On the metal ion binding properties of orotidine

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Abstract

The stability constants of the 1:1 complexes formed between orotidinate (Or^-) and Cu^{2+} or Zn^{2+} were determined by potentiometric pH titrations in solvent mixtures (vol./vol.) consisting of 70% 1,4-dioxane and 30% water at 25 °C and $I = 0.1$ M ($NaNO_3$). Orotidine is first deprotonated at the carboxylic acid residue in position 6 ($pK^H_{H(Or)} = 2.36$) and next at the H(N-3) site ($pK^H_{Or} = 10.67$). The latter site plays no role in the physiological pH range, i.e. the obvious site for metal ion coordination is the carboxylate group at position 6. For comparison we have studied therefore also the stabilities of the 1:1 complexes formed between Cu^{2+} or Zn^{2+} and the simple carboxylates (CA^-): $HCOO^-$, CH_3COO^-, CH_2ClCOO^-, $CHCl_2COO^-$ and CHF_2COO^-. Plots of log $K^M_{M(CA)}$ versus $pK^H_{H(CA)}$ indicated that the stability of the $M(Or)^+$ complexes is largely governed by the basicity of the corresponding carboxylate groups, but there are also indications that in the mentioned solvent mixture some (macro)chelate formation may occur, i.e. the carboxylate-coordinated metal ion is possibly interacting with a further site of the Or^- ligand (probably the 'ether' oxygen of the ribosyl residue which is accessible in the less favored *anti* conformation). The upper limit for the formation degree of such a chelate is 45%. It should be pointed out that in aqueous solution ($I = 0.1$ M, $NaNO_3$; 25 °C) the basicity of the carboxylate group is strongly reduced ($pK^H_{H(Or)} = 0.5 \pm 0.3$) and consequently the metal ion affinity of this group is also expected to be considerably smaller; indeed, estimations of the stability of the orotidinate complexes of Cu^{2+}, Zn^{2+} or Mg^{2+} confirm this expectation. Some relevant points following from the present results regarding biological systems are indicated.

Introduction

Orotidine and its derivatives play an important role as intermediates in the metabolism of pyrimidine-nucleotides [1], and these enzyme-dependent reactions also depend, at least in part, on the presence of metal ions [2]. So far only the coordinating properties of orotic acid have been considered somewhat [3, 4], but to our knowledge there is no detailed study available on the metal ion binding properties of orotidine. Therefore, we decided to tackle this problem in the course of our studies on nucleoside [5] and nucleotide complexes [6–8].

Orotidine and its derivatives exist in solution predominantly in the *syn* conformation [9–11], i.e. the carbonyl O-2 and N-3 are above the ribose ring [11, 12], while the carboxylate group at position 6 is pointing away (see Fig. 1)**. Considering that a carboxylate group, especially if solvated, is much larger than a carbonyl group, this *syn* conformation is actually the one to be expected.

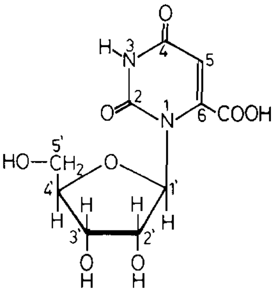

Fig. 1. Chemical structure of orotidine, H(Or), shown in its dominating *syn* conformation [9–11].

During the course of the present study it turned out that the basicity of the carboxylate group of orotidine is very low, but that this group still governs the metal ion coordinating properties of this nucleoside in the physiological pH range. N-3 is becoming accessible for metal ions only after depro-

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**Abbreviations: A, anion; CA^- and H(CA), carboxylate ligand and carboxylic acid, respectively; M^{2+}, divalent metal ion; Or^- and H(Or), orotidinate and orotidine, respectively.

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tonation of the H(N-3) site, and this reaction occurs only in the alkaline pH range.

Experimental

Materials

Orotidine (=3-β-D-ribofuranosyl orotic acid) was obtained from Sigma-Aldrich Co., St. Louis, MO, U.S.A., and difluoroacetic acid (98%) from Aldrich Chemie GmbH & Co. KG, Steinheim, F.R.G. Formic acid, acetic acid, chloroacetic acid and dichloroacetic acid (all *pro analysi*) were purchased from Fluka Chemie AG, Buchs, Switzerland. The disodium salt of ethylenediamine-*N,N,N',N'*-tetraacetic acid (Na_2H_2EDTA), potassium hydrogen phthalate, HNO_3, NaOH (Titrisol), the nitrate salts of Na^+, Cu^{2+} and Zn^{2+} (all *pro analysi*), and 1,4-dioxane (*reinst*) were from Merck AG, Darmstadt, F.R.G. All solutions were prepared with distilled CO_2-free water.

The exact concentrations of the solutions with orotidine or one of the carboxylic acids were determined by titrations with NaOH. Its titer was measured with potassium hydrogen phthalate, and the concentrations of the stock solutions of Cu^{2+} and Zn^{2+} were established with EDTA.

Potentiometric pH titrations

The pH titrations were carried out with a Metrohm E536 potentiograph, E655 dosimat and 6.0202.100 (JC) macro glass electrodes. The buffer solutions (pH 4.64, 7.00 and 9.00) used for calibration were also from Metrohm AG, Herisau, Switzerland; the buffers of this company are based on the scale of the U.S. National Bureau of Standards [13, 14]. The direct pH meter readings were used in the calculations of the acidity constants, i.e. these constants are so-called practical, 'mixed' or Brønsted constants. If so desired, the given negative logarithms of the mixed acidity constants for aqueous solutions (25 °C; $I=0.1$ M, $NaNO_3$) may be converted into the corresponding concentration constants by subtracting 0.02 log unit (H. Sigel *et al.*, results to be published); this conversion term, which contains the junction potential of the electrode and the activity coefficient of H^+ [15], is close to that given by others, e.g. 0.036 log unit (25 °C; $I=0.1$ M, KNO_3 [16]) or 0.039 log unit (25 °C; $I=0.5$ M, KCl [17]), but rather different from that often employed, i.e. 0.11 log unit (25 °C; $I=0.1$ M [18]). It should be noted that for stability constants of metal ion complexes no conversion is necessary.

The direct pH-meter readings were also used in the calculations for the acidity constants when 1,4-dioxane–water mixtures were employed as solvent. This means, no 'corrections' were applied for the change in solvent from water to the dioxane–water mixtures, though correction factors have been published [19].

