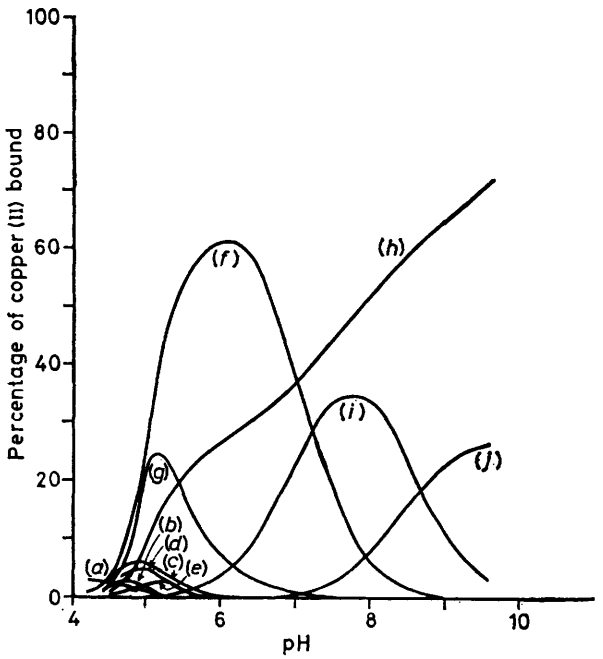

FIGURE 5 Species distribution as a function of pH for Cu^{II}-glycylglycyl-L-histidine Curves: (*a*) $Cu^{II}H_2L$, (*b*) $Cu^{II}HL$, (*c*) $Cu^{II}L$, (*d*) $Cu^{II}H_2L_2$, (*e*) $Cu^{II}HL_2$, (*f*) $Cu^{II}L_2$, (*g*) $Cu^{II}H_{-1}L$, (*h*) $Cu^{II}H_{-2}L$, (*i*) $Cu^{II}H_{-1}L_2$, (*j*) $Cu^{II}H_{-2}L_2$

peptide hydrogens in the formation of the major species $MH_{-2}L$.[8,17]

Spectral measurement confirms the presence of several species in the pH region 4.7—6.0. Small but significant absorption (broad peaks) was observed between 600 and 800 nm in addition to the lower wavelength absorption peaks. This high wavelength absorption diminishes as the pH gradually increases, as was observed in the case of albumin. Eventually only the lower wavelength absorption which maximizes in the vicinity of 520—525 nm remains. Since no appreciable amounts of any of the minor species were detected in the low pH region, the individual spectrum for these species was not computed due to the possible large error introduced.

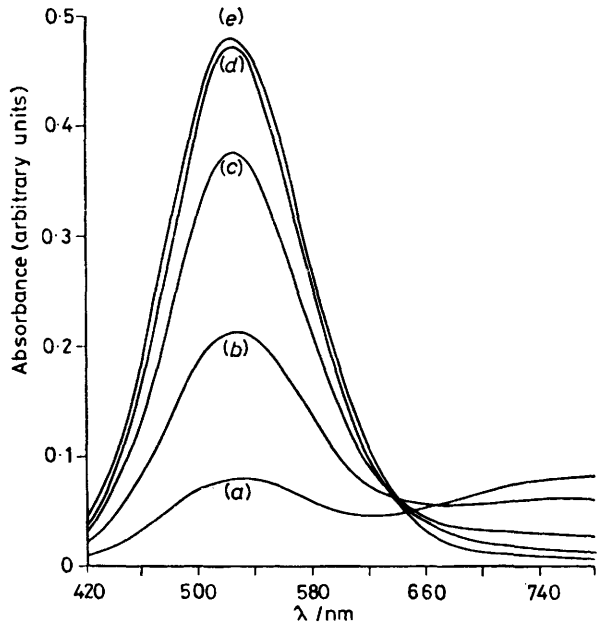

FIGURE 6 Visible absorption spectra as a function of pH for the Cu^{II}-glycylglycyl-L-histidine system in 0.15 mol dm^{-3} NaCl at 25 °C. $[Cu^{II}] = 5.12 \times 10^{-3}$ mol dm^{-3}, $[L_t] = 5.52 \times 10^{-3}$ mol dm^{-3}, light-path = 1 cm. pH 4.72 (*a*), 4.93 (*b*), 5.22 (*c*), 6.26 (*d*), and 7.92 (*e*)

