

A quantitative study of the biotransformation of insulin-enhancing VO²⁺ compounds

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Abstract Potentiometric (pH titrations) and spectroscopic (electron paramagnetic resonance) methods have been used to determine the thermodynamic stability constants of the various VO²⁺ complexes formed after the interaction of four insulin-enhancing vanadium compounds, [VO(6-mepic)₂], *cis*-[VO(pic)₂(H₂O)], [VO(acac)₂], and [VO(dhp)₂], where 6-mepic, pic, acac, and dhp indicate the deprotonated forms of 6-methylpicolinic acid, picolinic acid, acetylacetone, and 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone, with high molecular mass [human serum apotransferrin (hTf) and human serum albumin (HSA)] and low molecular mass (lactate) components of blood serum. In particular, log β values for the formation of (VO)hTf (13.0 ± 0.5), (VO)₂hTf (25.5 ± 0.5), (VO)HSA (9.1 ± 1.0), (VO)₂^dHSA (20.9 ± 1.0), *cis*-VO(dhp)₂(hTf) (25.5 ± 0.6), *cis*-VO(dhp)₂(HSA) (25.9 ± 0.6), (VO)hTf(lact) (14.5 ± 0.8), (VO)₂hTf(lact)₂ (28.5 ± 0.8), (VO)hTf(pic) (15.6 ± 0.8), and (VO)₂hTf(pic)₂ (30.4 ± 0.8) were determined. The values of the stability constants were used to compare the calculated

composition of ternary and quinary systems with that recently proposed by some of us through electron paramagnetic resonance and density functional theory methods (Sanna et al. in *Inorg. Chem.* 49:174–187, 2010) and to predict the distribution of VO²⁺ ion in blood serum when one of the four insulin-enhancing vanadium compounds studied, [VO(carrier)₂], is administered.

Keywords Vanadium · Insulin-enhancing drugs · Stability constants · Transport in blood serum

Introduction

One of the most important applications of vanadium compounds is their potential use in the therapy of patients suffering from type II diabetes mellitus [1–6], which affects 150 million people worldwide according to the estimates of the World Health Organization [7]. Since type II diabetes is accompanied by an increasing lack of response to insulin, insulin injections can ultimately become ineffective and alternative methods of treatment are desirable. About 30 years ago, an *in vitro* study on the insulin-enhancing action of vanadate was published, revealing the vanadate-stimulated uptake and degradation of glucose by adipocytes (fat cells), the stimulation of glycogenesis in the liver, and the inhibition of hepatic gluconeogenesis [8]; these effects were shortly thereafter related to the reduction of vanadate to vanadyl (VO²⁺), and to the action of VO²⁺ ion as an inhibitor of a cellular phosphatase [9]. Vanadium species have the advantage over insulin of possible oral administration.

After the initial use of vanadium(IV) and vanadium(V) inorganic salts, not easily applicable as antidiabetic drugs because of their toxicity and very low absorption rate,

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several peroxovanadium(V) complexes with (N,N), (N,O), and (O,O) ligands [10, 11] and neutral vanadyl species (VOL_2 , where L is an organic carrier) with bidentate anionic ligands were tested [2–6]. Most of these compounds stimulate glucose intake into cells, inhibit free fatty acids release and gluconeogenesis, stimulate lipogenesis, and lower or restore blood glucose level. Which is the active species, vanadyl (VO^{2+}) or vanadate (H_2VO_4^-), and the mechanism of action of such compounds are two questions still remaining to be answered: it seems, however, that the effect is based on the phosphate-like activity and/or interference with phosphatases [8–12] and mainly on the inhibition of protein tyrosine phosphatase, which is responsible for the inhibition of the signal transduction pathways and glucose transport into the cells in the absence of insulin [2, 3, 13–16].

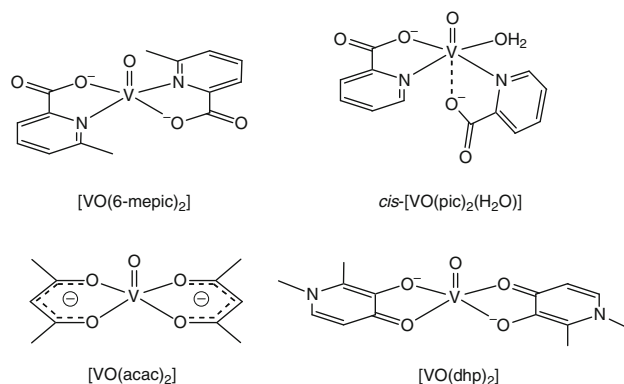
VO^{2+} complexes with VOL_2 stoichiometry were found to be very effective and well tolerated in all the animal models of diabetes and resulted in a more reliable glucose lowering than VOSO_4 [7]. Among them, $[\text{VO}(\text{ethylmalto})_2]$, or BEOV, is undergoing phase IIa clinical trials [17], and *cis*- $[\text{VO}(\text{pic})_2(\text{H}_2\text{O})]$ and $[\text{VO}(6\text{-mepic})_2]$ (pic and 6-mepic indicate picolinate and 6-methylpicolinate) are strong inhibitors of the mobilization of the fatty acids and are effective in the treatment of rats affected by diabetes induced by streptozotocin [19–22].

An important factor in assessing the mechanism of action of an insulin-enhancing vanadium compound is its interaction with serum components present under physiological conditions; these include high molecular mass components, such as human serum apotransferrin (hTf) and human serum albumin (HSA), and low molecular mass components of the blood serum; among these latter, some of us demonstrated that only lactate and citrate can interact with the binary complexes of transferrin and albumin to form mixed species generally indicated as $\text{VO}^{2+}\text{-hTf-lact}$, $\text{VO}^{2+}\text{-hTf-citr}$, $\text{VO}^{2+}\text{-HSA-lact}$, and $\text{VO}^{2+}\text{-HSA-citr}$, where lact and citr indicate lactate and citrate, respectively [23]. Both high molecular mass and low molecular mass components can partly or fully displace the original carrier L^- of the insulin-enhancing agent VOL_2 to yield binary or ternary species [24–27], which could be the pharmacologically active species or, at least, the main way that vanadium is delivered to cells. The existence of a mixed complex between $[\text{VO}(\text{mal})_2]$, where mal denotes the deprotonated form of maltol, and albumin was proposed by Willsky et al. [28], who observed that at least one of the maltol ligand remains coordinated to vanadium without specifying the stoichiometry of the species; subsequently, Liboiron et al. [26] reported the formation of adducts with $[\text{VO}(\text{mal})_2(\text{HSA})]$ stoichiometry, with one imidazole nitrogen of a histidine residue that occupies the fourth equatorial position of VO^{2+} ion. These suggestions appear to be in agreement with in

vivo blood circulation monitoring–EPR measurements in rats [29] and with the recent demonstration that in conditions close to the physiological ones the bis-chelated complexes with *cis*-octahedral geometry can form ternary species such as *cis*- $\text{VO}(\text{pic})_2(\text{HSA})$, *cis*- $\text{VO}(\text{dhp})_2(\text{HSA})$, and *cis*- $\text{VO}(\text{dhp})_2(\text{hTf})$, in which a histidine nitrogen replaces the water molecule in the equatorial plane [dhp is the deprotonated form of 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone] [30]. These last insights were based on the interpretation of EPR spectra of the ternary systems $\text{VO}^{2+}/\text{hTf}/\text{carrier}$ and $\text{VO}^{2+}/\text{HSA}/\text{carrier}$ and on their comparison with model systems containing 1-methylimidazole (1-MeIm), which coordinates VO^{2+} ion like an imidazole nitrogen of a histidine residue [26]; these data also allowed an explanation of the results of Jakusch et al. [31], who proposed the compositions $(\text{VO})\text{hTf}(\text{dhp})$, $(\text{VO})_2\text{hTf}(\text{dhp})$, and $(\text{VO})_2\text{hTf}(\text{dhp})_2$, with dhp bound to one lobe or both lobes of the protein, for the mixed species formed by transferrin and 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone.

In this work, we studied the biospeciation in the presence of the two most important high molecular mass components (transferrin and albumin) and one low molecular mass component (lactate) of four among the most representative insulin-enhancing vanadium compounds, two with $\text{VO}(\text{N}_2\text{O}_2)$ coordination and two with $\text{VO}(\text{O}_4)$ coordination: $[\text{VO}(6\text{-mepic})_2]$ [32, 33], $[\text{VO}(\text{pic})_2(\text{H}_2\text{O})]$ [6, 14], $[\text{VO}(\text{acac})_2]$ [34, 35], and $[\text{VO}(\text{dhp})_2]$ [36] (acac is the deprotonated form of acetylacetonone) (Scheme 1).

To quantitatively describe the biotransformation of an insulin-enhancing vanadium drug, it is necessary to know the stability constants associated with the several complexes that are formed. The values of $\log K$ (or $\log \beta$) can be used to calculate the percent composition of a system with a high number of components or with a low concentration of VO^{2+} , in conditions for which the characterization of the species



Scheme 1 Insulin-enhancing vanadium compounds studied in this work

present in solution should be difficult with any instrumental technique.

