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On the Transport of Vanadium in Blood Serum

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The complexation of the VO^{2+} ion in several systems that can model the physiological conditions of its transport in blood serum was studied using electron paramagnetic resonance (EPR) spectroscopy. Particularly, the ternary systems formed by (i) VO^{2+} and two high-molecular-mass components of blood serum, human serum apo-transferrin (hTf) and human serum albumin (HSA); (ii) VO^{2+}, hTf, and bL; and (iii) VO^{2+}, HSA, and bL, where bL is one of the six most important low-molecular-mass bioligands of the blood serum (bL = lactate, citrate, oxalate, phosphate, glycine, or histidine), were examined. The results indicate that, in aqueous solution, transferrin is a stronger binder than albumin, and at the physiological ratio, most of the VO^{2+} ion is present as $(VO)_2$hTf, and a small amount as $(VO)_2{}^{d}$HSA, the dinuclear species formed by albumin where the two metal ions are interacting and the spin state *S* is 1. Among the bL ligands, only lactate and citrate are able to bind VO^{2+} in the presence of transferrin or albumin, the others not interacting at all. Finally, the quaternary systems formed by (i) VO^{2+}, hTf, HSA, and lactate and (ii) VO^{2+}, hTf, HSA, and citrate were studied. In these cases, the results suggest that the predominant species is $(VO)_2$hTf, followed by the mixed complexes VO^{2+}−hTf−lactate or VO^{2+}−hTf−citrate, whereas $(VO)_2{}^{d}$HSA and $[(VO)_2(citrH_{-1})_2]^{4-}$ are minor components at physiological pH. The conclusions of this study give new insights on how the VO^{2+} ion distributes among the blood serum components and is transported in the plasma toward the target sites in the organism.

Introduction

Vanadium plays a number of roles in biological systems.[1] It is present in two enzymes, vanadium-dependent haloperoxidases[2] and nitrogenase,[3] and is accumulated by tunicates (ascidians or sea squirts)[4] and by some species of the mushroom genus *Amanita*.[5]

It is commonly accepted that vanadium plays an essential role in higher animals, even if its biochemical functions still remain unclear.[6] On the basis of its ability to inhibit many phosphate-metabolizing enzymes, such as phosphatases, ribonuclease, and ATPases,[7] it most likely acts as a regulator in phosphate metabolism. Moreover, in the human organism, vanadium compounds show insulin-enhancing activity.[8] The lungs, liver, and kidneys seem to be the target organs.[9]

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For the vanadium concentration in human blood plasma, values in the range 1−750 nM have been reported in the literature,[10,11] and an average of approximately 200 nM can be considered acceptable.[12] However, the tissue concentrations can go up to 6 μM.[12,13] About 90% of the vanadium present in the blood is associated with the plasma fraction,[6a] and over the past few years, it has been demonstrated that it is transported in the blood in the +IV oxidation state in the VO^{2+} form, almost independently of the initial state.[6,9,14] If vanadate appears in the blood, it is quickly converted to the VO^{2+} ion in the erythrocytes by glutathione,[15] or in the plasma by reductants such as ascorbate,[16] catecholamines,[17] and cysteine.[18] In vivo blood circulation monitoring−electron paramagnetic resonance (BCM-EPR) studies on rats confirm these data,[19] and the binding of VO^{2+} to the bioligands can further stabilize the +IV state and prevent its oxidation to vanadium(V).

It is not fully clear in which form the VO^{2+} ion is transported to the target organs in the organism. The most probable candidates among the bioligands of blood serum are human serum apo-transferrin (hTf) and human serum albumin (HSA), indicated as high-molecular-mass (hmm) components.[20,21] Chasteen and co-workers found that the ratio between the first association constant of VO^{2+} toward hTf and HSA is K_1(hTf)/K_1(HSA) ~ 6.[21] Subsequent results suggest that all of the VO^{2+} ion is bound to transferrin,[22] and recent data indicate that there are at least 4 orders of magnitude between K_1(hTf) and K_1(HSA).[14,23] However, given the uncertainty of the values of K_1(hTf) and K_1(HSA), the higher concentration of albumin in the blood[24] could compensate the lower affinity toward VO^{2+} with respect to transferrin. Nevertheless, when the VO^{2+} ion is absorbed, it can meet many other bioligands with a low-molecular-mass (lmm components) present in the biological fluids, like nucleotides, inorganic and organic phosphates, citrate, and lactate, and undergo several transformations to form ternary species with transferrin or albumin or the corresponding binary complexes.[22]

Therefore, in order to shed light on the transport of vanadium in the organism, it is essential to know the interaction between the VO^{2+} ion and hTf and HSA in their binary and ternary systems. Similarly, it is necessary to know the behavior of the ternary systems formed by one hmm (transferrin or albumin) and one lmm component. Finally, the quaternary systems formed by transferrin, albumin and one, lmm component must be studied. Electron paramagnetic resonance (EPR) spectroscopy is an excellent technique for studying VO^{2+} complexes and, particularly, complicated systems like those with proteins.[25] Recently, we demonstrated that the recording of EPR spectra through the repeated acquisition of weak signals, due to the low concentrations used, is essential in obtaining a good signal-to-noise ratio.[26]

The binary systems VO^{2+}-hTf and VO^{2+}-HSA have been widely studied in the literature.[20,21,26−33] Ternary VO^{2+}-hTf-HSA systems have been recently examined by Kiss et al.,[23] but only NMR relaxation studies at one ratio (VO^{2+}/hTf/HSA = 1:1:1) and one concentration (1 mM) have been reported. Therefore, these systems were also examined through EPR spectroscopy by our research group.[26]

In this work, the ternary system VO^{2+}-hTf-HSA has been studied in conditions which could better model the physiological ones, that is with the same molar ratio between transferrin and albumin present in the blood serum. Moreover, the ternary systems VO^{2+}-hTf-bL and VO^{2+}-HSA-bL, where bL indicates the six more important low-molecular-mass bioligands of the serum, have been examined. When the observations reported in the literature are followed,[22] citrate, lactate, oxalate, and phosphate seem to be the most likely potential VO^{2+} binders since they contain basic and negatively charged O donor groups; herein, we added glycine and histidine as the more representative amino acids, the first for its high concentration and the second for its high affinity toward VO^{2+}, the binding strength of other lmm serum components, such as sulfate, being negligible relative to these ligands. The six binary systems VO^{2+}-bL have been used as a reference in the interpretation of the results obtained here; for the coordination geometry and the thermodynamic stability constants of the species formed at physiological pH, the data reported in the literature have been considered.[34−40]

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The results of this study can help to develop an understanding of how the VO^{2+} ion 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.[41]

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}).[21,42]

$NaHCO_3$, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), lactate, citrate, oxalate, phosphate, glycine and histidine were of the highest grade available from Aldrich and were used as received.

