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New Developments in the Comprehension of the Biotransformation and Transport of Insulin-Enhancing Vanadium Compounds in the Blood Serum

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The possible biotransformations in the blood serum of four representative *insulin-enhancing* vanadium compounds, $[VO(6\text{-mepic})_2]$, *cis*-$[VO(pic)_2(H_2O)]$, $[VO(acac)_2]$, and $[VO(dhp)_2]$, where 6-mepic, pic, acac, and dhp indicate the deprotonated forms of 6-methylpicolinic and picolinic acids, acetylacetone, and 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone, were examined. In particular, the behavior of the quinary systems formed by the *insulin-enhancing* species, human serum apo-transferrin (hTf), human serum albumin (HSA), and lactate (lact) or citrate (citr) at physiological pH and conditions was studied. The results indicate that, besides the case in which the ligand is very weak like 6-mepic, the *carrier* can interact in some form with VO^{2+} ion until its intake into the cell. In fact with stronger ligands like pic, acac, and dhp, VO^{2+} is transported not only by transferrin but also as $[VO(carrier)_2]$ and as mixed species VO^{2+}−hTF−*carrier*. There are two ways in which the undissociated form of a bis-chelated complex can interact with transferrin, one "specific" when the *carrier* possesses a carboxylate group and behaves like a synergistic anion, and another "non-specific" when an imidazole nitrogen of a histidine residue from hTf replaces an equatorially coordinated water molecule giving rise to a ternary species with *cis*-octahedral geometry and *cis*-$VO(carrier)_2$(hTf) stoichiometry. It is found that also albumin can participate in the transport of an *insulin-enhancing* compound forming a mixed species *cis*-$VO(carrier)_2$(HSA), when the *carrier* stabilizes in aqueous solution the *cis*-octahedral form, or the dinuclear compound $(VO)_2{}^d$HSA, when the *carrier* forms unstable complexes. These insights were confirmed through density functional theory (DFT) calculations.

Introduction

Among the various functions of vanadium in the biological systems,[1] those in the human organism are of relevant importance. It is commonly accepted that vanadium plays an essential role, even if its biochemical functions still remain unclear.[2] On the basis of its ability to inhibit many phosphate-metabolizing enzymes, such as phosphatases, ribonuclease, and ATPases,[3] it is probable that vanadium is involved in the regulation of phosphate metabolism.

More than one hundred years ago, it was discovered that vanadium can improve the state of patients suffering from diabetes mellitus.[4] One century later, the effect of vanadium on the glucose metabolism was confirmed through in vitro studies.[5] From 1980, many articles were published on the synthesis, reactivity, transformations, and biochemical activity of new *insulin-enhancing* vanadium compounds.[6] After the initial use of vanadium(IV) and vanadium(V) inorganic salts, it was found that neutral VO^{2+} complexes with bidentate anionic ligands (VOL_2) were more effective, better tolerated, and resulted in reliable glucose-lowering in

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all the animal models of diabetes than $VOSO_4$. In particular, [VO(ethylmaltolato)$_2$] or BEOV has arrived at phase IIa of the clinical trias.[7] The mechanism of action, based on the phosphate-like activity and/or interference with phosphatases,[3,5,8] and the active species, oxovanadium (VO^{2+}) or vanadate ($H_2VO_4^-$), are two questions still discussed. Over the past years, it has been suggested that vanadium is present in the blood serum in the oxidation state +IV, almost independently of the initial state,[2,9,10] and that vanadate is quickly converted to VO^{2+} ion in the erythrocytes by glutathione,[11] and in the plasma by several reductants.[12] These findings are confirmed by in vivo blood circulation monitoring-electron paramagnetic resonance (BCM-EPR) studies on rats;[13] moreover, it has been shown that, when vanadium is administered intravenously to rats or dogs, ~77% of the plasma fraction is bound to transferrin as V^{IV}, regardless of the form (vanadium(IV) or vanadium(V)) initially injected.[14]

It is not fully clear which vanadium concentration is necessary to observe *insulin-enhancing* activity: in some articles it is reported that it must be higher than 1 μM and lower than 10 μM,[7a,15,16] but elsewhere it is found that the maximum activity is for higher concentration, approximately in the range 100−400 μM.[1b,17] Another question under debate is in which form VO^{2+} ion is transported to the target organs in the human body: both the high-molecular-mass (hmm), like human serum apo-transferrin (hTf) and human serum albumin (HSA), and the low-molecular-mass (lmm) components of the blood serum can partly or fully displace the original *carrier* L of the *insulin-enhancing* agent VOL_2 to yield ternary species with transferrin or albumin or the corresponding binary complexes.[18−20]

Chasteen et al. found that the first association constants toward VO^{2+} of hTf and HSA are comparable (K_1(hTf)/K_1(HSA) ~6).[19] Their value has been improved subsequently by Kiss and co-workers, whose results suggest that there are at least 4 orders of magnitude difference between K_1(hTf) and K_1(HSA),[10,21] with the consequence that all of VO^{2+} ion should be bound to transferrin.[10,20,21] These assumptions have been recently reaffirmed.[22] Nevertheless, the possible existence of mixed species between the *insulin-enhancing* compound [VO(maltolato)$_2$] and transferrin or albumin was proposed by Willsky et al.,[23] whereas Orvig and co-workers reported that the formation of adducts between [VO(maltolato)$_2$] and albumin could be so important in the transport of the drug to be considered the pharmacologically active species.[24] These suggestions appear to be in agreement with in vivo BCM-EPR measurements in rats, which indicate that [VO(6-methylpicolinato)$_2$] reacts with albumin and that VO^{2+} is bound to HSA under serum conditions.[25]

Kiss and co-workers very recently proposed the formation of ternary complexes of transferrin with maltol (mal) and 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone (dhp), and attributed to them the composition (VO)hTf(mal), (VO)hTf(dhp), (VO)$_2$hTf(mal), (VO)$_2$hTf(dhp), (VO)$_2$hTf(mal)$_2$, and (VO)$_2$-hTf(dhp)$_2$ with the *carrier* ligand bound to one or both the lobes of the protein.[26] Moreover, they redetermined the values of log K_1 and log K_2 for the formation of (VO)hTf and (VO)$_2$hTf, 13.4 and 11.8, respectively.[26] The conditional binding constant value of albumin (log K_1) was set to ~10 if measured with spectroscopic methods,[10,21] and ~9 with ultrafiltration studies.[26]

The questions remaining open are how and where the *carrier* ligand can be coordinated by VO^{2+}−hTf species and if albumin and its ternary complexes may take part to the biotransformation and transport of *insulin-enhancing* drugs. In the previous studies, no particular emphasis has been given to the potential role of lmm components in the biospeciation of such species. However, we recently demonstrated that, among the six most important lmm components of the blood serum (lactate, citrate, oxalate, phosphate, glycine, and histidine), only lactate (lact) and citrate (citr), from here denoted as bL, can interact with the binary complexes of transferrin and albumin to form mixed species indicated as VO^{2+}−hTf−bL or VO^{2+}−HSA−bL;[27] they replace in the first case the bicarbonate ion in the active site of transferrin with a COO^- group, behaving as synergistic anions,[28] and in the second one the amino acid residues of albumin polypeptide chain coordinated to VO^{2+} with the (COO^-, O^-) set.[27] Therefore, the possible presence of these complexes and of

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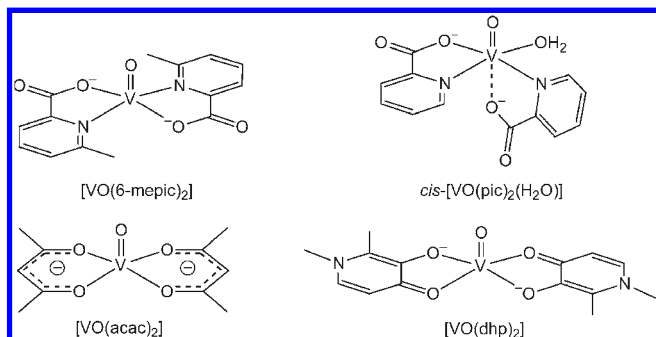
Scheme 1. *Insulin-Enhancing* Vanadium Compounds Studied in This Work



ternary species formed by one lmm component (lactate or citrate) and one *carrier* molecule like $[VO(carrier)(bLH_{-1})]^{x-}$ (with $x = 1$ or 3 if bL is lactate or citrate) cannot be neglected in the study of the biotransformations of an *insulin-enhancing* compound in the blood serum.

In this work, we studied the biospeciation in the presence of the two most important hmm components (transferrin and albumin) and lmm components (lactate and citrate) of four among the most representatives *insulin-enhancing* vanadium compounds, two with $VO(N_2O_2)$ and two with $VO(O_4)$ coordination: $[VO(6\text{-mepic})_2]$,[29] *cis*-$[VO(pic)_2(H_2O)]$,[6d,30] $[VO(acac)_2]$,[31] and $[VO(dhp)_2]$,[32] where 6-mepic, pic, acac, and dhp indicate the deprotonated form of 6-methylpicolinic and picolinic acids, acetylacetone, and 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone (Scheme 1).

The objectives of this work are as follows:

(i) The study of the ternary systems formed by $[VO(6\text{-mepic})_2]$, *cis*-$[VO(pic)_2(H_2O)]$, $[VO(acac)_2]$, and $[VO(dhp)_2]$ with transferrin or albumin to put in evidence the eventual presence of mixed species.

(ii) The study of the quaternary systems formed by one *insulin-enhancing* agent, transferrin, and one of the two lmm components (lactate or citrate) to understand if also a bL ligand can participate to the VO^{2+} binding.

(iii) The study of the quinary systems formed by the *insulin-enhancing* compound, transferrin, albumin, and lactate or citrate in the physiological conditions, that is, at pH 7.4 and using the ratio between transferrin, albumin, and bL of the blood serum.

The technique used in this work is electron paramagnetic resonance (EPR) spectroscopy, which revealed to be an excellent tool to study the biospeciation of VO^{2+} ion in these complicated systems;[33] in particular, when the spectra are recorded through the repeated acquisition of weak signals, it is possible to use a vanadium concentration in the range necessary to observe *insulin-enhancing* effects.[27,34]

The results of this study can help to understand how a VO^{2+} *insulin-enhancing* compound distributes among the blood serum components and is transported in the plasma toward the target sites in the organism.

Experimental Section

Chemicals. Water was deionized prior to use through the purification system Millipore Milli-Q Academic. VO^{2+} solutions were prepared from $VOSO_4 \cdot 3H_2O$ following literature methods.[35]

Human serum apo-transferrin and human serum albumin were obtained from Sigma. Apo-transferrin (98%) was obtained as a lyophilized powder with a molecular weight of 76−81 kDa (Sigma T4283). Albumin (97−99%), containing only trace amounts of fatty acids, was crystallized and lyophilized with a molecular weight of 66 kDa (Sigma A9511). The concentration of the protein solutions was estimated from their UV absorption (ε_{280}(hTf) = 92 300 M^{-1} cm^{-1}; ε_{278}(HSA) = 42 000 M^{-1} cm^{-1}).[19,36]

6-Methylpicolinic and picolinic acids, acetylacetone, 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone, lactic and citric acids, 1-methylimidazole (1-MeIm), $NaHCO_3$, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were of the highest grade available and were used as received.

