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Interaction between the low molecular mass components of blood serum and the VO(IV)–DHP system (DHP = 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone)

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In order to estimate the impact of the low molecular mass (l.m.m.) VO(IV) binders of blood serum on the potentially insulin-enhancing drug $[VO(DHP)_2]$ [DHP = 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone], the speciation in the binary system VO(IV)–DHP and in the ternary systems VO–DHP–ligand B (B = oxalate, lactate, citrate or phosphate) was studied by pH-potentiometry at 25.0 °C and at an ionic strength $I = 0.2$ mol dm^{-3} (KCl). The binding modes of the complexes formed were determined by spectroscopic (electronic absorption and EPR) techniques. DHP was found to form stable mono and bis complexes *via* the coordination of (O,O) chelate(s). Through displacement of the oxo group of VO(IV), the tris complex is also formed, especially at a high excess of ligand. The results in the ternary systems demonstrate that, at physiological pH, none of the B ligands can compete with DHP; $[VO(DHP)_2]$ therefore seems to remain almost completely intact, even in the presence of citrate, the strongest competitor among these B ligands. These findings indicate that, for DHP, unlike maltol or picolinic acid, ternary complex formation and thus transformation reactions with the l.m.m. binders of biofluids, is almost negligible. From among the three carrier molecules, only DHP can efficiently compete with serum transferrin for binding of VO(IV).

Introduction

Vanadium is a beneficial trace element in numerous biological systems. It has been known since the early 1980s that vanadium compounds display insulin-enhancing features both *in vitro* and *in vivo*;[1,2] great efforts have therefore been made to prepare vanadium(IV) complexes with high biological activity, low toxicity and good absorption properties.[3]

Bismaltolato-oxovanadium(IV)[4] is one of the most promising compounds exhibiting the properties above. Substitution of the O of the pyrone ring in maltol by N–R yields pyridinone derivatives. These ligands have very similar chelating capabilities to those of maltol, but by changing the side-chain on the nitrogen in the ring, their hydrophilic/hydrophobic balance, and thus their *in vivo* transport properties, may be finely tuned.[5] 1,2-Dimethyl-3-hydroxy-4(1*H*)-pyridinone (DHP) (see Scheme 1), also called deferiprone, is one of the best-known pyridinone ligands; it is currently used in the treatment of thalassaemia (iron-overload).

The insulin-enhancing properties of $[VO(DHP)_2]$ were tested *in vitro* by Sakurai *et al.*,[6] who found that $[VO(DHP)_2]$ is effective in terms of free fatty acid release from isolated rat adipocytes and has a significantly better insulin-mimetic activity than $VOSO_4$.

The complex $[VO(DHP)_2]$ has been characterised in the solid state.[7] Spectroscopic (VIS, EPR and EXAFS) evidence indicates that the bis complex is five coordinate and has square-pyramidal geometry.

DHP forms stable complexes with many divalent and trivalent metal ions,[8] including V(V), in solution.[9] VO(IV) has been found to readily form various mono and bis complexes $[VOA]^+$, $[VOH_{-1}A]$† and $[VOA_2]$, the bis complex being predominant in the pH range 4–8.[10,11] By means of simultaneous potentiometric and spectrophotometric titrations, Taylor[11] detected and characterised the formation of a minor non-oxo species $[VA_3]$. Under aerobic conditions, VO(IV) complexes of DHP were oxidised slowly but completely to the violet–blue V(V) species.[11]

After the oral administration of $[VO(DHP)_2]$, it may interact with many other potential VO(IV) binders present in the extra- or intracellular biofluids. These latter molecules may displace DHP partially or completely from the coordination sphere of the metal ion. Accordingly, the possibility of the formation of ternary complexes has to be considered in the speciation characterisation of $[VO(DHP)_2]$ in biological fluids. Such ternary complexes might be of great importance in the adsorption and transport processes of $[VO(DHP)_2]$, and even in its physiological activity.

The VO(IV)-binding abilities of the low molecular mass (l.m.m.) components (ligand B) of blood serum, such as oxalate

† A negative stoichiometric number for H in the formula indicates a proton, which dissociates only from the complex but neither from the ligand nor from the metal aqua ion separately. This proton can liberate either from a water molecule in the coordination sphere of the metal ion bound by ligand(s), or from a very weakly acidic group of the bound ligand, such as the alcoholic-OH group of citric acid.

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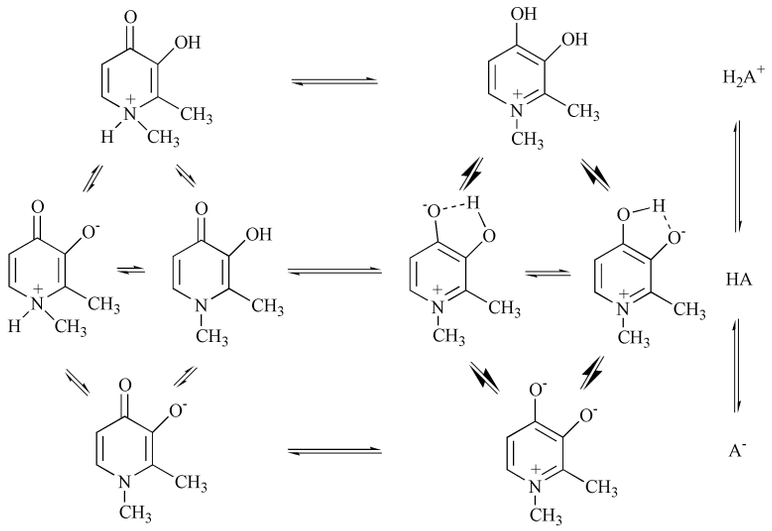

Scheme 1

(B^{2-}), lactate (B^{-}), citrate (B^{3-}) or monophosphate (B^{3-}), have been the subject of several studies.[12–16]

Due to the low proton competition at the carboxylic groups, oxalate binds VO(IV) strongly in the acidic pH range, yielding [VOB] and $[VOB_2]^{2-}$. In the neutral or weakly basic pH range, dinuclear mixed hydroxo species are also formed.[12,16]