Preparation of the Solutions. The solutions were prepared by dissolving in ultrapure water an amount of $VOSO_4 \cdot 3H_2O$ in order to obtain a metal ion concentration between 8.8×10^{-5} and 3×10^{-3} M. Argon was bubbled through the solution to ensure the absence of oxygen and avoid the oxidation of VO^{2+} ions. To the solution was added an appropriate amount of HEPES and $NaHCO_3$. The final concentration of HEPES and $NaHCO_3$ was 0.1 M and 2.5×10^{-2} M, respectively. EPR studies performed on model systems prove that HEPES does not interact with the VO^{2+} ion under the conditions used for the experiments.

To 1 mL of this solution, again carefully purged with argon, was added an amount of human serum apo-transferrin or human serum albumin in order 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, the pH was adjusted to ca. 7.4.

The ratio between hTf and HSA and between such proteins and the low-molecular-mass components (lactate, citrate, oxalate, phosphate, glicine, and histidine) in the examined solutions was the same as that of the blood serum to model the biological conditions well. In order to obtain satisfactory signal-to-noise instrumental ratios, a vanadium concentration higher than the physiological one (200 nM)[12] but in the range necessary to observe insulin-enhancing activity (approximately 1−400 μM) was used.[12,43]

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

When the samples were transferred into the EPR tubes (120 K), spectra were immediately measured. Only low-temperature EPR spectra were measured in order to minimize the oxidation of the VO^{2+} ion to vanadium(V), which otherwise would happen very quickly, with a half-time between 5 and 13 min at room temperature.[21] Moreover, as noticed in the literature,[44] an anisotropic EPR spectrum allows for 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 relationship,[28,44] and the presence of minor species in solution with respect to an isotropic spectrum.

To increase the signal-to-noise ratio, in these systems, signal averaging was used.[26]

The spectra were simulated with the computer program Bruker WinEPR SimFonia.[45] In all of the simulations, second-order effects were taken into account; the line width along the *x*, *y*, and *z* axes was set to 1 mT and the ratio Lorentzian/Gaussian, affecting the line shape, to 1.

As usually done for the analysis of EPR spectra,[46] in all of 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 EPR spectra are reported in the Supporting Information (Figures S1−S19), where a number of comparisons of the spectra of the more representative systems (Figures S20−S22) is also shown. When such a comparison is made, it is clear that is very difficult to observe the differences between them, and any analysis is precluded (for example, in Figure S21, the spectra of the binary VO^{2+}-hTf, ternary VO^{2+}-hTf-lactate, and quaternary VO^{2+}-hTf-HSA-lactate systems are reported).

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
	B_1	1.941	170.5	1.941	170.3	1.938	170.0
	B_2	1.935	171.8	1.937	172.4		
ref		26	26	28, 48	28, 48	29	29
VO^{2+}-HSA	$(VO)_x{}^m$HSA	1.947	164.6	1.939	172.8	1.927	166.5
	$(VO)_2{}^d$HSA[b]	1.981	80[c]				
ref		26	26	32	32	33	33

[a] Values measured in 10^{-4} cm^{-1}. [b] *D* value for dinuclear $(VO)_2{}^d$HSA 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 and Discussion

The Binary System VO^{2+}-Transferrin. The EPR spectrum of a frozen aqueous solution containing VO^{2+} and transferrin at physiological pH is composed of two sets of resonances (A and B)[27,28] whose relative intensity is strongly pH-dependent.[47] The B signals are further split into two components, indicated as B_1 and B_2, that can be resolved not only in the Q-band[48] but also in the X-band EPR spectra.[26] 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 however must have certain features, such as a carboxylate and a withdrawing electron group.[49]

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The Binary System VO^{2+}-Albumin. From an examination of the EPR spectra recorded for the VO^{2+}-HSA system, Chasteen and Francavilla distinguished one "strong" and five "weak" binding sites having different EPR parameters.[32] The authors suggested that the "strong" site is probably the primary binding site for Cu^{2+}, whereas the binding at the "weak" sites probably occurs with only carboxyl groups. These results were subsequently confirmed by Willsky et al.[30] and Liboiron et al.[33]

Recently, we demonstrated that, when a VO^{2+}/HSA ratio of 1:1 or lower is used, EPR spectra are characterized by the presence of signals attributable to a dinuclear species, denoted as $(VO)_2{}^{d}$HSA,[50] in which the two VO^{2+} ions are interacting and the spectrum is characteristic of an $S = 1$ spin state. When the ratio is increased to 2:1, 4:1, and 8:1, the EPR resonances are attributable to a multinuclear complex $(VO)_x{}^{m}$HSA with an $S = 1/2$ state,[50] indicating that the metal ions coordinated by albumin are not interacting.[26]

The EPR spectrum of $(VO)_2{}^{d}$HSA is displayed in Figure 1. The presence and the position of all of the signals attributable to such a complex can be detected only with signal averaging adding a considerable number of spectra (see the Experimental Section). It consists of two sets of signals corresponding to the perpendicular and parallel orientations of the magnetic field with respect to the V=O bond, separated by D and $2D$, respectively, with D the zero-field splitting parameter. Each set is further split into 15 lines due to the interaction of the unpaired electron with two vanadium nuclei ($I = 7/2$), for a total value of 60 transitions (30 for the parallel and 30 for the perpendicular part of the spectrum: in Figure 1, the first eight and the last six lines of the parallel components are indicated). The hyperfine coupling constant, which can be measured in the outer part of the spectrum, in the parallel region, is one-half of the value which would be observed for the monomeric species having the same equatorial coordination mode. From an analysis of the spectrum, a D value of 631×10^{-4} cm^{-1} can be measured, whereas g and A are 1.981 and 80×10^{-4} cm^{-1} (Table 1), respectively. The very high g value and the very low A value confirm the dinuclear arrangement of such a species.