Preparation of the *Insulin-Enhancing* Compounds. $[VO(6\text{-mepic})_2]$, *cis*-$[VO(pic)_2(H_2O)]$, $[VO(acac)_2]$, and $[VO(dhp)_2]$ were prepared according to the procedures established in the literature.[30,31b,32,37−40]

Preparation of the Solutions. The solutions were prepared dissolving in ultrapure water the *insulin-enhancing* compound to obtain a VO^{2+} concentration between 8.8×10^{-5} and 1×10^{-3} M and adding the opportune amount of lactate and citrate. Argon was bubbled through the solutions to ensure the absence of oxygen and avoid the oxidation of VO^{2+} ion. To the solution was added an appropriate amount of HEPES and $NaHCO_3$ to obtain a final concentration 0.1 M and 2.5×10^{-2} M, respectively. Electron paramagnetic resonance (EPR) studies performed on model systems prove that HEPES does not interact with VO^{2+} ion in the conditions used for the experiments.

To 1 mL of this solution, again carefully purged with argon, an amount of transferrin or albumin was added to obtain a concentration between 4.4×10^{-5} and 2.5×10^{-4} M for transferrin, or in the range $2.5{-}7.5 \times 10^{-4}$ M for albumin; readily, pH was adjusted to about 7.4.

The ratio between hTf and HSA and between such proteins and bL (lactate and citrate) in the examined solutions was the same of the blood serum to model the biological conditions well. A vanadium concentration in the range of that necessary to observe *insulin-enhancing* activity (approximately 1−400 μM) was used.[1b,15−17]

EPR spectra of all the model system (VO^{2+}-*carrier*, VO^{2+}-bL, and VO^{2+}-*carrier*-bL) were recorded with the same molar ratio used with transferrin or albumin but with a higher VO^{2+} concentration (1.0×10^{-3} M).

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The spectra of the system with 1-methylimidazole were recorded with a ratio of 1/2/4 between VO^{2+}, *carrier*, and 1-MeIm. The ratio between VO^{2+} and 1-methylimidazole (1/4) was set 16 times lower than that used in the systems with albumin to simulate the presence of 16 histidine residue in the polypeptide chain.[41]

EPR Spectroscopy. EPR spectra were recorded with an X-band (9.4 GHz) Bruker EMX spectrometer. Anisotropic spectra were recorded on frozen solutions at 120 K. The addition of DMSO was not necessary, and no improvement in the resolution of the spectra was obtained.

When the samples were transferred into the EPR tubes, the spectra were immediately measured. Only low temperature EPR spectra were measured to minimize the oxidation of VO^{2+} ion to vanadium(V), which otherwise would happen very quickly, with a half-time between 5 and 13 min at room temperature.[19] Moreover, as noticed in the literature,[42] an anisotropic EPR spectrum allows getting more information on the symmetry and the coordination geometry of a VO^{2+} complex, the identity of the equatorial donors through the application of the "additivity rule",[33,42,43] and the presence of minor species in solution with respect to an isotropic spectrum.

To increase the signal-to-noise ratio, signal averaging was used.[27,34]

As usually done for the analysis of the EPR spectra,[43] in all the figures reported in the text only the high field region, the part more sensitive to the identity and the amount of the several species in solution, is presented. The complete spectra are reported in the Supporting Information (Figures S10–S29).

DFT Calculations. All the calculations presented in this paper were performed with Gaussian 03 program (revision C.02)[44] and density functional theory (DFT) methods.[45] The hybrid exchange-correlation B3LYP,[46,47] and the half-and-half functional BHandHLYP, as incorporated in the Gaussian 03 software, were used.

As demonstrated in the literature, DFT simulations are a valid tool for predicting EPR parameters of VO^{2+} complexes.[48] Using the BHandHLYP functional and 6-311g(d,p) basis set it is possible to calculate the ^{51}V hyperfine coupling constant along the z axis (A_z) with a mean deviation from the experimental value lower than 3%.[49]

The geometries of the VO^{2+} complexes investigated were first pre-optimized at the B3LYP/sto-3g level and further optimized at the B3LYP/6-311g level of theory. For all the structures, minima were verified through frequency calculations. The optimized structures were used to calculate the values of ^{51}V A_{iso}, A_x, A_y, and A_z at the BHandHLYP/6-311g(d,p) level of theory. A_{iso} and A_z values (as well as A_x and A_y) are negative, but in the literature their absolute value is usually reported. This must kept in mind when positive values are discussed.

Table 1. EPR Parameters of the VO^{2+} Sites Measured at Physiological pH in the Binary Systems VO^{2+}-hTf and VO^{2+}-HSA

system	site	g_z	A_z[a]	g_z	A_z[a]	g_z	A_z[a]
VO^{2+}-hTf	A	1.937	168.3	1.938	168.0	1.938	168.0
	B1	1.941	170.5	1.941	170.3	1.938	170.0
	B2	1.935	171.8	1.937	172.4		
ref		34	34	33, 51	33, 51	52	52
VO^{2+}-HSA	$(VO)_x{}^mHSA$	1.947	164.6	1.939	172.8	1.927	166.5
	$(VO)_2{}^dHSA$[b]	1.981	80[c]				
ref		34	34	55	55	24	24

[a] Values measured in 10^{-4} cm^{-1}. [b] D value for dinuclear $(VO)_2{}^dHSA$ of 631×10^{-4} cm^{-1}. [c] Hyperfine coupling constant, measured in the parallel region of the spectrum, equal to one-half of the value which would be observed for the mononuclear species having the same equatorial coordination mode.

Results

(1). Binary and Ternary Systems of VO^{2+} Ion with Transferrin and Albumin. (a). VO^{2+}-Transferrin. The binary system VO^{2+}-hTf has been widely studied in the literature.[18,19,23,33,34,50–54] The EPR spectrum of frozen aqueous solution containing VO^{2+} and transferrin at physiological pH is composed by two sets of resonances, indicated as A and B,[33,50] with the B signals further split into two components, B_1 and B_2.[34,51,52] The spectral parameters are reported in Table 1. 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, as a carboxylate and an electron withdrawing group.[28]

(b). VO^{2+}-HSA. Several papers have been also devoted to the examination of the binary system VO^{2+}-HSA.[23,24,34,55] Initially, the presence of one "strong" and five "weak" binding sites with different EPR parameters has been proposed, with the "strong" one associated to the primary site for Cu^{2+}.[23,24,55] However, we recently demonstrated that in equimolar solution or with an excess of albumin, EPR spectra are characterized by the presence of signals attributable to a dinuclear species (denoted as $(VO)_2{}^dHSA$) with a spin state $S = 1$, whereas with an excess of VO^{2+} EPR resonances are assigned to a multinuclear complex (denoted as $(VO)_x{}^mHSA$) characterized by

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Table 2. EPR Parameters of the Species Formed in the Ternary Systems VO^{2+}-hTf-*carrier*

system[a,b]	g_z	A_z[c]	species	*carrier* donors
VO^{2+}-hTf-pic	~1.944	~167	VO^{2+}−hTf−pic	COO^-
	1.947	159.8	*cis*-$[VO(pic)_2(OH)]^-$	(N, COO^-); (N, COO^{-ax}); OH^-
VO^{2+}-hTf-acac	1.948	166.4	$[VO(acac)_2]$	($O^{\delta-}$, $O^{\delta-}$); ($O^{\delta-}$, $O^{\delta-}$)
VO^{2+}-hTf-dhp	1.940	166.1	*cis*-$[VO(dhp)_2(H_2O)]$	(CO, O^-); (CO, O^{-ax}); H_2O
	1.947	163.3	*cis*-$VO(dhp)_2$(hTf)	(CO, O^-); (CO, O^{-ax}); N_{His}[d]
	1.951	158.3	$[VO(dhp)_2]$	(CO, O^-); (CO, O^-)

[a] In all the systems $(VO)_2$hTf is also formed. [b] Concentration (M): $5.0 \times 10^{-4}/2.5 \times 10^{-4}/1.0 \times 10^{-3}$. [c] Values measured in 10^{-4} cm^{-1}. [d] N_{His} belonging to a histidine residue of transferrin.

a state $S = 1/2$.[34] The detection of only one set of EPR resonances at the physiological pH suggests that the five-six different metal ions bound by albumin have the same coordination: we called this site "high pH".[34] Its experimental A_z value (165×10^{-4} cm^{-1}, Table 1) allows for ruling out the (NH_2, N^-, N^-, N_{imid}) coordination, suggesting that the binding sites are relatively non-specific with respect to that of Cu^{2+} or Ni^{2+};[56] we propose that the coordination of three or four imidazole nitrogens, or of two imidazole nitrogen plus a carboxylate group belonging to an aspartate or a glutamate residue, should be more probable.[34]

(c). VO^{2+}-hTf-HSA. The ternary system VO^{2+}-hTf-HSA has been examined by Kiss and co-workers,[21,26] which stressed the impossibility of applying the biological ratio (1/17, with transferrin ~37 μM and albumin ~630 μM[57]). Instead, we recently measured EPR spectra using the physiological ratio between hTf and HSA and a VO^{2+} concentration in the range suitable to show *insulin-enhancing* effects (VO^{2+} 8.8×10^{-5} M, hTf 4.4×10^{-5} M, HSA 7.5×10^{-4} M) and found that transferrin is stronger than albumin, but this latter one partially compensates the lower affinity toward VO^{2+} with its higher concentration, forming the dinuclear species $(VO)_2{}^d$HSA;[27] such a species is favored by the ratio between albumin and VO^{2+} ion higher than 1 (see above). We approximately quantified its amount, on the basis of the EPR signal intensity and of the spectra simulations, around 10%. Therefore, the presence of $(VO)_2{}^d$HSA must be taken into account in the interpretation of the systems containing albumin.

(2). Ternary Systems of $[VO(carrier)_2]$ with Transferrin or Albumin. (a). $[VO(6\text{-mepic})_2]$-hTf. At the physiological pH the binary system formed by VO^{2+} ion and 6-methylpicolinic acid with a metal to ligand molar ratio of 1/2 shows extensive hydrolysis.[37] This is due to the weakness of 6-methylpicolinate as a ligand. Because of its low strength, 6-mepic is not able to compete with transferrin in the complexation of VO^{2+} ion. This is demonstrated by a comparison of EPR spectra (Figure S1 of the Supporting Information) recorded in the same experimental conditions of the systems with $VO^{2+}$and hTf on one hand and with VO^{2+}, hTf, and 6-methylpicolinate on the other, which shows that these are substantially coincident and confirms that all of the VO^{2+} ion is bound to transferrin as $(VO)_2$hTf.

