32. Zinc Complexes of Cysteine, Histidine, and Derivatives Thereof: Potentiometric Determination of Their Compositions and Stabilities

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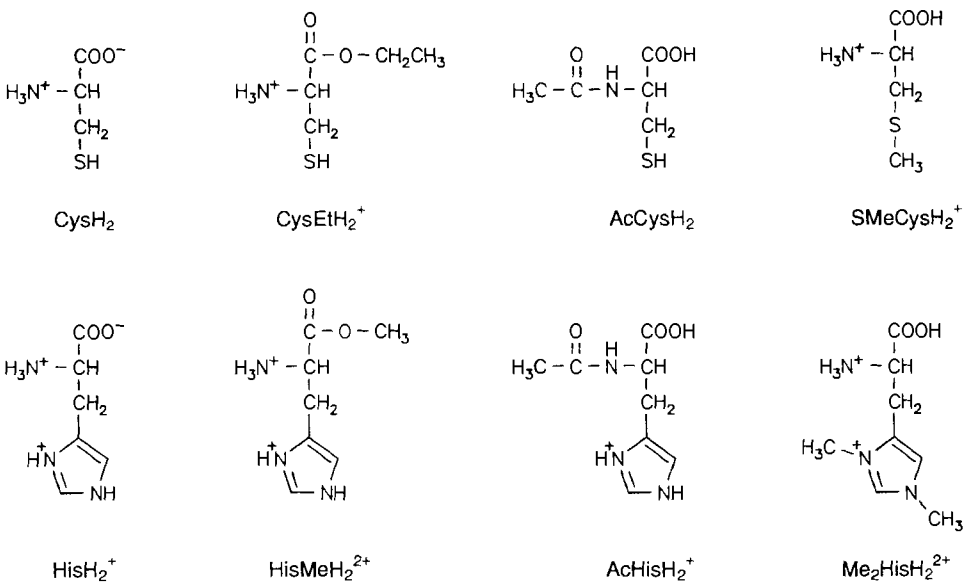
The stabilities of Zn complexes of cysteine and histidine have been determined together with those of three derivatives of each in which one of their three donor functions (carboxyl, amino, and mercapto and imidazole, respectively) has been blocked. Using potentiometric titrations of aqueous solutions, the 1:1 and 1:2 complexes of all four cysteine- and all four histidine-derived ligands are observed among the various species present (ligands, 1:1 and 1:2 complexes, and protonated derivatives thereof). All cysteine-derived complexes are more stable than the corresponding histidine-derived complexes by 1–2 orders of magnitude for the 1:1 composition and by 1–6 orders of magnitude for the 1:2 composition. For the cysteine series, the sequence of stabilities is cysteine > cysteine ethyl ester ≫ $N(\alpha)$-acetylcysteine ≫ *S*-methylcysteine. For the histidine series, the corresponding sequence is histidine > histidine methyl ester > *N*,N(imidazole)-dimethylhistidine > $N(\alpha)$-acetylhistidine. The order of stabilities can be explained by the relative strengths of the Zn–S *vs.* Zn–N coordination, by charge effects, and by chelate ring sizes.

Introduction. – The importance of Zn for all kinds of life processes [1–3] is generating new activities in the once-neglected field of Zn-coordination chemistry. One aspect of this should be the study of Zn-amino acid and peptide complexes. While much information on such types of complexes was gathered early-on for the popular metals like Co and Cu [4–6], the corresponding body of knowledge for Zn-containing systems is still limited [7] [8]. In contrast, the knowledge on the structure and function of Zn-containing enzymes [1–3] is unusually high, and the discovery of the 'zinc fingers' his triggered intensive studies on the interaction of medium-sized proteins with Zn ions [9] [10].

As part of our program on the coordination chemistry of Zn with relation to its biological functions [11] [12], we have set out to elucidate the molecular structures and reactivities of Zn complexes of small peptides. This involves preparative peptide chemistry, crystal structure determinations, NMR investigations, and stability measurements of the Zn complexes. The two central peptide building blocks for these studies are the amino acids cysteine and histidine which are the predominant ligating units for Zn in proteins.