For multinuclear $(VO)_x{}^{m}$HSA species,[50] only one set of EPR resonances is observed at physiological pH, suggesting that the five to six different metal ions coordinated by albumin have the same coordination.[26] This binding sites appear relatively nonspecific with respect to that interacting with Cu^{2+} or Ni^{2+}.[51] For this reason, the spectra are more influenced by the experimental procedure in comparison with those recorded with transferrin. Chasteen and Orvig proposed the presence of an imidazole nitrogen in the first coordination sphere of the VO^{2+} ion of the "strong" site;[32,33] Orvig has also proved that

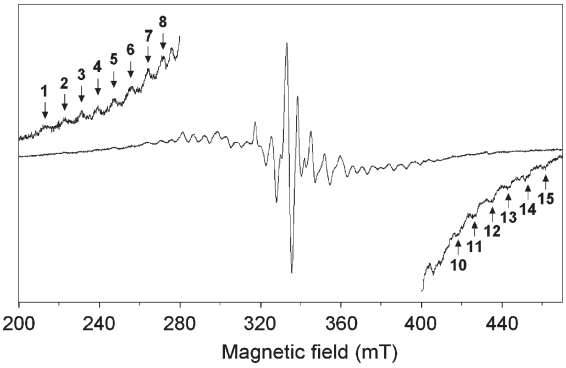

Figure 1. X-band anisotropic EPR spectrum of the $(VO)_2{}^{d}$HSA complex recorded at a pH of 7.4 in a frozen aqueous solution containing 7.5×10^{-4} M VO^{2+} and 7.5×10^{-4} M HSA. The signals reported in the outer regions have been obtained by adding 20 and 40 scans, respectively; the arrows **1**−**8** and **10**−**15** indicate the resonances at low and high fields corresponding to the two parallel components of the spectrum. Reprinted from ref 26. Copyright 2009 Elsevier.

albumin binds VO^{2+} with an imidazole nitrogen of a histidine residue when the ternary complex, [VO(maltolato)$_2$HSA], between the insulin-enhancing agent [VO(maltolato)$_2$] and the protein is formed.[33] We recently proposed 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, can be compatible with EPR parameters,[26] even if other combinations could be possible. However, on the basis of the experimental A_z value (165×10^{-4} cm^{-1}), the coordination of the donor set (NH_2, N^-, N^-, N_{imid}), whose A_z is 151×10^{-4} cm^{-1},[52] can be ruled out.

The Ternary System VO^{2+}-Transferrin-Albumin. Recently, we studied the ternary system VO^{2+}-transferrin-albumin using a molar ratio of 2:1:1.[26] However, several aspects must be taken into account when the interaction of hTf and HSA with vanadium in the organism is mimicked: (i) Human serum albumin is present in blood serum at a concentration of 630 μM, while human serum transferrin is 37 μM, so that their molar ratio should be about 17.[22,24] (ii) The vanadium concentration should be as low as possible (the average concentration in human plasma is, according to Rehder, around 200 nM[12]), compatible with its instrumental detection. (iii) The solubility of albumin in aqueous solution is about 7.5×10^{-4} M.

To try to overcome these limitations, we planned two different experiments, besides that previously described, in which HSA and hTf were present at the same concentration (Figure 2b).[26] In these two experiments, the ratio between HSA and hTf (17:1) was the same as that present in blood serum, and two different metal concentrations were used, the solubility of HSA (7.5×10^{-4} M) limiting the concentration of hTf present in aqueous solution (4.4×10^{-5} M). The VO^{2+} concentration (8.8×10^{-5} M in the first and 3×10^{-3} M in the second one) was selected to completely saturate the binding sites of transferrin and albumin (that, as was previously observed,[26,32,33] can bind up to 5−6 equiv of metal ions). The molar ratio VO^{2+}/hTf/HSA was 2:1:17 and 4:0.06:1, respectively.

(50) In this work, $(VO)_2{}^{d}$HSA indicates the VO^{2+}−HSA species, observed when the VO^{2+}/HSA ratio is ≤1, in which the two VO^{2+} ions are interacting and the EPR spectrum is characteristic of a dinuclear complex with a spin state $S = 1$, and $(VO)_x{}^{m}$HSA (where $x = 5-6$ and the superscript m denotes a multinuclear complex) is the VO^{2+}−HSA species, detected when the VO^{2+}/HSA ratio is >1, in which the VO^{2+} ions are not interacting.

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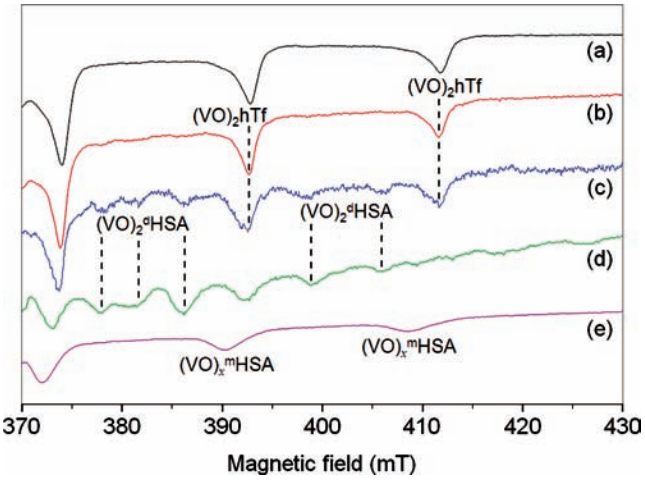

Figure 2. High-field region of the X-band anisotropic EPR spectra recorded at a pH of 7.4 in frozen solutions (120 K) containing different ratios of VO^{2+}/hTf/HSA: (a) 2:1:0 (VO^{2+}, 5.0×10^{-4} M), (b) 2:1:1 (VO^{2+}, 5.0×10^{-4} M), (c) 2:1:17 (VO^{2+}, 8.8×10^{-5} M), (d) 1:0:1 (VO^{2+}, 7.5×10^{-4} M), and (e) 4:0:1 (VO^{2+}, 1.0×10^{-3} M). HEPES (0.1 M) and HCO_3^- (2.5×10^{-2} M) in all cases.