(b). *cis*-$[VO(pic)_2(H_2O)]$-hTf. Differently from what is observed with 6-mepic, at the physiological pH in the binary system VO^{2+}-picolinate with metal to ligand molar ratio of 1/2, VO^{2+} is distributed between the bis-chelated species *cis*-$[VO(pic)_2(H_2O)]$ and *cis*-$[VO(pic)_2(OH)]^-$, with a lower concentration of the hydrolytic complex $[(VO)_2(OH)_5]^-$.[58]

In the presence of transferrin, an examination of the X-band anisotropic EPR spectra reveals that most of VO^{2+} ion is present in the form $(VO)_2$hTf, but the enlargement of the $M_I = 7/2$ transition toward lower fields indicates with no doubt that picolinate interacts with this complex (Figure S2 of the Supporting Information). The value of the ^{51}V anisotropic hyperfine coupling constant along the *z* axis, A_z, is about 167×10^{-4} cm^{-1} (Table 2). A similar behavior is shown by lactate and citrate, that form ternary complexes characterized by an identical value of A_z,[27] and that share with picolinate the presence of a carboxylate group and the features of synergistic anions.[28] The comparison of such an A_z value with 168.3×10^{-4} cm^{-1} measured for the A site of transferrin, indicates that in the two cases the equatorial donors have comparable strength. Therefore, it is probable that picolinate forms a ternary complex in which it replaces, at least partly, bicarbonate ion: in analogy with lactate and citrate, we will indicate it as VO^{2+}−hTf−pic, to put in evidence that we do not know its exact stoichiometry and if picolinate binds on both the A and B sites of transferrin.

(c). $[VO(acac)_2]$-hTf. Potentiometric and spectroscopic studies indicate that in the system VO^{2+}-acetylacetone, around the physiological pH, VO^{2+} ion is present as neutral complex $[VO(acac)_2]$ with very low hydrolysis degree.[38,39] The spectral parameters of $[VO(acac)_2]$ are listed in Table 2.[39]

A comparison of the anisotropic EPR spectra recorded at pH 7.4 on the systems VO^{2+}-hTf, VO^{2+}-acac, and VO^{2+}-hTf-acac reveals that in the ternary system two different species can be distinguished, one present in higher concentration, $(VO)_2$hTf, and another in lower amount, $[VO(acac)_2]$ (Figure S3 of the Supporting Information). Examining the intensities of EPR signals it is possible to attribute to them a percent concentration of 75−80 and 15−20%, respectively.

For this system, the spectroscopic measurements suggest that no mixed complexes are formed, and this is in agreement with the impossibility of acetylacetonate to act as a synergistic anion.

(d). $[VO(dhp)_2]$-hTf. Potentiometric data show that in the binary system with a VO^{2+} concentration of 1.0 mM and dhp of 2.0 mM the species $[VO(dhp)_2]$ predominates in solution in the pH range 5−8,[40] indicating that among the four examined

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ligands 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone is surely the strongest one. The bis-chelated complex is present in two isomeric forms in aqueous solution, the predominant $[VO(dhp)_2]$ and the minor *cis*-$[VO(dhp)_2(H_2O)]$;[40,59] their percent amount is around 80 and 20%, respectively, and EPR parameters are reported in Table 2.

Anisotropic EPR spectra recorded on aqueous solutions at pH 7.4 in the ternary system with VO^{2+}, hTf, and dhp show a species not observed in the binary systems, VO^{2+}-hTf and VO^{2+}-dhp. The considerably lower value of A_z in comparison with that of the A site of $(VO)_2$hTf (Table 2) suggests that this is a ternary complex. The existence of such a species has been first proposed by our research group[60] and subsequently confirmed by Jakusch et al.[26] These authors supposed that three different ternary complexes are formed with composition (VO)hTf(dhp), $(VO)_2$hTf(dhp), and $(VO)_2$hTf$(dhp)_2$ and determined the stability constants for such species. They assumed that the metal coordination in these complexes is the same and measured for this binding mode the following parameters: g_z = 1.940 and A_z = 164.5 × 10^{-4} cm^{-1}.[26] They also observed that the ternary complex formation was not competitive with the synergistic anion and, for this reason, proposed that also bicarbonate is in the first coordination sphere of VO^{2+} ion.[26]

What remains to be explained is the coordination mode of the metal ion in these complexes. Jakusch et al. reported that O partly replace N donors because the EPR signals are sharper,[26] and supposed that dhp enters in the two Fe^{3+} binding sites: if this was true, then all the ligands could in principle form ternary complexes with transferrin. Instead, we demonstrated that this is not the case (for example, acetylacetonate does not form ternary complexes with hTf) and so a different explanation is needed.

To interpret our and their data, we examined the ternary system formed by VO^{2+} ion, dhp, and 1-MeIm, that coordinates VO^{2+} ion exclusively through the imidazole nitrogen atom and can represent a valid model for the binding to a metal ion of the histidine nitrogen of a protein.[24] As it is possible to observe in Figure 1c, VO^{2+} forms in the presence of dhp and 1-MeIm only one species at pH 7.4, with A_z value (163.0 × 10^{-4} cm^{-1}) intermediate between those of $[VO(dhp)_2]$ and *cis*-$[VO(dhp)_2(H_2O)]$, interpretable as a mixed complex in which an imidazole nitrogen replaces the water molecule in the equatorial position of the *cis* isomer, with stoichiometry *cis*-$[VO(dhp)_2$(1-MeIm)]. Significantly, the A_z value for the ternary complex formed by VO^{2+}, hTf, and dhp is practically coincident with that of *cis*-$[VO(dhp)_2$(1-MeIm)] (cf. traces b and c of Figure 1): the stoichiometry of such a species is, therefore, *cis*-$VO(dhp)_2$(hTf). This new interpretation explains why there no competition between dhp and HCO_3^-:[26] the *carrier*, not possessing the structural features of a synergistic anion, cannot occupy the active sites of iron.

This hypothesis has been proved by performing simulations with density functional theory (DFT) methods on *cis*-$[VO(dhp)_2$(1-MeIm)]. The optimized structure for the species *cis*-$[VO(dhp)_2$(1-MeIm)] is shown in Figure 2a; the simulation suggest that the plane of the aromatic ring is arranged almost parallel to the V═O direction, and the dihedral angle measures 4.2°.

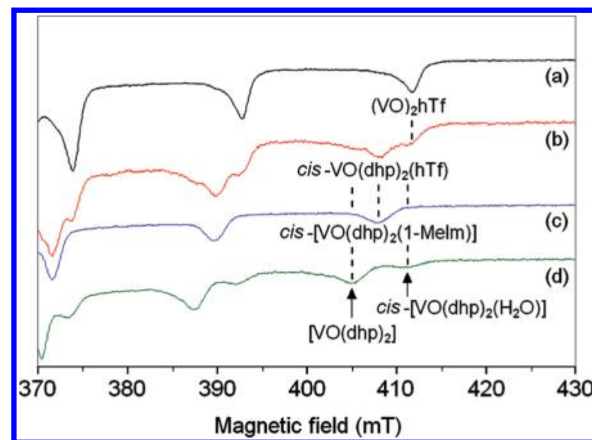

Figure 1. High field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing: (a) VO^{2+}/hTf 2/1 (VO^{2+} 5.0 × 10^{-4} M); (b) VO^{2+}/dhp/1-MeIm 1/2/4 (VO^{2+} 1.0 × 10^{-3} M); (c) VO^{2+}/hTf/dhp 2/1/4 (VO^{2+} 5.0 × 10^{-4} M), and (d) VO^{2+}/dhp 1/2 (VO^{2+} 1.0 × 10^{-3} M). HEPES 0.1 M and HCO_3^- 2.5 × 10^{-2} M in all the cases.

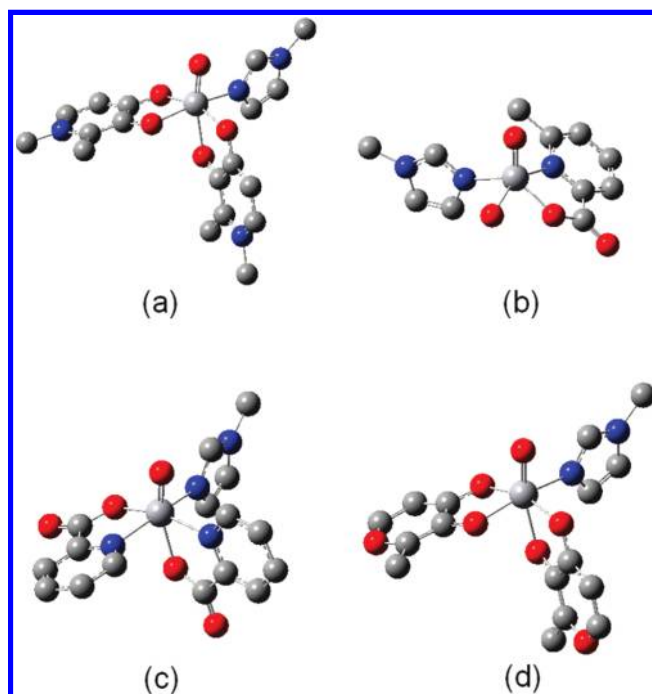

Figure 2. Calculated structures with DFT method at the level of theory B3LYP/6-311g of the complexes: (a) *cis*-$[VO(dhp)_2$(1-MeIm)], (b) [VO(6-mepic)(1-MeIm)(OH)], (c) *cis*-$[VO(pic)_2$(1-MeIm)], and (d) *cis*-$[VO(mal)_2$(1-MeIm)]. The hydrogen atoms have been omitted for clarity.

EPR parameters obtained from the simulations are reported in Table 3. As expected on the basis of the "additivity rule" and the contribution of an imidazole nitrogen with the ring almost parallel to the V═O bond,[61] A_z value calculated is lower than that of *cis*-$[VO(dhp)_2(H_2O)]$ and in significant agreement with experimental

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Table 3. EPR Parameters Calculated at the Level of Theory BHandHLYP/6-311g(d,p) for the Species Formed by 1-MeIm and OH^- with 6-mepic, pic, dhp, and mal[a]

complex	A_{iso}^{calcd}	T_x^{calcd}	T_y^{calcd}	T_z^{calcd}	A_x^{calcd}	A_y^{calcd}	A_z^{calcd}	A_z^{exptl}	$A_z^{exptl\ b}$	$\%\|A_z\|^{c}$
[VO(6-mepic)(1-MeIm)(OH)]	−92.9	32.2	36.0	−68.2	−60.6	−56.9	−161.1	−162.9	−162.9	−1.1
cis-[VO(pic)$_2$(1-MeIm)]	−90.0	31.8	34.8	−66.6	−58.2	−55.2	−156.6	−158.8	−159.7	−1.4
cis-[VO(pic)$_2$(OH)]$^-$	−87.7	30.3	37.1	−67.4	−57.4	−50.6	−155.1	−159.8		−2.9
cis-[VO(dhp)$_2$(1-MeIm)]	−92.9	32.4	35.4	−67.8	−60.5	−57.5	−160.7	−163.0	−162.1[d]	−1.4
cis-[VO(dhp)$_2$(OH)]$^-$	−95.8	34.1	35.2	−69.3	−61.7	−60.6	−165.1	−163.6		+0.9
cis-[VO(mal)$_2$(1-MeIm)]	−94.7	32.2	35.2	−67.4	−62.5	−59.5	−162.1	−164.8	−163.2	−1.6
cis-[VO(mal)$_2$(OH)]$^-$	−96.0	33.9	34.9	−68.8	−62.1	−61.1	−164.8	−167.2		−1.4

[a] All the A values measured in 10^{-4} cm^{-1}. [b] A_z^{exptl} of the complex with albumin instead of 1-MeIm. [c] Absolute percentage deviation from the experimental value for calculated as: $100 \times (|A_z|^{calcd} - |A_z|^{exptl})/|A_z|^{exptl}$. [d] A_z^{exptl} of the complex with transferrin is -163.3×10^{-4} cm^{-1}.