This paper reports information which had to be gathered to lay a basis for bonding and stability discussions. Potentiometric methods were applied to compare the properties of Zn-cysteine and Zn-histidine complexes with those of cysteine and histidine derivatives of reduced functionality, respectively. The aim of the study was to obtain quantitative information on preferred sets of donor atoms of these amino acids. Therefore, three

Scheme 1. *The Ligands Used, Shown in Their Diprotonated Forms*



derivatives each were used in which one of the three functional groups of the amino acid is blocked at a time, making two sets of three different bidentate chelating ligands available. The compounds used and their terminology are shown in *Scheme 1*. All materials used were optically pure and derived from the L-amino acids.

There is information in the literature, in addition to that on the two amino acids and their Zn complexes [13–16], on pK_a values and Zn-complex stabilities of cysteine methyl ester [17], histidine methyl ester [18], and $N(\alpha)$-acetylhistidine [19], whereas the values for cysteine ethyl ester, $N(\alpha)$-acetylcysteine, *S*-methylcysteine and *N*,*N*(imidazole)-dimethylhistidine are reported here for the first time. We chose to redetermine all values, because there was a wide spread of measuring techniques, solution compositions, concentrations, ionic strengths, and the presence of possibly ligating anions, and because the optical purity of the ligands was not defined in all cases. We found good agreement between our results and those reported for histidine and histidine methyl ester.

Results and Discussion. – The blocking of one acidic group in each of the amino-acid derivatives induces systematic changes in all pK_a values. *Table 1* gives the data for the cysteine system. There is a general trend for all pK values in the order Ac-

Table 1. *p*K_a *Values of the Cysteine Derivatives* (Mixed pK_a values at $I = 0.1$ $NaClO_4$ and 25.0°)

	Cys[a])	CysEt[b])	AcCys	SMeCys
COOH	1.82 (2)	–	3.08 (2)	1.94 (4)
SH	8.24 (2)	6.71 (2)	9.62 (2)	–
NH_3^+	10.36 (3)	9.18 (2)	–	8.83 (3)

[a]) Reported [14]: 8.13 (–) for SH and 10.11 (–) for NH_3^+.
[b]) Reported for cysteine methyl ester [16]: 6.52 (2) and 9.15 (1).

Cys > Cys > CysEt, while for SMeCys the pK of the carboxylic group is similar to, but that of the ammonium group smaller than that for cysteine. Most of this can be explained in terms of the charges of the species involved. Thus, the characteristic destabilization of the amino-acid COOH function due to the neighboring NH_3^+ is not operating in AcCys and hence its reduced acidity. *Vice versa*, the ammonium function in CysEt is not stabilized by a neighboring COO^- and hence its increased acidity. The deprotonation of the SH group in CysEt produces a neutral species, in Cys a uninegative and in AcCys a dinegative species, with a concomitant increase in the pK of *ca.* 1.5 units for each step. Similarly, the lower pK_a value of the NH_3^+ function in SMeCys relative to that in Cys can be related to the charges of the molecules[1]).

Table 2. *p*K$_a$ *Values of the Histidine Derivatives* (Mixed pK_a values at $I = 0.1$ KNO_3 and 25.0°)

	His[a])	HisMe[b])	AcHis[c])	Me_2His
COOH	1.77 (2)	–	2.94 (2)	1.96 (4)
NH^+	6.14 (2)	5.41 (3)	7.11 (3)	–
NH_3^+	9.24 (2)	7.35 (2)	–	7.83 (2)

[a]) Reported [15]: 6.08 (–) for NH^+ and 9.20 (–) for NH_3^+.
[b]) Reported [17]: 5.35 (3) and 7.30 (3).
[c]) Reported [18]: 7.08 (–) for NH^+.