In the first experiment (VO^{2+}, 8.8×10^{-5} M; hTf, 4.4×10^{-5} M; HSA, 7.5×10^{-4} M), the intensity of the anisotropic EPR spectra is very weak (due to the low VO^{2+} concentration), and so a lot of scans are necessary to distinguish the signals of the metal complexes. Nevertheless, the spectrum shows the presence of the two sets of resonances due to VO^{2+} coordinated to the A and B sites of the transferrin as complex $(VO)_2$hTf,[20,26−28,33] while a small fraction of vanadium is bound to HSA, forming the dinuclear species $(VO)_2{}^d$HSA,[26,50] whose characteristic signals (Figure 2d) can be distinguished. We would highlight that these conditions can model the physiological ones very well because of the low vanadium concentration; therefore, the spectra could be also used to interpret the studies on the transport of insulin-enhancing drugs, for which a vanadium concentration in the range of 1−400 μM ($0.01-4 \times 10^{-4}$ M) is needed.[12,43]

The results obtained in this study confirm that transferrin is stronger than albumin and add new information that the higher concentration of albumin is able to compensate for the lower affinity toward the VO^{2+} ion only partially. Indeed, a comparison between the spectra recorded for the VO^{2+}-hTf-HSA system with ratios of 2:1:1 and 2:1:17 (Figures 2b and c) show that, at the physiological ratio, the amount of $(VO)_2{}^d$HSA complex increases, but albumin coordinates only a small fraction of the total vanadium present in solution. This amount has been approximately quantified on the basis of the signal intensity and of the spectra simulations and is not higher than 10%. The simulation of the spectrum measured for the ternary system VO^{2+}-hTf-HSA with a ratio of 2:1:17 is shown in Figure 3, where the total and the independent signals of three sites of transferrin (A, B_1, and B_2) and of $(VO)_2{}^d$HSA species formed by albumin are presented.

In the second experiment (VO^{2+}, 3.0×10^{-3} M; hTf, 4.4×10^{-5} M; HSA, 7.5×10^{-4} M), the VO^{2+}/HSA ratio was set to 4:1 to obtain the best spectra of $(VO)_x{}^m$HSA.[26,33] With such ratios, transferrin can coordinate only a small amount of the vanadium present in solution (8.8×10^{-5} M,

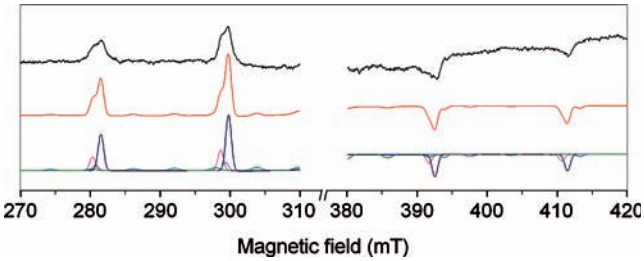

Figure 3. Low- and high-field regions of the X-band anisotropic EPR spectrum recorded at a pH of 7.4 in a frozen solution of the system VO^{2+}-hTf-HSA with a ratio of 2:1:17 (VO^{2+}, 8.8×10^{-5} M): (a) experimental spectrum; (b) simulated spectrum; and (c) independent signals due to A_1 (blue), B_1 (pink), and B_2 (sky-blue) sites of transferrin and $(VO)_2{}^d$HSA species (green) of albumin. The simulations were performed using the parameters reported in Table 1 and considering percentage amounts of $(VO)_2$hTf and $(VO)_2{}^d$HSA of 90 and 10%, respectively.

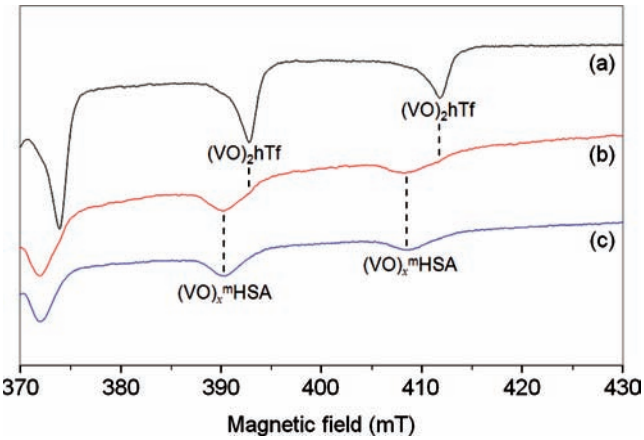

Figure 4. High-field region of the X-band anisotropic EPR spectra recorded at a pH of 7.4 in frozen solutions (120 K) containing different ratios of VO^{2+}/hTf/HSA: (a) 2:1:0 (VO^{2+}, 5.0×10^{-4} M), (b) 4:0.06:1 (VO^{2+}, 3.0×10^{-3} M), and (c) 4:0:1 (VO^{2+}, 1.0×10^{-3} M). HEPES (0.1 M) and HCO_3^- (2.5×10^{-2} M) in all cases.

that is, not more than 2 equiv of vanadium per equivalent of hTf), and a large fraction of metal ion is available for the albumin binding. The presence of $(VO)_2$hTf species formed by transferrin is observable as a shoulder at high field of the resonances attributable to $(VO)_x{}^m$-HSA. However, the ratio between the vanadium unbound to hTf and HSA is very close to 4:1, and under these experimental conditions, as we previously observed in the binary system VO^{2+}-HSA, only the resonances of $(VO)_x{}^m$HSA are observed and not those attributable to $(VO)_2{}^d$HSA (cf., Figures 2c and 4b).[26] The results indicate that the transferrin present in aqueous solution (4.4×10^{-5} M) is able to completely bind the maximum possible concentration of VO^{2+} (8.8×10^{-5} M), whereas the remaining portion of VO^{2+} ions can coordinate to albumin.