It should be emphasized that in our calculation procedures the ionic product of water (K_w) and the hydrogen ion activity (γ) (to be more exact: the mentioned 'combined' term for converting the measured data into H^+ concentration) do *not* enter into the calculations, because we evaluate the *differences* in NaOH consumption between two corresponding solutions, i.e. always solutions with and without ligand are titrated (see below). The advantage of this procedure is (aside from not needing K_w or γ values) that impurities in the solvent or in the salts, as well as systematic errors, etc., cancel.

The calculations for the equilibrium constants were carried out with a Hewlett-Packard Vectra 60PC desk-computer connected with a Brother M-1509 printer and a Hewlett-Packard 7475A plotter.

Titrations in aqueous solutions

Due to the different acidities of the carboxylic acids somewhat different experimental conditions had to be employed, but the ionic strength (I) was always adjusted to 0.1 M by adding the necessary amount of $NaNO_3$. All titrations were carried out at 25 °C under N_2. In the case of orotidine three independent pairs of titrations were made and for the carboxylic acids at least six such titration pairs were performed and the results averaged. The details were as follows.

(i) The acidity constants $K^H_{H(Or)}$ and K^H_{Or} of orotidine were determined by titrating 10 ml of aqueous 0.0475 M HNO_3 (plus $NaNO_3$; $I=0.1$ M) in the presence and absence of 8 mM Or^- with 2.5 ml 0.2 M NaOH (i.e. the ionic strength varied slightly). The difference of such a pair of titrations was evaluated in the pH range 1.4–10.8 by a curve-fit procedure using a Newton-Gauss non-linear least-squares program and by taking into account the species H^+, H(Or), Or^- and $(Or\text{–}H)^{2-}$.

(ii) The acidity constants $K^H_{H(CA)}$ of difluoroacetic acid, dichloroacetic acid and chloroacetic acid were determined by titrating 25 ml of aqueous 0.0148 M HNO_3 (plus $NaNO_3$) in the presence and absence of 5.6 mM CA^- (this concentration was predetermined in 70% dioxane–water) with 2 ml 0.2 M NaOH. The differences of the titration pairs were evaluated in the pH range 2–3.5 for CHF_2COOH and $CHCl_2COOH$, and in the pH range 2.2–4.4 for $CH_2ClCOOH$, in the way indicated above by taking into account the species H^+, H(CA) and CA^-.

(iii) The acidity constant K^H_{HCOOH} of formic acid was determined by titrating 50 ml of solutions containing half of the reagent concentrations given in (ii) with 2 ml 0.2 M NaOH. The evaluation was carried out in the pH range 2.3–5.5.

(iv) The acidity constant $K^{H}_{CH_3COOH}$ of acetic acid was measured by titrating 50 ml of aqueous 0.9 mM HNO_3 (plus $NaNO_3$) in the presence and absence of 0.52 mM CH_3COO^- with 1 ml 0.05 M NaOH. The evaluation was carried out in the pH range 3.5 to 6.

Titrations in 70% (vol./vol.) dioxane–water mixtures

The acidity constants $K^{H}_{H(Or)}$ and K^{H}_{Or} of H(Or) and $K^{H}_{H(CA)}$ of H(CA) were determined by titrating 50 ml of 7.2 mM HNO_3 and $NaNO_3$ ($I=0.1$ M; 25 °C) in a solvent mixture consisting of 70% (vol./vol.) dioxane and 30% water in the presence and absence of 2.8 mM Or^- or CA^- under N_2 with 2.5 ml (in the case of Or^-) or 2 ml (in the case of CA^-) of 0.2 M NaOH and by using the differences in NaOH consumption between such pairs of titrations for the calculations. The acidity constants were calculated by the above mentioned curve-fit procedure within the pH range determined by the lowest point of neutralization reached by the experimental conditions (pH about 2.2) and about 97% neutralization for the equilibrium $H(CA)/CA^-$. In the case of Or^- the evaluation was carried on up to pH 12 to allow also the calculation of K^{H}_{Or} for the equilibrium $Or^-/(Or{-}H)^{2-}$. All the acidity constants given are the averages of at least six independent pairs of titrations.

The conditions for the determination of the stability constants $K^{M}_{M(Or)}$ or $K^{M}_{M(CA)}$ for the $M(Or)^+$ or $M(CA)^+$ complexes ($I=0.1$ M; 25 °C) were the same as given in the preceding paragraph for the acidity constants except that $NaNO_3$ was now replaced by 0.0333 M $Cu(NO_3)_2$ or $Zn(NO_3)_2$ (i.e. Or or $CA{:}M^{2+}=1{:}12$). The stability constants $K^{M}_{M(CA)}$ (or $K^{M}_{M(Or)}$) were computed for each pair of titrations by taking into account the species H^+, H(CA), CA^-, M^{2+} and $M(CA)^+$ [20]. Throughout, the data were collected (every 0.1 pH unit) from about 5% complex formation to a neutralization degree of about 85% or to the beginning of the hydrolysis of $M(aq)^{2+}$, which was evident from the titrations without ligand. The values calculated individually for $\log K^{M}_{M(CA)}$ or $\log K^{M}_{M(Or)}$ showed no dependence on pH. All stability constants given are the averaged results of at least five independent pairs of titrations.

Results and discussion

A view on the structure of orotidine in Fig. 1 immediately indicates that the important binding site for metal ions in this molecule is most likely the carboxylate group at position 6. Therefore, we included for reasons of comparison also the following simple carboxylic acids in our study: formic acid (HCOOH), acetic acid (CH_3COOH), chloroacetic acid ($CH_2ClCOOH$), dichloroacetic acid ($CHCl_2COOH$) and difluoroacetic acid (CHF_2COOH).

1. Definition of equilibrium constants

The mentioned carboxylic acids, H(CA), may be deprotonated according to equilibrium (1):

$$H(CA) \rightleftharpoons CA^- + H^+ \quad (1a)$$

$$K^{H}_{H(CA)} = [H^+][CA^-]/[H(CA)] \quad (1b)$$

Provided a large excess of metal ions, M^{2+}, over the concentration of the carboxylates is employed in the experiments, only the following complex forming reaction will be of significance:

$$M^{2+} + CA^- \rightleftharpoons M(CA)^+ \quad (2a)$$

$$K^{M}_{M(CA)} = [M(CA)^+]/([M^{2+}][CA^-]) \quad (2b)$$

It is evident that for orotidine, H(Or), exactly the same equilibria may be formulated, i.e. in eqns. (1) and (2) the symbol CA should simply be replaced by Or.