The general trends in pK_a values for the histidine derivatives (*cf. Table 2*) are completely analogous. For all values, the sequence of magnitudes is AcHis > His > HisMe, and for Me_2His the pK(COOH) is similar to and the pK(NH_3^+) is smaller than that for His. A detailed inspection reveals that all pK values for the histidine derivatives are smaller than the corresponding ones for the cysteine derivatives. This may also be related to charge effects: in each case the charge of the histidine species is one unit more positive than that of the corresponding cysteine species. With this in mind, the higher acidity of the histidine imidazole NH^+ function compared to that of the cysteine SH function is also not so much an intrinsic but rather a charge-related property.

The Zn complexes in solutions of the cysteine derivatives and their stabilities are listed in *Table 3*. *Fig. 1* shows as an example the pH-dependent Zn-species distribution in solutions with a Zn/AcCys concentration ratio of *ca.* 1:2. The species distributions show

Table 3. *Stability Constants* (log K) *for the Zn Complexes of Cysteine Derivatives* ($I = 0.1$ $NaClO_4$ and 25.0°)

	Cys[a])	CysEt[b])	AcCys	SMeCys
[ZnL]	8.97 (2)	7.42 (6)	4.90 (13)	4.30 (4)
[ZnLH]	–	12.70 (10)	–	–
[ZnL_2]	17.86 (1)	16.33 (1)	11.48 (2)	7.82 (8)
[ZnL_2H]	–	–	18.39 (4)	–
[ZnL_2OH]	–	–	2.71 (3)	–

[a]) Reported [14]: 9.04 (–) and 17.54 (–).
[b]) Reported for cysteine methyl ester [16]: 12.74 (47) for [ZnLH] and 15.91 (3) for [ZnL_2].

[1]) As discussed in [20], the pK_a values given for the SH and NH_3^+ groups of Cys and CysEt in *Table 1* can be interpreted as mixed deprotonation constants involving both these groups. This does not touch the following discussion.

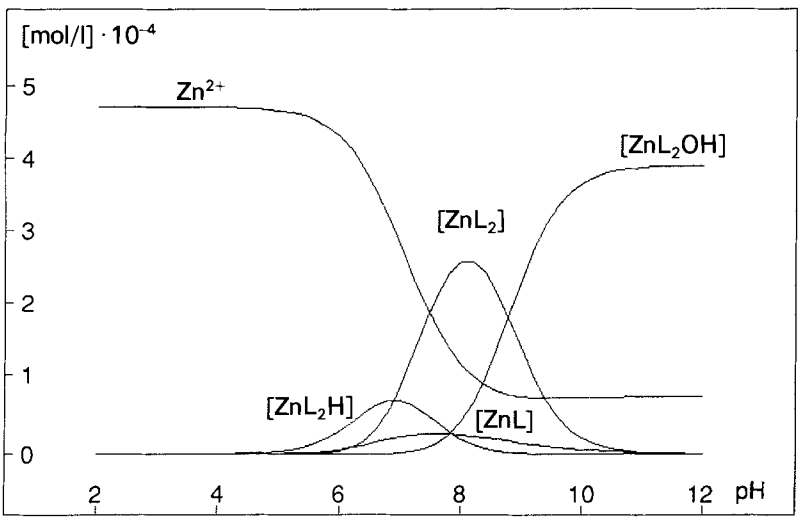


Fig. 1. *Zn-Species distribution curves for Zn salt/*N(α)*-acetylcysteine solutions.* Initial concentrations: $Zn(ClO_4)_2$, $3.82 \cdot 10^{-4}$M; $AcCysH_2$ $8.00 \cdot 10^{-4}$M.