Chasteen et al. [25] found that in vitro transferrin binds VO^{2+} ion approximately 6 times more strongly than albumin. Recently Jakusch et al. [31] calculated the values of 13.4 for $\log K_1(\text{hTf})$ and 11.8 for $\log K_2(\text{hTf})$, whereas the binding constant of albumin for (VO)HSA [$\log K_1(\text{HSA})$] was around 10 if it was measured with spectroscopic methods [37, 38] and 9 in ultrafiltration studies [31]. The 3–4 orders of magnitude between $K_1(\text{hTf})$ and $K_1(\text{HSA})$ implies that all of VO^{2+} ion should be bound to transferrin [31, 37–39]. However, their results were based on the approximation that ternary complex formation between VO^{2+} , hTf, and dhp could be neglected [30, 31]. Therefore, we believe that $\log K_1(\text{hTf})$ and $\log K_2(\text{hTf})$ must be recalculated in a system where the formation of mixed species is lacking: for example, that containing acetylacetonate is surely a valid choice, since acac does not form ternary complexes with hTf [30]. For the same reason, the value of $\log K_1(\text{HSA})$, measured in a system where the formation of *cis*-VO(pic)₂(HSA) is detected [30, 37], must be rediscussed and redetermined.

Moreover, here we report for the first time the stability constants of the (1) dinuclear species formed by albumin, (VO)₂^dHSA [40], (2) ternary species formed by transferrin with lactate or picolinate, where the two ligands replace the bicarbonate ion in the active site of transferrin with a COO⁻ group [23], and (3) ternary species formed by transferrin or albumin with the carrier, VO(6-mepic)(HSA)(OH), *cis*-VO(pic)₂(HSA), *cis*-VO(dhp)₂(hTf), and *cis*-VO(dhp)₂(HSA) [30]. In the examination of the biospeciation of an insulin-enhancing agent in the blood serum, besides the above-mentioned stability constants, those reported in the literature for the insulin-enhancing drugs in their original form [VO(carrier)₂] [41–44], for the mixed species formed by carrier and lactate such as [VO(carrier)(lactH₋₁)]⁻ [41, 44], and for the binary complexes formed by VO^{2+} and lactate [45] must be taken into account.

Finally, the mixed species formed at physiological pH by 1-MeIm were used as models for the coordination of transferrin or albumin through an imidazole nitrogen belonging to a surface residue of histidine [26, 30]. The stability constants for such species were compared with those of the analogous complexes formed by hTf and HSA [30].

We believe that the results of this study can help to quantitatively estimate the biotransformation of an insulin-enhancing VO^{2+} compound in blood serum and to identify and evaluate the importance at physiological conditions of the various species present, some of which could be revealed to be the pharmacologically active form.

Materials and methods

Chemicals

Water was deionized prior to use through a Millipore Milli-Q Academic purification system. VO^{2+} solutions were prepared from VOSO₄·3H₂O following literature methods [46].

HSA and hTf were obtained from Sigma. Apotransferrin (98%) was obtained as a lyophilized powder with a molecular mass of 76–81 kDa (Sigma T4283). Albumin (97–99%), containing only trace amounts of fatty acids, was crystallized and lyophilized with a molecular mass of 66 kDa (Sigma A9511). The concentrations of the protein solutions were estimated from their UV absorption [$\epsilon_{280}(\text{hTf}) = 92,300 \text{ M}^{-1} \text{ cm}^{-1}$; $\epsilon_{278}(\text{HSA}) = 42,000 \text{ M}^{-1} \text{ cm}^{-1}$] [25, 47].

6-Methylpicolinic acid, picolinic acid, acetylacetone, 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone, lactic acid, 1-MeIm, NaHCO₃, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were of the highest grade available and were used as received.

Preparation of the solutions

The solutions were prepared by dissolving VOSO₄·3H₂O and the organic carrier in ultrapure water. The vanadium concentration used was in the range necessary to observe insulin-enhancing activity (approximately 1–400 μM) [7, 48–50]. When desired, lactate was also added. Argon was bubbled through the solutions to ensure the absence of oxygen and avoid the oxidation of VO^{2+} ion. Appropriate amounts of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and NaHCO₃ were added to the solution to obtain final concentrations of 1.0×10^{-1} and $2.5 \times 10^{-2} \text{ M}$, respectively.

To 1 mL of these solutions, again carefully purged with argon, an amount of transferrin or albumin was added to obtain the correct hTf to VO^{2+} and HSA to VO^{2+} ratios; the pH was adjusted to approximately 7.4.

To calculate the stability constants of the several species, the following solutions at pH around 7.4 were considered: (1) 1:1:4 $\text{VO}^{2+}/\text{hTf}/\text{acac}$ ($2.5 \times 10^{-4} \text{ M VO}^{2+}$) and 1:1:10 $\text{VO}^{2+}/\text{hTf}/\text{acac}$ ($2.5 \times 10^{-4} \text{ M VO}^{2+}$) to calculate $\log \beta_1$ of (VO)hTf; (2) 2:1:5 $\text{VO}^{2+}/\text{hTf}/\text{acac}$ ($5.0 \times 10^{-4} \text{ M VO}^{2+}$) and 2:1:15 $\text{VO}^{2+}/\text{hTf}/\text{acac}$ ($5.0 \times 10^{-4} \text{ M VO}^{2+}$) to calculate $\log \beta_2$ of (VO)₂hTf; (3) 4:1:10 $\text{VO}^{2+}/\text{HSA}/\text{pic}$ ($1.0 \times 10^{-3} \text{ M VO}^{2+}$) and 4:1:8:20 $\text{VO}^{2+}/\text{HSA}/\text{pic}/1\text{-MeIm}$ ($1.0 \times 10^{-3} \text{ M VO}^{2+}$) to calculate $\log \beta$ of (VO)HSA; (4) 1:1:2:5 $\text{VO}^{2+}/\text{HSA}/\text{pic}/1\text{-MeIm}$ ($7.5 \times 10^{-4} \text{ M VO}^{2+}$) to calculate $\log \beta$ of (VO)₂^dHSA; (5) 2:1:5 $\text{VO}^{2+}/\text{hTf}/\text{dhp}$ ($5.0 \times 10^{-4} \text{ M VO}^{2+}$) to calculate $\log \beta$ of *cis*-VO(dhp)₂(hTf); (6) 4:1:10 $\text{VO}^{2+}/\text{HSA}/\text{dhp}$ ($1.0 \times 10^{-3} \text{ M VO}^{2+}$) to calculate $\log \beta$ of *cis*-VO(dhp)₂(HSA);

(7) 1:1:40.8 VO²⁺/hTf/lact (2.5 × 10⁻⁴ M VO²⁺) to calculate log β of (VO)hTf(lact); (8) 2:1:40.8 VO²⁺/hTf/lact (VO²⁺ 5.0 × 10⁻⁴ M) to calculate log β of (VO)₂hTf(lact)₂; (9) 1:1:2.5 VO²⁺/hTf/pic (2.5 × 10⁻⁴ M VO²⁺) to calculate log β of (VO)hTf(pic), and (10) 2:1:5 VO²⁺/hTf/pic (5.0 × 10⁻⁴ M VO²⁺) to calculate log β of (VO)₂hTf(pic)₂.

EPR spectra of all the model systems (VO²⁺/carrier, VO²⁺/lact, and VO²⁺/carrier/lact) were recorded with the same molar ratio used with transferrin or albumin.

EPR spectroscopy

EPR spectra were recorded on frozen solutions at -150 °C with an X-band (9.4-GHz) Bruker EMX spectrometer. The addition of dimethyl sulfoxide was not necessary and no improvement in the resolution of the spectra was obtained. All the solutions were degassed before measuring the anisotropic spectra. After the samples had been transferred into the EPR tubes, the spectra were immediately recorded. Only low-temperature EPR spectra were measured to minimize the oxidation of VO²⁺ ion to vanadium(V). To increase the signal to noise ratio, signal averaging was used [23, 30, 40]. During the time needed to record the spectra of the frozen-solution samples no appreciable decrease of the signal intensity was observed.

A power saturation study was undertaken and the results showed that with the microwave power used in this study (10 dB) the signals were not saturated. No correction for the phase of the signal for each individual spectrum was needed because it was adjusted by the autotune routine of the acquisition program. This was periodically checked and the phase was always the one giving the highest intensity signals.

The values of A_z were calculated through simulations with the computer program Bruker WinEPR SimFonia [51].

The intensity of the EPR signals was measured in the M_I = -7/2, 5/2, 7/2 parallel transitions through the double integration of the first-derivative spectra. The examination of the other parallel resonances or of the perpendicular resonances was not possible for the extended superimposition of the signals belonging to the species present in aqueous solution.

The signals of the species (VO)₂^dHSA (S = 1) have lower intensity with respect to (VO)HSA (S = 1/2) [40], and therefore the values obtained with double integration for the two complexes are not directly comparable. To determine the amount of VO²⁺ bound as (VO)₂^dHSA complex in a system containing both (VO)₂^dHSA and (VO)HSA species, the solution containing VO²⁺, picolinic acid, and 1-MeIm, with the pH adjusted to approximately 7.4, was divided into two fractions: anisotropic EPR

spectra were recorded for the first fraction, where an appropriate amount of HSA was added to form the species (VO)₂^dHSA, *cis*-[VO(pic)₂(1-MeIm)], and [VO(pic)₂(OH)]⁻, and for the second one. In this way the vanadium concentrations in both samples were exactly the same and the fraction present as (VO)₂^dHSA can be obtained from the difference between the intensity signals of the mononuclear species. This procedure was validated on systems in which the relative amounts of mononuclear and dinuclear complexes were known, for example, those containing tartaric or citric acid [52].

The complete EPR spectra in Figs. 2, trace c, 3, trace a, and 5, traces c and d, and in Figs. 3, trace c and 4, traces a and b are reported in the supporting information of [23] and [30], respectively, whereas those in Figs. 2, traces a, b, and d, 3, traces b and d, 4, trace c, and 5, traces a and b are shown in Figs. S1–S7. The microwave frequency used was around 9.4 GHz.