Lactate forms the complexes $[VOB]^{+}$ and $[VOB_2]$ below pH ≈ 5, with involvement of the carboxylate and the protonated alcoholic hydroxyl group. After deprotonation and coordination of the alcoholate-O^{-}, depending upon the metal-to-ligand ratio and pH, $[VOH_{-1}B]$, $[VOH_{-1}B_2]$ and $[VOH_{-2}B_2]^{2-}$ are present in solution.[13]

Citrate prefers to form dinuclear complexes with VO(IV), which predominate up to pH ≈ 8. In these species, citrate acts as a bridging ligand with tridentate coordination *via* two carboxylates and the alcoholate group.[14]

Phosphate is a rather weak VO(IV) binder, with formation of a precipitate in the pH range 4–8.5. Below pH ≈ 4, protonated 1 : 1 species are present in solution; in the basic pH range, $[VOB]^{-}$, $[(VO)_2H_{-2}B_2]^{4-}$ and $[VOH_{-2}B]^{3-}$ can be detected, besides the binary hydroxo complexes of the metal ion.[15,17]

To the best of our knowledge, no data have been published on ternary complex formation in the VO–DHP (ligand A) system.

The goal of the present work was to study the impact of the l.m.m. VO(IV) binders of blood serum on the potential drug $[VO(DHP)_2]$, which exhibits insulin-enhancing properties. Accordingly, the solution equilibria and structures of the VO(IV) complexes formed either in the binary system with DHP or in the ternary systems with DHP and oxalate, lactate, citrate or phosphate have been investigated. pH potentiometry was used to determine the stoichiometries and stability constants of the species formed, and spectroscopic (electronic absorption and EPR) measurements were made to establish the most probable structures of the complexes present in solution. (The stability data obtained were used to model the speciation of VO(IV) under the conditions existing in blood serum.)

Experimental

General

Elemental analysis (C, H, N) was carried out with a Perkin-Elmer 240 B elemental analyser. Thermogravimetric data were obtained with a Perkin-Elmer TGS-2 apparatus under a nitrogen flow. ^{1}H NMR measurements were performed on a Bruker AM 360 instrument in D_2O. Melting points are uncorrected.

Reagents

All ligands except DHP were Aldrich or Fluka products of puriss. quality and were used as received. Their purities were checked and the exact concentrations of the prepared stock solutions were determined by the Gran method.[18] A VO(IV) stock solution was prepared according to Nagypál and Fábián[12] and standardised for metal ion concentration by permanganate titration and for hydrogen ion concentration by pH potentiometry, using the appropriate Gran function.

3-Hydroxy-1,2-dimethyl-4(1*H*)-pyridinone (DHP). DHP was synthesised by slightly modified literature methods.[19–21] Methylammonium chloride (13.0 g, 193 mmol) was dissolved in water (15 ml) and chilled. To this, KOH (10.89 g, 194 mmol) dissolved in water (50 ml) was added at 0 °C. To a suspension of maltol (5.0 g, 39.7 mmol) in water (60 ml) at 50 °C, the methylamine solution was added dropwise under nitrogen over 4 h. The reaction mixture was further stirred and kept at 50 °C for 6 h. The solution was concentrated *in vacuo* (50 ml) and left to stand in a refrigerator overnight. The crude product obtained was filtered and recrystallised twice from hot methanol using charcoal. Yield: 1.39 g (25%). Mp.: 263–265 °C (dec.) [ref. 19: 260 °C (dec.)]. ^{1}H NMR (360 MHz, D_2O): δ 7.60 (1 H, d, ^{6}H, $J_{5,6}$ 7.0 Hz), 6.47 (1 H, d, ^{5}H, $J_{5,6}$ 7.0 Hz), 3.75 (3 H, s, $^1CH_3$), 2.38 (3 H, s, $^2CH_3$).

$[VO(DHP)_2]$. DHP (234 mg, 1.68 mmol) was suspended in water (3 ml) and heated at 50–60 °C while argon was bubbled through the solution. VO(IV) solution (4 ml, c = 0.205 mol dm^{-3}) and solid NaOH (67 mg, 1.68 mmol) were added. A deep-blue microcrystalline solid formed from the green solution upon chilling. It was filtered off, washed with water and acetone and dried *in vacuo*. Yield: 242 mg, 86%. Found: C, 48.72; H, 4.31; N, 7.99; V, 14.5; calc. for $C_{14}H_{16}N_2O_5V$: C, 48.99; H, 4.70; N, 8.16; V, 14.84%. IR: 980 cm^{-1} (ν_{VO}). Thermogravimetric analysis demonstrated the absence of water and a residue of V_2O_5 (calc. 26.5; found 25.8%).

Potentiometric measurements

The stability constants of the proton and VO(IV) complexes of the ligands were determined by pH-potentiometric titration of 25.0 ml samples. The ionic strength was adjusted to 0.20 M KCl in each solution studied. In all cases, the temperature was 25.0 ± 0.1 °C. The DHP concentration was in the range 2–6 mM and the molar ratios in the VO(IV)–DHP system were 0 : 1, 1 : 1, 1 : 2 and 1 : 4. For the ternary systems, the ratios of metal ion, DHP and the B ligand were 1 : 1 : 1, 1 : 1 : 2, 1 : 2 : 1 and 1 : 2 : 2. All

the titrations were performed over the range pH 2–11, or until precipitation occurred, with a carbonate-free KOH solution of known concentration (~0.2 mol dm^{-3}), under a purified argon atmosphere. The reproducibility of the titration points included in the evaluation was within 0.005 pH units throughout the whole pH range.

pH was measured with a Radiometer pHM 84 instrument equipped with a Metrohm combined electrode (type 6.0234.110), calibrated for hydrogen ion concentration according to Irving *et al.*[22] A pK_w value of 13.76 ± 0.01 was determined and used for the calculations. The concentration stability constants $\beta_{pqr} = [M_pH_qA_r]/[M]^p[H]^q[A]^r$ were calculated with the aid of the PSEQUAD computer program.[23] The uncertainties (σ values) in the stability constants are given in parentheses in the Tables.

During the calculations, the following VO(IV)–hydroxo complexes were assumed: $[VO(OH)]^+$ (log $\beta_{1-10} = -5.94$) and $[\{VO(OH)\}_2]^{2+}$ (log $\beta_{2-20} = -6.95$) calculated from the data published by Henry *et al.*,[24] the Davies equation being used to take into consideration the different ionic strengths, $[VO(OH)_3]^-$ (log $\beta_{1-30} = -18.0$) and $[(VO)_2(OH)_5]^-$ (log $\beta_{2-50} = -22.5$) with data taken from ref. 25.