Table 4. EPR Parameters of the Species Formed in the Ternary Systems VO^{2+}-HSA-*carrier* and VO^{2+}-*carrier*-1-MeIm.[a]

system[a,b]	g_z	A_z^{c}	species	*carrier* donors
VO^{2+}-HSA-6-mepic	1.947	162.9	VO(6-mepic)(HSA)(OH)	(N, COO$^-$); N$_{His}$; OH$^-$[d]
VO^{2+}-6-mepic-1-MeIm	1.950	162.9	[VO(6-mepic)(1-MeIm)(OH)]	(N, COO$^-$); N$_{imid}$; OH$^-$
VO^{2+}-HSA-pic	1.950	159.7	*cis*-VO(pic)$_2$(HSA)	(N, COO$^-$); (N, COO^{-ax}); N$_{His}$[d]
	1.948	160.3	*cis*-[VO(pic)$_2$(OH)]$^-$	(N, COO$^-$); (N, COO^{-ax}); OH$^-$
VO^{2+}-pic-1-MeIm	1.943	158.8	*cis*-[VO(pic)$_2$(1-MeIm)]	(N, COO$^-$); (N, COO^{-ax}); N$_{imid}$
VO^{2+}-HSA-acac	1.950	166.5	[VO(acac)$_2$]	(O$^{\delta-}$, O$^{\delta-}$); (O$^{\delta-}$, O$^{\delta-}$)
VO^{2+}-HSA-dhp	1.940	166.2	*cis*-[VO(dhp)$_2$(H$_2$O)]	(CO, O$^-$); (CO, O^{-ax}); H$_2$O
	1.947	162.1	*cis*-VO(dhp)$_2$(HSA)	(CO, O$^-$); (CO, O^{-ax}); N$_{His}$[d]
	1.950	157.4	[VO(dhp)$_2$]	(CO, O$^-$); (CO, O$^-$)
VO^{2+}-dhp-1-MeIm	1.947	163.0	*cis*-[VO(dhp)$_2$(1-MeIm)]	(CO, O$^-$); (CO, O^{-ax}); N$_{imid}$

[a] In all the systems (VO)$_x^m$HSA is also formed. [b] Concentration (M): $1.0 \times 10^{-3}/0.25 \times 10^{-4}/2.0 \times 10^{-3}$ (HSA); $1.0 \times 10^{-3}/2.0 \times 10^{-3}/4.0 \times 10^{-3}$ (1-MeIm). [c] Values measured in 10^{-4} cm^{-1}. [d] N$_{His}$ belonging to a histidine residue of albumin.

values measured for *cis*-[VO(dhp)$_2$(1-MeIm)] and *cis*-VO(dhp)$_2$(hTf).

Differently from the other three systems just described, with 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone the mixed species *cis*-VO(dhp)$_2$(hTf) is the predominant complex, the two isomers [VO(dhp)$_2$] and *cis*-[VO(dhp)$_2$(H$_2$O)] are present in intermediate concentration, and (VO)$_2$hTf exists but in a minor amount. Regarding the relative amount of these species in aqueous solution at pH 7.4, however, our results are in agreement with those recently published.[26]

(e). [VO(6-mepic)$_2$]-HSA. Anisotropic EPR spectra recorded at physiological pH on the ternary system formed by VO^{2+}, HSA, and 6-mepic show the presence, besides (VO)$_x^m$HSA and the hydrolytic species of 6-methylpicolinate, of a species countersigned by $g_z = 1.947$ and $A_z = 162.9 \times 10^{-4}$ cm^{-1} (Table 4). The spectral parameters are intermediate between those of mono- and bis-chelated complexes ($A_z = 168 \times 10^{-4}$ cm^{-1} and 161×10^{-4} cm^{-1}, respectively) of 6-mepic,[37] suggesting that this could be a mixed species in which the VO^{2+} ion is coordinated by 6-methylpicolinate with the donor set (N, COO$^-$) and by albumin.

In this case too, the results have been compared with those obtained in the ternary system containing 1-methylimidazole instead of albumin. EPR spectra recorded for this system show, at pH 7.4, the formation of only one species having $g_z = 1.950$ and $A_z = 162.9 \times 10^{-4}$ cm^{-1} (Figure S4 of the Supporting Information). The similarity of these parameters with those measured for the mixed complex formed by albumin suggests that HSA binds the VO^{2+} ion with a histidine nitrogen atom. DFT simulation for [VO(6-mepic)(1-MeIm)(OH)] (Figure 2b) gives an absolute A_z value of 161.1×10^{-4} cm^{-1} (Table 3), suggesting that with albumin a species with stoichiometry VO(6-mepic)(HSA)(OH) is formed; in such a complex, only one 6-methylpicolinate, a ligand not particularly strong, is coordinated to VO^{2+} ion.

(f). *cis*-[VO(pic)$_2$(H$_2$O)]-HSA. Anisotropic EPR spectra of the ternary system VO^{2+}-HSA-pic show the coexistence of two species, one of which is surely the complex (VO)$_x^m$HSA (Figure 3).[24,34,55] However, for a correct interpretation of the data, a careful examination of the ternary system VO^{2+}-pic-1-MeIm is necessary.

The results can be interpreted considering the following experimental observations: (i) in the binary system VO^{2+}-pic, at pH 7.4, the complex *cis*-[VO(pic)$_2$(OH)]$^-$ is present;[58] (ii) on the basis of the "additivity rule", the replacement of the equatorial OH$^-$ in such a species with an imidazole nitrogen atom, in which the ring plane is parallel to the double bond V=O, should not significantly change the EPR parameters;[33,61] (iii) the A_z value for *cis*-[VO(pic)$_2$(1-MeIm)] should be lower of $4-6 \times 10^{-4}$ cm^{-1} with respect to that measured for *cis*-[VO(pic)$_2$(H$_2$O)] (165×10^{-4} cm^{-1});[33,61] (iv) a comparison of the spectra of the systems VO^{2+}-pic-HSA and VO^{2+}-pic-1-MeIm shows very small differences with A_z values of 159.7 and 158.8×10^{-4} cm^{-1}, respectively (Table 4). Therefore, even if A_z for *cis*-[VO(pic)$_2$(OH)]$^-$ and *cis*-VO(pic)$_2$(HSA) should be similar, our conclusion is that an imidazole donor from albumin replaces, at least partly, the equatorial OH$^-$ ion in the hydroxo species leading to the formation of the mixed complex *cis*-VO(pic)$_2$(HSA).

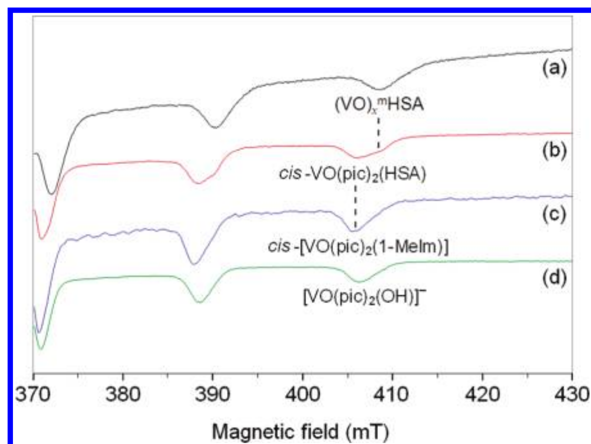


Figure 3. High field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing (a) VO^{2+}/HSA 4/1 (VO^{2+} 1.0 × 10^{-3} M), (b) VO^{2+}/HSA/pic 4/1/8 (VO^{2+} 1.0 × 10^{-3} M), (c) VO^{2+}/pic/1-MeIm 1/2/4 (VO^{2+} 1.0 × 10^{-3} M), and (d) VO^{2+}/pic 1/2 (VO^{2+} 1.0 × 10^{-3} M). HEPES 0.1 M and HCO_3^- 2.5 × 10^{-2} M in all the cases.

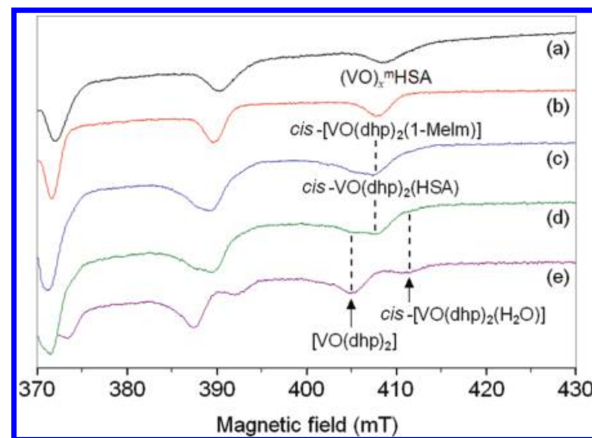


Figure 4. High field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing: (a) VO^{2+}/HSA 4/1 (VO^{2+} 1.0 × 10^{-3} M); (b) VO^{2+}/dhp/1-MeIm 1/2/4 (VO^{2+} 1.0 × 10^{-3} M), (c) VO^{2+}/HSA/dhp 4/1/8 (VO^{2+} 1.0 × 10^{-3} M), (d) sum of the spectra obtained in the systems VO^{2+}/dhp/1-MeIm (trace b) and VO^{2+}/dhp (trace e), and (e) VO^{2+}/dhp 1/2 (VO^{2+} 1.0 × 10^{-3} M). HEPES 0.1 M and HCO_3^- 2.5 × 10^{-2} M in all the cases.