a somewhat irregular behavior. Only for cysteine and *S*-methylcysteine are both the ZnL and the ZnL_2 complex observed with certainty. In solutions of cysteine ethyl ester and of *N*(α)-acetylcysteine, the $[ZnL_2]$ complex prevails, and the [ZnL] complex could be included in the computations only at rather low concentration (*i.e.* significance) levels. Instead, complexes of the protonated ligands and even hydroxo species had to be taken into account to fit the data. For CysEt, a model involving no [ZnL] complex but a $[ZnL_2H]$ species instead ($\log K = 21.04(5)$) gave similar agreement between observed and calculated titration curves and the same value for $\log K([ZnL_2])$. Although this agrees with literature data for CysMe [17], we cannot rule out that the uncertainties are in part due to the Zn-catalyzed hydrolysis of CysEt. Furthermore, the $[ZnL_2H]$ species for AcCys which would correspond to an acid with $pK_a \approx 7$ would not be primarily expected, but alternative models did not give a satisfactory fit of the data. In addition, it should be mentioned that, due to the tendency of mercaptides to form oligonuclear complexes, such species might be taken into consideration for the Zn/cysteine system [16]. However, under our measurement conditions, no evidence for them could be found, and larger changes of the concentrations of the reagents which might improve their detectability were not made. Accordingly, in view of the above-mentioned limitations, we limit the discussion to the values obtained with certainty for all cysteine derivatives, *i.e.* the $\log K$ values for $[ZnL_2]$.

Of all amino acids, cysteine forms the most stable complexes with Zn [14], as expressed by the $\log K$ values of *Table 3*. Inspection of *Table 3* also reveals that the mercapto S^- is primarily responsible for this, while the contribution of the COOH function is insignificant. Blocking of the mercapto S-atom (SMeCys) reduces *K* for $[ML_2]$ by ten orders of magnitude, while blocking of the COOH group (CysEt) accounts for only one $\log K$ unit. Blocking of the amino function (AcCys) also has significant effects (six orders of magnitude), but it stays four orders of magnitude behind the effect of blocking the SH group. Even the fact that the charges of the ligands vary along the series cannot alter this

argumentation. As seen from the comparison of the $[ZnCys_2]$ and $[Zn(CysEt)_2]$ complexes, changing the charge accounts for about one log unit in stability, just like for the pK_a values. So taking this into account for a comparison of the $[Zn(AcCys)_2]$ and $[Zn(SMeCys)_2]$ complexes still leaves a difference in stabilities of three orders of magnitude.

The interpretation of the measurements for the histidine derivatives was much easier as the titration curves could be fitted very well by considering only the [ZnL] and the $[ZnL_2]$ complexes, which is in agreement with NMR investigations of histidine/$Zn(NO_3)_2$ solutions [21]. *Table 4* lists the stability values, and *Fig. 2* gives a characteristic Zn-species distribution, taken for the Zn/HisMe system and a Zn/L concentration ratio of *ca.* 1:2 again. Both the stability constants for the [ZnL] and for the $[ZnL_2]$ complexes show the same trend with the sequence of magnitudes being His > HisMe > Me_2His > AcHis.

There are significant differences between the cysteine and the histidine series concerning both the magnitude and the succession of the log K values along the series. Thus, the $[ZnHis_2]$ complex is more than five orders of magnitude less stable than the $[ZnCys_2]$ complex. Secondly, blocking the imidazole N functions (Me_2His) does not cause more of an effect than blocking the amino functions (AcHis). Actually, the AcHis complexes are

Table 4. *Stability Constants* (log K) *for the Zn Complexes of Histidine Derivatives* (I = 0.1 KNO_3 and 25.0°)

	His[a])	HisMe[b])	AcHis[c])	Me_2His
[ZnL]	6.62 (2)	4.45 (4)	2.91 (12)	3.53 (6)
$[ZnL_2]$	12.03 (2)	8.66 (4)	5.74 (20)	6.65 (10)

[a]) Reported [15]: 6.61 (–) and 11.95 (–).
[b]) Reported [17]: 4.46 (8) and 8.66 (8).
[c]) Reported [18]: 2.50 (–) and 4.80 (–).