Spectroscopic measurements

Anisotropic X-band EPR spectra (9.15 GHz) were recorded at 140 K in aqueous solutions, using a Varian E-9 instrument. As usual, the samples for low temperature measurements contained a few drops of DMSO to ensure good glass formation in frozen solutions.

Absorption spectra were recorded with a Hewlett-Packard HP 8453 spectrophotometer. All manipulations and titrations were performed under an atmosphere of purified argon. Aqueous solutions of the VO(IV)–DHP–B ligand systems at ratios of 1 : 1 : 0, 1 : 2 : 0, 1 : 1 : 1 and 1 : 2 : 2, and a VO(IV) concentration of 0.004 mol dm^{-3} (practically the same as for the pH-metric speciation studies) were studied over the pH range 2–11 by EPR and VIS spectroscopy. The stability constant of the tris complex formed in the VO(IV)–DHP system was also determined from VIS absorbance data measured in the wavelength range 350–800 nm at different pH values with the aid of the PSEQUAD[23] computer program. The $c_{VO(IV)}$ was 0.0005 mol dm^{-3} and the metal ion to ligand ratio was 1 : 10 or 1 : 20.

Results and discussion

Protonation processes of DHP

The stability constants calculated for the proton complexes of DHP are listed in Table 1.

When the differences in the experimental conditions are taken into consideration, the log K values determined for the proton complexes are in good agreement with the published data.[5,11] Comparison with maltol (log $K = 8.44$)[16] shows that the log K_{HA} value for DHP relates to the negatively charged oxygen on the pyridinone ring. The other protonation may take place either on the ring N or on the other oxygen on the pyridinone ring, yielding a highly delocalised π electron system on $[H_2A]^+$ (see Scheme 1).

The results of ^{1}H NMR titration of the ligand in the pH range 2.2–5.6 reveal that the chemical shifts relating to the ring hydrogens in positions 5 and 6 change the most (Fig. 1).

The very slight change in the chemical shifts of the methyl protons strongly suggest that the protonation of [HA] takes place mainly on the O rather than on the ring N. The ring proton in position 5 (closest to the O site assumed to be protonated) displays the largest chemical shift in the protonation pH range, suggesting changes at the O site, but the significant ring electron delocalisation makes this interpretation uncertain.

Table 1 Stability constants of proton (log K) and oxovanadium(IV) (log β) complexes of DHP at 25.0 °C and at $I = 0.20$ mol dm^{-3} (KCl) (standard deviations are in parentheses)

Complex	log K/log β
log K (HA)	9.76(1)
log K (H_2A)	3.70(1)
$[VOA]^+$	12.18(1)
$[VOA_2]$	22.83(2)
$[VOH_{-1}A_2]^-$	12.24(3)
$[VOH_2A_3]^+ = [VA_3]^+$	38.5(1)
$[(VO)_2H_{-2}A_2]^{2-}$	16.43(8)
Fitting[a]	0.00861
No. of points	96
log K_{VOA_2}	10.65
log (K_{VOA}/K_{VOA_2})	1.53
$[VO]^{2+} + HA = [VOA]^+ + H^+$	2.42
$[VOA]^+ + HA = [VOA_2] + H^+$	0.89
$[VOA_2] + H_2A^+ = [VOH_2A_3]^+$	2.2(1)[b]

[a] The average difference between the calculated and experimental titration curves expressed in cm^3 of the titrant. [b] A value of 2.01(8) can be obtained from spectrophotometric titrations.

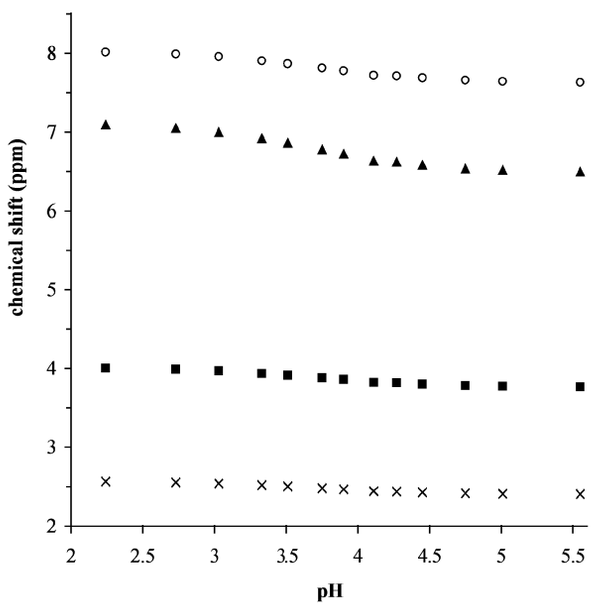

Fig. 1 ^{1}H NMR chemical shifts of the *N*-methyl hydrogens (■), the methyl hydrogens in position 2 (×) and the ring hydrogens in positions 5 (▲) and 6 (○) of DHP in the pH range 2.2–5.6.

VO(IV)–DHP system

The potentiometric titrations indicated a strong metal ion–ligand interaction: a metal-to-ligand ratio as low as 1 : 1.5 was high enough to prevent hydrolysis of the metal ion and thus precipitate formation. The data in Table 1 and the speciation curves in Fig. 2 indicate a complexation scheme involving the formation of mono, bis and tris V(IV) complexes. The spectral parameters of the complexes formed in the system are presented in Table 2.