Analogous results have been obtained for the mixed complex formed by $[VO(mal)_2]$ with albumin or 1-methylimidazole, for which a decrease of 6−8 × 10^{-4} cm^{-1} in the A_z value going from *cis*-$[VO(mal)_2(H_2O)]$ (171 × 10^{-4} cm^{-1})[62] to *cis*-$[VO(mal)_2(HSA)]$ (163 × 10^{-4} cm^{-1}) or *cis*-$[VO(mal)_2(1\text{-MeIm})]$ (165 × 10^{-4} cm^{-1}) is observed;[24] moreover, the A_z value of the adduct with albumin shows parameters comparable with those of *cis*-$[VO(mal)_2(OH)]^-$ (167 × 10^{-4} cm^{-1}).[62]

These findings have been demonstrated through DFT simulations on *cis*-$[VO(pic)_2(1\text{-MeIm})]$, *cis*-$[VO(pic)_2(OH)]^-$, and, for comparison, on *cis*-$[VO(mal)_2(1\text{-MeIm})]$ and *cis*-$[VO(mal)_2(OH)]^-$ complexes. The optimized structures of *cis*-$[VO(pic)_2(1\text{-MeIm})]$ and *cis*-$[VO(mal)_2(1\text{-MeIm})]$ are shown in Figures 2c and 2d, and the calculated EPR parameters in Table 3. The results indicate that the 1-methylimidazole coordinates in the equatorial plane with V−N distances of 2.109 and 2.139 Å and with bond angles O═V−N of 94.7 and 89.3°, respectively. As already noticed for *cis*-$[VO(dhp)_2(1\text{-MeIm})]$, the plane of the aromatic ring disposes almost parallel to the V═O direction, and the dihedral angles are −5.5° (pic) and 4.0° (mal).

From an examination of Table 3 it can be concluded that the absolute values of A_z calculated for *cis*-$[VO(carrier)_2(1\text{-MeIm})]$ differ less than 1.6% in comparison with the experimental ones and that these increase in the order $|A_z|$ (pic) < $|A_z|$ (dhp) < $|A_z|$ (mal), in agreement with what is experimentally observed. Moreover, DFT results confirm our supposition, showing that the A_z values for the complexes $[VO(carrier)_2(1\text{-MeIm})]$ and *cis*-$[VO(carrier)_2(OH)]^-$ are very similar and that, therefore, the contribution to A_z of an imidazole nitrogen, when the ring plane is almost parallel to the V═O bond, is comparable to that of an OH^- ion. Chasteen and Smith et al. empirically calculated the values of 38.7 and 38.9 × 10^{-4} cm^{-1} for the contribution of equatorial OH^- and N_{imid} donors.[33,61]

(g). $[VO(acac)_2]$-HSA. Anisotropic EPR spectra (Figure S5 of the Supporting Information) recorded at the physiological pH on the ternary system VO^{2+}-HSA-acac show the presence of the bis-chelated complex formed by acetylacetonate, $[VO(acac)_2]$, as the exclusive species. The examination of the ternary systems containing 1-methylimidazole, for which EPR spectra are practically indistinguishable from those of the binary system VO^{2+}-acac, confirms that there is no tendency to the formation of mixed species. This observation cannot be justified only with the stability of $[VO(acac)_2]$, but also considering that in such a species the absence of a water molecule coordinated in the equatorial position to be replaced by a monodentate donor like a histidine nitrogen prevents the formation of ternary complexes.

(h). $[VO(dhp)_2]$-HSA. Anisotropic EPR spectra of the ternary system VO^{2+}-HSA-dhp show the presence of a species not observable in the parent binary systems (Figure 4). The A_z value, very similar to that measured in the system VO^{2+}-hTf-dhp (Tables 2 and 4), induces us to suppose that an imidazole nitrogen belonging to a histidine residue of albumin replaces the water molecule in the equatorial position of *cis*-$[VO(dhp)_2(H_2O)]$ to form a species with stoichiometry *cis*-$VO(dhp)_2(HSA)$.

We believe that such a complex is analogous to *cis*-$VO(mal)_2(HSA)$[24] and *cis*-$VO(pic)_2(HSA)$ (see above). The comparison with the anisotropic EPR spectra recorded at pH 7.4 on the system containing VO^{2+}, dhp, and 1-MeIm, where only *cis*-$[VO(dhp)_2(1\text{-MeIm})]$ exists, further supports this attribution (Figure 4b). Interestingly, the sum of spectra recorded in the same experimental conditions on the systems VO^{2+}-dhp and VO^{2+}-dhp-1-MeIm (Figures 4b and 4e) gives a spectrum (Figure 4d) exactly coincident with that obtained in the system VO^{2+}-HSA-dhp, confirming that in this case the binding mode of albumin can be simply described as that of an imidazole ligand.

These conclusions are confirmed by DFT simulations (Table 3).

(3). Quaternary Systems of $[VO(carrier)_2]$ with Transferrin, Albumin and bL (Lactate or Citrate). **(a). $[VO(6\text{-mepic})_2]$-hTf-bL.** In the system with VO^{2+}, transferrin, 6-methypicolinate, and lactate or citrate, EPR anisotropic spectra recorded

(62) Buglyó, P.; Kiss, E.; Fabian, I.; Kiss, T.; Sanna, D.; Garribba, E.; Micera, G. *Inorg. Chim. Acta* **2000**, *306*, 174–183.

at pH 7.4 are indistinguishable from those of the ternary system VO^{2+}-hTf-lact or VO^{2+}-hTf-citr (Figure S6 of the Supporting Information), suggesting that the presence of 6-methypicolinate does not change the complexation equilibria. Therefore, in the quaternary system, VO^{2+} ion is distributed among $(VO)_2$hTF (~80−85%) and VO^{2+}−hTf−lact or VO^{2+}−hTf−citr (~10−15%).

(b). *cis*-[VO(pic)$_2$(H$_2$O)]-hTf-bL. In the quaternary systems VO^{2+}-hTf-pic-lact and VO^{2+}-hTf-pic-citr at molar ratio 2/1/4/40.8 and 2/1/4/2.68 four different species, that can be characterized by comparison with EPR spectra recorded on the respective ternary systems, coexist. Most of VO^{2+} is bound to transferrin as $(VO)_2$hTf and a consistent part as mixed species VO^{2+}−hTf−bL and VO^{2+}−hTf−pic, in which lactate or citrate and picolinate insert into the specific sites of iron replacing bicarbonate. The fourth species in both the systems is the ternary complex formed by *carrier* and bL, $[VO(pic)(lactH_{-1})]^-$ and $[VO(pic)(citrH_{-1})]^{3-}$, where lactate and citrate coordinate vanadium with the (COO^-, O^-) and (COO^-, O^-, COO^{-ax}) donor sets.[37]

EPR spectra with citrate are shown in Figure 5. It is worth noticing that the shoulders at lower field of the characteristic resonances of $(VO)_2$hTF increase in intensity with respect to those detected in the ternary systems

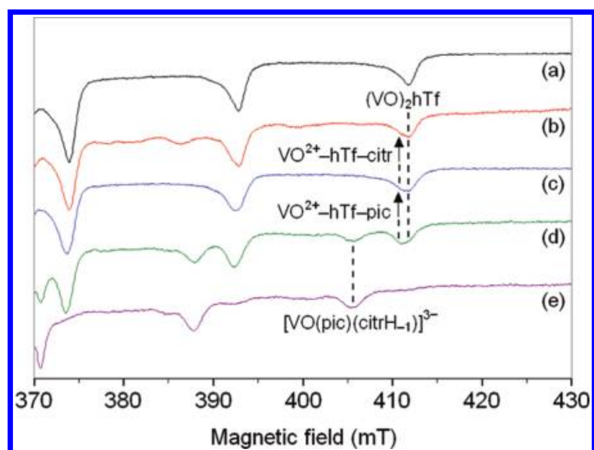


Figure 5. High field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing (a) VO^{2+}/hTf 2/1 (VO^{2+} 5.0 × 10^{-4} M), (b) VO^{2+}/hTf/citr 2/1/2.68 (VO^{2+} 5.0 × 10^{-4} M), (c) VO^{2+}/hTf/pic 2/1/4 (VO^{2+} 5.0 × 10^{-4} M), (d) VO^{2+}/hTf/pic/citr 2/1/4/2.68 (VO^{2+} 5.0 × 10^{-4} M), and (e) VO^{2+}/pic/citr 1/2/1.34 (VO^{2+} 1.0 × 10^{-3} M). HEPES 0.1 M and HCO_3^- 2.5 × 10^{-2} M in all the cases.

demonstrating that these are due to the sum of the signals belonging to the two species VO^{2+}−hTf−citr and VO^{2+}−hTf−pic. Moreover, the signals of $[VO(pic)(citrH_{-1})]^{3-}$ complex are easily observable (Table 5).

(c). [VO(acac)$_2$]-hTf-bL. In the quaternary systems with lactate or citrate, the results of EPR measurements indicate that at physiological pH the formation of mixed species between transferrin and acetylacetonate is disfavored. In solution the two species $(VO)_2$hTF and the *insulin-enhancing* compound in its original form [VO(acac)$_2$] predominate.

The spectra recorded on the quaternary system with lactate are represented in Figure S7 of the Supporting Information. The lmm component contributes to the biospeciation of VO^{2+} ion forming the mixed species VO^{2+}−hTf−lact (or VO^{2+}−hTf−citr) and a low amount of $[VO(acac)(lactH_{-1})]^-$ (or $[VO(acac)(citrH_{-1})]^{3-}$). These latter complexes were not previously characterized in the literature but their binding modes and composition have been assigned on the basis of a comparison with ternary systems containing VO^{2+}, acac, and lact or citr; the proposed coordination modes are ($O^{\delta-}$, $O^{\delta-}$); (COO^-, O^-) and ($O^{\delta-}$, $O^{\delta-}$); (COO^-, O^-, COO^{-ax}), respectively (Table 5). However, the participation of these ternary species in the biospeciation of [VO(acac)$_2$] seems to be less important than in the systems with picolinate.

(d). [VO(dhp)$_2$]-hTf-bL. The comparison between the quaternary VO^{2+}-hTf-dhp-bL and ternary VO^{2+}-hTf-dhp systems shows a substantial coincidence of the spectral pattern. The species present in aqueous solution at pH 7.4 are $(VO)_2$hTF, the two isomers of *insulin-enhancing* agent [VO(dhp)$_2$] and *cis*-[VO(dhp)$_2$(H$_2$O)] in their relative amount,[40,59] and the mixed species formed by transferrin and dhp, *cis*-VO(dhp)$_2$(hTf), see above. However, the presence of VO^{2+}−hTf−lact or VO^{2+}−hTf−citr, whose EPR resonances fall close to those of $(VO)_2$hTf, cannot be excluded (Figure S8 of the Supporting Information).

(4). Quinary Systems of [VO(*carrier*)$_2$] with Transferrin, Albumin and bL (Lactate or Citrate). After the examination of the quaternary systems with hTf, we here describe those quinary formed by [VO(*carrier*)$_2$], by both the hmm components of the blood serum, transferrin and albumin, and one lmm component, lactate or citrate (bL).