TABLE 1

Values of pK_a and log stability constants (β_{pqr}) of complex species $M_pH_qL_r$ (M = Cu^{II}, L = glycylglycyl-L-histidine anion) in 0.15 mol dm^{-3} NaCl at 25 °C, standard deviations are shown in parentheses

			This work		Other work
			pK_a	log β_{pqr}	pK_a
p	*q*	*r*			
0	3	1	2.83 (0.011)		2.72,[a] 2.43,[b] 2.84,[c] 2.84 [d]
0	2	1	6.92 (0.008)		6.74,[a] 6.52,[b] 6.87,[c] 6.99,[c] 6.87 [d]
0	1	1	8.14 (0.024)		8.04,[a] 7.51,[b] 8.01,[c] 8.23,[c] 8.22 [d]
1	2	1		16.72 (0.025)	
1	1	1		11.78 (0.018)	11.70,[b] 12.24 [f]
1	0	1		7.55 (0.035)	9.220,[a] 7.04,[b] 7.59 [f]
1	−1	1		2.68 (0.029)	3.648,[a] 2.53 [f]
1	−2	1		−1.92 (0.099)	−1.991,[a] −2.11,[b] −1.98 [e]
1	2	2		25.81 (0.031)	
1	1	2		20.64 (0.014)	
1	0	2		16.68 (0.041)	
1	−1	2		9.73 (0.013)	8.70 [b]
1	−2	2		1.43 (0.188)	1.30 [b]

[a] 25 °C, I = 0.15 mol dm^{-3}; ref. 4. [b] 37 °C, I = 0.15 mol dm^{-3}; ref. 10. [c] 21 °C; ref. 9. [d] 25 °C, I = 0.16 mol dm^{-3}; ref. 8. [e] 25 °C, I = 0.1 mol dm^{-3}; ref. 11. [f] 25 °C, I = 0.1 mol dm^{-3}; ref. 12.

The predominant species $MH_{-1}L$, $MH_{-2}L$, ML_2, and $MH_{-1}L_2$ all seem to have a computed maximal absorption around 525 nm which indicates that all four nitrogens are ligating around the central Cu^{II} ion. However, $MH_{-2}L_2$ has a calculated $\lambda_{max.}$ at *ca.* 560 nm. Judging from its occurrence at a slightly higher pH, a hydroxyl group is likely to be participating in the co-ordination, substituting in one of the nitrogen-ligand sites during the formation of $MH_{-2}L_2$ from $MH_{-1}L_2$.

The observed species are somewhat different from those published earlier by us[4] as well as by other groups.[10-12] The existence of five species, namely, MHL, ML, $MH_{-2}L$, $MH_{-1}L_2$, and $MH_{-2}L_2$ were found by Agarwal and Perrin.[10] As seen in Table 1, their corresponding stability constants are comparable to the present findings. On the other hand, Sakurai and Nakahara [11,12] found ex-

TABLE 2

Spectral characteristics of the complex species $M_pH_qL_r$ (M = Cu^{II}, L = glycylglycyl-L-histidine anion) in 0.15 mol dm^{-3} NaCl at 25 °C

Species	$\lambda_{max.}$/nm	$\varepsilon \pm 5$/dm^3 mol^{-1} cm^{-1}
$MH_{-1}L$	520	195
$MH_{-2}L$	525	112
ML_2	525	78
$MH_{-1}L_2$	525	84
$MH_{-2}L_2$	560	60

clusively the species $MH_{-2}L$ at pH 6. Based on the available information, it appears that the species distribution may be concentration dependent. It is not surprising to detect large amounts of bis complexes when the ligand is present in excess. The difference in profile is due mainly to the different concentration range of Cu^{II} and peptide used in the titration, and perhaps even more to the molar ratio of peptide to Cu^{II}. Most of the concentrations used in our work were in the range of 10^{-4} mol dm^{-3} which is about one order of magnitude lower than that of Agarwal and Perrin.[10] These authors do not provide the peptide to Cu^{II} molar ratio used in their investigation. In our case, the ratios varied from 2 : 1 to 10 : 1. One should also note the difference in temperature between these studies (25 °C *versus* 37 °C), a factor which influences the results. Furthermore, a set of strict criteria must be maintained to establish the purity of the peptide for titration since this particular peptide is found from our experience to be very easily contaminated and decomposed. This observation is consistent with the results of the *X*-ray crystallographic investigation of the copper(II) complex of glycylglycyl-L-histidine. The peptide in the complex was shown to have undergone decarboxylation.[18]