Potentiometric measurements of VO²⁺/carrier/1-MeIm systems

The stability constants of the proton and VO²⁺ complexes were determined by pH-potentiometric titrations on 25.00-mL samples. The ligand to metal molar ratio was between 1:1 and 8:1 and the VO²⁺ concentration was 0.001–0.004 M. Titrations were performed from pH 2.0 until precipitation or very extensive hydrolysis occurred by adding carbonate-free KOH of known concentration (approximately 0.2 M KOH) [53]. The pH was measured with a Metrohm 6.0234.100 combined electrode, calibrated for hydrogen ion concentration by the method of Irving et al. [54]. The water ionization constant, pK_w, was 13.76 ± 0.01 under the conditions employed. Measurements were carried out at 25.0 ± 0.1 °C and at a constant ionic strength of 0.20 M KCl with a Radiometer pHM 84 instrument and a Metrohm 715 Dosimat burette. Purified argon was bubbled through the samples to ensure the complete absence of oxygen and carbon dioxide. The number of experimental points used in the calculations was 150–200 for each system and the reproducibility of the points included in the evaluation was within 0.005 pH units in the whole pH range measured.

The stability constants, β_{pqr} = [(VO)_pB_qH_r]/[VO]^p[B]^q[H]^r for the binary complexes and β_{pqrs} = [(VO)_pA_qB_rH_s]/[VO]^p[A]^q[B]^r[H]^s for the ternary species, where VO stands for VO²⁺ ion, A and B are the deprotonated forms of the carrier and 1-MeIm, respectively, and H is the proton, were calculated with the aid of the PSEQUAD program [55]. The values reported for the species formed in the binary systems with 6-methylpicolinate, picolinate, and dhp, determined in identical conditions, were used to estimate log β for the ternary complexes with 1-MeIm

[41, 42, 44]. Standard deviations were calculated by assuming random errors. The conventional notation was used: a negative sign for protons indicates either the dissociation of groups which do not deprotonate in the absence of VO^{2+} coordination or hydroxo ligands. Hydroxo complexes of VO^{2+} were taken into account and the following species were assumed: $[\text{VO}(\text{OH})]^+$ ($\log \beta = -5.94$), $[(\text{VO})_2(\text{OH})_2]^{2+}$ ($\log \beta = -6.95$), with stability constants calculated from the data of Henry et al. [56] and corrected for the different ionic strengths by use of the Davies equation [57], $[\text{VO}(\text{OH})_3]^-$ ($\log \beta = -18.0$), and $[(\text{VO})_2(\text{OH})_5]^-$ ($\log \beta = -22.0$) [58, 59].

Results

The $\text{VO}^{2+}/1\text{-MeIm}$ and $\text{VO}^{2+}/\text{carrier}/1\text{-MeIm}$ systems

In the literature it has been suggested that 1-MeIm can be considered as a valid model for the binding of an imidazole nitrogen belonging to a histidine residue present in a polypeptide chain [26]. Therefore, in this work the interaction of 1-MeIm with VO^{2+} ion and the insulin-enhancing compounds $[\text{VO}(\text{carrier})_2]$ was studied with the combined application of potentiometric (pH titrations) and spectroscopic (EPR) techniques. The stability constants of proton and VO^{2+} complexes are reported in Table 1.

The $\text{p}K_a$ value of 1-MeIm (indicated as HB^+ in the fully protonated form in aqueous solution) is slightly higher than that of imidazole, 6.95 [60], owing to the presence of an electron-releasing methyl group on nitrogen in position 1 of the aromatic ring.

As expected, 1-MeIm is a weak ligand towards VO^{2+} and even at a ligand to metal ratio of 8:1 the hydrolysis starts around pH 4.5. Therefore, the points below pH 4.0 were used for the calculations and from these data only $\log \beta$ for $[\text{VOB}]^{2+}$ can be determined (Table 1); in this

Table 1 Stability constants ($\log \beta$) of proton, binary VO^{2+} complexes of 1-methylimidazole (*1-MeIm*) and ternary complexes of $\text{VO}^{2+}/1\text{-MeIm}$ with the deprotonated forms of 6-methylpicolinic acid (*6-mepic*), picolinic acid (*pic*), and 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone (*dhp*) at $25.0 \pm 0.1^\circ\text{C}$ and $I = 0.20$ M (KCl)

Species	1-MeIm	6-mepic	pic	dhp
HB^+	7.11 (1)	–	–	–
$[\text{VOB}]^{2+}$	5.06 (6)	–	–	–
$[\text{VOAB}]^+$	–	9.2 (2)	–	–
$[\text{VOABH}_{-1}]$	–	3.82 (7)	–	–
<i>cis</i> - $[\text{VOA}_2\text{B}]$	–	–	14.96 (4)	25.40 (2)

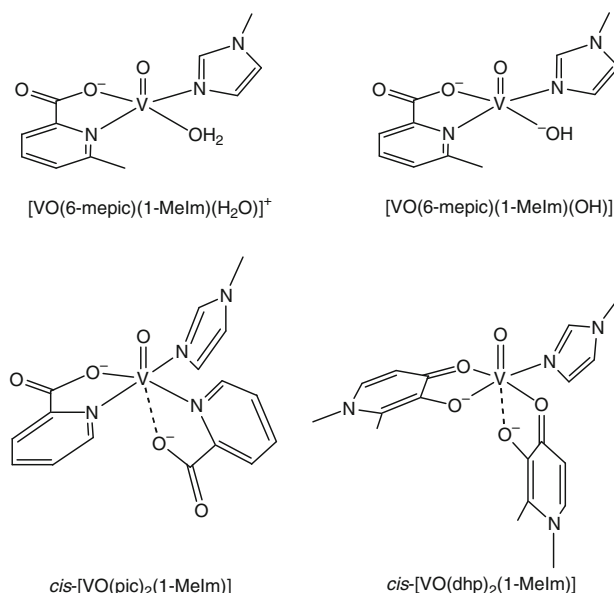
The uncertainties (3σ values) of the protonation and stability constants are given in parentheses

A carrier, B 1-MeIm

complex the aromatic nitrogen behaves as a monodentate donor. Its A_z value ($176 \times 10^{-4} \text{ cm}^{-1}$) agrees well with the data in the literature [61]. Only at very high ratio (100:1) can the EPR signals of $[\text{VOB}]^{2+}$, $[\text{VOB}_2]^{2+}$, $[\text{VOB}_3]^{2+}$, and $[\text{VOB}_4]^{2+}$, in which one, two, three, or four nitrogens occupy the equatorial positions, be revealed [61].

The potentiometric and spectroscopic study of the ternary systems $\text{VO}^{2+}/6\text{-mepic}/1\text{-MeIm}$, $\text{VO}^{2+}/\text{pic}/1\text{-MeIm}$, and $\text{VO}^{2+}/\text{dhp}/1\text{-MeIm}$ shows that mixed complexes are formed. In particular, $[\text{VO}(6\text{-mepic})(1\text{-MeIm})(\text{H}_2\text{O})]^+$ and $[\text{VO}(6\text{-mepic})(1\text{-MeIm})(\text{OH})]$ are detected in the system with 6-methylpicolinate (A_z 168×10^{-4} and $163 \times 10^{-4} \text{ cm}^{-1}$, respectively), with the organic carrier coordinating the metal ion with the (N, COO^-) donor set, a monodentate imidazole nitrogen, and a water or a hydroxide ligand completing the equatorial position of vanadium; the deprotonation of the water molecule takes place with a $\text{p}K$ of 5.4.

In the ternary systems containing picolinate and dhp, $[\text{VO}(\text{pic})_2(1\text{-MeIm})]$ and $[\text{VO}(\text{dhp})_2(1\text{-MeIm})]$ are observed. Their A_z value measured in the EPR spectra is lower than that of *cis*- $[\text{VO}(\text{pic})_2(\text{H}_2\text{O})]$ and *cis*- $[\text{VO}(\text{dhp})_2(\text{H}_2\text{O})]$ (159×10^{-4} versus $165 \times 10^{-4} \text{ cm}^{-1}$ for picolinate [42] and 163×10^{-4} versus $166 \times 10^{-4} \text{ cm}^{-1}$ for 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone [44]); this suggests that in the mixed species the equatorial water molecule has been replaced by the imidazole nitrogen of 1-MeIm. Recent density functional theory results also confirm this attribution [30]. The structures of the ternary complexes formed by 1-MeIm are shown in Scheme 2. As an example, the distribution curves as a function of pH for the species formed with dhp are depicted in Fig. 1.



Scheme 2 Ternary complexes formed by 1-MeIm with 6-mepic, pic, and dhp

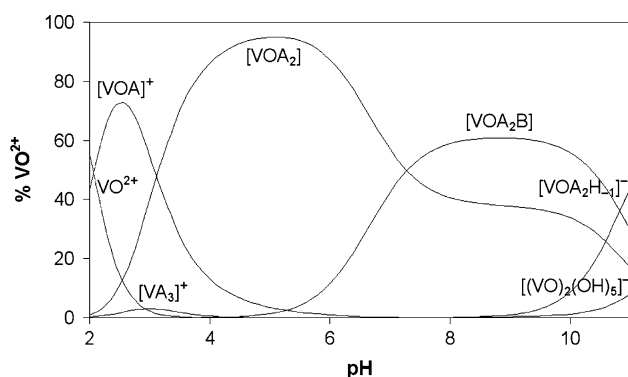


Fig. 1 Species distribution for the VO^{2+} /dhp/1-MeIm system as a function of pH with a molar ratio of 1:2:4 and a VO^{2+} concentration of 1 mM

Determination of the stability constants for (VO)hTf and $(\text{VO})_2\text{hTf}$

The binary system VO^{2+} /hTf has been widely studied through spectroscopic techniques [24, 25, 28, 40, 62–67]. EPR results suggest that there are two binding sites, called A and B [62, 63], with B being further split into B_1 and B_2 [40, 65, 66]. Analogously to Fe^{3+} , VO^{2+} needs HCO_3^- for the binding to the A and B sites of the protein; bicarbonate can be replaced by other anions, called synergistic anions, which must have certain structural features, such as a carboxylate and an electron-withdrawing group [68, 69].