For the recording of the EPR spectra two experimental conditions have been used. In the first case, the molar

Table 5. EPR Parameters of the Ternary Species Formed in the Quaternary Systems VO^{2+}-hTf-*carrier*-bL[a]

system[a,b]	g_z	A_z[c]	species	*Carrier*/bL donors
VO^{2+}-hTf-6-mepic-lact	~1.939	~167	VO^{2+}−hTf−lact	COO^-
VO^{2+}-hTf-6-mepic-citr	~1.939	~167	VO^{2+}−hTf−citr	COO^-
VO^{2+}-hTf-pic-lact	~1.939	~167	VO^{2+}−hTf−lact	COO^-
	1.951	156.8	$[VO(pic)(lactH_{-1})]^-$	(N, COO^-); (COO^-, O^-)
VO^{2+}-hTf-pic-citr	~1.939	~167	VO^{2+}−hTf−citr	COO^-
	1.951	158.4	$[VO(pic)(citrH_{-1})]^{3-}$	(N, COO^-); (COO^-, O^-, COO^{-ax})
VO^{2+}-hTf-acac-lact	~1.939	~167	VO^{2+}−hTf−lact	COO^-
	1.950	156.8	$[VO(acac)(lactH_{-1})]^-$	($O^{\delta-}$, $O^{\delta-}$); (COO^-, O^-)
VO^{2+}-hTf-acac-citr	~1.939	~167	VO^{2+}−hTf−citr	COO^-
	1.948	157.5	$[VO(acac)(citrH_{-1})]^{3-}$	($O^{\delta-}$, $O^{\delta-}$); (COO^-, O^-, COO^{-ax})

[a] In all the systems $(VO)_2$hTf is also formed. [b] Concentration (M): 5.0 × 10^{-4}/2.5 × 10^{-4}/1.0 × 10^{-3}/1.02 × 10^{-2} (lact); 5.0 × 10^{-4}/2.5 × 10^{-4}/1.0 × 10^{-3}/6.69 × 10^{-4} (citr). [c] Values measured in 10^{-4} cm^{-1}.

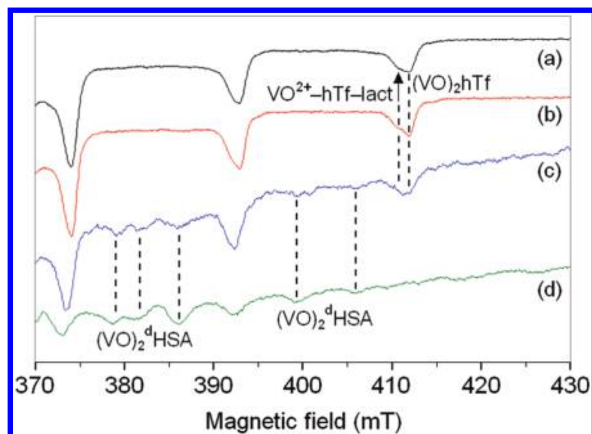


Figure 6. High field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing: (a) VO^{2+}/hTf/lact 2/1/40.8 (VO^{2+} 5.0 × 10^{-4} M), (b) VO^{2+}/hTf/6-mepic/lact 2/1/4/40.8 (VO^{2+} 5.0 × 10^{-4} M), (c) VO^{2+}/hTf/HSA/6-mepic/lact 2/1/3/4/40.8 (VO^{2+} 5.0 × 10^{-4} M), and (d) VO^{2+}/HSA 1/1 (VO^{2+} 7.5 × 10^{-4} M). HEPES 0.1 M and HCO_3^- 2.5 × 10^{-2} M in all the cases.

ratio between the components VO^{2+}/hTf/HSA/*carrier* was 2/1/3/4, with the concentration of lactate and citrate 40.8 and 2.68 times that of transferrin: this procedure has the advantage to obtain a good signal-to-noise ratio, but the disadvantage to not consider the physiological ratio between transferrin and albumin, that is, 1/17. In the second case, the ratio was 2/1/17/4/40.8 with lactate and 2/1/17/4/2.68 with citrate: in these experiments it is necessary to decrease the VO^{2+} concentration to 8.8 × 10^{-5} M because of the low solubility of albumin in aqueous solution (7.5 × 10^{-4} M), and very weak signals in spite of high number of recorded spectra are detected.

(a). [VO(6-mepic)$_2$]-hTf-HSA-bL. EPR anisotropic spectrum recorded at pH 7.4 in the quinary system VO^{2+}-hTf-HSA-6-mepic-lact with molar ratio 2/1/3/4/40.8 is reported in Figure 6, together with some other ones presented for comparison. Its behavior is very similar to the quaternary system VO^{2+}-hTf-HSA-lact,[27] and the resonances of the predominant complex $(VO)_2$hTF, of the mixed species VO^{2+}−hTf−lact, and, in low concentration, of $(VO)_2{}^d$HSA can be observed. As expected, the dinuclear species formed by albumin is present in higher amount than in the system where the ratio between VO^{2+}, hTf, and HSA is 2/1/1,[34] in agreement with the observation that $(VO)_2{}^d$HSA is favored by an excess of albumin. The percentage amounts of $(VO)_2$hTF, VO^{2+}−hTf−lact and $(VO)_2{}^d$HSA are comparable with those calculated previously for the system VO^{2+}-hTf-HSA-lact.[27]

The spectra recorded on the system VO^{2+}-hTf-HSA-6-mepic-lact with ratio 2/1/17/4/40.8 and with VO^{2+} concentration of 8.8 × 10^{-5} M are very similar, except for the lower signal-to-noise ratio and for a decrease of the amount of $(VO)_2{}^d$HSA, disfavored by the lower concentration of the metal ion.

EPR spectra of the system VO^{2+}-hTf-HSA-6-mepic-citr (2/1/3/4/2.68) do not show appreciable differences with those containing lactate, even if the presence of a low amount of $[(VO)_2(citrH_{-1})_2]^{4-}$[63] cannot be excluded.[27]

(63) Kiss, T.; Buglyó, P.; Sanna, D.; Micera, G.; Decock, P.; Dewaele, D. *Inorg. Chim. Acta* **1995**, *239*, 145–153.

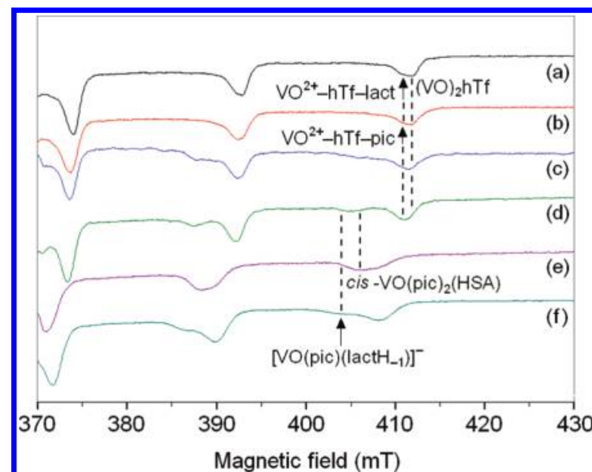


Figure 7. High field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing (a) VO^{2+}/hTf/lact 2/1/40.8 (VO^{2+} 5.0 × 10^{-4} M), (b) VO^{2+}/hTf/pic 2/1/4 (VO^{2+} 5.0 × 10^{-4} M), (c) VO^{2+}/hTf/pic/lact 2/1/4/40.8 (VO^{2+} 5.0 × 10^{-4} M), (d) VO^{2+}/hTf/HSA/pic/lact 2/1/3/4/40.8 (VO^{2+} 5.0 × 10^{-4} M), (e) VO^{2+}/HSA/pic 4/1/8 (VO^{2+} 1.0 × 10^{-3} M), and (f) VO^{2+}/pic/lact 1/2/20.4 (VO^{2+} 1.0 × 10^{-3} M). HEPES 0.1 M and HCO_3^- 2.5 × 10^{-2} M in all the cases.

The results suggest that 6-methylpicolinate, the weakest among the four *carriers* studied, is completely removed from the first coordination sphere of VO^{2+} by the hmm and lmm components of the blood serum, particularly transferrin.

(b). *cis*-[VO(pic)$_2$(H$_2$O)]-hTf-HSA-bL. EPR spectra of the quinary system VO^{2+}-hTf-HSA-pic-lact with molar ratio 2/1/3/4/40.8, shown in Figure 7, reveal that most of VO^{2+} is bound to transferrin in the form $(VO)_2$hTF and one part as mixed species VO^{2+}−hTf−lact and VO^{2+}−hTf−pic, with lactate and picolinate behaving as synergistic anions.[28]

Besides these complexes, the M_I = 5/2, 7/2 transitions centered around 405 and 387 mT are observed; the broad band and the value of the magnetic field of resonance, intermediate between those of $[VO(pic)(lactH_{-1})]^-$ and *cis*-$VO(pic)_2$(HSA), induce us to think that there is the contemporaneous presence of a small amount of these two species.

In this system the signals attributable to $(VO)_2{}^d$HSA are not observable because picolinate binds VO^{2+} ion more strongly than 6-methylpicolinate, favoring the formation of mixed species.

Similar results are found in the quinary system containing citrate: besides $(VO)_2$hTF and the mixed species VO^{2+}−hTf−citr and VO^{2+}−hTf−pic, the resonances of $[VO(pic)(citrH_{-1})]^{3-}$[37] and *cis*-$VO(pic)_2$(HSA) are observed.

(c). [VO(acac)$_2$]-hTf-HSA-bL. As already observed above, acetylacetonate is not able to form mixed complexes with transferrin and albumin, because it is not a synergistic anion, the square pyramidal geometry precludes the presence of an equatorial water molecule which can be replaced by a monodentate donor, and [VO(acac)$_2$] is a relatively stable species. In the ternary systems with hTf and HSA, $(VO)_2$hTF and [VO(acac)$_2$] in the first case, and exclusively [VO(acac)$_2$] in the second one, are present. Therefore, since albumin does not form with acac mixed species, we expect to detect in the quinary system the same species observed in the quaternary system VO^{2+}-hTf-acac-bL (Figure S7 of the Supporting Information).

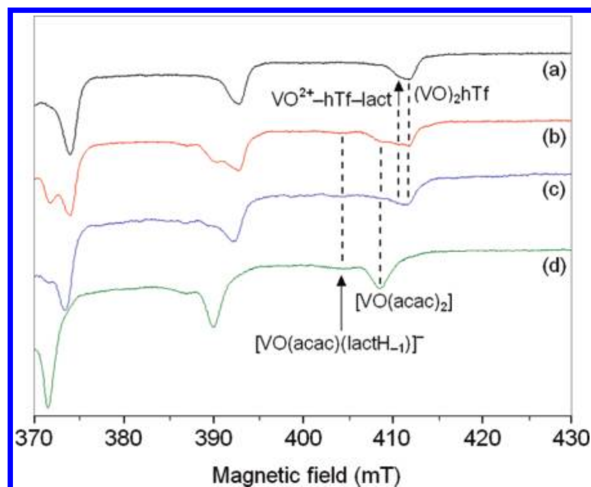


Figure 8. High field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing (a) VO^{2+}/hTf/lact 2/1/40.8 (VO^{2+} 5.0 × 10^{-4} M), (b) VO^{2+}/hTf/acac/lact 2/1/4/40.8 (VO^{2+} 5.0 × 10^{-4} M), (c) VO^{2+}/hTf/HSA/acac/lact 2/1/3/4/40.8 (VO^{2+} 5.0 × 10^{-4} M), and (d) VO^{2+}/acac/lact 1/2/20.4 (VO^{2+} 1.0 × 10^{-3} M). HEPES 0.1 M and HCO_3^- 2.5 × 10^{-2} M in all the cases.