However, in the case of orotidine also a further deprotonation reaction has to be expected. This expectation is based on the experience with uridine and thymidine derivatives, which have a structure similar to that of orotidine (Fig. 1); indeed, decarboxylation of orotidine at position 6 gives uridine, and addition of a methyl group to uridine in position 5 results in thymidine. These two latter mentioned nucleosides may be deprotonated at the H(N-3) site in the alkaline pH region [12, 21]. Hence after deprotonation of orotidine according to equilibrium (1), the following reaction (3) may also occur.

$$Or^- \rightleftharpoons (Or{-}H)^{2-} + H^+ \quad (3a)$$

$$K^{H}_{Or} = [H^+][(Or{-}H)^{2-}]/[Or^-] \quad (3b)$$

After some preliminary experiments in aqueous solution it became immediately clear that H(Or) is very acetic (eqn. (1)) and also that the affinity of Or^- for metal ions (eqn. (2)) is rather low. As it is well known that solvents with a lower polarity than water considerably favor carboxylate-metal ion interactions [22–24], we decided to use 70% (vol./vol.) 1,4-dioxane–water mixtures in those experiments aimed to obtain an overview on the coordinating properties of orotidine.

2. Complex formation of orotidinate and carboxylates in 70% (vol./vol.) dioxane–water as solvent

The mole fraction of dioxane in a mixture (vol./vol.) of 70% 1,4-dioxane and 30% water corresponds to 0.331, and it has a dielectric constant, ϵ, of only 18.6 [25], i.e. a value considerably lower than that of water ($\epsilon = 78.5$). For this solvent mixture the acidity constants of H(Or) and H(CA) according to equilibria (1) and (3), as well as the stability constants of the corresponding $\mathrm{M(Or)^+}$ and $\mathrm{M(CA)^+}$ complexes according to equilibrium (2) with $\mathrm{Cu^{2+}}$ and $\mathrm{Zn^{2+}}$ were determined by potentiometric pH titrations at $I = 0.1$ M ($\mathrm{NaNO_3}$) and 25 °C. The results are summarized in Table 1.

The acidity constants obtained for the carboxylic acids are in the order expected from previous results [22, 23]; however, it is evident that H(Or) is the strongest among all these acids, i.e. the basicity of the carboxylate group of orotidinate is very low. On the other hand, ionization of the H(N-3) unit according to equilibrium (3) occurs with $\mathrm{p}K_\mathrm{a} = 10.67$ only in the alkaline pH range and hence, this site will play no role in the formation of metal ion complexes in the pH range studied here. Indeed, this pH range is below pH 5.5 for all metal ion complexes considered; naturally, the range used for the calculations of the stability constants varied with the ligands, as well as with the kind of metal ion.

To facilitate the evaluation of the equilibrium data, use is made of the well-known correlation between complex stability and ligating group basicity which is expected for structurally related ligands [26]. Therefore in Fig. 2 the values of log $K^{\mathrm{M}}_{\mathrm{M(CA)}}$ for the $\mathrm{Cu(CA)^+}$ and $\mathrm{Zn(CA)^+}$ complexes are plotted versus the negative logarithms of the acidity constants, $\mathrm{p}K^{\mathrm{H}}_{\mathrm{H(CA)}}$. It is immediately obvious, at least to a first approximation, that all data points fit on straight lines (broken lines in Fig. 2; see also Section 3) and that orotidinate largely behaves as a simple carboxylate ligand, i.e. the stability of the $\mathrm{Cu(Or)^+}$ and $\mathrm{Zn(Or)^+}$ complexes is clearly governed by the basicity of the carboxylate group. Should a further binding site be of importance, then this should be reflected [26] by a stability higher than expected on the basis of the $\mathrm{p}K^{\mathrm{H}}_{\mathrm{H(Or)}}$ value for $\mathrm{H(Or)^+}$. This means, in such a case the data points in Fig. 2 for $\mathrm{Cu(Or)^+}$ and $\mathrm{Zn(Or)^+}$ should be significantly above their corresponding reference lines. This is clearly not the case.

3. A more detailed appraisal of the stability of the orotidinate complexes in 70% (vol./vol.) dioxane–water as solvent

Calculation of the least-squares lines through all six data points available for each of the two metal ion systems (see Table 1) in the log $K^{\mathrm{M}}_{\mathrm{M(CA)}}$ versus $\mathrm{p}K^{\mathrm{H}}_{\mathrm{H(CA)}}$ plots results in the broken lines shown in Fig. 2. The corresponding straight-line equations are those given in relations (4) and (5) for the $\mathrm{Cu^{2+}}$ and $\mathrm{Zn^{2+}}$ systems, respectively (the error limits correspond to 1σ).

$$\log K^{\mathrm{Cu}}_{\mathrm{Cu(CA)}} = (0.426 \pm 0.019) \times \mathrm{p}K^{\mathrm{H}}_{\mathrm{H(CA)}} + (1.029 \pm 0.087) \tag{4}$$

$$\log K^{\mathrm{Zn}}_{\mathrm{Zn(CA)}} = (0.287 \pm 0.017) \times \mathrm{p}K^{\mathrm{H}}_{\mathrm{H(CA)}} + (0.915 \pm 0.077) \tag{5}$$

With the aid of eqns. (4) and (5) and the value for $\mathrm{p}K^{\mathrm{H}}_{\mathrm{H(Or)}}$ ($=2.36$) one may calculate that the stability of $\mathrm{Cu(Or)^+}$ is 0.10 log unit larger than expected on the basicity of the carboxylate group; the point for $\mathrm{Zn(Or)^+}$ is also 0.05 log unit above the reference line. These differences are certainly small, but still they are larger than the standard deviations (1σ) of all points of the reference lines, i.e. 0.026 log unit for the $\mathrm{Cu^{2+}}$ systems and 0.024 log unit for the $\mathrm{Zn^{2+}}$ system.