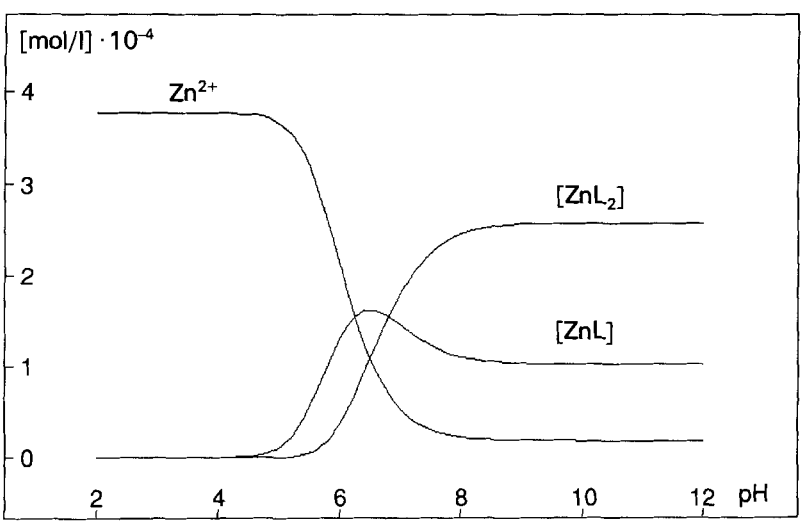


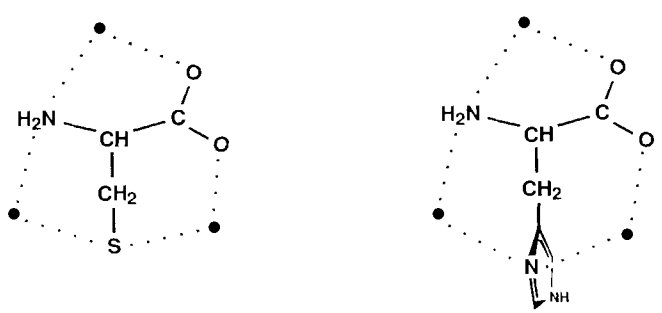
Fig. 2. *Zn-Species distribution curves for Zn salt/histidine methyl ester solutions.* Initial concentrations: $Zn(NO_3)_2$, $3.76 \cdot 10^{-4}$M; HisMe, $7.80 \cdot 10^{-4}$M.

slightly less stable than the Me_2His complexes. Thirdly, blocking the COOH group (HisMe) reduces the $[ZnL_2]$ complex stability by more than three $\log K$ units, unlike the situation in the cysteine series. All this underlines the dominating importance of the SH group, but it also points to a different coordination behavior of the histidine-derived ligands. While the primary ligating units are, in accordance with all experience, the imidazole and amino groups, the COOH group seems to participate as well. This may mean higher than tetrahedral coordination for $[ZnHis_2]$ in solution, and it means that the (O,N) ligand combination of $[Zn(Me_2His)_2]$ is at least as favorable as that of $[Zn(AcHis)_2]$. If one invokes the charge difference between AcHis and Me_2His, as in AcCys and SMeCys before, the stability situation turns even more in favor of the Me_2His complexes, thereby making the contribution of the COOH group (in $Zn(Me_2His)_2$) almost as important as the contribution of the imidazole group (in $Zn(HisMe)_2$).

According to the crystal structures of $[ZnCys_2]$ [22], $[ZnHis_2]$ [23], and $[Zn(SMeCys)_2]$ [24], all complexes considered here can be discussed primarily in terms of a tetrahedral coordination with all ligands being bidentate and chelating, notwithstanding the occasional presence of weak additional Zn–O(carboxylate) coordination in the solid state. This is supported by the solid-state structures of $[Zn(CysEt)_2]$ [22], $[Zn(CysGlyNH_2)_2]$ [25], and $[Zn(HisMe)_2(BPh_4)_2]$ [26]. But according to our structure determinations of $ZnCl_2 \cdot His \cdot HCl$ ($ZnCl_3O$ ligation with histidine only O-bonded) [27] and $[Zn(HisGly)_2]$ (coordination polymer with octahedral ZnN_4O_2 coordination) [26], it can be taken only as a first approximation for the histidine-containing complexes, as is also indicated by the above discussion. However, even limiting the discussion to tetrahedral Zn coordination and bidentate ligation allows to draw some further conclusions.