The first values reported in the literature for the binding constants for binding to the two lobes of transferrin were by Kiss et al. [39], who used a linear free-energy relationship between $\log K_1(\text{hTf})$ and the stability constant for the binding of hydroxide ion, $\log K_{\text{OH}}$ [69]: values of 13.2 ± 1.6 and 12.2 ± 1.6 were obtained for $\log K_1$ and $\log K_2$, with a rather high uncertainty degree [39]. Such values were subsequently corrected to 14.3 ± 0.6 and 11.7 ± 0.6 through the examination of the partial displacement reaction between (VO)hTf and $[\text{VO}(\text{dhp})_2]$; these measurements were based on the assumption that VO^{2+} , hTf, and dhp do not form ternary species [66]. Recently, $\log K_1$ and $\log K_2$ were fixed at 13.4 and 11.8, respectively; in this last work, however, the authors attributed the stoichiometries (VO)hTf(dhp), $(\text{VO})_2\text{hTf}(\text{dhp})$, and $(\text{VO})_2\text{hTf}(\text{dhp})_2$ to the mixed species detected in solution at physiological pH [31]. In contrast, some of us recently proposed that the species formed under such conditions is *cis*- $\text{VO}(\text{dhp})_2(\text{hTf})$, with an imidazole nitrogen of a histidine residue of the transferrin chain occupying the fourth equatorial position [30]. Therefore, incorrect knowledge of the composition of the ternary system certainly affects the estimated values of the stability constants relating to the VO^{2+} -hTf interaction.

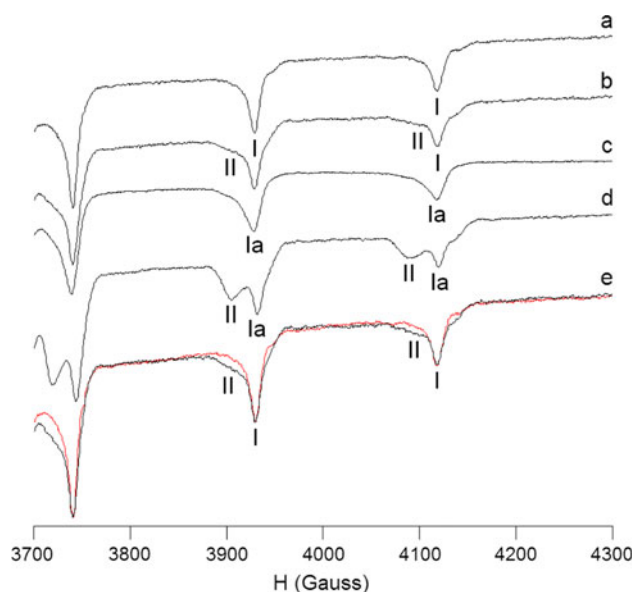


Fig. 2 High-field region of the X-band anisotropic EPR spectra recorded at physiological pH on aqueous solutions containing *a* 1:1 VO^{2+} /hTf (2.5×10^{-4} M VO^{2+}), *b* 1:1:4 VO^{2+} /hTf/acac (2.5×10^{-4} M VO^{2+}), *c* 2:1 VO^{2+} /hTf (5.0×10^{-4} M VO^{2+}), and *d* 2:1:15 VO^{2+} /hTf/acac (5.0×10^{-4} M VO^{2+}). *e* is the superimposition of the spectra reported in traces *b* (black) and *a* (red). *I*, *Ia*, and *II* indicate the resonances of (VO)hTf, $(\text{VO})_2\text{hTf}$, and $[\text{VO}(\text{acac})_2]$, respectively

To get correct values of $\log \beta_1$ and $\log \beta_2$ for the formation of (VO)hTf and $(\text{VO})_2\text{hTf}$, a system where mixed species are not observed must be chosen. It was recently found that in the ternary VO^{2+} /hTf/acac system only the formation of binary complexes between VO^{2+} and transferrin, on one hand, and between VO^{2+} and acetylacetonate, on the other, can be detected [30].

To estimate $\log \beta_1$ for the formation of (VO)hTf, EPR spectra at different molar ratios were recorded; in particular the system VO^{2+} /hTf/acac with ratios of 1:1:4 and 1:1:10 was considered (see “Materials and methods”). The results are presented in Fig. 2.

As it is possible to see from an examination of Fig. 2, both (VO)hTf and $[\text{VO}(\text{acac})_2]$ species exist, with the relative amount depending on the molar ratio of transferrin to acetylacetonate.

The displacement reaction is



and $\log \beta_1$ (equal to $\log K_1$) can be calculated through the equation

$$\begin{aligned} \log \beta_1 &= \log \frac{[(\text{VO})\text{hTf}]}{[\text{VO}^{2+}][\text{hTf}]} \\ &= \log \left(\frac{[\text{VO}(\text{acac})_2]}{[\text{VO}^{2+}][\text{acac}]^2} \times \frac{[(\text{VO})\text{hTf}][\text{acac}]^2}{[\text{VO}(\text{acac})_2][\text{hTf}]} \right) \\ &= \log \beta_2[\text{VO}(\text{acac})_2] + \log K_D \end{aligned} \quad (2)$$

where K_D indicates the constant for the displacement reaction. The value of $\log \beta_2$ for the formation of $[\text{VO}(\text{acac})_2]$ is 16.27 [43], whereas K_D can be determined by measuring the ratio of the concentrations of $(\text{VO})\text{hTf}$ and $[\text{VO}(\text{acac})_2]$, in turn calculated through the integration of the respective EPR signals.

The relative amount of $(\text{VO})\text{hTf}$ and $[\text{VO}(\text{acac})_2]$ is calculated from the first, seventh, and eighth parallel transitions ($M_I = -7/2, 5/2$ and $7/2$, respectively). It must be kept in mind, moreover, that the effective concentration of the deprotonated form of acetylacetonate can be determined from the experimental one ($[\text{acac}]^{\text{exptl}}$) through the following equation:

$$[\text{acac}] = \frac{[\text{acac}]^{\text{exptl}}}{[1 + 10^{-(\text{pH}-\text{p}K_a)}]} = \frac{[\text{acac}]^{\text{exptl}}}{[1 + 10^{-(\text{pH}-8.76)}]} \quad (3)$$

For example, in the system $\text{VO}^{2+}/\text{hTf}/\text{acac}$ with a molar ratio of 1:1:4 and a concentration of the metal ion of 2.5×10^{-4} M, the amounts of $(\text{VO})\text{hTf}$ and $[\text{VO}(\text{acac})_2]$ measured in the $M_I = 5/2, 7/2$ parallel transitions were 73.9 and 26.1% and 73.7 and 26.3%, respectively. From these data, the values of -3.28 and -3.29 for $\log K_D$ and of 12.99 and 12.98 for $\log \beta_1$ can be determined. Using the data obtained for two different ratios (1:1:4 and 1:1:10, see “Materials and methods”), the mean value for $\log \beta_1$ is 13.0 ± 0.5 .

To estimate $\log \beta_2$, the systems with molar ratios of 2:1:5 and 2:1:15 for VO^{2+} , hTf , and acetylacetonate were studied. From the percentages of $[\text{VO}(\text{acac})_2]$ with respect to $(\text{VO})_2\text{hTf}$, calculated from $M_I = -7/2, 5/2$ and $7/2$ resonances in the EPR spectra, a mean value of 25.5 ± 0.5 is obtained.

The final values for $\log \beta_1$ and $\log \beta_2$ are summarized below:

$$\log \beta_1[(\text{VO})\text{hTf}] = 13.0 \pm 0.5 \quad (4)$$

$$\log \beta_2[(\text{VO})_2\text{hTf}] = 25.5 \pm 0.5 \quad (5)$$

The binding constants calculated exhibit reasonably good agreement with the values recently reported by Jakusch et al. [31] ($\log \beta_1 = 13.4$ and $\log \beta_2 = 25.2$). Our data suggest, however, that the affinities of the two sites are very similar ($\log K_1 = 13.0$ and $\log K_2 = 12.5$), in agreement with the observation that the two sites of transferrin differ by approximately one unit in their $\log K$ values [69]. For Fe^{3+} , the site associated with the higher value of $\log K$ (site A) has been identified as the C-terminal [69].

Determination of the stability constants for $(\text{VO})\text{HSA}$ and $(\text{VO})_2^{\text{d}}(\text{HSA})$

Several papers have been devoted to the examination of the binary system $\text{VO}^{2+}/\text{HSA}$ [26, 28, 38, 40, 70]. In

equimolar solution or with an excess of albumin, the EPR spectra are characterized by the presence of signals attributable to a dinuclear species [denoted as $(\text{VO})_2^{\text{d}}\text{HSA}$] with spin state $S = 1$, whereas with an excess of VO^{2+} , the EPR resonances are assigned to a multinuclear complex [denoted as $(\text{VO})_x^{\text{m}}\text{HSA}$, with $x = 5-6$] characterized by an $S = 1/2$ spin state [40].

In the multinuclear species $(\text{VO})_x^{\text{m}}\text{HSA}$ the presence of one “strong” and five “weak” binding sites with different EPR parameters was firstly proposed, with the “strong” binding site associated with the primary site for Cu^{2+} [26, 28, 70]. The detection of only one set of EPR resonances at physiological pH when the VO^{2+} to HSA ratio is higher than 1 suggests that the five to six different metal ions bound by albumin have the same coordination and we called this site “high pH” [40]. Its experimental A_z value ($165 \times 10^{-4} \text{ cm}^{-1}$) allows the $(\text{NH}_2, \text{N}^-, \text{N}^-, \text{N}_{\text{imid}})$ coordination to be ruled out, suggesting that the binding sites are relatively nonspecific with respect to that of Cu^{2+} or Ni^{2+} [71]; the coordination of three or four imidazole nitrogens, or of two imidazole nitrogens plus a carboxylate group belonging to an aspartate or a glutamate residue, has been proposed [40].