It is seen in Fig. 2 that at pH ≈ 2, more than half of the metal ion is already complexed in the form of $[VOA]^+$ ($g_{||} = 1.939$; $A_{||} = 170 \times 10^{-4}$ cm^{-1}). The predicted $A_{||}$ value is in accordance with the equatorial (O,O) coordination of the ligand. In contrast with the maltolate system,[16] the mono-chelated species here is clearly detectable by EPR (**I** in Fig. 3). At a 1 : 1 ratio, the assumption of a dimer with stoichiometry $[(VO)_2H_{-2}A_2]^{2-}$ improved the fit significantly. This species can be rationalised in terms of the deprotonation of a coordinated water molecule and dimerisation of the resulting $[VOA(OH)]^-$. This EPR-silent complex is formed at around pH 5.0; with increasing pH, precipitation occurred at a 1 : 1 ratio. At a metal-to-ligand ratio of

Table 2 Spectral parameters and chelating sets for oxovanadium(IV) complexes formed in the VO(IV)–DHP (A) binary and the VO(IV)–DHP–ligand B ternary systems

Species	$g_{\|\|}$	$10^4A_{\|\|}/cm^{-1}$	Chelating sets
VO(IV)–DHP binary system			
$[VOA]^+$	1.939	170	(O^-, O^-)
$[VOA_2]$ (*trans*)	1.950	157	(O^-, O^-); (O^-, O^-); H_2O_{ax}
$[VOA_2]$ (*cis*)	1.940	166	(O^-, O^-); (O^-, O^-_{ax}); H_2O_{eq}
$[VOH_{-1}A_2]^-$ (*cis*)	1.942	164	(O^-, O^-); (O^-, O^-_{ax}); OH^-_{eq}
VO(IV)–DHP–oxalic acid ternary system			
$[VOAB]^-$ (*trans*)	1.942	167	(O^-, O^-); (COO^-, COO^-)
VO(IV)–DHP–lactic acid ternary system			
[VOAB] (*trans*)	1.944	167	(O^-, O^-); (COO^-, OH)
VO(IV)–DHP–citric acid ternary system			
$[VOHAB]^-$	1.944	166	(O^-, O^-); (COO^-, OH, COO^-_{ax})
$[VOAB]^{2-}$	1.944	166	(O^-, O^-); (COO^-, OH, COO^-_{ax})
$[VOH_{-1}AB]^{3-}$	1.947	159	(O^-, O^-); $(COO^-, O^-, COO^-_{ax.})$ and (O^-, O^-); $(COO^-, COO^-_{ax})OH^-_{eq}$
VO(IV)–DHP–phosphoric acid ternary system			
$[VOH_2AB]$	1.940	169	(O^-, O^-); $(H_2PO_4^-)$; H_2O_{ekv}

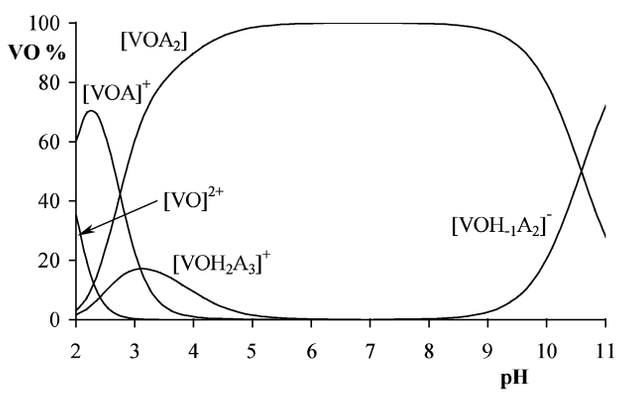

Fig. 2 Speciation curves of the complexes formed in the VO(IV)–DHP system at a 1 : 4 metal-to-ligand ratio. $c_{VO(IV)} = 0.001$ mol dm^{-3}, $I = 0.2$ mol dm^{-3} and $T = 25$ °C.

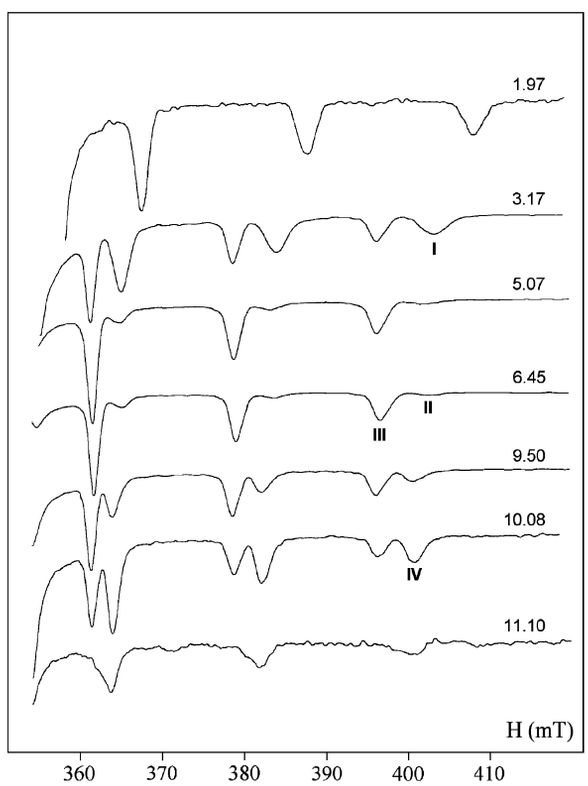

Fig. 3 High-field parallel region of the EPR spectra recorded at 140 K of aqueous solutions of the VO(IV)–DHP system at a 1 : 2 metal ion-to-ligand ratio, as a function of pH. $c_{VO(IV)} = 0.004$ mol dm^{-3}.

1 : 2 or at higher ligand excess, the bis complex $[VOA_2]$ is the predominant species in the pH range 4–10. The EPR spectra (Fig. 3) indicate one major species ($g_{||} = 1.950$; $A_{||} = 157 \times 10^{-4}$ cm^{-1}, **III** in Fig. 3) and a minor one ($g_{||} = 1.940$; $A_{||} = 166 \times 10^{-4}$ cm^{-1}, **II** in Fig. 3): the ratio of these two complexes remains the same in the pH range 4–9.