As can be seen from *Scheme 2*, the three modes of bidentate ligation for both ligand types give rise to differently sized chelate rings. For cysteine, the most stable (*N*,*S*) ligation produces a five-membered, the secound best (*O*,*S*) ligation a six-membered, and the worst (*N*,*O*) ligation a five-membered ring. For histidine, the most stable (*N*,*N*) ligation results in a six-membered, the second best ($N(\alpha)$,*O*) ligation in a five-membered, and the worst (*N*(im),*O*) ligation in a seven-membered ring. This underlines once again the dominance of the Zn–S coordination which, even with the unfavorable six-membered chelate ring ($[Zn(AcCys)_2]$), is preferred over the Zn–N coordination in a five-membered chelate ring ($[Zn(SMeCys)_2]$). On the other hand, it helps to understand the different sequence of stabilities for the histidine-derived complexes where the best combination ($[ZnHis_2]$) already suffers from the six-membered chelate ring, while the low stability of

Scheme 2. *Possible Modes of Chelation for the Cysteine- and Histidine-Derived Ligands*



the Zn-AcHis complexes finds its explanation in their very unfavorable seven-membered chelate rings.

The stability values in *Tables 3* and *4* and the geometrical effects outlined above give rise to a hierarchy of stability factors for all the complexes discussed here. First comes the preferred Zn–S bonding, second the chelate ring size, and third the charge of the ligand. Of these, the chelate effect will become less important and disappear as one moves on from amino acid to peptide derivatives. As a consequence, all Zn complexes of cysteine- and histidine-derived peptides should have lower stabilities than [$ZnCys_2$] or [$ZnHis_2$], unless favorable peptide folding brings chelate effects back in. On the other hand, the *N*(imidazole) contribution which comes out unusually low in some of the examples here should, for the peptide complexes, show more of the prominence that it has in nature.

Conclusion. – The three possible chelation modes for both cysteine and histidine towards Zn have been evaluated by alternatively blocking one of the three coordination sites and determining the complex stabilities. For cysteine derivatives, the (*N*,*S*) donor set is preferred (cysteine, cysteine ethyl ester), followed by the (*O*,*S*) donor set (*N*(α)-acetylcysteine) and the (*N*,*O*) donor set (*S*-methylcysteine). For histidine derivatives, the differentiation is not so pronounced, with the (*N*,*N*) donor set (histidine, histidine methyl ester) being preferred over the two (*N*,*O*) donor sets (*N*(α)-acetylhistidine, *N*,*N*(imidazole)-dimethylhistidine). The order of complex stabilities results firstly from the strength of the Zn–S interaction which is not matched by the strength of the Zn–N(imidazole) interaction, and secondly from charge and chelate ring-size influences. Since chelate effects are not as important for Zn-peptide complexes, different stability sequences may be expected for those. The data reported in this paper establish the reference standard for our planned stability determinations of Zn complexes of peptides containing terminal cysteine and histidine residues.

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Experimental. – *Materials.* All reagents were anal. grade. Cysteine and histidine were used as supplied by *Aldrich*. Cysteine ethyl ester [28], *N*(α)-acetylcysteine [29], *S*-methylcysteine [30], histidine methyl ester [31], *N*(α)-acetylhistidine [32], and *N*,*N*(imidazole)-dimethylhistidine [33] were prepared according to the published procedures and their purity checked by HPLC. All soln. were made up under a N_2 atmosphere in H_2O which was decarbonated and triply distilled. The standard solns. of NaOH (0.200M), HNO_3 (0.100M), and $HClO_4$ (0.100M) were kept for no longer than three weeks, those of the tris buffer ($4 \cdot 10^{-3}$M) for no longer than 3 d. Stock solns. of $NaClO_4$ (1.0M), KNO_3 (1.0M), $Zn(ClO_4)_2$ (0.100M), and $Zn(NO_3)_2$ (0.100M) were kept under N_2. Solns. of the ligands ($4 \cdot 10^{-3}$M) were made up prior to use. The exact concentrations of the Zn salt, acid, and NaOH solns. were determined titrimetrically (EDTA, NaOH, kalium hydrogen phthalate).