These results indicate that the affinities of these five to six sites towards VO^{2+} ion are very similar and, therefore, only a mean value for the association constant of $(\text{VO})_x^{\text{m}}\text{HSA}$ can be calculated. The real values of $\log \beta_x$ for the five to six equilibrium steps should differ only slightly from each other (in our opinion, not more than 0.1–0.2 log units) and from the mean value (around 0.3–0.4 log units). Using the same notation as Jakusch et al. [31, 37, 38] on grounds of expediency [40], such a mean value will refer to the species $(\text{VO})\text{HSA}$.

To determine the stability constant for $(\text{VO})\text{HSA}$, a ternary system containing albumin and a ligand forming a binary complex with comparable stability must be studied. For this purpose, Kiss et al. [37] used picolinic acid, assuming that the formation of ternary species between albumin and picolinate was negligible. In the system $\text{VO}^{2+}/\text{HSA}/\text{pic}$ in aqueous solution, at pH 7.4, the species $(\text{VO})_2^{\text{d}}\text{HSA}$ [23, 40], $(\text{VO})\text{HSA}$ [23, 31, 37, 40], and $\text{cis}-[\text{VO}(\text{pic})_2(\text{OH})]^-$ [42] exist and we recently demonstrated that also a mixed complex with the composition $\text{cis}-\text{VO}(\text{pic})_2(\text{HSA})$ is formed, having a histidine nitrogen bound in the equatorial position and two picolinate ligands in an equatorial–equatorial and equatorial–axial arrangement [30]. As mentioned above, the presence of $(\text{VO})_2^{\text{d}}\text{HSA}$ or $(\text{VO})\text{HSA}$ depends on the VO^{2+} to HSA ratio (a ratio lower than 1 favors the first complex, and a ratio higher than 1 favors the second species).

For this reason, the value of the stability constant obtained by Kiss et al. ($\log K_1 = 10 \pm 1$ through EPR measurements [37, 38] and $\log K_1$ approximately 9 through

ultrafiltration studies [31]) must be recalculated: in fact, they supposed that no ternary species exist in aqueous solution at physiological conditions.

The first system from which we calculated $\log \beta$ for VO(HSA) is $\text{VO}^{2+}/\text{HSA}/\text{pic}$ with a molar ratio of 4:1:10. In these experimental conditions, only (VO)HSA, *cis*-[VO(pic)₂(OH)][−], and *cis*-VO(pic)₂(HSA) exist in solution (see Fig. 3 in [30]). We do not know the exact value of $\log \beta$ for *cis*-VO(pic)₂(HSA), but in the first approximation it can be considered equal to that of the mixed complex *cis*-[VO(pic)₂(1-MeIm)], whose coordination mode is the same [(N, COO[−]); (N, COO^{−ax}); N_{imid}]; the similarity between $\log \beta$ of *cis*-[VO(dhp)₂(1-MeIm)], *cis*-VO(dhp)₂(HSA), and *cis*-VO(dhp)₂(hTf) (see below) supports this assumption, which, of course, results in a rather high value of the uncertainty on the stability constant. By using $\log \beta$ for [VO(pic)₂(OH)][−] (5.13) and *cis*-VO(pic)₂(HSA) (14.96), one can calculate that for (VO)HSA. The result is $\log \beta[(\text{VO})\text{HSA}] = 9.1 \pm 1.0$.

The second method to calculate the value of $\log \beta[(\text{VO})\text{HSA}]$ is the analysis of the system $\text{VO}^{2+}/\text{HSA}/\text{pic}/1\text{-MeIm}$. It is possible to convert *cis*-VO(pic)₂(HSA) into *cis*-[VO(pic)₂(1-MeIm)], whose $\log \beta$ is known (14.96, Table 1), using an excess of 1-MeIm so that the nitrogen of 1-MeIm replaces that of the histidine in the equatorial position of the octahedral VO^{2+} ion. In this way, only *cis*-[VO(pic)₂(OH)][−], *cis*-[VO(pic)₂(1-MeIm)], and (VO)HSA are present in aqueous solution at equilibrium. From the knowledge of the stability constants of the first two complexes [42] (Table 1), a value for $\log \beta_1[(\text{VO})\text{HSA}]$ of 9.0 ± 1.0 is found.

It can be observed that the values of $\log \beta_1$ determined with the two different methods are comparable. The mean of the two values gives

$$\log \beta_1[(\text{VO})\text{HSA}] = 9.1 \pm 1.0 \quad (6)$$

If the EPR spectra of the quaternary system $\text{VO}^{2+}/\text{HSA}/\text{pic}/1\text{-MeIm}$ are recorded with a ratio of VO^{2+} to HSA of 1:1, the characteristic resonances of (VO)₂^dHSA with $S = 1$ appear (Fig. 3, trace b).

In this case, the presence in solution of (VO)₂^dHSA, [VO(pic)₂(OH)][−], and *cis*-[VO(pic)₂(1-MeIm)] must be considered. From the stability constants of *cis*-[VO(pic)₂(OH)][−] and *cis*-[VO(pic)₂(1-MeIm)] [42] (Table 1) and from their relative amounts, as evaluated by the double integration of the respective EPR signals, the following value for $\log \beta[(\text{VO})_2^d\text{HSA}]$ is calculated:

$$\log \beta[(\text{VO})_2^d\text{HSA}] = 20.9 \pm 1.0 \quad (7)$$

This is the first time that $\log \beta$ for the formation of (VO)₂^dHSA has been reported in the literature.

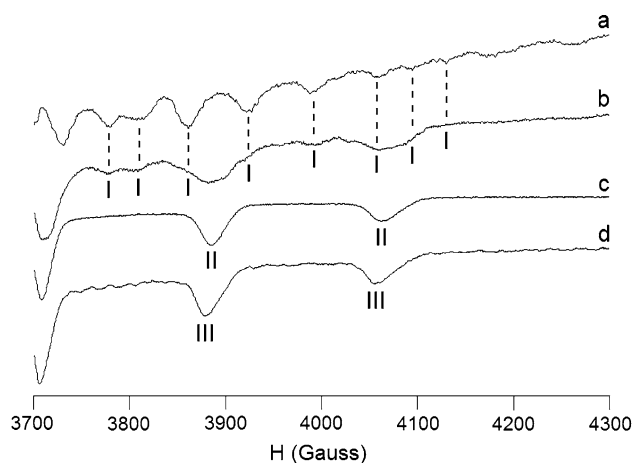


Fig. 3 High-field region of the X-band anisotropic EPR spectra recorded at physiological pH in aqueous solutions containing a 1:1 $\text{VO}^{2+}/\text{HSA}$ (7.5×10^{-4} M VO^{2+}), b 1:1:2:5 $\text{VO}^{2+}/\text{HSA}/\text{pic}/1\text{-MeIm}$ (7.5×10^{-4} M VO^{2+}), c 1:2:4 $\text{VO}^{2+}/\text{pic}/1\text{-MeIm}$ (1.0×10^{-3} M VO^{2+}), and d 1:2.5 $\text{VO}^{2+}/\text{pic}$ (1.0×10^{-3} M VO^{2+}). I (and dotted lines), II, and III indicate the resonances of $(\text{VO})_2^d\text{HSA}$, *cis*-[VO(pic)₂(1-MeIm)], and *cis*-[VO(pic)₂(OH)][−], respectively

Determination of the stability constants for *cis*-VO(dhp)₂(hTf) and *cis*-VO(dhp)₂(HSA)

Jakusch et al. [31] recently proposed for the mixed species formed by transferrin and 1,2-dimethyl-3-hydroxy-4(1H)-pyridinone stoichiometries of (VO)hTf(dhp), (VO)₂hTf(dhp), and (VO)₂hTf(dhp)₂, with dhp bound to one lobe or both the lobes of the protein. Recent EPR evidence suggests, however, that in the ternary system $\text{VO}^{2+}/\text{hTf}/\text{dhp}$, at physiological conditions, a mixed complex *cis*-VO(dhp)₂(hTf) is formed, in which the equatorial water molecule of the parent *cis*-[VO(dhp)₂(H₂O)] is replaced by a histidine nitrogen of hTf according to the reaction [30]



The $\log \beta$ value of VO(dhp)₂(hTf) can be calculated through the equation

$$\begin{aligned} \log \beta[\text{VO}(\text{dhp})_2(\text{hTf})] &= \log \beta_2(\text{VO}(\text{dhp})_2) + \log K_D \\ &= 22.83 + \log K_D \end{aligned} \quad (9)$$

The comparison of the spectra recorded for the binary $\text{VO}^{2+}/\text{dhp}$ and ternary $\text{VO}^{2+}/\text{hTf}/\text{dhp}$ system is shown in Fig. 4.