The conclusions of such a series of experiments are not in full agreement with the results reported in the literature.[14,22,23] In fact, our measurements suggest that transferrin is really stronger than albumin in VO^{2+} binding, but albumin can coordinate a small fraction of vanadium as dinuclear species $(VO)_2{}^d$HSA. The difference between our results and the previous ones probably depends on the fact that the presence of such a dinuclear complex has never been taken into account.

Table 2. Expected Percent Distribution of the VO^{2+} Species in the Ternary System VO^{2+}-hTf-HSA[a]

ratio VO^{2+}/hTf/HSA	2:1:1[b]	2:1:17[c]	4:0.06:1[d]
(VO)hTf	6.1	15.2	0.0
$(VO)_2$hTf	87.5	57.5	2.8
$(VO)^{m}$HSA[e]	6.2	27.2	21.7
$(VO)_2(OH)_5$	0.1	0.0	74.9
$VO(OH)_3$	0.1	0.1	0.6

[a] Calculated on the basis of the stability constant reported in refs 14 and 53. [b] VO^{2+}, 5.0×10^{-4} M. [c] VO^{2+}, 8.8×10^{-5} M. [d] VO^{2+}, 3.0×10^{-3} M. [e] In ref 14, only the first association constant for the VO^{2+}−HSA species is reported: thus, this complex must be indicated as $(VO)^{m}$HSA, with $x = 1$.

Recently, Kiss and co-workers redetermined the values of the stability constants for hTf ($\log K_1 = 14.3 \pm 0.6$ and $\log K_2 = 11.7 \pm 0.6$)[53] and measured the value of K_1 for HSA ($\log K_1 = 10.0 \pm 1.0$);[14,23] this allows for a comparison of the results obtained with EPR spectroscopy with those expected on the basis of the thermodynamic stability constants (Table 2).

The values in Table 2 are in partial agreement with those reported in this work. The aspects that have to be highlighted are as follows: (i) When the real ratio in solution between VO^{2+} and HSA is lower than 1 (systems with VO^{2+}/hTf/HSA ratios of 2:1:1 and 2:1:17), we observe $(VO)_2{}^{d}$HSA and not $(VO)_x{}^{m}$HSA; therefore the stability constant for the formation of the dinuclear species $(VO)_2{}^{d}$HSA formed by albumin must be considered. (ii) In the experiment with a VO^{2+}/hTf/HSA ratio of 2:1:17, the calculated amount of VO^{2+} bound to albumin is too high (27.2%); this means that the ratio $\log[K_1(\text{hTf})/K_1(\text{HSA})]$ is probably greater than 4.3. (iii) In the experiment with a VO^{2+}/hTf/HSA ratio of 4:0.06:1, the extent of hydrolysis is overestimated; a higher value of $\log K_1$ for $(VO)^{m}$HSA and the presence of an adequate value for $(VO)_2{}^{d}$HSA could allow for reconciliation between literature and EPR data. (iv) It has been reported in the literature that there are five or six sites available for VO^{2+} for each albumin molecule;[32,33] we recently demonstrated that, at physiological pH, these sites are equivalent because only one set of EPR resonances is detected.[26] Therefore, it should be desirable that a value for the subsequent binding of the VO^{2+} ion to form $(VO)_x{}^{m}$HSA complexes (with $x = 5-6$) to albumin be provided.

The Ternary Systems VO^{2+}-Transferrin-bL. The various lmm bioligands present in blood serum are characterized by a concentration variable in a wide range, 10 μM to 25 mM (the concentration of the six bioligands studied in this work is as follows: lactate, 1.51 mM; citrate, 99.0 μM; oxalate, 9.20 μM; phosphate, 1.10 mM; glycine, 2.30 mM; histidine, 77.0 μM).[22,24] VO^{2+} shows a different affinity toward these ligands essentially due to the type of donor atoms involved in the metal coordination. In fact, it is well-known that it forms stable complexes with oxygen donors, particularly if these are negatively charged, as in the case of carboxylates, phenolates, or alcoholates. Its affinity for nitrogen donors is much lower, and higher ligand-to-metal molar ratios are necessary for the formation of VO^{2+} complexes. As an example, bis-chelated complexes are formed in aqueous solutions containing VO^{2+} and lactic acid with a ligand-to-metal molar ratio of 2, while in the case of amino acids without donor groups in the side chain, a much higher ratio (for example, that for glycine is higher than 50) must be used. The situation is more favorable if the amino acids have donor atoms in the side chain and can act as tridentate ligands, as in the case of histidine.

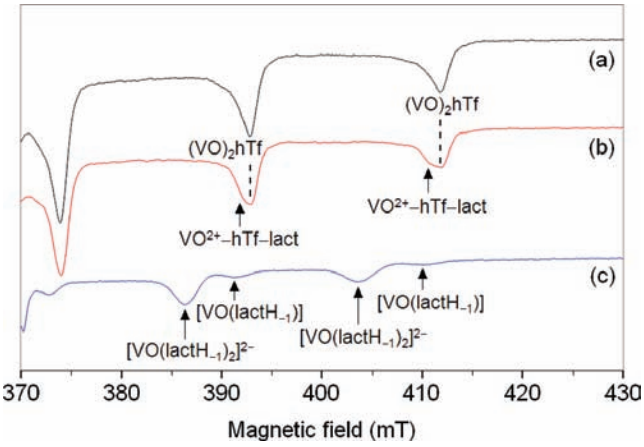

Figure 5. High-field region of the X-band anisotropic EPR spectra recorded at a pH of 7.4 in frozen solutions (120 K) containing (a) 2:1 VO^{2+}/hTf (VO^{2+}, 5.0×10^{-4} M), (b) 2:1:40.8 VO^{2+}/hTf/lactate (VO^{2+}, 5.0×10^{-4} M), and (c) 1:20.4 VO^{2+}/lactate (VO^{2+} 5.0×10^{-4} M). HEPES (0.1 M) and HCO_3^- (2.5×10^{-2} M) in all cases.