This is just what is observed (Figure 8): $(VO)_2$hTF and [VO(acac)$_2$] are the predominant and secondary species, whereas the presence of lactate leads to the formation of the ternary complexes VO^{2+}−hTf−lact and [VO(acac)(lactH$_{-1}$)]$^-$. As in the system with picolinate, the dinuclear species of albumin $(VO)_2{}^d$HSA is not detected.

In Figure S9 of the Supporting Information the spectra of the system with lactate in physiological conditions, that is using a ratio 2/1/17/4/40.8 between VO^{2+}, hTf, HSA, acac, and lactate, are reported. They are less intense than those presented in Figure 8 for the lower concentration of VO^{2+} (8.8 × 10^{-5} vs 5.0 × 10^{-4} M), but confirm the results just described: the main species in aqueous solution are $(VO)_2$hTF and [VO(acac)$_2$]. The expected increase of concentration of $(VO)_2{}^d$HSA is not observed, both for the low signal-to-noise ratio and, probably, for the lower concentration of the metal ion.

Analogous behavior is shown by the quinary system containing citrate: in this case besides $(VO)_2$hTF and [VO(acac)$_2$], the species VO^{2+}−hTf−citr, [VO(acac)(citrH$_{-1}$)]$^{3-}$ and, in low concentration, [$(VO)_2$(citrH$_{-1}$)$_2$]$^{4-63}$ are formed.

(d). [VO(dhp)$_2$]-hTf-HSA-bL. Anisotropic EPR spectra recorded in aqueous solution at pH 7.4 on the quinary systems VO^{2+}-hTf-HSA-dhp-lact (Figure 9) and VO^{2+}-hTf-HSA-dhp-citr with molar ratio 2/1/3/4/40.8 and 2/1/3/4/2.68 shows the same ternary species observed in the systems VO^{2+}/dhp/1-MeIm, VO^{2+}/hTf/dhp, and VO^{2+}/HSA/dhp, in which transferrin and/or albumin replace the water molecule coordinated in the equatorial position of *cis*-[VO(dhp)$_2$(H_2O)] with an imidazole nitrogen of a histidine residue.

From an examination of Figure 9, it is evident that $(VO)_2$hTf is not the predominating species like in the previous systems, but that in solution the complexes formed by the *carrier* mainly exist: particularly, *cis*-VO(dhp)$_2$(hTf) and *cis*-VO(dhp)$_2$(HSA), and the undissociated form of the *insulin-enhancing* compound present in the two isomers, [VO(dhp)$_2$] and *cis*-[VO(dhp)$_2$(H_2O)].[40,59] Also the ternary species VO^{2+}−hTf−lact is

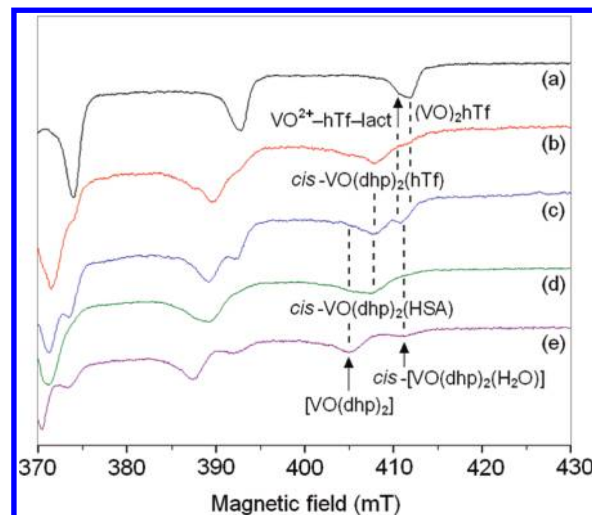


Figure 9. High field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing (a) VO^{2+}/hTf/lact 2/1/40.8 (VO^{2+} 5.0 × 10^{-4} M), (b) VO^{2+}/hTf/dhp/lact 2/1/4/40.8 (VO^{2+} 5.0 × 10^{-4} M), (c) VO^{2+}/hTf/HSA/dhp/lact 2/1/3/4/40.8 (VO^{2+} 5.0 × 10^{-4} M), (d) VO^{2+}/HSA/dhp 4/1/8 (VO^{2+} 1.0 × 10^{-3} M), and (e) VO^{2+}/dhp 1/2 (VO^{2+} 1.0 × 10^{-3} M). HEPES 0.1 M and HCO_3^- 2.5 × 10^{-2} M in all the cases.

observed, while $(VO)_2{}^d$HSA is not detected in solution in these experimental conditions.

Discussion

(1). Binding of [VO(*carrier*)$_2$] to Transferrin and Albumin. (a). Binding to Transferrin. The results of this work suggest that there are two possible modes for the binding of an *insulin-enhancing* agent of stoichiometry [VO(*carrier*)$_2$] to transferrin.

The first binding mode can be called "specific" and is observed when the organic *carrier* shows the features of a synergistic anion;[28] particularly, it seems that the presence of a carboxylate group in its structure is necessary to realize this type of coordination. In this case, the *carrier* replaces bicarbonate in the active sites of iron and binds VO^{2+} ion through its COO^- function. The situation is represented by picolinate, but it has been already evidenced for lactate and citrate.[27] This binding mode can be revealed by the shoulders at lower field with respect to the M_I = 5/2 and 7/2 transitions of $(VO)_2$hTf species (see Figures 5 and 7 in the case of picolinate) and by A_z value slightly lower than that of site A of transferrin.

The second binding mode is called "non-specific" and is realized when the *carrier* is not a synergistic anion and stabilizes the bis-chelated *cis*-octahedral geometry in aqueous solution. For this species, transferrin occupies one of the four equatorial positions, replacing a water molecule, with an imidazole nitrogen of a histidine residue, probably situated on the surface of the protein. In this case, the A_z value for the complex *cis*-VO(*carrier*)$_2$(hTf) is different with respect to that of $(VO)_2$hTF, around 4−6 × 10^{-4} cm^{-1} lower than that of the corresponding *cis*-[VO(*carrier*)$_2$(H_2O)] species and comparable with that of the hydroxo complex *cis*-[VO(*carrier*)$_2$(OH)]$^-$.

On this basis, the recent detection of a ternary complex in the system VO^{2+}-hTf-maltol reported by Kiss and coworkers can be easily rationalized.[26] They simply found

cis-VO(mal)$_2$(hTf) and the A_z value measured (165.5 × 10^{-4} cm^{-1}) is 5.5 × 10^{-4} cm^{-1} lower than *cis*-[VO(mal)$_2$-(H$_2$O)] and close to [VO(mal)$_2$(OH)]$^-$.[62] The possibility of "specific" binding of maltol to transferrin can be ruled out for the lacking of a carboxylate group.

(b). Binding to Albumin. When an *insulin-enhancing* compound is present in aqueous solution in bis-chelated *cis*-octahedral arrangement, albumin can participate to its transport forming *cis*-VO(*carrier*)$_2$(HSA) species, analogous to that just described for transferrin. As demonstrated through the study of the model systems with 1-methylimidazole, a histidine nitrogen could insert in the fourth equatorial position of *cis*-[VO(*carrier*)$_2$(H$_2$O)], as shown by Orvig and co-workers for maltol.[24] Therefore, the involvement of albumin in the transport of an *insulin-enhancing* compound is not exclusively observed when the *carrier* is weak like with 6-methylpicolinate (in this case a low amount of (VO)$_2$^{d}HSA is formed), but also when the *carrier* is strong, as 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone: for such systems, indeed, it is not important the capacity of albumin to replace a ligand molecule but the possibility for a protein donor to bind in the fourth equatorial position of a *cis* structure to yield a mixed complex with composition *cis*-VO(*carrier*)$_2$(HSA).

From these considerations, we can affirm that the species *cis*-VO(*carrier*)$_2$(hTf) and *cis*-VO(*carrier*)$_2$(HSA) are equivalent: this is demonstrated by the comparison between the two adducts formed by dhp, whose A_z value is 163 for *cis*-VO(dhp)$_2$(hTf) and 162 × 10^{-4} cm^{-1} for *cis*-VO(dhp)$_2$(HSA), see Tables 2 and 4.

Scheme 2. Possible Geometries of an *Insulin-Enhancing* Compound with [VO(*carrier*)$_2$] Composition in Aqueous Solution: (a) Square Pyramidal and (b) *cis*-Octahedral

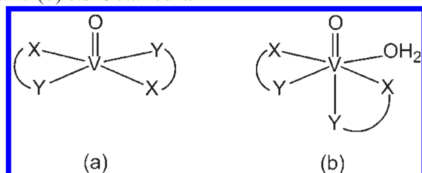
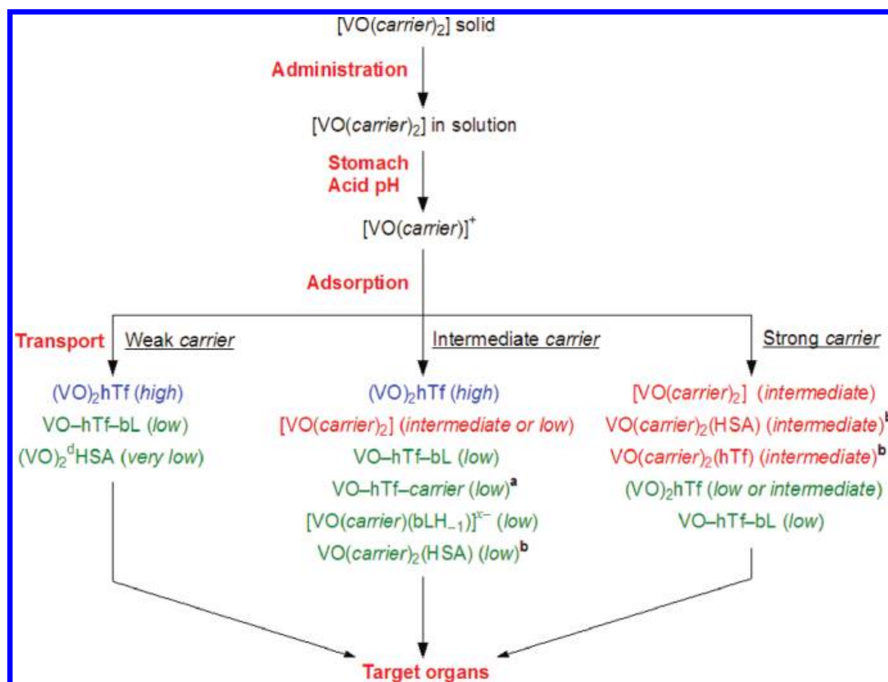