In the system with a VO^{2+} , hTf, and dhp ratio of 2:1:5 and a VO^{2+} concentration of 5.0×10^{-4} M, the amounts of the bis-chelated species of dhp and of VO(dhp)₂(hTf) are 54.2 and 45.8% in the $M_I = 5/2$ transition, and 55.9 and 44.1% in the $M_I = 7/2$ transition. If, as supposed, transferrin coordinates the metal ion with an imidazole nitrogen, it must be taken into account that the number of histidine residues exposed on the protein surface is 12 [72], so the effective concentration of histidine involved in the

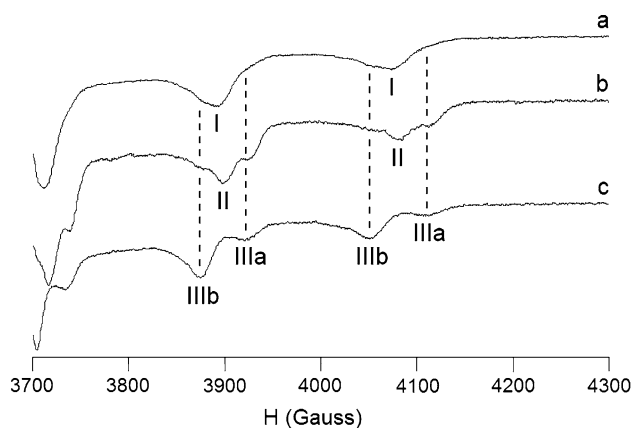


Fig. 4 High-field region of the X-band anisotropic EPR spectra recorded at physiological pH on aqueous solutions containing a 4:1:10 VO^{2+} /HSA/dhp (1.0×10^{-3} M VO^{2+}), b 2:1:5 VO^{2+} /hTf/dhp (5.0×10^{-4} M VO^{2+}), and c 1:2.5 VO^{2+} /dhp (1.0×10^{-3} M VO^{2+}). I, II, IIIa (and dotted lines), and IIIb (and dotted lines) indicate the resonances of *cis*- $\text{VO}(\text{dhp})_2(\text{HSA})$, *cis*- $\text{VO}(\text{dhp})_2(\text{hTf})$, *cis*- $[\text{VO}(\text{dhp})_2(\text{H}_2\text{O})]$, and $[\text{VO}(\text{dhp})_2]$, respectively

complexation is 3.0×10^{-3} M. These data allow the value of $\log \beta$ for the formation of $\text{VO}(\text{dhp})_2(\text{hTf})$ to be calculated:

$$\log \beta[\text{VO}(\text{dhp})_2(\text{hTf})] = 25.5 \pm 0.6 \quad (10)$$

It is worth noting that this value is very close to that obtained by pH-potentiometry for the mixed complex $[\text{VO}(\text{dhp})_2(1\text{-MeIm})]$, $\log \beta = 25.40$ (Table 1), indicating that it is only the number of histidine residues present on the protein surface which influences the formation and stability of such a kind of species. Moreover, this result proves that 1-MeIm is a good choice to model the interaction between a metal ion and the histidine residues of a protein.

In the ternary system VO^{2+} /HSA/dhp, a mixed species analogous to that described for transferrin with stoichiometry *cis*- $\text{VO}(\text{dhp})_2(\text{HSA})$, in which a histidine nitrogen is equatorially bound to vanadium, is observed [30].

In Fig. 4, trace b is the spectrum recorded for the ternary VO^{2+} /HSA/dhp system. The molar ratio used was 4:1:10 with a VO^{2+} concentration of 1.0×10^{-3} M. The equations used and the discussion for the calculation of $\log \beta$ are similar to those just given for transferrin. The only difference that must be considered is that, in the case of albumin, the number of histidine residues on the protein surface is six [73] for an effective concentration of 1.5×10^{-3} M. The value of $\log \beta$ is

$$\log \beta[\text{VO}(\text{dhp})_2(\text{HSA})] = 25.9 \pm 0.6 \quad (11)$$

In this case too the stability constant for the formation of $\text{VO}(\text{dhp})_2(\text{HSA})$ is comparable to that of $[\text{VO}(\text{dhp})_2(1\text{-MeIm})]$ and is similar to that found for $\text{VO}(\text{dhp})_2(\text{hTf})$. Of course, this depends on the fact that the structure of the

three species is the same, with the two dhp ligands adopting an equatorial–equatorial and an equatorial–axial arrangement and an imidazole nitrogen (belonging to 1-MeIm or to a histidine residue present on the surface of transferrin or albumin) occupying the fourth equatorial position. The small difference between the $\log \beta$ values for $\text{VO}(\text{dhp})_2(\text{hTf})$ and $\text{VO}(\text{dhp})_2(\text{HSA})$ can be attributed to a different exposure of the histidine residues on the protein surface and/or to charge and solvation effects.

Determination of the stability constants for $(\text{VO})\text{hTf}(\text{lact})$ and $(\text{VO})_2\text{hTf}(\text{lact})_2$

It has long been known that ligands with a carboxylate group, behaving as synergistic anions, can replace bicarbonate in the iron sites of transferrin [24, 68, 69]. Lactate is one of these ligands and in this work we were able to measure the stability constants of the mixed complexes formed with transferrin. From EPR spectra, it is not possible to determine the exact stoichiometry of such species, indicated generally as $\text{VO}^{2+}\text{-hTf-lact}$. However, according to chemical considerations and literature comments [31], the stoichiometries $(\text{VO})\text{hTf}(\text{lact})$ and $(\text{VO})_2\text{hTf}(\text{lact})_2$, with the first being detected when the VO^{2+} to hTf ratio is 1:1 and the second when the ratio is 2:1, can be proposed. Since lactate replaces, at least partly, bicarbonate in the sites occupied by vanadium, the determination of the stability constant of $(\text{VO})_2\text{hTf}(\text{lact})_2$, in which only one lactate anion is bound to VO^{2+} , is not possible.

As previously reported [68], it is very difficult to remove bicarbonate from solutions containing transferrin and a residual fraction always remains, affecting the binding of lactate. The procedure for removing completely HCO_3^- ion was recently described [26], but it is not particularly useful in the present experiments. In fact, if bicarbonate was completely removed from a solution containing lactate, only the VO^{2+} /hTf/lact ternary species would be obtained (since a synergistic anion is needed for the VO^{2+} binding to hTf), and $\log \beta$ for such a mixed complex could not be determined. In this work the evaluation of the stability constants by EPR spectroscopy was based on displacement reactions: thus, we need two species to be present in solution, and $\log \beta$ for one of them must be known. Therefore, the present experiments can be considered as a competition between bicarbonate and lactate and, for this reason, a large excess of the latter is necessary to observe the coordination.

EPR spectra recorded in the VO^{2+} /hTf system with or without lactate are shown in Fig. 5. The coordination of lactate can be revealed by shoulders at lower field with respect to the $M_I = 5/2$ and $7/2$ transitions of $(\text{VO})_2\text{hTf}$ and by an A_z value slightly lower than that of site A of transferrin, around $167 \times 10^{-4} \text{ cm}^{-1}$ [23, 30]. From the

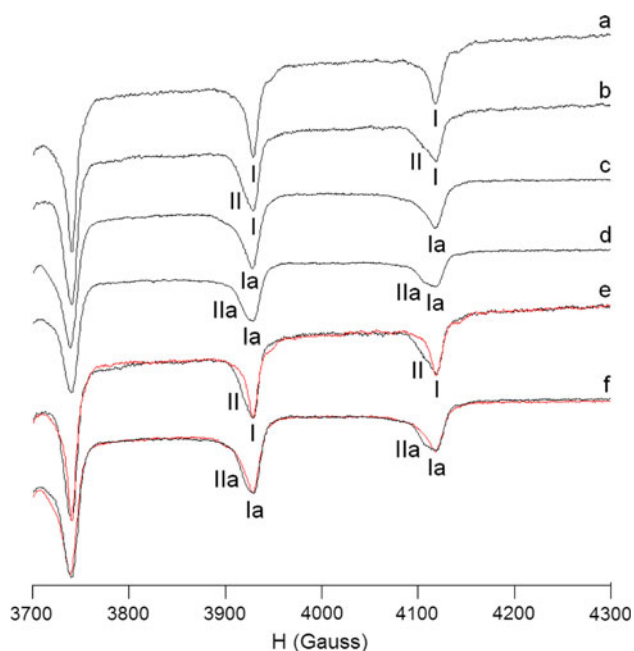


Fig. 5 High-field region of the X-band anisotropic EPR spectra recorded at physiological pH on aqueous solutions containing *a* 1:1 $\text{VO}^{2+}/\text{hTf}$ ($2.5 \times 10^{-4} \text{ M VO}^{2+}$), *b* 1:1:40.8 $\text{VO}^{2+}/\text{hTf}/\text{lact}$ ($2.5 \times 10^{-4} \text{ M VO}^{2+}$), *c* 2:1 $\text{VO}^{2+}/\text{hTf}$ ($5.0 \times 10^{-4} \text{ M VO}^{2+}$), and *d* 2:1:40.8 $\text{VO}^{2+}/\text{hTf}/\text{lact}$ ($5.0 \times 10^{-4} \text{ M VO}^{2+}$). *e* is the superimposition of the spectra reported in traces *b* (black) and *a* (red) and *f* is the superimposition of the spectra reported in traces *d* (black) and *c* (red). *I*, *Ia*, *II*, and *IIa* indicate the resonances of $(\text{VO})\text{hTf}$, $(\text{VO})_2\text{hTf}$, $(\text{VO})\text{hTf}(\text{lact})$, and $(\text{VO})_2\text{hTf}(\text{lact})_2$, respectively

double integration of the spectral lines, it was possible to estimate the relative amount of $(\text{VO})\text{hTf}$ (with HCO_3^- bound to vanadium in the active sites of transferrin) and $(\text{VO})\text{hTf}(\text{lact})$ (VO^{2+} to hTf ratio of 1:1) and of $(\text{VO})_2\text{hTf}$ (HCO_3^- bound to VO^{2+}) and $(\text{VO})_2\text{hTf}(\text{lact})_2$ (VO^{2+} to hTf ratio of 2:1). In traces *e* and *f* in Fig. 5 the enlargement of the spectral lines of $(\text{VO})\text{hTf}$ and $(\text{VO})_2\text{hTf}$, due to the presence of the mixed complexes of lactate, is easily observable. The experimental data allow one to calculate the following values of $\log \beta$ for the formation of such species:

$$\log \beta[(\text{VO})\text{hTf}(\text{lact})] = 14.5 \pm 0.8 \quad (12)$$

$$\log \beta[(\text{VO})_2\text{hTf}(\text{lact})_2] = 28.5 \pm 0.8 \quad (13)$$

Determination of the stability constants for $(\text{VO})\text{hTf}(\text{pic})$ and $(\text{VO})_2\text{hTf}(\text{pic})_2$

Analogously to lactate, picolinate possesses the features of a synergistic anion [68, 69] and, therefore, forms ternary complexes with VO^{2+} and hTf [30]. In this case too, the stoichiometry of such species depends on the VO^{2+} to hTf ratio and can be considered as $(\text{VO})\text{hTf}(\text{pic})$ when the ratio is 1 and $(\text{VO})_2\text{hTf}(\text{pic})_2$ when it is 2.