For these reasons, in this study, lactate, citrate, oxalate, phosphate, glycine, and histidine have been selected, some of them for their high concentration in blood serum (lactate, phosphate, and glycine) and some others for their high affinity toward VO^{2+} (lactate, citrate, oxalate, and histidine). In particular, citrate, lactate, and histidine are able to form stable complexes, even with low ligand-to-metal ratios, and therefore could effectively compete with transferrin and albumin for VO^{2+} coordination.

The experiments were performed using the same ratio between hTf and the bioligands as found in blood serum; concentrations of hTf of 2.5×10^{-4} M and of VO^{2+} of 5.0×10^{-4} M, in order to saturate the two binding sites of the protein, of $NaHCO_3$ of 25 mM, necessary for the metal ion binding, and of HEPES of 0.1 M, to buffer the pH at a physiological value of 7.4, were also added.[26,33,49] The ternary systems VO^{2+}-hTf-bL have been compared with the corresponding binary systems VO^{2+}-hTf and VO^{2+}-bL to verify the eventual formation of ternary complexes and the distribution of the metal ion between the different ligands.

The system VO^{2+}-hTf-lactate was studied using a ratio of 2:1:40.8, that is, the physiological between transferrin and lactate. The spectra measured are displayed in Figure 5 and clearly show that, besides the complex $(VO)_2$hTf, a species with a low amount not observable in the binary systems VO^{2+}-hTf and VO^{2+}-lactic is formed. These results can be explained by postulating the formation of a ternary complex between VO^{2+}, hTf, and lactate (indicated as VO^{2+}−hTf−lactate), in which lactate replaces bicarbonate, behaving as a synergistic anion.[49] In the corresponding binary system VO^{2+}-lactate, measured under the same conditions, the mono- and,

(53) Kiss, T.; Jakusch, T.; Bouhsina, S.; Sakurai, H.; Enyedy, E. A. *Eur. J. Inorg. Chem.* **2006**, 3607–3613.

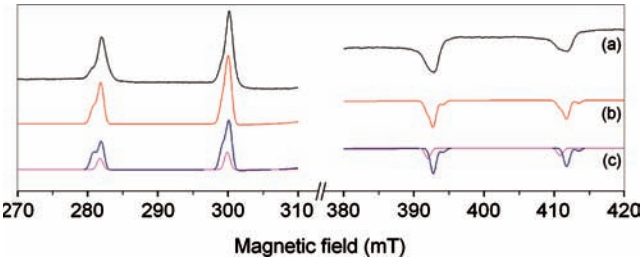

Figure 6. Low- and high-field regions of the X-band anisotropic EPR spectrum recorded at a pH of 7.4 in a frozen solution of the system VO^{2+}/hTf/lactate with a ratio of 2:1:40.8 (VO^{2+}, 5.0×10^{-4} M): (a) experimental spectrum, (b) simulated spectrum, and (c) independent signals due to $(VO)_2$hTf (blue) and to mixed species VO^{2+}−hTf−lactate (pink). The simulations were performed using the parameters reported in Table 3 and considering percentage amounts of $(VO)_2$hTf and VO^{2+}−hTf−lactate of 80 and 20%, respectively.

mainly, the bis-chelated species with (COO^-, O^-) and 2× (COO^-, O^-) coordination can be observed (Figure 5c).[34,40] Therefore, even if lactate is present at a very high ratio in comparison with hTf (1.0×10^{-2} M vs 2.5×10^{-4} M, for a molar ratio of 40.8), it is not able to compete with transferrin for the formation of binary complexes. The comparison between experimental and simulated spectra of the ternary system VO^{2+}-hTf-lactate is presented in Figure 6, with the signals due to $(VO)_2$hTf and VO^{2+}-hTf-lactate species separately shown in trace c. On the basis of the result of the simulations, the percentage amounts of $(VO)_2$hTf and VO^{2+}−hTf−lactate in solution at physiological pH are about 80 and 20%, respectively.

EPR parameters of the ternary VO^{2+}−hTf−lactate species are reported in Table 3. However, only on the basis of the spectral parameters is it not possible to advance a hypotheses on the coordination mode of the complex.

The system VO^{2+}-hTf-citrate was measured at a ratio of 2:1:2.68 among the three components, with the ratio of hTf/citrate equal to that in blood serum. Citrate is present in much lower concentration in comparison with lactate (6.7×10^{-4} M vs 1.0×10^{-2} M) and, analogously to the latter, can replace bicarbonate as the synergistic anion.[49] The EPR spectra (Figure 7) are similar to those recorded with lactate and show the presence of a ternary complex VO^{2+}−hTf−citrate (Figure 7b); besides this species, in aqueous solution, there is a high concentration of $(VO)_2$hTf and a small amount of the dimer formed by citrate, $[(VO)_2(citrH_{-1})_2]^{4-}$ (Figure 7c).[35] The formation of such a dinuclear complex rather than mononuclear species is favored by the low ratio between citrate and VO^{2+} (concentration of 6.7×10^{-4} M vs 5.0×10^{-4} M, respectively). Citrate has a higher negative charge than lactate or bicarbonate, and probably for this reason its ternary complex with transferrin is present at a lower concentration in comparison to that previously described for lactate (10 vs 20%). The simulation (Figure S23 of the Supporting Information) suggests that the percentages of $(VO)_2$hTf, VO^{2+}−hTf−citrate, and $[(VO)_2(citrateH_{-1})_2]^{4-}$ in solution are about 85, 10, and 5%, respectively.

In the ternary systems VO^{2+}-hTf-bL (bL = oxalate, phosphate, glycine, and histidine), the following ratios have been used: oxalate, 2:1:0.2; phosphate, 2:1:29.7; glycine, 2:1:62.2; and histidine, 2:1:2.1. The concentration of oxalate is too low for this ligand to compete with transferrin for vanadium binding, and as expected, the EPR spectra of the ternary system (Figure 8c) are perfectly superimposable with those of the binary system VO^{2+}-hTf (Figure 8a).