Indeed, a careful consideration of the data plotted in Fig. 2 indicates that the data points for ligands 1 through 4 fit for both metal ions more nicely on

TABLE 1. Negative logarithms of the acidity constants (eqns. (1) and (3)) of the protonated carboxylate ($\mathrm{CA^-}$) and orotidinate ($\mathrm{Or^-}$) ligands considered in this study and logarithms of the stability constants of some corresponding $\mathrm{M(CA)^+}$ and $\mathrm{M(Or)^+}$ complexes (eqn. (2)) as determined by potentiometric pH titrations in a solvent mixture (vol./vol.) consisting of 70% 1,4-dioxane and 30% water at 25 °C and $I \simeq 0.1$ M ($\mathrm{NaNO_3}$)[a]

No.	Ligand	$\mathrm{p}K^{\mathrm{H}}_{\mathrm{H(CA)}}$ or $\mathrm{p}K^{\mathrm{H}}_{\mathrm{H(Or)}}$	$\mathrm{p}K^{\mathrm{H}}_{\mathrm{Or}}$	log $K^{\mathrm{Cu}}_{\mathrm{Cu(CA)}}$ or log $K^{\mathrm{Cu}}_{\mathrm{Cu(Or)}}$	log $K^{\mathrm{Zn}}_{\mathrm{Zn(CA)}}$ or log $K^{\mathrm{Zn}}_{\mathrm{Zn(Or)}}$
1	$\mathrm{CH_3COO^-}$	6.68 ± 0.01		3.90 ± 0.03	2.89 ± 0.01
2	$\mathrm{HCOO^-}$	5.35 ± 0.02		3.32 ± 0.01	2.41 ± 0.01
3	$\mathrm{CH_2ClCOO^-}$	4.72 ± 0.01		3.01 ± 0.01	2.23 ± 0.01
4	$\mathrm{CHCl_2COO^-}$	3.25 ± 0.02		2.32 ± 0.04	1.78 ± 0.01
5	$\mathrm{CHF_2COO^-}$	3.10 ± 0.04		2.33 ± 0.02	1.85 ± 0.02
6	$\mathrm{Or^-}$	2.36 ± 0.05	10.67 ± 0.02	2.13 ± 0.05	1.64 ± 0.04

[a] The errors given are *three times* the standard error of the mean value or the sum of the probable systematic errors, whichever is larger.

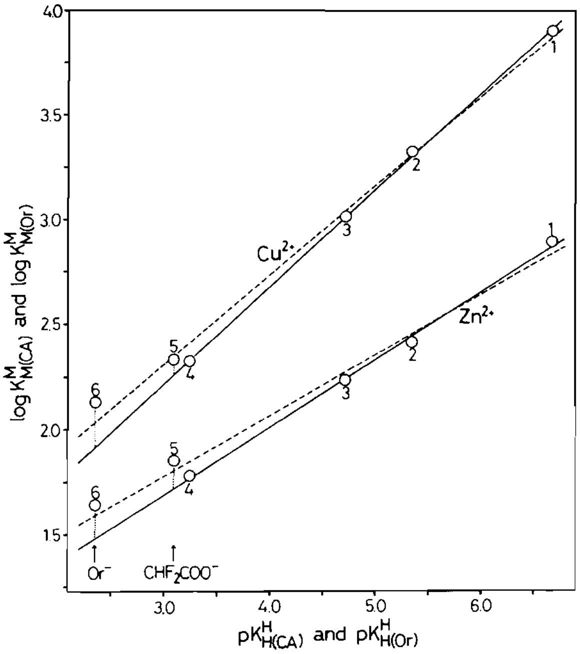


Fig. 2. Relationship between $\log K^{M}_{M(CA)}$ or $\log K^{M}_{M(Or)}$ and $pK^{H}_{H(CA)}$ or $pK^{H}_{H(Or)}$ for the $M(CA)^+$ and $M(Or)^+$ complexes of Cu^{2+} and Zn^{2+} in 70% (vol./vol.) dioxane–water at 25 °C and $I = 0.1$ M ($NaNO_3$); the plotted data are those of Table 1. The ligand systems are identified by the numbers used also in Table 1: CH_3COO^- (1), $HCOO^-$ (2), CH_2ClCOO^- (3), $CHCl_2COO^-$ (4), CHF_2COO^- (5) and Or^- (6). The solid least-squares lines are drawn each through the four data points (○) of the systems (1) through (4) (see eqns. (6) and (7)); the broken regression lines are drawn each through the data points of all six systems (see eqns. (4) and (5)). For the significance of the vertical dotted lines see the discussion in Section 3.

straight lines. Calculation of the corresponding least-squares regression lines leads to the solid lines shown in Fig. 2; the results of these calculations are summarized in eqns. (6) and (7).

$$\log K^{Cu}_{Cu(CA)} = (0.462 \pm 0.008) \times pK^{H}_{H(CA)} + (0.828 \pm 0.039) \qquad (6)$$

$$\log K^{Zn}_{Zn(CA)} = (0.322 \pm 0.012) \times pK^{H}_{H(CA)} + (0.718 \pm 0.061) \qquad (7)$$

The standard deviation (1σ) from the reference line of the four points considered in the calculation is now only 0.007 log unit for the Cu^{2+} systems and 0.012 log unit for the Zn^{2+} systems. In addition, the stability increase of $Cu(Or)^+$ and $Zn(Or)^+$ by 0.21 ± 0.05 and 0.16 ± 0.06 (3σ) log units, respectively, appears now as significant (see the vertical dotted lines in Fig. 2).

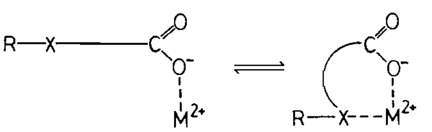
However, it has also to be emphasized that comparison of eqns. (4) with (6) and (5) with (7) shows that the terms of the corresponding straight lines agree within *two* standard deviations. Moreover, it is difficult to measure exact stability constants by potentiometric pH titrations for ligand systems with values for pK_a below about 3.5 due to the resulting small depression of the buffer region of the ligand. This fact could cast doubts on the validity of the constants determined for the $M(Or)^+$ complexes. These doubts could only have been overcome by including a ligand with a pK_a value close to that of H(Or) into the measurements and by showing that the data of this ligand fit exactly on the reference lines (eqns. (6) and (7)); unfortunately, such a carboxylic acid was not available to us.

Our attempts to solve this problem by including trihalogenated acetic acid derivatives into this study failed, because already the complexes of $Cu(CHF_2COO)^+$ and $Zn(CHF_2COO)^+$, are also somewhat more stable than expected on the basis of eqns. (6) and (7), i.e. by 0.07 ± 0.03 and 0.13 ± 0.04 (3σ) log units, respectively (see Fig. 2). These results for the difluoroacetate complexes could be explained by assuming the formation of a small fraction (i.e. below 30%; cf. ref. 26) of five-membered chelates involving the fluorine atom, as well as by the assumption that one of the metal ion-coordinated water molecules within $M(CHF_2COO)^+$ forms a hydrogen bond to one of the fluorine atoms leading to a seven-membered ring (see also below in Section 4). The indicated problem regarding the participation of halogen atoms in chelate formation has been addressed before [27–29] and to our knowledge has not yet definitely been solved for solvents containing water.