Considering the aforementioned difficulties encountered with this peptide, subsequent work in this laboratory has been carried out with the peptide derivatives, namely, glycylglycyl-L-histidine-*N*-methylamide [19-22] and L-aspartyl-L-alanyl-L-histidine-*N*-methylamide.[21-25] In both cases, the major species present in solution shows similar copper(II) binding properties as those of human albumin. Moreover, an *X*-ray crystallographic investigation has demonstrated the complexation of the intact glycylglycyl-L-histidine-*N*-methylamide with copper(II) ion involving four nitrogens in a square plane.[20] These peptide derivatives with no available terminal-carboxyl groups seem to be more appropriate models in simulating the copper(II) binding characteristics of the native protein.

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REFERENCES

[1] P. Z. Neumann and A. Sass-Kortsak, *J. Clin. Invest.*, 1967, **46**, 646.
[2] B. Sarkar and Y. Wigfield, *Can. J. Biochem.*, 1968, **46**, 601.
[3] A. G. Bearn and H. G. Kunkel, *Proc. Soc. Exp. Biol. Med.*, 1954, **88**, 44.
[4] S. Lau, T. P. A. Kruck, and B. Sarkar, *J. Biol. Chem.*, 1974, **249**, 5878.
[5] T. Peters, jun., and F. A. Blumenstock, *J. Biol. Chem.*, 1967, **242**, 1574.
[6] T. P. A. Kruck and B. Sarkar, *Inorg. Chem.*, 1975, **14**, 2383.
[7] S. Lau and B. Sarkar, *Can. J. Chem.*, 1975, **53**, 710.
[8] G. F. Bryce, R. W. Roeske, and F. R. N. Gurd, *J. Biol. Chem.*, 1965, **240**, 3837.
[9] A. Yokoyama, H. Aiba, and H. Tanaka, *Bull. Chem. Soc. Jpn.*, 1974, **47**, 112.
[10] R. P. Agarwal and D. D. Perrin, *J. Chem. Soc., Dalton Trans.*, 1977, 53.
[11] T. Sakurai and A. Nakahara, *Inorg. Chim. Acta*, 1979, **34**, L245.
[12] T. Sakurai and A. Nakahara, *Inorg. Chem.*, 1980, **19**, 847.
[13] A. Avdeef, and K. N. Raymond, *Inorg. Chem.*, 1979, **18**, 1605.
[14] Y. Kuroda and H. Aiba, *J. Am. Chem. Soc.*, 1979, **101**, 6837.
[15] B. Sarkar and T. P. A. Kruck, *Can. J. Chem.*, 1973, **51**, 3541.
[16] B. Sarkar, *J. Indian Chem. Soc.*, 1977, **54**, 117.
[17] R. J. Sundberg and R. B. Martin, *Chem. Rev.*, 1974, **74**, 471.
[18] P. de Meester and D. J. Hodgson, *J. Am. Chem. Soc.*, 1976, **98**, 7086.
[19] T. P. A. Kruck, S. Lau, and B. Sarkar, *Can. J. Chem.*, 1976, **54**, 1300.
[20] N. Camerman, A. Camerman, and B. Sarkar, *Can. J. Chem.*, 1976, **54**, 1309.
[21] B. Sarkar, in ' Metal–Ligand Interaction in Organic Chemistry and Biochemistry,' eds. B. Pullman and N. Goldblum, D. Reidel Publishers, Dordrecht, Holland, 1977, pp. 193.
[22] G. Arena, E. Rizzarelli, and B. Sarkar, *Inorg. Chim. Acta*, 1979, **37**, L555.
[23] K. S. N. Iyer, S. Lau, S. H. Laurie, and B. Sarker, *Biochem. J.*, 1978, **169**, 61.
[24] B. Sarkar, H. B. F. Dixon, and D. Webster, *Biochem. J.*, 1978, **173**, 895.
[25] J.-P. Laussac and B. Sarkar, *J. Biol. Chem.*, 1980, **255**, 7563.