Phosphate is present in a large excess in comparison with transferrin, but it acts only as a monodentate ligand and cannot compete with hTf. However, the replacement of bicarbonate with phosphate as the synergistic anion is, in principle, possible but, if it takes place, gives spectra not distinguishable from those formed with bicarbonate. Therefore, it can be affirmed that the presence of phosphate is not necessary for the transport of vanadium in blood serum because the concentration of bicarbonate is high enough.

Table 3. EPR Parameters of the VO^{2+} Species Formed in the Ternary Systems VO^{2+}−hTf−bL

system[a]	species	g_z	A_z[b]
VO^{2+}-hTf-lactate	$(VO)_2$hTf	[c]	[c]
	VO^{2+}−hTf−lactate[d]	∼1.939	∼167
VO^{2+}-hTf-citrate	$(VO)_2$hTf[e]	[c]	[c]
	VO^{2+}−hTf−citrate[d]	∼1.939	∼167
	$[(VO)_2(citrH_{-1})_2]^{4-}$[e]	1.952	77[f]
VO^{2+}-hTf-oxalate	$(VO)_2$hTf	[c]	[c]
VO^{2+}-hTf-phosphate	$(VO)_2$hTf	[c]	[c]
VO^{2+}-hTf-glycine	$(VO)_2$hTf	[c]	[c]
VO^{2+}-hTf-histidine	$(VO)_2$hTf	[c]	[c]

[a] Concentrations: VO^{2+}, 5.0×10^{-4} M; hTf, 2.5×10^{-4} M; lactate, 1.0×10^{-2} M; citrate, 6.7×10^{-4} M; oxalate, 6.2×10^{-5} M; phosphate, 7.4×10^{-3} M; glycine, 1.6×10^{-2} M; histidine, 5.2×10^{-4} M. [b] Values measured in 10^{-4} cm^{-1}. [c] EPR parameters of the three sites of transferrin: $g_z = 1.937$, $A_z = 168 \times 10^{-4}$ cm^{-1} (site A); $g_z = 1.941$, $A_z = 171 \times 10^{-4}$ cm^{-1} (site B_1); $g_z = 1.935$, $A_z = 172 \times 10^{-4}$ cm^{-1} (site B_2). [d] Ternary complex formed by VO^{2+}, hTf, and bL. [e] Zero-field splitting of 666×10^{-4} cm^{-1}. [f] For a dinuclear species, the coupling constant along the z axis is one-half of the value which would be observed for the corresponding mononuclear complex species having the same equatorial coordination mode.

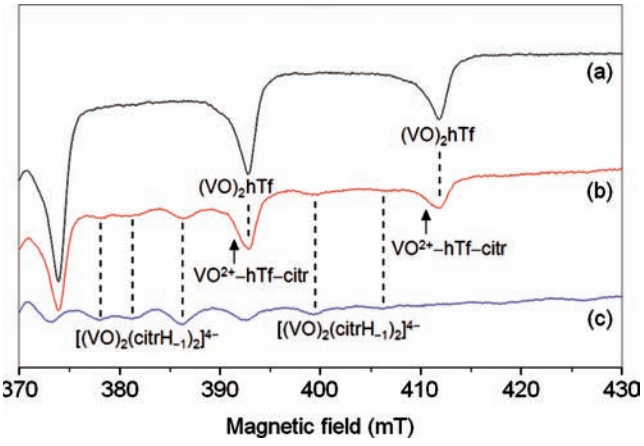

Figure 7. High-field region of the X-band anisotropic EPR spectra recorded at a pH of 7.4 in frozen solutions (120 K) containing (a) 2:1 VO^{2+}/hTf (VO^{2+}, 5.0×10^{-4} M), (b) 2:1:2.68 VO^{2+}/hTf/citrate (VO^{2+}, 5.0×10^{-4} M), and (c) 1:1.34 VO^{2+}/citrate (VO^{2+}, 5.0×10^{-4} M). HEPES (0.1 M) and HCO_3^- (2.5×10^{-2} M) in all cases.

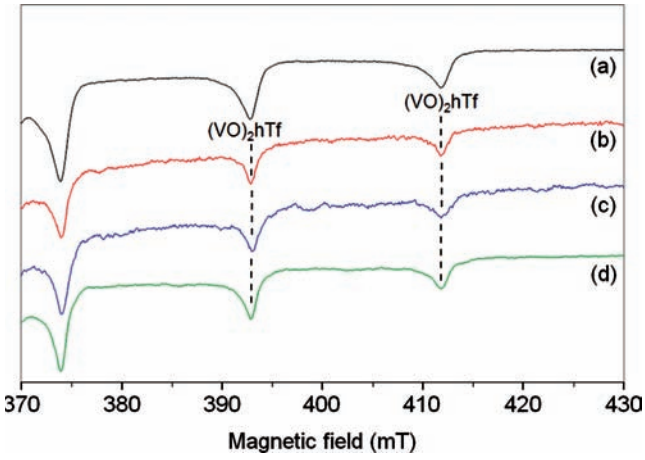

Figure 8. High-field region of the X-band anisotropic EPR spectra recorded at a pH of 7.4 in frozen solutions (120 K) containing (a) 2:1 VO^{2+}/hTf (VO^{2+}, 5.0×10^{-4} M), (b) 2:1:62 VO^{2+}/hTf/Gly (VO^{2+}, 5.0×10^{-4} M), (c) 2:1:0.248 VO^{2+}/hTf/oxalate (VO^{2+}, 5.0×10^{-4} M), and (d) 2:1:2.08 VO^{2+}/hTf/His (VO^{2+}, 5.0×10^{-4} M). HEPES (0.1 M) and HCO_3^- (2.5×10^{-2} M) in all cases.