The measured EPR data above suggest that the major bis-chelated species is in the *trans* form. In solution, the complex is six coordinate,[16] the two ligand molecules occupy the four equatorial sites and there is a water molecule in the sixth apical [*trans* to the oxo group of VO(IV)] position. The EPR parameters of this complex are similar to those of the bis-chelated species formed by catechol ($g_{||} = 1.947$; $A_{||} = 154 \times 10^{-4}$ cm^{-1}).[26] In the case of catechol, all four O atoms are deprotonated and the similarity of the spectral parameters of the two complexes means that DHP is a strong ligand and that the resonance form of the ligand in which the two O atoms are negatively charged is important. The minor EPR signal present in this pH range should belong to the *cis* isomer of the $[VOA_2]$ complex in which a water molecule, in the *cis* position with respect to the V=O bond, is coordinated in the equatorial plane and one of the two ligands has an axial-equatorial coordination mode. Its EPR parameters are $g_{||} = 1.940$ and $A_{||} = 166 \times 10^{-4}$ cm^{-1} (see Table 2). As was found earlier for the 5-membered ring chelation of VO(IV) by (O,O)-donor ligands,[26] the increased basicity of the ligands makes the *trans* coordination mode more preferred in the complex $[VOA_2]$. The high value of the deprotonation constant of $[VOA_2]$ ($pK_{VOA_2} = 10.59$) indicates[26] that the proton is released from a water molecule weakly bound in the axial position. The measured EPR parameters of $[VOH_{-1}A_2]^-$ ($g_{||} = 1.942$; $A_{||} = 164 \times 10^{-4}$ cm^{-1}, **IV** in Fig. 3), which differ from those of the *trans* isomer **III** of $[VOA_2]$ (*vide supra*) however, strongly suggest the *cis* geometry of this species, with an OH^- occupying an equatorial position. These observations can be explained by a structural rearrangement of the *trans*-$[VOA_2]$ to form $[VOH_{-1}A_2]^-$ with a *cis* configuration during the deprotonation process.

The complex $[VO(DHP)_2]$ precipitates from aqueous solutions containing VO(IV) concentrations higher than 0.05 mol dm^{-3}. Thermogravimetric analysis of the solid compound

indicates the absence of water coordinated to vanadyl ion. The EPR spectrum of the solid dissolved in a weakly coordinating solvent such as DMF shows only the presence of the *trans* isomer with *x*,*y* anisotropy ($g_x = 1.988$, $g_y = 1.977$ and $A_x = 50 \times 10^{-4}$ cm^{-1}, $A_y = 55 \times 10^{-4}$ cm^{-1}). The values $g_z = 1.950$ and $A_z = 157 \times 10^{-4}$ cm^{-1} are observed, coincident with those measured in aqueous solution. If the solid is dissolved in water, a small amount of *cis*-[$VO(H_2O)A_2$] is also observed.

The intense deep-blue colour of the samples in the pH range 2.0–4.5, especially at larger ligand excess, strongly suggests the formation of a tris complex with non-oxo 3 × (O,O)-type coordination, as previously found for catecholates.[27] At around pH 4.5, tris complex formation has a direct pH effect; complexation is enhanced with decreasing pH, according to the reaction $VOA_2 + HA + H^+ = VA_3^+ + H_2O$. This gives an opportunity to calculate the stability constant of this species directly from the potentiometric data. At below pH 4, the free ligand starts to protonate and therefore the tris complex formation process no longer has a pH effect. Furthermore, this process is restricted to a relatively narrow pH range, due to the amount of [VOA_2] decreasing as the solution becomes more acidic. Since the components involved in the speciation calculations are VO^{2+}, H^+ and A^-, the tris complex was defined as [VOH_2A_3]$^+$ ([VA_3]$^+$·H_2O), as in previous cases.[27–29]

The formation of the tris complex was also monitored by spectrophotometric titration of samples with different V(IV)–DHP ratios (see Experimental). Treatment of the data obtained from visible spectra with PSEQUAD[23] results in log β_{123} = 38.30(2) for the tris complex, which is in good agreement with the constant found from pH potentiometry [38.5(1)]. The log *K* value of 2.2 calculated for the process of formation of [VOH_2A_3]$^+$ ([VA_3]$^+$·H_2O) from [VOA_2] (see Table 1) lies in the range observed for various catechol derivatives (tiron: 2.03; dopamine: 2.2, D,L-epinephrine: 2.3, D,L-norepinephrine: 2.2, L-Dopa: 2.5).[27,29] The striking difference in ability to form tris complexes between DHP and maltol may be attributed to the delocalised structure of deprotonated DHP and to the more pronounced basicity of the deprotonated ligand form.

The EPR spectra of aqueous solutions containing VO(IV) and DHP with a ligand to metal molar ratio of 25 or 50 indicated formation of the tris-chelated species; this was always found to be present, under these experimental conditions, together with the bis-chelated complex. The spectrum of the pure tris-chelated species can be obtained by dissolving the solid complex [VOA_2] in concentrated acetic acid containing an excess of ligand. The same procedure was used previously[30] and was explained by considering the need for an extra proton to form a water molecule (*vide supra*).

The experimental and simulated EPR spectra are shown in Fig. 4.

The EPR spectrum of the tris-chelated species (the characteristic parameters are $g_x = 1.917$, $g_y = 1.920$, $g_z = 1.985$ and $A_x = 120 \times 10^{-4}$ cm^{-1}, $A_y = 103 \times 10^{-4}$ cm^{-1} and $A_z = 10 \times 10^{-4}$ cm^{-1}) is indicative of a d_{z^2} ground state because $g_z \sim g_e > g_x$, g_y and $A_z \ll A_x$, A_y. This is the case for hexacoordinated non-oxo complexes of V(IV) with geometry distorted towards the trigonal prismatic as a consequence of the steric requirements of the ligand molecules.[31] In this spectrum, the A_z value is too low to be resolved, it does not affect the simulated spectrum if its value remains below 10×10^{-4} cm^{-1} (A_z could be measured only by an examination of the isotropic spectrum recorded at room temperature, through the relationship $A_z = 3A_0 - A_x - A_y$. In our case, the spectrum at room temperature is not completely isotropic and it can not be used to calculate the g_z and A_z values). *x*,*y* anisotropy can be measured, as demonstrated by an $A_x - A_y$ value of 17×10^{-4} cm^{-1}. This effect can be attributed to a splitting of the $d_{x^2-y^2}$ and d_{xy} orbitals, which should have the same energy for regular geometry, between that of a trigonal prism and that of an octahedron.