Measurements. All NaOH titrations were done under N_2 in thermostatted vessels at 25.0°. The fully automatic pH titration unit consisted of a combined glass electrode (*Metrohm UX*), a digital pH-meter (*Metrohm 652*), a digital burette (*Metrohm Dosimat 665*), an interface unit (*Twix 32 bit I/O*), and a *80286 PC*. The exact calibration was done daily by first using commercial buffer solns. (*Merck*) of pH 4 and 7 and then titrating 50 ml of $8 \cdot 10^{-4}$M *tris* (tris(hydroxymethyl)methylamine) and HNO_3 ($1.3 \cdot 10^{-3}$M) with NaOH (0.200M). Values of the apparent activity coefficient α (0.957), pK_a of tris (8.167), and pK_w (13.86) were taken as reference from [34] and used for final readjustment of the experimental pH readings. The above values closely corresponded to results obtained in the present study from *tris* titrations with carefully calibrated electrode systems. During the measurements, the waiting period between successive NaOH additions was adjusted automatically by the data-acquisition program according to the pH changes. For $CysEtH_2$, the waiting time was limited to 60 s, because Zn ions catalyze the hydrolysis of the

ester, and hence the measurement had to be done in a short time. For the same reason, the titration curves for the highest Zn concentration gave the poorest fit between observed and calculated data.

The measurements were carried out with solns. (50 ml) containing 0.100M $NaClO_4$ for all cysteine derivatives and 0.100M KNO_3 for all histidine derivatives. As the contribution of the species under investigation is very low ($\leq 4\%$), the ionic strength can be considered constant and equal to 0.1M. The starting concentration of the ligands was always *ca.* $8 \cdot 10^{-4}$M with a *ca.* 50% excess of $HClO_4$ for all cysteine derivatives and of HNO_3 for all histidine derivatives. For the measurements of the Zn-complex stabilities, $Zn(ClO_4)_2$ was used for the cysteine derivatives and $Zn(NO_3)_2$ for the histidine derivatives. Three different mixtures each were titrated representing Zn/L ratios of *ca.* 1:1, 1:2, and 1:4 in addition to the titration of the Zn-free solution. For each of the 16 reagent combinations, the measurement was repeated, until three consistent titration curves were obtained for one identical set of conditions. For the determination of the pK_a values less than 2, the concentration of the ligands was $1.6 \cdot 10^{-3}$M with a *ca.* twofold excess of HNO_3. The concentration of the NaOH soln. was 1.00M for these titrations. During the NaOH titrations of AcHis and Me_2His, precipitation of $Zn(OH)_2$ occurred around pH 8.5 and above.

Computations. All calculations were done using the TITFIT program [35] which also handles the data acquisition. Mixed stability constants based on the readjusted pH readings (*cf.* above) are given in this paper. These may be converted into concentration constants using $\alpha = 0.957$. As discussed in [34], α does not correspond to the single ion activity coefficient of H^+, but may be used to convert pH readings based on electrodes calibrated with NBS buffers into proton concentrations: $[H^+] = 10^{-pH}/\alpha$.

For each system, pK_a values of the uncomplexed ligands were first determined from independent measurements. Based thereupon, complex stability constants were obtained from batch calculations consisting of three titration curves with different $[Zn^{2+}]/[L]$ ratios. Calculations were always started with a model consisting of the species [ZnL] and $[ZnL_2]$. Protonated or partially hydrolyzed species $[ZnL_nH_x]$ were included as necessary to obtain a reasonable fit. All complexes are described by overall formation constants related to $Zn^{2+} + nL + xH^+ \rightarrow ZnL_nH_x$ ($x < 0$ for hydrolyzed species). The detection limit for minor species amounts to 1–5% of the total analytical concentration for our experiments. Uncertainties of all equilibrium constants are given by twice their standard errors. For AcHis and Me_2His, due to the above-mentioned precipitation of $Zn(OH)_2$, the measurements and computations were confined to the pH range 2.5–8.5.