4. Some considerations on possible structures of orotidinate complexes in 70% (vol./vol.) dioxane–water as solvent

As it seems to us that the indicated, though small, increased stability of the $M(Or)^+$ complexes is most likely real (see Section 3 and Fig. 2), some further discussion on the possible origin of the increased stability is warranted. But still, in view of the difficulties outlined in the last two paragraphs of the preceding Section 3, one has also to note that the following should be considered as a 'limiting' evaluation, i.e. the formation degree of chelates in $M(Or)^+$ complexes could be as low as zero, but hardly larger than shown by the calculations below.

An increased complex stability of $M(Or)^+$ (or any other) complexes can arise only, if aside from the carboxylate group, a further site is at least partially participating in complex formation. This means, an intramolecular equilibrium of the following type has to be considered.



(8)

If the 'open' species in equilibrium (8) is designated as $M(Or)^+_{op}$ and the 'closed' one as $M(Or)^+_{cl}$, the dimensionless intramolecular equilibrium constant K_I can be defined according to eqn. (9) and calculated with eqn. (10) (for details see ref. 26 or 30).

$$K_I = [M(Or)^+_{cl}]/[M(Or)^+_{op}] \tag{9}$$

$$K_I = \frac{K^M_{M(Or)}}{K^M_{M(Or)_{op}}} - 1 = 10^{\log \Delta} - 1 \tag{10}$$

The difference $\log \Delta = \log K^M_{M(Or)} - \log K^M_{M(Or)_{op}}$ corresponds to the increased stability of 0.21 (±0.05) attributed in the preceding Section 3 to $Cu(Or)^+$ and of 0.16 (±0.06) log unit for $Zn(Or)^+$. From these data and eqns. (9) and (10) follow the percentages of the closed species [30], i.e. 38±7% for $Cu(Or)^+_{cl}$ and 31±10% for $Zn(Or)^+_{cl}$; the limiting values for the maximal formation degree of (macro)chelates are <45% for $Cu(Or)^+_{cl}$ and <40% for $Zn(Or)^+_{cl}$.

The most likely candidate for the backbinding of a carboxylate-coordinated M^{2+} to another binding atom of orotidinate (eqn. (8)) appears to be the 'ether' oxygen of the ribosyl ring (see Fig. 1). This oxygen atom could be reached in a seven-membered chelate by a carboxylate-coordinated metal ion provided orotidinate is turned into the less stable *anti* conformation (see 'Introduction'). As the energy barrier between the *syn* and *anti* conformations can be considerable [31], this structural change does not appear as very favorable, which would be in agreement with the low formation degree of the closed species. Indeed, into this described picture fits well the estimated [11] population of 14% for the *anti* conformation of orotidinate in aqueous solution.

5. Acidity constants of H(Or) and the H(CA) species in aqueous solution, and relation between the acidity constants valid in water and in 70% (vol./vol.) dioxane–water

The acidity constants of O ligands are clearly dependent upon the polarity of the solvent [13, 32]: the basicity of O ligands (with a negative charge) increases markedly as the dielectric constant decreases [22, 23, 32]. Indeed, as indicated in the final paragraph of Section 1, this was the reason why in the studies described so far, i.e. in Sections 1 to 4, 70% (vol./vol.) dioxane–water mixtures were used as solvent.

To develop at least a feeling for the situation in water the acidity constants $K^H_{H(CA)}$ (eqn. (1)), of acetic acid, formic acid and the halogenoacetic acid derivatives used previously were also measured by potentiometric pH titrations in this solvent (Table 2 [33]). The same experiments were carried out with orotidine. However, as only the pH range 1.4 to 10.8 could be evaluated (see 'Experimental') the formation degree of the species H(Or) was very low and consequently only a rough estimate for the corresponding acidity constant could be obtained: $pK^H_{H(Or)} = 0.7 \pm 0.3$.

Therefore we attempted also a different approach. The acidity constants listed in Table 2 for the carboxylic acids allow construction of a plot of $pK^H_{H(CA)}$ values valid for aqueous solution versus $pK^H_{H(CA)}$ values valid for solvent mixtures (vol./vol.) consisting of 70% dioxane and 30% water. From Fig. 3 it is evident that the available five data pairs fit well on a straight line; the corresponding straight-line equation is given in relation (11) (the error limits correspond to 1σ).

$$pK^H_{H(CA)/H_2O} = (1.013 \pm 0.052) \times pK^H_{H(CA)/70\%Diox} - (2.063 \pm 0.250) \tag{11}$$

The standard deviation (1σ) of the five data points from the reference line is 0.060 log units. With $pK^H_{H(Or)} = 2.36$ (±0.05) for 70% (vol./vol.) dioxane–

TABLE 2. Comparison of the negative logarithms of the acidity constants (eqn. (1)) of some carboxylic acids and of orotidine (Fig.1) as determined by potentiometric pH titrations in aqueous solutions and in a solvent mixture (vol./vol.) consisting of 70% 1,4-dioxane and 30% water at 25 °C and $I \approx 0.1$ M ($NaNO_3$). The value for the deprotonation of the H(N-3) unit of orotidinate (Or^-) is also given (entry No. 7; eqn. (3))[a]

No.	Acid	$pK^H_{H(CA)}$ or $pK^H_{H(Or)}$ for the acids in	
		Water	70% Dioxane[b] (vol./vol.)
1	CH_3COOH	4.57 ± 0.01[c]	6.68 ± 0.01
2	HCOOH	3.58 ± 0.01[c]	5.35 ± 0.02
3	$CH_2ClCOOH$	2.71 ± 0.02	4.72 ± 0.01
4	$CHCl_2COOH$	1.22 ± 0.08	3.25 ± 0.02
5	CHF_2COOH	1.01 ± 0.05	3.10 ± 0.04
6	H(Or)	0.5 ± 0.3[d]	2.36 ± 0.05
7	Or^-	9.12 ± 0.02[e]	10.67 ± 0.02[e]

[a] The errors given are *three times* the standard error of the mean value or the sum of the probable systematic errors, whichever is larger. [b] These constants are from Table 1. [c] These values are from ref. 33; they agree within the error limits with the determinations carried out during this study. [d] For the determination of this value see text in Section 5. [e] These values refer to pK^H_{Or}, i.e. the deprotonation of the H(N-3) unit, and they correspond to eqn. (3).