Table 6. Percent Distribution of the Species Formed from the Biotransformation of an *Insulin-Enhancing* Compound in the Corresponding Quinary Systems

species[a]	[VO(6-mepic)$_2$]	*cis*-[VO(pic)$_2$(H$_2$O)]	[VO(acac)$_2$]	[VO(dhp)$_2$]
(VO)$_2$hTf	80−85	65−70	70−75	10−15[b]
VO^{2+}−hTf−bL	10−15	10−15[c]	5−10	10−15[b]
(VO)$_2$^{d}HSA	0−5			
VO^{2+}−hTf−*carrier*		10−15[c]		
[VO(*carrier*)(bLH$_{-1}$)]$^{x-}$		5−10	0−5	
[VO(*carrier*)$_2$]			15−20	40−45 [d]
cis-VO(*carrier*)$_2$(HSA)		5−10		40−45 [e]
cis-VO(*carrier*)$_2$(hTf)				40−45 [e]

[a] bL is lactate or citrate. [b] The value refers to the sum of (VO)$_2$hTf and VO^{2+}−hTf−bL, that cannot be distinguished from the examination of the EPR spectra. [c] The value refers to the sum of VO^{2+}−hTf−bL and VO^{2+}−hTf−*carrier*, that cannot be distinguished from the examination of the EPR spectra. [d] Present as an equilibrium mixture of [VO(dhp)$_2$] and *cis*-[VO(dhp)$_2$(H$_2$O)]. [e] The value refers to the sum of *cis*-VO(*carrier*)$_2$(HSA) and *cis*-VO(*carrier*)$_2$(hTf), that cannot be distinguished from the examination of the EPR spectra.

Scheme 3. Possible Transformations of an *Insulin-Enhancing* Compound [VO(*carrier*)$_2$] in the Blood Serum[a]



[a] In brackets the concentration of the several species formed is indicated with high, intermediate, low, and very low. **a** If the *carrier* is a synergistic anion. **b** If the *insulin-enhancing* compound assumes the *cis*-octahedral geometry in aqueous solution.

(2). Biotransformation of an *Insulin-Enhancing* Compound [VO(*carrier*)$_2$]. From the results obtained in this study, it can be affirmed that the biotransformations of an *insulin-enhancing* compound [VO(*carrier*)$_2$] depend not only on the strength of the *carrier* but also on the geometry that bis-chelated species assumes in aqueous solution, usually square pyramidal or *cis*-octahedral with a water molecule in the fourth equatorial position (Scheme 2).

Among the *carriers* studied in this work, the strength increases in the order 6-mepic < pic < acac ≪ dhp. About the geometry, [VO(6-mepic)$_2$] and [VO(acac)$_2$] are square pyramidal both in the solid state and in the aqueous solution,[37,39] *cis*-[VO(pic)$_2$(H$_2$O)] is *cis*-octahedral as solid and in water,[58,64] whereas [VO(dhp)$_2$] is a penta-coordinated species distorted toward the trigonal bipyramid in the solid phase but is in equilibrium with *cis*-[VO(dhp)$_2$(H$_2$O)] in aqueous solution.[40,59]

If the *carrier* is weak, like 6-methylpicolinate, it cannot compete with transferrin for the VO^{2+} complexation. Independently of its geometry in water, vanadium is mainly transported as (VO)$_2$hTf, secondarily as mixed species with lactate and citrate VO^{2+}−hTf−bL, and, finally, as dinuclear complex (VO)$_2^{d}$HSA in very low amount.[27] The percentage amount of the several species, calculated from the intensity of the EPR signals belonging to each complex, is listed in Table 6; the values are comparable with those recently reported for the VO^{2+}-hTf-HSA system.[27]

If the *carrier* has an intermediate strength, like picolinate or acetylacetonate, the percentage of vanadium bound to transferrin as (VO)$_2$hTf is lower, because the *insulin-enhancing* compound in its original form [VO(*carrier*)$_2$] (as for acac) can survive, mixed species with transferrin in which the *carrier* replaces bicarbonate in the specific sites of Fe^{3+} (as for VO^{2+}−hTf−pic) can be formed, and ternary complexes with *carrier* and bL (for example $[VO(pic)(bLH_{-1})]^{x-}$ or $[VO(acac)(bLH_{-1})]^{x-}$, with $x = 1$ if bL is lactate and 3 if it is citrate), can be originated. However, it must be highlighted that the formation of species like VO^{2+}−hTf−pic is dependent on the capacity of the *carrier* to behave as a synergistic anion, in its turn connected with the presence of one carboxylate group in the structure.[28] Moreover, if the *insulin-enhancing* compound stabilizes the *cis*-octahedral geometry in aqueous solution, mixed complexes with albumin like *cis*-VO(*carrier*)$_2$(HSA) can be formed, in which albumin binds the fourth equatorial position with an imidazole nitrogen of a histidine residue. This finding confirms the hypothesis of Orvig and co-workers, which were able to detect a species of this type with maltol, *cis*-octahedral in water,[62,65] for which a stoichiometry *cis*-VO(mal)$_2$(HSA) has been proposed.[24] The approximate percentages of the species formed in the systems with picolinate and acetylacetonate are shown in Table 6.

Finally, if the *carrier* is strong, like dhp, the amount of VO^{2+} in the (VO)$_2$hTf form significantly decreases and contemporaneously that of [VO(*carrier*)$_2$] increases (Table 6). In principle, ternary transferrin complexes with the *carrier* in the iron active sites are possible if this can behave as a synergistic anion; if, instead, it is not provided with a carboxylate group, like dhp (but this should happen for most of the strong ligands), the binding in the specific sites of Fe^{3+} is precluded, and the formation of mixed species depends on the tendency of the *insulin-enhancing* compound to form *cis*-octahedral species in aqueous solution, in which a histidine nitrogen could replace an equatorial water. For such *carriers*, species like $[VO(carrier)(bLH_{-1})]^{x-}$ are less important. Among the strong *carriers*, analogous results are expected for 2-hydroxypyridine-*N*-oxide, *cis* in aqueous solution,[66] whereas for 2-mercaptopyridine-*N*-oxide, square pyramidal,[66] we do not expect the formation of adducts with albumin.

The processes discussed are represented in Scheme 3. The *insulin-enhancing* compound can undergo partial dissociation in the stomach and transforms into mono-chelated species [VO(*carrier*)]$^{+}$; after the absorption in the small intestine, it can react with the bioligands of the blood serum, transferrin rather than albumin among hmm, and lactate and citrate rather than oxalate, phosphate, glycine and histidine among lmm components. The form with which it reaches the target organs essentially depends, as illustrated above, on the strength of the *carrier* and on the geometry assumed by the bis-chelated species in aqueous solution.

Conclusions

The results of this work provide new insights on the biotransformation of an *insulin-enhancing* compound and on its transport in a human organism to the target sites.

The interpretation of the data of an apparently too complicated system like a quinary one (with the *insulin-enhancing* compound, transferrin, albumin and lactate or citrate) was possible through the comparison with the measurements on simpler systems. It is important to highlight that each VO^{2+} species is countersigned by a precise value of its EPR parameters, and this allows for its identification also in the presence of other complexes. With the method of the signal averaging, we were able to record in the quinary systems interpretable signals using conditions very close to the physiological ones: for example EPR spectrum in Figure S9 (trace c) of the Supporting Information, was obtained with [VO(acac)$_2$] 88 μM, that is with a concentration high enough to show *insulin-enhancing* activity, hTf 44 μM, HSA 748 μM and lactate 1.80 mM (cf. with the values of hTf 37 μM, HSA 630 μM and lactate 1.51 mM in the blood serum[20,57]). Therefore, we are close to studying the biospeciation of an *insulin-enhancing* compound in the same conditions that exist in a human organism. EPR spectroscopy can be considered an excellent technique to examine the biotransformations of the pharmacologically active complexes formed by paramagnetic ions, and could be applied to other cases, for example chemotherapeutic copper compounds.[67]

Save the case of a very weak ligand like 6-methypicolinate, the organic *carrier* can interact with VO^{2+} until the intake

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into the cells. This could explain the significant difference in toxicity and pharmacological activity of the several compounds. Therefore, not only transferrin can transport an *insulin-enhancing* compound, but mixed species like VO^{2+}−hTf−bL, VO^{2+}−hTf−*carrier*, *cis*-VO(*carrier*)$_2$(hTf), *cis*-VO(*carrier*)$_2$(HSA), and, secondarily, [VO(*carrier*)(bLH$_{-1}$)]$^{x-}$ could be important.

Moreover, differently from what was recently proposed,[10,21,26] albumin can also participate in these processes. In the physiological conditions, with the vanadium concentration necessary to observe *insulin-enhancing* effect, the ratio between albumin and VO^{2+} ion much higher than 1 favors the formation of $(VO)_2{}^{d}$HSA rather than $(VO)_x{}^{m}$HSA;[27,34] such a species must be taken into account in the case of weak *carriers* like 6-methylpicolinate. The role of albumin could become particularly important when the *insulin-enhancing* compound is present in *cis*-octahedral arrangement in aqueous solution; in these situations, it can replace the equatorial water molecule to form *cis*-VO(*carrier*)$_2$(HSA). Orvig and co-workers proposed that adducts of this type could be the pharmacologically active form for some *insulin-enhancing* agent.[24] Our results confirm these observations.

These insights can be supported by another important observation. If in the human organism, only 70% of the transferrin sites are free, its effective concentration is 25.9 μM, and such an amount can bind around 51.8 μM of VO^{2+}. This maximum value, of course, decreases if transferrin coordinates other metal ions.[68] Therefore, if the concentration of an *insulin-enhancing* compound is higher than some tens of μM, hTf is not able to bind all of VO^{2+}, and the organic *carriers* and lmm bioligands must be necessarily involved in its transport.

The picture obtained from this work is more complicated than expected (see Table 6 and Scheme 3). The limitation lies in the fact that those studied are only model systems. However, since EPR signals characteristic of a specific species can be undoubtedly observed in the physiological conditions mimicked, the presence of such complexes must be considered in the possible biotransformations of an *insulin-enhancing* compound. Of course, it is desirable that other studies, for example ex vivo anion exchange chromatographic separation measurements,[10,26] could confirm our results.

Note Added after ASAP Publication. This article was released ASAP on November 30, 2009, with errors in the section numbering and in a table footnote citation. The correct version was posted on December 2, 2009.

Supporting Information Available: EPR spectra of the systems VO^{2+}-hTf-6-mepic, VO^{2+}-hTf-pic, VO^{2+}-hTf-acac, VO^{2+}-HSA-6-mepic, VO^{2+}-HSA-acac, VO^{2+}-hTf-6-mepic-lact, VO^{2+}-hTf-acac-lact, VO^{2+}-hTf-dhp-lact, and VO^{2+}-hTf-HSA-acac-lact (Figures S1−S9), and all the complete EPR spectra (Figures S10−S29). This material is available free of charge via the Internet at http://pubs.acs.org.

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