The procedure for the determination of the stability constants of such species is the same as described for lactate. The results of the measurements give the following values for the stability constants of $(\text{VO})\text{hTf}(\text{pic})$ and $(\text{VO})_2\text{hTf}(\text{pic})_2$:

$$\log \beta[(\text{VO})\text{hTf}(\text{pic})] = 15.6 \pm 0.8 \quad (14)$$

$$\log \beta[(\text{VO})_2\text{hTf}(\text{pic})_2] = 30.4 \pm 0.8 \quad (15)$$

A comparison of the $\log \beta$ values measured for the mixed complexes formed by lactate and picolinate allows the following comments to be made: (1) the values of $\log K$ for the two systems are comparable (14.5 and 14.0 for lactate and 15.6 and 14.8 for picolinate), with the picolinate ligand showing a slightly greater capability to act as synergistic anion and (2) the difference between the $\log K$ values for the two complexation steps is, as expected, less than one log unit (0.5 for lactate and 0.8 for picolinate) and similar to that found for the binary species of transferrin (0.5). This indicates that for a ligand to behave as synergistic anion, it is more important that it fulfills a structural requirement (such as the presence of a carboxylate group and the possibility of being stabilized in the pocket occupied by bicarbonate) rather than being a strong donor [24, 69].

Stability constants for $\text{VO}(\text{6-mepic})(\text{HSA})(\text{OH})$ and *cis*- $\text{VO}(\text{pic})_2(\text{HSA})$

The other two species that an insulin-enhancing compound can form in the presence of albumin are $\text{VO}(\text{6-mepic})(\text{HSA})(\text{OH})$ and *cis*- $\text{VO}(\text{pic})_2(\text{HSA})$, as confirmed by density functional theory calculations [30].

For these two species we were not able to calculate the stability constants from the examination of EPR spectra; thus, in a first approximation, the value of $\log \beta$ for the analogous complexes formed by 1-MeIm [3.82 for $\text{VO}(\text{6-mepic})(\text{HSA})(\text{OH})$ and 14.96 for *cis*- $\text{VO}(\text{pic})_2(\text{HSA})$, Table 1] will be used.

Discussion

The values of the stability constants reported in this work can be used to calculate the distribution of the species formed in some complicated systems like those recently discussed by some of us [23, 30, 40].

As a first example, we will compare the data predicted for the ternary $\text{VO}^{2+}/\text{hTf}/\text{HSA}$ system with different molar ratios, 2:1:1 and 2:1:17, with those experimentally obtained by EPR spectroscopy [30, 40]. The results are listed in Table 2, together with the percent amount of the species expected on the basis of the $\log \beta$ values calculated in [31].

Table 2 Predicted and measured percent distribution of the VO²⁺ species in the ternary system VO²⁺/hTf/HSA

Ratio VO ²⁺ /hTf/HSA	2:1:1 ^a			2:1:17 ^b		
	Predicted ^c	Predicted ^d	Measured ^e	Predicted ^c	Predicted ^d	Measured ^f
(VO)hTf + (VO) ₂ hTf	97.1	94.3 (97.7)	~95	84.0	79.7 (92.3)	~90
(VO)HSA	0.4	5.6 (1.7)	~0	3.1	20.2 (7.4)	~0
(VO) ₂ ^d HSA	2.5	–	~5	12.9	–	~10
(VO) ₂ (OH) ₅ + VO(OH) ₃	0.0	0.1 (0.6)	~0	0.0	0.1 (0.3)	~0

hTf human serum apotransferrin, *HSA* human serum albumin

^a 5.0 × 10⁻⁴ M VO²⁺

^b 8.8 × 10⁻⁵ M VO²⁺

^c Values predicted from the stability constants reported in this work

^d Values predicted from the stability constants reported in [31]; the data were obtained using log β₁[(VO)HSA] = 10 and, in parentheses, using with log β₁[(VO)HSA] = 9

^e Values measured from the EPR spectra and reported in [40]

^f Values measured from the EPR spectra and reported in [23]

It can be noticed that the experimental spectroscopic data are better fitted by the values reported in this work, mainly for the system with the physiological ratio of hTf to HSA (1:17). In particular, it emerges that the presence of the dinuclear species (VO)₂^dHSA should be considered, otherwise the fraction of VO²⁺ not complexed by transferrin will be incorrectly attributed to (VO)HSA.

Subsequently, the values of the thermodynamic stability constants reported above can be used to evaluate in which form the insulin-enhancing VO²⁺ compounds are transported in the human body. EPR spectra on the quinary systems formed by an insulin-enhancing agent with [VO(carrier)₂] composition (in particular, [VO(6-mepic)₂], *cis*-[VO(pic)₂(H₂O)], [VO(acac)₂], and [VO(dhp)₂]), by both the high molecular mass components of blood serum (transferrin and albumin), and one low molecular mass component (lactate or citrate) were presented recently [30]. The spectroscopic results obtained when the molar ratio of the components VO²⁺/hTf/HSA/carrier/lact was 2:1:3:4:40.8 could be compared with those expected on the basis of the log β values calculated in this work. The data are reported in Table 3.

In Table 3 it is observed that the predicted and measured percent values of the main species are in good agreement. In particular, the following comments can be made:

1. As suggested by the EPR spectra, in all the systems the presence of (VO)hTf(lact) and (VO)₂hTf(lact)₂ is expected, and their calculated amounts are in satisfactory agreement with those obtained experimentally [30].
2. As observed in the EPR spectra, in the system with 6-methylpicolinate a small amount of (VO)₂^dHSA is predicted [30].
3. The importance of mixed species formed by carrier and low molecular mass bioligands must not be

underestimated, as demonstrated by the case of picolinate: the presence of [VO(pic)(lactH₋₁)]⁻ species in solution can be clearly detected by EPR spectroscopy [30] and is expected on the basis of the thermodynamic stability constants.

4. The data for dhp agree well with the EPR results obtained in the ternary systems VO²⁺/dhp/hTf and VO²⁺/dhp/HSA, which can be explained by considering in solution an equal amount of *cis*-[VO(dhp)₂(hTf)] or *cis*-[VO(dhp)₂(HSA)] and [VO(dhp)₂]; only a small percentage of (VO)hTf, (VO)₂hTf, (VO)hTf(lact), and (VO)₂hTf(lact)₂ is expected [30].
5. In the system with dhp, the EPR spectra do not allow *cis*-VO(dhp)₂(HSA) to be distinguished from *cis*-VO(dhp)₂(hTf) owing to their similar A_z values [30]; from the stability constants calculated in this work, the mixed species formed by albumin should be the predominant species at physiological conditions (hTf to HSA ratio of 1:17).
6. In the systems with picolinate and dhp, only the sum of (VO)hTf(lact) and (VO)₂hTf(lact)₂, (VO)hTf(pic), and (VO)₂hTf(pic)₂, on one hand, and that of (VO)hTf and (VO)₂hTf, (VO)hTf(lact), and (VO)₂hTf(lact)₂, on the other, can be measured from EPR spectra owing to the similarity of their A_z value [30]; the calculated sums are, however, in very good agreement with those measured experimentally.
7. With the stability constants of the previous model for the species formed by dhp, in the system VO²⁺/hTf/HSA/dhp/lact with ratio 2:1:3:4:40.8 only [VO(dhp)₂] (53.7%) and the ternary species (VO)hTf(dhp), (VO)₂hTf(dhp), and (VO)₂hTf(dhp)₂ (sum 45.8%) are expected [31]. The EPR spectra reported in Fig. 4, instead, clearly show the presence of (VO)hTf, (VO)₂hTf, and the mixed species formed by lactate,

Table 3 Predicted and measured (in *parentheses*) percent distribution of the main species formed from the biotransformation of the insulin-enhancing VO²⁺ agents in the quinary system VO²⁺/hTf/HSA/dhp/lact

Species	[VO(6-mepic) ₂]	<i>cis</i> -[VO(pic) ₂ (H ₂ O)]	[VO(acac) ₂]	[VO(dhp) ₂]
(VO)hTf + (VO) ₂ hTf	83.2 (80–85)	70.5 (65–70)	78.6 (70–75)	7.8 (10–15) ^a
(VO)hTf(lact) + (VO) ₂ hTf(lact) ₂	9.6 (10–15)	8.8 (10–15) ^b	9.8 (5–10)	2.3 (10–15) ^a
(VO) ₂ ^d HSA	1.5 (0–5)	0.4 (0–0)	0.5 (0–0)	0.0 (0–0)
(VO)HSA	0.5 (0–0)	0.3 (0–0)	0.3 (0–0)	0.0 (0–0)
(VO)(6-mepic)(HSA)(OH)	0.0 (0–0)	–	–	–
(VO)hTf(pic) + (VO) ₂ hTf(pic) ₂	–	6.8 (10–15) ^b	–	–
[VO(carrier)(lactH ₋₁)] ^{x-}	–	9.4 (5–10)	(0–5) ^c	–
[VO(carrier) ₂]	0.1 (0–0)	1.0 (0–0)	7.9 (15–20)	50.9 (40–45) ^d
<i>cis</i> -VO(dhp) ₂ (hTf)	–	–	–	4.3 (40–45) ^e
<i>cis</i> -VO(carrier) ₂ (HSA)	–	0.2 (5–10)	–	34.6 (40–45) ^e
[VO(lactH ₋₁) ₂] ²⁻	5.1 (0–0)	2.6 (0–0)	2.9 (0–0)	0.1 (0–0)