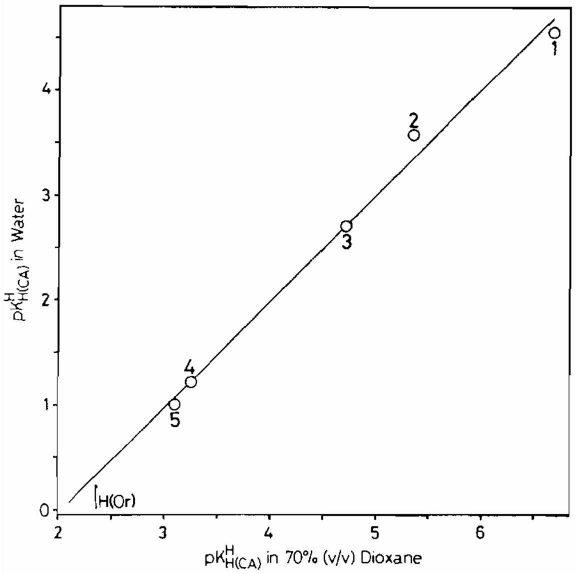


Fig. 3. Relationship between $pK^H_{H(CA)}$ in water and $pK^H_{H(CA)}$ in 70% (vol./vol.) dioxane–water for the carboxylic acids of Table 2 at 25 °C and $I = 0.1$ M ($NaNO_3$). The acids are identified by the numbers used also in Table 2: CH_3COOH (1), HCOOH (2), $CH_2ClCOOH$ (3), $CHCl_2COOH$ (4) and CHF_2COOH (5); the least-squares line through these five data points gives the result summarized in eqn. (11). The intercept of the vertical arrow in the lower left hand corner of the figure ($= pK^H_{H(Or)}$ in 70% dioxane) with the regression line gives $pK^H_{H(Or)} = 0.33 \pm 0.06$ (1σ) for the deprotonation of orotidine in water (see also text in Section 5).

water and eqn. (11) one obtains the acidity constant for H(Or) in water: $pK^H_{H(Or)} = 0.3 \pm 0.3$ (see also legend for Fig. 3).

Evidently the two values obtained for $pK^H_{H(Or)}$ of orotidine in water agree within their error limits. As both results are estimations, we conclude that the best value presently available is their average, i.e. $pK^H_{H(Or)} = 0.5 \pm 0.3$, and this value with its estimated error limit is listed in Table 2.

It is very interesting to note that the acidity constants measured in the two mentioned solvents for the deprotonation of the H(N-3) site of orotidinate fit also on the relation shown in Fig. 3. We have therefore plotted all the acidity constants listed in Table 2 as shown in Fig. 4; the resulting straight-line equation is summarized in relation (12).

$$pK^H_{HA/H_2O} = (1.045 \pm 0.025) \times pK^H_{HA/70\%Diox} - (2.152 \pm 0.143) \qquad (12)$$

The standard deviation (1σ) from the reference line of the seven data points is 0.059 log unit and the correlation coefficient $R = 0.999$. All terms in eqns. (11) and (12) agree within *one* standard deviation.

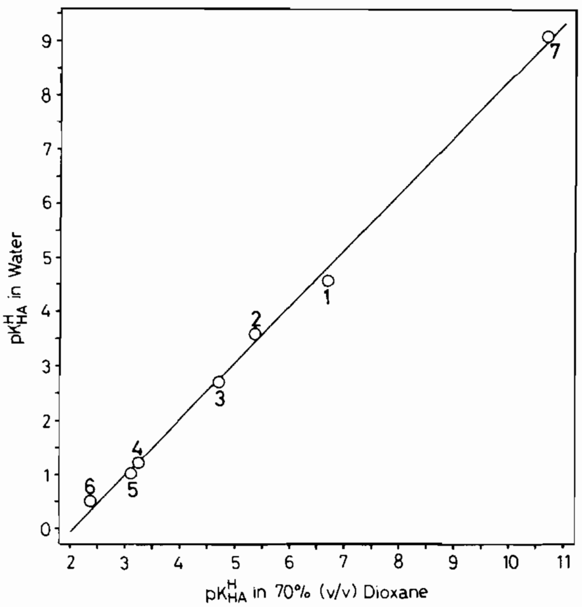


Fig. 4. Relationship between pK^H_{HA} in water and pK^H_{HA} in 70% (vol./vol.) dioxane–water for all the seven acids listed in Table 2, i.e. $pK^H_{HA} = pK^H_{H(CA)}$, $pK^H_{H(Or)}$ and pK^H_{Or} ($I = 0.1$ M, $NaNO_3$; 25 °C). The acids are identified by the numbers used also in Table 2: CH_3COOH (1), HCOOH (2), $CH_2ClCOOH$ (3), $CHCl_2COOH$ (4), CHF_2COOH (5), H(Or) (6) and Or^- (7); the least-squares line through these seven data points gives the result summarized in eqn. (12). The average standard deviation for the vertical distance of the points from the straight line corresponds to 0.06 log units.

It is remarkable that the straight line of Fig. 4 covers nearly 10 log units on both pK_a scales and the deprotonation of $-COOH$ and $=NH$ groups is influenced to the same extent by a change in the polarity of the solvent. This indicates that the kind of atom to which the proton is bound is of minor importance; the more important point appears to be that in all these cases the proton is released from an uncharged group leading to an anionic site.

Though not in the focus of this study, it may be pointed out that eqn. (12) offers the possibility to calculate for an acid from its known acidity constant valid for one solvent the corresponding acidity constant for the other solvent. For example, this procedure may be of interest for all pK_a values of the H(N-1) site of inosine and guanosine or the H(N-3) site of uridine and thymidine. Moreover, it is evident that more such relations may be constructed for other solvent mixtures as well.

6. Estimation of the stability of the Cu^{2+}, Zn^{2+} and Mg^{2+} complexes of orotidinate in aqueous solution

Knowledge of the acidity constant for the carboxylic acid side chain of orotidine (Fig. 1), i.e. $pK^H_{H(Or)} = 0.5 \pm 0.3$, should now also allow an estimation of the stability of $M(Or)^+$ complexes as defined in eqn. (2). It should be recalled at this point that it is the basicity of the carboxylate group which (largely) determines the stability of the $M(Or)^+$ species in 70% (vol./vol.) dioxane–water (see Sections 2 to 4 and Fig. 2), and in the presence of an increased water activity, as it applies to an aqueous solution, this will be even more true. Hence, the construction of $\log K^{M}_{M(CA)}$ versus $pK^{H}_{H(CA)}$ plots for carboxylic acid systems in an aqueous solution should here be of help [26]. It should be pointed out that estimation of the stability for some $M(Or)^+$ complexes in aqueous solution is highly desirable because direct experimental data will not be easily obtained.