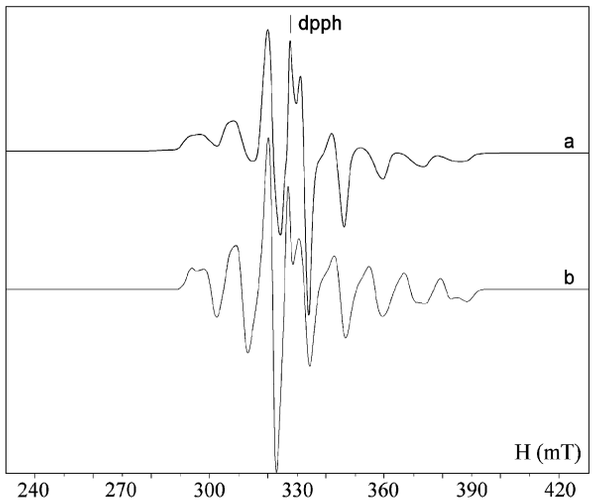

Fig. 4 Experimental (a) and simulated (b) anisotropic EPR spectrum of tris-chelated complex [$V(DHP)_3$]$^+$. The simulated spectrum was obtained with the computer program Bruker WINEPR SimFonia.

Comparison of the strength of the VO(IV) binding to DHP with that to maltol indicates that DHP is more effective. This is not only due to the more basic character of the donor groups of DHP. The calculated constants for the proton displacement reactions of DHP (2.42 and 0.89, see Table 1) are about two orders of magnitude higher than those of the corresponding processes for maltol (0.25 and −0.84, respectively).[16] Interestingly, we have found no evidence for the formation of any protonated complexes in the VO(IV)–DHP system, although their formation range would be below the pH range for [HA] protonation. This supports the assumption that in the [H_2A]$^+$ form of the free ligand the second proton is mainly situated on the O rather than on the N, and thus the [HA] form of the ligand may exist as a zwitterion with an internal H-bond between the two O atoms (see Scheme 1).

Ternary systems

As a consequence of their high affinities for hard metal ions, oxalate, citrate, lactate and phosphate are the most probable l.m.m. binders for VO(IV) in blood serum. Their concentrations in the serum are as follows: oxalate ~ 0.01 mmol dm^{-3}, citrate ~ 0.1 mmol dm^{-3}, lactate ~ 1.51 mmol dm^{-3} and phosphate ~ 1.10 mmol dm^{-3}.[32] The stability data and spectral parameters obtained for these ternary systems are listed in Tables 2 and 3. The protonation and VO(IV) complex formation constants for the corresponding VO(IV)–B ligand systems were taken from earlier literature reports[13–16] on work carried out under the same conditions.

Oxalate. The potentiometric titration curves obtained for the VO(IV)–DHP–oxalate system could be fitted with the assumption of a single new complex: [VOAB]$^-$. The formation of this species is favoured in the pH range 3–7 and is predominant at a 1 : 1 : 1 ratio. However, as may be seen from the species distribution curves depicted in Fig. 5 at a 1 : 2 : 2 ratio, at pH > 5, the DHP complexes predominate.

The slightly favoured formation of the mixed complex is reflected by the derived equilibrium constants relating to the reaction $VOA^+ + B^{2-} = VOAB^-$ (log $K = 5.33$), which indicates that the binding of an oxalate (B) to the complex [VOA]$^+$ of DHP is not significantly more favoured than that to its own binary complex (log $K_{VOB_2} = 5.03$).[16] This is the case for the binding of DHP (A) to [VOB] (log $K = 10.86$) as compared to binding to [VOA]$^+$ (log $K_{VOA_2} = 10.65$). The EPR parameters confirm the formation of a new species, [VOAB]$^-$, in this pH range ($g_{||} = 1.942$; $A_{||} = 167 \times 10^{-4}$ cm^{-1}). The very similar donor atom sets in the equatorial plane imply that basically the same

Table 3 Stability constants of mixed ligand VO(IV) complexes of DHP with some B ligands at 25.0 °C and at $I = 0.20$ mol dm^{-3} (KCl) (standard deviations are in parentheses)

Complex	Oxalate (B^{2-})	Lactate (B^{-})	Citrate (B^{3-})	Phosphate (B^{3-})
$[VOABH_2]$	—	—	—	32.33(3)
[VOABH]	—	—	22.28(3)	—
[VOAB]	17.60(3)	14.62(3)	18.66(2)	—
$[VOABH_{-1}]$	—	—	10.25(3)	—
Fitting[a]	0.0132	0.0101	0.0123	0.00975
No. of points	232	214	260	160

[a] The average difference between the calculated and experimental titration curves expressed in cm^3 of the titrant.

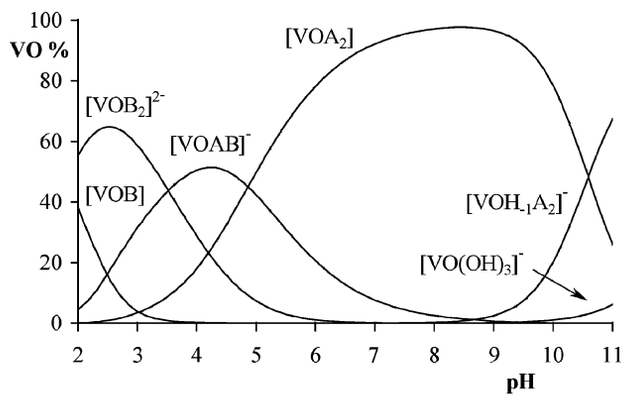

Fig. 5 Speciation curves of the complexes formed in the VO(IV)–DHP (A)–oxalate (B) system at a 1 : 2 : 2 metal-to-ligand ratio. $c_{VO(IV)} = 0.001$ mol dm^{-3}, $I = 0.2$ mol dm^{-3} and $T = 25$ °C.

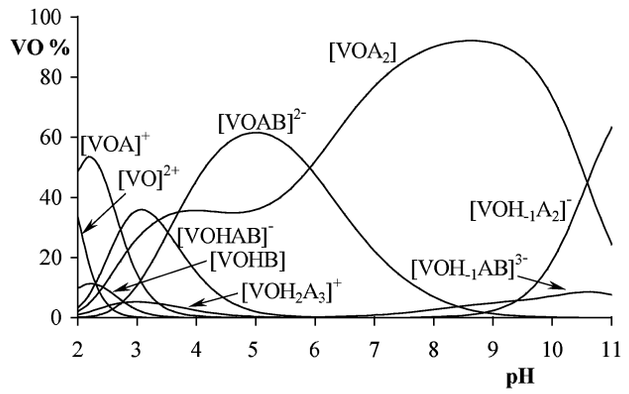

Fig. 6 Speciation curves of the complexes formed in the VO(IV)–DHP (A)–citrate (B) system at a 1 : 2 : 2 metal-to-ligand ratio. $c_{VO(IV)} = 0.001$ mol dm^{-3}, $I = 0.2$ mol dm^{-3} and $T = 25$ °C.