Fig. 3 and *4* show representative titration curves for the ligands cysteine ethyl ester and *N,N*(imidazole)-dimethylhistidine.

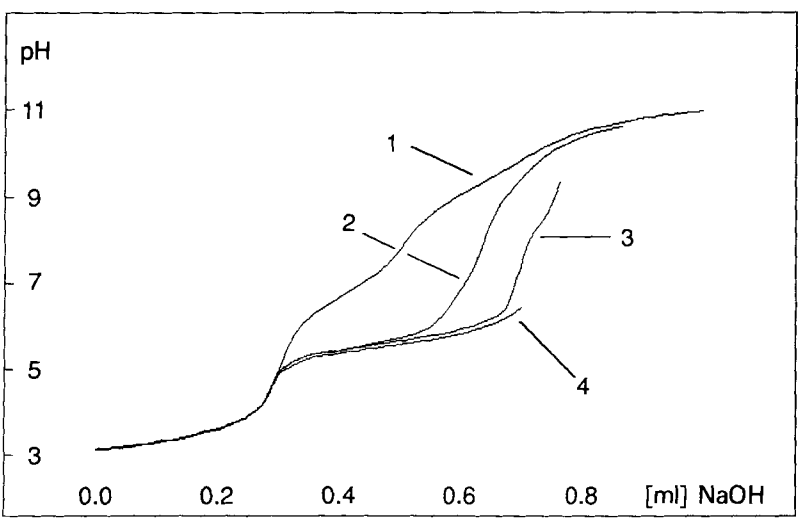

Fig. 3. *Potentiometric titration curves for cysteine ethyl ester* ($8 \cdot 10^{-4}$M) *in the presence of* $HClO_4$ ($1 \cdot 10^{-3}$M) *and* $NaClO_4$ ($I = 0.1$). *1*) In the absence of Zn^{2+}; *2–4*) in the presence of $Zn(ClO_4)_2$; *2*) $2.39 \cdot 10^{-4}$M, *3*) $4.78 \cdot 10^{-4}$M, *4*) $9.56 \cdot 10^{-4}$M).

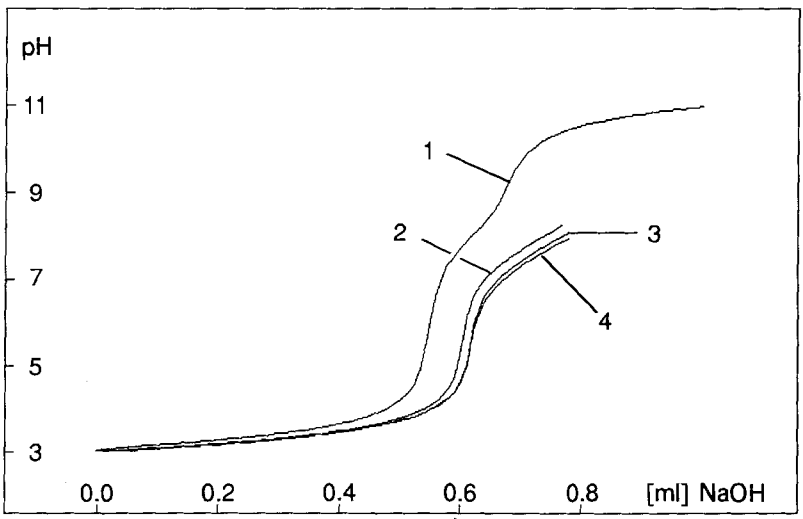

Fig. 4. *Potentiometric titration curves for* N,N*(imidazole)-dimethylhistidine* ($8 \cdot 10^{-4}$M) *in the presence of* HNO_3 ($1.56 \cdot 10^{-3}$M) *and* KNO_3 ($I = 0.1$). *1*) In the absence of Zn^{2+}; *2–4*) in the presence of $Zn(NO_3)_2$; *2*) $2.41 \cdot 10^{-4}$M, *3*) $4.80 \cdot 10^{-4}$M, *4*) $7.22 \cdot 10^{-4}$M).

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