The relationship between $\log K^{M}_{M(CA)}$ and $pK^{H}_{H(CA)}$ for a number of carboxylate complexes of Cu^{2+} and Zn^{2+} in aqueous solution is shown in Fig. 5 [34–42]. The corresponding straight-line equations are those given in relations (13) and (14) for the Cu^{2+} and Zn^{2+} systems, respectively (the error limits correspond to 1σ).

$$\log K^{Cu}_{Cu(CA)} = (0.169 \pm 0.027) \times pK^{H}_{H(CA)} + (1.024 \pm 0.118) \qquad (13)$$

$$\log K^{Zn}_{Zn(CA)} = (0.024 \pm 0.034) \times pK^{H}_{H(CA)} + (0.933 \pm 0.149) \qquad (14)$$

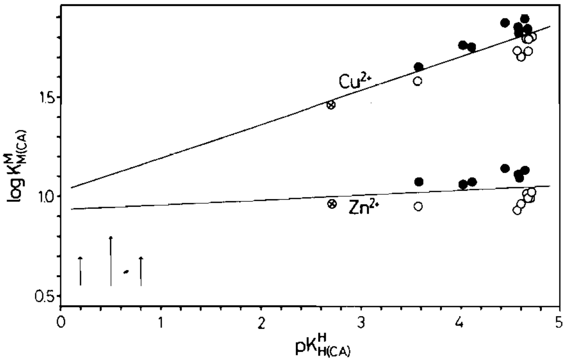


Fig. 5. Relationship between $\log K^{M}_{M(CA)}$ and $pK^{H}_{H(CA)}$ for carboxylate complexes of Cu^{2+} and Zn^{2+} in aqueous solution at 25 °C and $I = 0.1$ M ($NaNO_3$), with a data-set of phenylalkanecarboxylates including $HCOO^-$ and CH_3COO^- (●; the constants are from Table I in ref. 34; see also Table S1 in ref. 23), a data-set of isoalkanecarboxylates also including $HCOO^-$ and CH_3COO^- (○; these constants are from Table I in ref. 33), and the constants for the corresponding CH_2ClCOO^- systems (⊗; $pK^{H}_{H(CA)} = 2.71 \pm 0.02$, from Table 2; $\log K^{Cu}_{Cu(CA)} = 1.46 \pm 0.13$, average of the constants given in refs. 35–38, see also refs. 39 and 40; and $\log K^{Zn}_{Zn(CA)} = 0.96$ from ref. 41 as given in ref. 42). The solid least-squares lines are drawn through all the available data for the Cu^{2+} and Zn^{2+} systems; the corresponding results are summarized in eqns. (13) and (14), respectively. The three vertical arrows in the lower left hand corner of the figure represent the positions of $pK^{H}_{H(Or)} = 0.5 \pm 0.3$ (from Table 2); see also the discussion in Section 6.

With these straight-line equations and $pK^{H}_{H(Or)} = 0.5 \pm 0.3$ (see lower left hand corner in Fig. 5) one obtains for the stability of the Cu^{2+} and Zn^{2+} complexes of orotidinate (by taking into account the error limit of ±0.3 log unit in the pK_a value) the following (preliminary) results.

$$\log K^{Cu}_{Cu(Or)} = 1.11 \pm 0.05$$

$$\log K^{Zn}_{Zn(Or)} = 0.95 \pm 0.01$$

Another kind of relationship is shown in Fig. 6 where the logarithms of the stability constants of $M(CA)^+$ complexes, $\log K^{M}_{M(CA)}$, in aqueous solution are plotted versus the corresponding data valid for 70% (vol./vol.) dioxane–water mixtures as solvent. This kind of evaluation is not completely independent of the one applied in Fig. 5, because a linear relationship between $\log K^{M}_{M(CA)}$ and $pK^{H}_{H(CA)}$ is obtained for aqueous solutions (Fig. 5) and also for 70% (vol./vol.) dioxane–water as solvent (see Fig. 2), there has to exist also a linear dependence between the log $K^{M}_{M(CA)}$ values valid for the two mentioned solvents. The advantage of this additional evaluation is that in practice experimental data different from those in Fig. 5 have to be applied and therefore a rather independent estimation for $\log K^{M}_{M(CA)}$ in water is achieved. The data pairs for the Cu^{2+} and Zn^{2+} complexes on which Fig. 6 is based are listed in Table 3. The resulting straight-line equations are summarized in expressions (15) and (16).

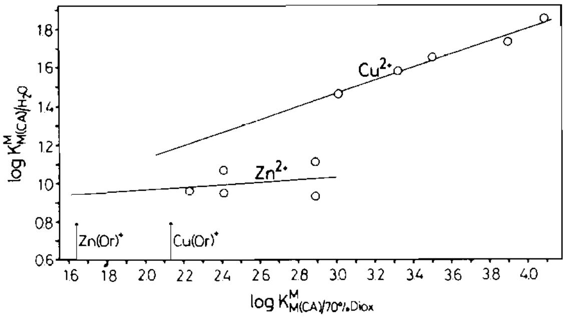


Fig. 6. Relationship between $\log K^{M}_{M(CA)}$ for carboxylate complexes of Cu^{2+} and Zn^{2+} in water and in 70% (vol./vol.) dioxane–water (25 °C; $I = 0.1$ M). The plotted stability constants are those listed in Table 3. The solid least-squares lines correspond to the straight-line eqns. (15) and (16). The two vertical arrows in the lower left hand corner of the Figure represent the positions of $\log K^{Zn}_{Zn(Or)} = 1.64$ and $\log K^{Cu}_{Cu(Or)} = 2.13$ (from Table 1); see also the discussion in Section 6.

TABLE 3. List of pairwise stability constants, log $K^{M}_{M(CA)}$, of some $M(CA)^+$ complexes valid for aqueous solution and for a solvent mixture (vol./vol.) consisting of 70% dioxane and 30% water at $I=0.1$ M and 25 °C

System	log $K^{M}_{M(CA)}$ for		Source or reference
	Water	70% Dioxane	
Cu^{2+}/CH_3COO^-	1.85 ± 0.05	4.09 ± 0.01	22/22
Cu^{2+}/CH_3COO^-	1.73 ± 0.04	3.90 ± 0.03	33/Table 1
$Cu^{2+}/HCOO^-$	1.65 ± 0.09	3.50 ± 0.01	22/22
$Cu^{2+}/HCOO^-$	1.58 ± 0.04	3.32 ± 0.01	33/Table 1
Cu^{2+}/CH_2ClCOO^-	1.46 ± 0.13	3.01 ± 0.01	[a]/Table 1
Zn^{2+}/CH_3COO^-	1.11 ± 0.02	2.89 ± 0.01	22/Table 1
Zn^{2+}/CH_3COO^-	0.93 ± 0.03	2.89 ± 0.01	33/Table 1
$Zn^{2+}/HCOO^-$	1.07 ± 0.05	2.41 ± 0.01	22/Table 1
$Zn^{2+}/HCOO^-$	0.95 ± 0.03	2.41 ± 0.01	33/Table 1
Zn^{2+}/CH_2ClCOO^-	0.96	2.23 ± 0.04	[b]/Table 1