EPR parameters can be expected for the *cis* and *trans* arrangements in the ternary complex. However, it is probable that there is a *trans* arrangement in the ternary species, since DHP forms almost exclusively *trans*-$[VOA_2]$ in the binary system. Above pH ≈ 7 and at a 1 : 1 : 1 ratio, the species $[VOA_2]$ is formed in parallel with oligonuclear hydroxo complexes; the partial hydrolysis of the metal ion is reflected in the decreased intensities of the EPR signals. At a 1 : 2 : 2 ratio, however, the bis complexes of DHP, $[VOA_2]$ and $[VOH_{-1}A_2]^-$, predominate.

Lactate. The potentiometric data on this system could be fitted by the assumption of a ternary complex [VOAB], which is formed in the pH range 3–4.5, with a maximum of 15% VO(IV) at pH 3.5. With increasing DHP concentration, the pH range of its formation is shifted in the more acidic direction and it becomes even less important. The EPR spectra may indicate a new species ([VOAB]) formed in parallel with the binary complex $[VOA]^+$ in this pH range, with parameters $g_{||} = 1.944$ and $A_{||} = 167 \times 10^{-4}$ cm^{-1}. The $A_{||}$ value is the same as found for the ternary complex with oxalate, which can be explained by the similar equatorial coordination set of the mixed complexes formed with oxalate (O^-, O^-); (COO^-, COO^-) and with lactate (O^-, O^-); (COO^-, OH). The substitution of a carboxylate group by a protonated alcoholic-OH group should not change the EPR parameters very much. Neither potentiometry nor spectroscopy support the formation of $[VOH_{-1}AB]^-$ with deprotonation and coordination of the hydroxyl group of the lactate. This is probably due to the significantly higher metal ion-binding strength of DHP at acidic pH, as reflected in the fact that at a ratio of 1 : 2 : 2, similarly as for the oxalate system, the bis complexes of DHP become the predominant species, *i.e.* lactate is not an efficient competitor at an excess of DHP.

Citrate. In general, citrate behaves as a tridentate ligand and is regarded as one of the most important l.m.m. binders, mainly for hard or borderline metal ions in the plasma. Potentiometry indicates that citrate prefers ternary complex formation much more strongly than do oxalic acid or lactic acid. The titration data can be fitted well with $[VOHAB]^-$ and $[VOAB]^{2-}$. The assumption of $[VOH_{-1}AB]^{3-}$ improved the fit slightly. As illustrated in Fig. 6, the ternary complexes predominate in the pH range 3–6. As DHP can bind to VO(IV) exclusively through the (O^-, O^-) donor set, only a change in the binding mode of citric acid can alter the composition of the mixed ligand complexes $[VOH_nAB]^{(2-n)-}$.

The EPR spectra of the VO–DHP–citrate system at a ratio of 1 : 1 : 1 reveal a new complex in the pH range 3–8, in addition to [VOHB] [carboxylate-bound VO(IV)–citrate species ($g_{||} = 1.937$; $A_{||} = 176 \times 10^{-4}$ cm^{-1}) [14]]. This new species, with spectral parameters of $g_{||} = 1.944$ and $A_{||} = 166 \times 10^{-4}$ cm^{-1}, is observed in the pH range where both $[VOHAB]^-$ and $[VOAB]^{2-}$ are indicated by potentiometry. Thus, it is reasonable that these two species have the same spectral features and that one transforms into the other *via* the simple deprotonation of a non-coordinating carboxylic group. The $A_{||}$ values measured for these species are again very similar to that of the mixed complex of lactate, suggesting the same coordination in the equatorial plane: (O^-, O^-)(COO^-, OH). Since citrate behaves as a tridentate ligand, the coordinated COO^- should be the one on the central carbon atom of the ligand, yielding a 5 + 6-membered joined chelate system with the protonated hydroxyl group of the citrate in the equatorial plane and one of the terminal COO^- groups should occupy the weak axial position. The other possible binding mode, with coordination by one of the terminal COO^- groups in the equatorial plane, would yield a 6-membered chelate with somewhat lower stability. (Concerning the numerous stability data reported in the literature for VO(IV) complexes, due to the size of the metal ion and their typical geometry, 5-membered chelate rings are usually more stable than 6-membered ones.) The pK of VOABH (3.60) is consistent with the dissociation of the other non-coordinated terminal carboxylic function, as it is comparable with those of the corresponding ternary maltolato (3.44 [16]), picolinato (3.63 [33]) and 6-methylpicolinato (3.14 [33]) ternary species with the coordination mode outlined above. At pH > 8, EPR and potentiometry indicate the formation of a new species, $[VOH_{-1}AB]^{3-}$. This complex may be formed either by deprotonation of the alcoholic-OH of the coordinated citrate yielding an equatorial (COO^-, O^-) binding mode, or

through partial displacement of citrate by an OH^-. Both donor atom sets would result in a very similar $A_{||}$ value in the EPR spectrum and, accordingly, they can not be distinguished in this way. The hindered formation of the species $[VOH_{-1}AB]^{3-}$ (Fig. 6) as compared to the corresponding ternary maltolato or picolinato complex (where the tridentate binding of citrate with a deprotonated alcoholate group has been proved)[15,33] and the occurrence of the EPR-silent binary VO(IV) dihydroxo-bridged species at 1 : 1 : 1 ratio at pH > 10.5, indicated by a decrease in the intensity of the EPR signals, however, suggest the formation of a mixed hydroxo species with equatorial (COO^-, OH^-) coordination, and the co-existence of the above two binding isomers of $[VOH_{-1}AB]^{3-}$ is therefore very probable.

Phosphate. As inorganic phosphate is the l.m.m. serum component with the second highest concentration,[32] it may have a significant impact on the transport properties of insulin-mimetic vanadium complexes. Although precipitation occurs in the VO(IV)–monophosphate system at pH ≈ 4,[15] two equivalents of DHP with respect to VO(IV) made the ternary system titratable in the whole pH range. To fit the potentiometric data, various chemically reasonable mixed ligand species with stoichiometries $[VOH_xAB]^{(2-n)-}$ and $[VOH_xA_2B]^{(3-n)-}$ were tested. pH potentiometry supports only the existence of $[VOH_2AB]$ in the pH range 2–4, with the parallel formation of $[VOA]^+$ and $[VOH_2B]^+$ at a ratio of 1 : 2 : 2 (see Fig. 7).