The values measured from the EPR spectra and reported in [30] are given in *parentheses*

lact lactate, *acac* deprotonated form of acetylacetone, *pic* picolinate, *6-mepic* 6-methylpicolinate, *dhp* deprotonated form of 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone

^a Owing to the similarity of *A*_z for (VO)hTf, (VO)₂hTf, (VO)hTf(lact), and (VO)₂hTf(lact)₂ and their low amount, only the sum of their concentrations can be measured from EPR spectra

^b Owing to the similarity of *A*_z for (VO)hTf(lact), (VO)₂hTf(lact)₂, (VO)hTf(pic), and (VO)₂hTf(pic)₂, only the sum of their concentrations can be measured from EPR spectra

^c log β for the species [VO(acac)(lactH₋₁)]⁻ is not available in the literature

^d Present as an equilibrium mixture of [VO(dhp)₂] and *cis*-[VO(dhp)₂(H₂O)]

^e Owing to the similarity of *A*_z of *cis*-[VO(dhp)₂(hTf)] and *cis*-[VO(dhp)₂(HSA)], only the sum of their concentrations can be measured from EPR spectra

(VO)hTf(lact) and (VO)₂hTf(lact)₂, besides [VO(dhp)₂] and *cis*-VO(dhp)₂(hTf) or *cis*-VO(dhp)₂(HSA) [30].

8. The only difference with the experimental data is the possible presence of a small amount of the bis-chelated species formed by lactate, [VO(lactH₋₁)₂]²⁻ [74].

In Table 4 the data expected on the basis of the stability constants reported in this work for the species formed after the biotransformation of [VO(6-mepic)₂], *cis*-[VO(pic)₂(H₂O)], [VO(acac)₂], and [VO(dhp)₂], when the concentration of the vanadium drug is in the range necessary to observe insulin-enhancing effects, 1–100 μM [7, 48–50], and that of the bioligands is as in the blood serum, are summarized.

Until now, it has been affirmed in the literature that when the concentration of the insulin-enhancing agent is between 1 and 10 μM and the carrier is weak (6-methylpicolinate) or of intermediate strength (picolinate and acac), almost all of the VO²⁺ ion exists in the forms (VO)hTf and (VO)₂hTf [31, 37–39]; moreover, the previous results indicate that also with maltol for a concentration lower than 10 μM all of the metal ion should be present in solution as binary species of transferrin [31]. Our data partly confirm these assumptions and suggest that when the concentration of [VO(6-mepic)₂], *cis*-[VO(pic)₂(H₂O)], [VO(acac)₂] is between 1 and 10 μM, most of the VO²⁺ is

bound as binary complex to transferrin, but 4–5% should be present in the form of (VO)hTf(lact) ternary species. When the carrier is strong (dhp), for concentrations around 10 μM the undissociated form of the insulin-enhancing agent and the mixed complex *cis*-VO(dhp)₂(HSA) become important, whereas the concentration of (VO)hTf and (VO)₂hTf decreases. For dhp, our results are different from those of Jakusch et al. [31], who found a smaller amount of the binary complexes of hTf and a larger amount of the supposed species (VO)hTf(dhp).

If the concentration of the insulin-enhancing compounds is around 100 μM, both with the weak and the intermediate-strength carriers, the amounts of the species formed by albumin increase, in particular the amount of (VO)₂^dHSA, favored by a lower ratio of HSA to VO²⁺ [40]; for these ligands, it is possible that mixed species formed by the carrier and low molecular mass bioligands, such as [VO(pic)(lactH₋₁)]⁻, or the undissociated form of the insulin-enhancing agent, such as [VO(acac)₂], can be important. With strong ligands, such as dhp, [VO(dhp)₂] and *cis*-VO(dhp)₂(HSA), in which approximately 85% of the VO²⁺ is bound, become prevalent, whereas the remaining metal ions exist as the binary complex of transferrin [10.8 and 3.2% of (VO)hTf and (VO)₂hTf, respectively].

Table 4 Predicted percent distribution of the main species formed from the biotransformation of the insulin-enhancing agents at physiological conditions

Concentration (μM)	[VO(6-mepic) ₂]			<i>cis</i> -[VO(pic) ₂ (H ₂ O)]			[VO(acac) ₂]			[VO(dhp) ₂]		
	1	10	100	1	10	100	1	10	100	1	10	100
(VO)hTf	93.3	80.9	2.2	93.2	80.4	2.5	93.3	80.9	2.4	91.3	48.9	10.8
(VO) ₂ hTf	1.9	14.9	69.1	1.9	14.7	68.0	1.9	14.9	68.6	1.9	4.8	3.2
(VO)hTf(lact)	4.5	3.9	0.1	4.5	3.9	0.1	4.5	3.9	0.1	4.4	2.3	0.5
(VO) ₂ hTf(lact) ₂	0.0	0.0	0.2	0.0	0.0	0.2	0.0	0.0	0.2	0.0	0.0	0.0
(VO) ₂ ^d HSA	0.0	0.0	23.2	0.0	0.0	18.6	0.0	0.0	19.0	0.0	0.0	0.0
(VO)HSA	0.2	0.2	3.8	0.2	0.2	3.4	0.2	0.2	3.4	0.2	0.1	0.1
VO(6-mepic)(HSA)(OH)	0.0	0.0	0.1	–	–	–	–	–	–	–	–	–
(VO)hTf(pic)	–	–	–	0.1	0.6	0.2	–	–	–	–	–	–
(VO) ₂ hTf(pic) ₂	–	–	–	0.0	0.0	0.2	–	–	–	–	–	–
[VO(carrier)(lactH ₋₁)] ^{-a}	–	–	–	0.0	0.1	5.0	b	b	b	–	–	–
[VO(carrier) ₂]	0.0	0.0	0.1	0.0	0.0	0.6	0.0	0.0	5.2	1.2	25.0	49.9
<i>cis</i> -VO(dhp) ₂ (hTf)	–	–	–	–	–	–	–	–	–	0.0	0.4	0.6
<i>cis</i> -VO(carrier) ₂ (HSA) ^c	–	–	–	0.0	0.0	0.1	–	–	–	0.9	18.4	34.9
[VO(lactH ₋₁) ₂] ²⁻	0.1	0.1	1.1	0.1	0.1	1.0	0.1	0.1	1.0	0.1	0.1	0.0
[VO(OH) ₃] ⁻	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0

^a Formed with pic and acac

^b $\log \beta$ for the species [VO(acac)(lactH₋₁)]⁻ is not available in the literature

^c Formed with pic and dhp

It could also be noticed that with increasing concentration of insulin-enhancing agent (from 1 to 100 μM) with respect to that of transferrin (37 μM), the percent amount of (VO)hTf decreases (e.g., from 93.3 to 2.2% for the weakest carrier, 6-methylpicolinate) and that of (VO)₂hTf increases (from 1.9 to 69.1% for 6-methylpicolinate).

Conclusions and outlook

In this work a quantitative study of the biotransformations in conditions mimicking blood serum of four among the most representative insulin-enhancing vanadium drugs, [VO(6-mepic)₂], *cis*-[VO(pic)₂(H₂O)], [VO(acac)₂], and [VO(dhp)₂], was presented. To achieve this goal, the determination of the stability constants of the several species formed after the interaction with high molecular mass and low molecular mass bioligands of the serum was necessary. For the first time in the literature, the value of $\log \beta$ for (VO)₂^dHSA, *cis*-VO(dhp)₂(hTf), *cis*-VO(dhp)₂(HSA), (VO)hTf(lact), (VO)₂hTf(lact)₂, (VO)hTf(pic), and (VO)₂hTf(pic)₂ was reported. The correctness of the stability constants measured was confirmed by comparison of the calculated percent amounts in the VO²⁺/hTf/HSA/carrier/lact

quinary system with those measured experimentally from the examination of the EPR spectra.

The data obtained allow a good picture of the real conditions of the organism to be provided when the concentration of the vanadium compound is that necessary to show insulin-enhancing effects. Differently from the conclusions reached previously in the literature, our results suggest that VO²⁺ ion is mainly bound to transferrin only for weak and intermediate-strength carriers and for concentrations in the range 1–10 μM ; in these situations, however, the possibility for the formation of mixed species with a low molecular mass component, such as lactate, must be taken into account. With strong carriers and in all the cases when the concentration approaches 100 μM , the binary complexes of albumin, the mixed species formed by the low molecular mass bioligands, and the ternary complexes formed when the carrier stabilizes the *cis*-octahedral arrangement in aqueous solution, for example, *cis*-VO(pic)₂(HSA), *cis*-VO(dhp)₂(hTf), and *cis*-VO(dhp)₂(HSA), can become important and must be considered.

Finally, we would like to stress that a future improvement of the values of $\log \beta$ with respect to those reported in this work, the determination of the stability constants not reported in this work, and in vivo studies could help to provide an even better description of the biotransformation

and transport of an insulin-enhancing vanadium agent in blood serum.

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