[a]Average of the constants given in refs. 35–38; see also ref. 39. [b]From ref. 41 as given in ref. 42.

$$\log K^{Cu}_{Cu(CA)/H_2O} = (0.335 \pm 0.031) \times \log K^{Cu}_{Cu(CA)/70\%Diox} + (0.461 \pm 0.110) \qquad (15)$$

$$\log K^{Zn}_{Zn(CA)/H_2O} = (0.063 \pm 0.148) \times \log K^{Zn}_{Zn(CA)/70\%Diox} + (0.843 \pm 0.382) \qquad (16)$$

With these expressions and log $K^{Cu}_{Cu(Or)/70\%Diox} = 2.13 \pm 0.05$ or log $K^{Zn}_{Zn(Or)} = 1.64 \pm 0.04$ (see Table 1) one obtains the following (also preliminary) results for the situation in water.

$$\log K^{Cu}_{Cu(Or)/H_2O} = 1.17 \pm 0.02$$

$$\log K^{Zn}_{Zn(Or)/H_2O} = 0.95 \pm 0.01$$

It is comforting to see that the two preliminary results obtained each for the stability of the $Cu(Or)^+$ and $Zn(Or)^+$ complexes agree so well. Our final estimates, based on the given data, for the stabilities of the mentioned complexes are listed in Table 4, together with their also estimated error limits.

TABLE 4. Summary of the estimated logarithms of the equilibrium constants for the reactions between orotidinate and H^+ or several M^{2+} in aqueous solutions with an ionic strength (*I*) close to 0.1 M and a temperature close to 25 °C

Equilibrium	log $K^{H}_{H(Or)}$ or log $K^{M}_{M(Or)}$
$H^+ + Or^- \rightleftharpoons H(Or)$	0.5 ± 0.3[a]
$Cu^{2+} + Or^- \rightleftharpoons Cu(Or)^+$	1.15 ± 0.2[b]
$Zn^{2+} + Or^- \rightleftharpoons Zn(Or)^+$	0.95 ± 0.2[b]
$Mg^{2+} + Or^- \rightleftharpoons Mg(Or)^+$	−0.1 ± 0.4[c]

[a]See Section 5 and Table 2. [b]Average of the two values given in the text of Section 6 and estimated error limit. [c]See the last paragraph in Section 6.

Considering that Mg^{2+} is a very important metal ion for enzymic systems involving nucleotides we endeavored to estimate also a value for the stability of $Mg(Or)^+$ in aqueous solution. Not many stability constants for $Mg(CA)^+$ complexes in water are available, but some information exists; for each of the following three carboxylate systems the first value refers to p$K^{H}_{H(CA)}$ (from Table 2) and the second one to log $K^{Mg}_{Mg(CA)}$: CH_2ClCOO^- (2.71/0.23 [38, 40]), $HCOO^-$ (3.58/0.34 [38, 40]) and CH_3COO^- (4.57/0.47 [38, 40], 0.51 [39, 43] or 0.55 [44]). These are in total five data pairs allowing the construction of a log $K^{Mg}_{Mg(CA)}$ versus p$K^{H}_{H(CA)}$ plot (which is analogous to those shown in Fig. 5); the resulting straight-line eqn. (17) contains the following terms.

$$\log K^{Mg}_{Mg(CA)} = (0.154 \pm 0.020) \times pK^{H}_{H(CA)} - (0.195 \pm 0.083) \qquad (17)$$

Together with p$K^{H}_{H(Or)} = 0.5 \pm 0.3$ (Table 2) one obtains for the stability of $Mg(Or)^+$ log $K^{Mg}_{Mg(Or)} = -0.12 \pm 0.05$; considering that this estimate is based only on few experimental data and only on a single evaluation method the actual error range may be rather large, and this is expressed in the final result listed in Table 4.

Some general conclusions

The estimations given for the stability of the orotidinate complexes with Mg^{2+}, Cu^{2+} and Zn^{2+} in aqueous solution are of relevance not only for principle reasons, but they should also be helpful in evaluating the structure of the complexes formed between orotidinate 5′-monophosphate (OMP^{3-}) and various divalent metal ions [45]. The main question for such $M(OMP)^-$ complexes will be: is the stability of these species simply determined by the basicity of the phosphate group or is the carboxylate residue also involved in complex formation?

Another important result of the presented analysis on the stability and structure of the $M(Or)^+$ complexes is that the main binding site of orotidinate is clearly the carboxylate in position 6 (see Sections 2 to 4); this group definitely dominates the stability of the $Cu(Or)^+$ and $Zn(Or)^+$ complexes. If some (macro)chelate formation occurs (eqn. (8)), then the formation degree of such a backbound species is relatively small: the upper limit being a formation degree of 45% (Section 4). It may further be added that the likelihood of such a chelate formation increases with a decreasing water concentration in the solvent mixture; for an aqueous solution itself it is expected that hardly any $M(Or)^+$ complexes exist in a chelated form.

A further point which warrants emphasis is that under conditions with a reduced solvent polarity the basicity of the carboxylate groups increases and therefore also their affinity for metal ions (Tables 2 and 3). That a reduced water activity will also favor the participation of other (polar) binding sites in metal ion coordination is evident. These facts should be taken into account in considerations focusing on the properties of active-site cavities of enzymes. For example, the enzyme carboxypeptidase A uses Zn^{2+} and the carboxylate group of Glu-270 in the catalyzed hydrolysis of peptides [46–48]; this carboxylate group shows an 'abnormally high' basicity [48], i.e. for the corresponding carboxylic acid residue holds $pK_a = 6.1$ [49]. It is evident (see Table 2) that this high basicity is simply the result of the reduced polarity, i.e. of a relatively small so-called 'effective or equivalent solution' dielectric constant, present in the active-site cavity of carboxypeptidase A [23, 24]*.

Acknowledgements

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*It may be pointed out that the value given in ref. 23 for the equivalent solution dielectric constant should be considered today rather as an upper limit, because it is now known [46, 50, 51] that only the *second* ligand binds to carboxypeptidase A at the intrinsic Zn^{2+}; in other words, the dielectric constant in the active-site cavity of carboxypeptidase A is below 70.

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