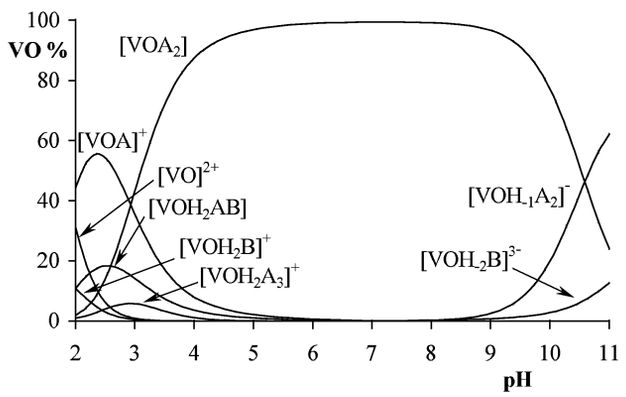

Fig. 7 Speciation curves of the complexes formed in the VO(IV)–DHP (A)–phosphate (B) system at 1 : 2 : 2 metal-to-ligand ratio. $c_{VO(IV)} = 0.001$ mol dm^{-3}, $I = 0.2$ mol dm^{-3} and $T = 25$ °C.

EPR also confirms the presence of the parent complexes and the broadening of the signal of $[VOA]^+$ with parameters $g_{||} = 1.940$; $A_{||} = 169 \times 10^{-4}$ cm^{-1} may indicate the parallel formation of some other ternary species, presumably with monodentate coordination of phosphate in the equatorial plane, which slightly decreases $A_{||}$ with respect to the mono-chelated species of DHP. At pH > 4, both potentiometry and EPR indicate the predominance of the bis complexes ($[VOA_2]$ and $[VOH_{-1}A_2]^-$) of DHP at a 1 : 2 : 2 ratio.

Conclusions

DHP forms a stable, neutral bis complex $[VOA_2]$ in solution which predominates over a wide pH range (pH 4–9); this complex may cross cell membranes effectively. The stability data and structural considerations of the ternary systems discussed above indicate that $[VO(DHP)_2]$, undergoes rather few transformations when it encounters the most important l.m.m. bioligands of the blood serum. This is illustrated by the data in Table 4, which provides the distribution of VO(IV) among the complexes of the carrier ligands and the l.m.m. components of the serum. The modelling calculations were performed at a VO(IV)–insulin-enhancing compound concentration of 10 μmol dm^{-3}, while the concentrations used for the serum

Table 4 Calculated species distribution of various insulin-enhancing VO(IV) compounds (10 μmol dm^{-3}) in the low molecular mass fraction of blood serum at pH 7.4

VO(IV) binder molecule	%VO(IV) bound		
	DHP	Maltol	Picolinic acid
Original carrier: VOA_2	71	0	0
Ternary complexes with:			
Citrate	29	14	42
Binary complexes with:			
Citrate	0	73	50
Lactate	0	5	3
Phosphate	0	8	5

bioligands were as follows:[32] phosphate 1.10 mmol dm^{-3}, citrate 99.0 μmol dm^{-3}, lactate 1.51 mmol dm^{-3} and oxalate 9.20 μmol dm^{-3}.

In contrast with the speciation of VO(IV) observed for maltol[16] or picolinic acid[33] as carrier ligand, these findings indicate that the original carrier complex $[VO(DHP)_2]$ remains predominant during its transport in the blood stream. This difference is due to the higher metal ion-binding strength of DHP, which makes $[VO(DHP)_2]$ stable enough almost not to take part in transformation reactions. The most effective competitor, citrate is the only l.m.m. ligand which is able to partly displace DHP from the bis complex.

If we take into account the interaction of VO(IV) with the high molecular mass components of the serum, the binding to albumin seems to be very weak,[34] but transferrin is known to be an efficient hard metal ion binder, although no binding constant has been reported in the literature for this interaction. As recently pointed out by Sun *et al.*,[35] there is a linear correlation (LFER) between the first transferrin-binding constants and the first hydroxide ion-binding constants of the metal ions. Using this relationship, we can predict a value of $\log K_1^* = 13.2 \pm 1.6$ for the stability constant of the VO(IV)–transferrin complex. With this value and the estimated value of $\log K_2^* = 12.2 \pm 1.6$ (the second transferrin-binding constant is generally one order of magnitude lower than the first one),[35] the complete speciation of VO(IV) can be calculated for the conditions in the serum. This is illustrated in Fig. 8, where, besides those for DHP,

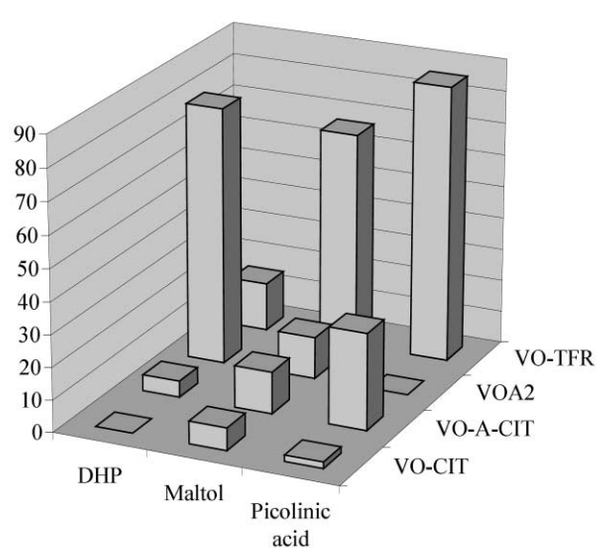

Fig. 8 Distribution of VO(IV) among the most important blood serum constituents. (Calculated for 100 μmol dm^{-3} of the insulin-mimetic complex and for serum component concentrations according to ref. 32).

the data for maltol and picolinic acid are also listed. Table 4 reveals that, of the three carriers, only DHP can efficiently compete with transferrin for the binding of VO(IV).

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