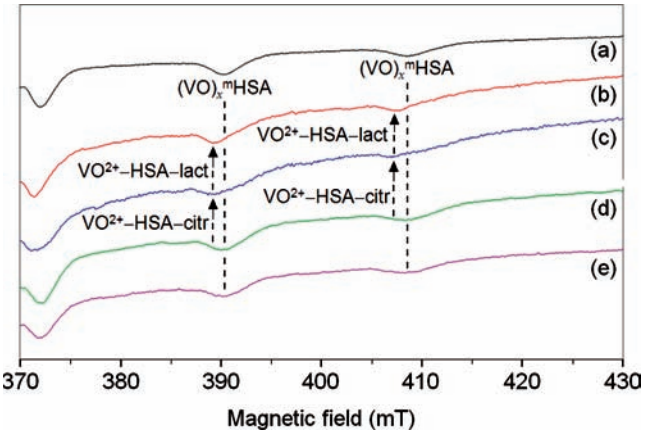

Figure 9. High-field region of the X-band anisotropic EPR spectra recorded at a pH of 7.4 in frozen solutions (120 K) containing (a) 4:1 VO^{2+}/HSA (VO^{2+}, 1.0×10^{-3} M), (b) 4:1:2.4 VO^{2+}/HSA/lactate (VO^{2+}, 1.0×10^{-3} M), (c) 4:1:0.156 VO^{2+}/HSA/citrate (VO^{2+}, 1.0×10^{-3} M), (d) 4:1:0.124 VO^{2+}/HSA/His (VO^{2+}, 1.0×10^{-3} M), and (e) 4:1:3.64 VO^{2+}/HSA/Gly (VO^{2+}, 1.0×10^{-3} M). HEPES (0.1 M) and HCO_3^- (2.5×10^{-2} M) in all cases.

The two amino acids examined, glycine and histidine, are present at a high ratio (Gly/VO^{2+}, 31.1:1) and in almost equimolar concentration (His/VO^{2+}, 1.05:1). EPR spectra show that none of them is able to compete effectively with transferrin for vanadium binding, and the experimental signals (Figure 8b,d) are the same as those observed in the binary system VO^{2+}-hTf.

The results obtained for the ternary systems VO^{2+}-hTf-bL can be summarized as follows: if the ratios present under physiological conditions are used, among the six lmm bioligands examined, only lactate and citrate are able to replace, at least partially, the synergistic anion bicarbonate at the two metal binding sites with the formation of ternary complexes VO^{2+}−hTf−lactate or VO^{2+}−hTf−citrate. Therefore, we believe that only these two bioligands must be taken into account when the transport of VO^{2+} ions in blood serum is considered. About the stoichiometry of these mixed species, no definitive answer can be given on the basis of our EPR data: the composition and the geometry of the ternary complexes discussed above can be the result of the replacement by lactate and citrate of the bicarbonate ion and, eventually, of other donors in the protein site, depending on their behavior as mono-, bi-, or tridentate (for citrate) ligands.

Ternary Systems VO^{2+}-Albumin-bL. In the ternary systems VO^{2+}-HSA-bL, the ratio VO^{2+}/HSA is the same as that used in the binary system (4:1), while the ratio HSA/bL is equal to that present in blood serum. Since albumin is present in the serum at a higher concentration in comparison with transferrin, all of the ratios bL/HSA are much lower than those previously examined with hTf. This means that, in the ternary systems, some of the bioligands are present at very low concentrations, and even if vanadium shows for them a high affinity, no competition with HSA can be expected.

The system VO^{2+}-HSA-lactate was studied using a ratio of 4:1:2.4 to keep it physiological between albumin and lactate. The anisotropic EPR spectrum at a pH of 7.4 shows the presence of the binary complex formed by albumin $(VO)_x{}^m$HSA (Figure 9a),[50] but a careful examination indicates that there are small differences (Figure 9b): with respect to the binary systems VO^{2+}-lactate and VO^{2+}-HSA,[26,34,40] the presence of a new species can be detected, interpreted as a ternary complex formed by albumin and lactate. A small amount of the mono chelated species [VO(lactH_{-1})] with (COO^-, O^-) coordination cannot be excluded (A_z = 168 × 10^{-4} cm^{-1}),[40] while the presence of the corresponding bis chelated complex $[VO(lactH_{-1})_2]^{2-}$ (A_z = 157 × 10^{-4} cm^{-1}) can be surely discarded.[40] The experimental results can be interpreted, from our point of view, assuming that most of the VO^{2+} is coordinated to HSA in the binary complex and the remaining part to HSA and lactate in a ternary species.

As in the case of the ternary complexes formed by lactate and citrate with transferrin, the exact composition and the donor set cannot be deduced only on the basis of our spectra. We believe that lactate could replace some donors present in the equatorial plane of $(VO)_x{}^m$HSA. For example, the set (COO^-, O^-) provided by lactate should contribute to the A_z value, with 77−78 × 10^{-4} cm^{-1};[28,54] if this set replaces two imidazole nitrogens with the ring parallel to the V=O bond (contribution to A_z around 80 × 10^{-4} cm^{-1}),[55] A_z should decrease by 2−3 × 10^{-4} cm^{-1}, exactly what is observed.

In the ternary systems VO^{2+}-HSA-citrate, the ratio 4:1:0.16 has been used. The citrate concentration is lower in comparison with that of lactate, and as a consequence, we expect a negligible effect on the VO^{2+} complexation. In fact, the EPR spectra of the ternary system (Figure 9c) are comparable with those obtained in the binary VO^{2+}-HSA (Figure 9a); nevertheless, it is possible to notice the presence of a small amount of the ternary species, whose EPR parameters are reported in Table 4 and are compatible, like in the system with lactate, with the replacement

(54) (a) Jakusch, T.; Buglyó, P.; Tomaz, A. I.; Costa Pessoa, J.; Kiss, T. *Inorg. Chim. Acta* **2002**, *339*, 119–128. (b) Garribba, E.; Lodyga-Chruscinska, E.; Micera, G.; Panzanelli, A.; Sanna, D. *Eur. J. Inorg. Chem.* **2005**, 1369–1382.

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Table 4. EPR Parameters of the VO^{2+} Species Formed in the Ternary Systems VO^{2+}-HSA-bL

system[a]	species	g_z	A_z[b]
VO^{2+}-HSA-lactate	$(VO)_x$[m]HSA	1.947	165
	VO^{2+}−hTf−lactate [c]	1.948	163
	$[VO(lactH_{-1})]$ [d]	1.942	168
VO^{2+}-HSA-citrate	$(VO)_x$[m]HSA	1.947	165
	VO^{2+}−hTf−citrate [c]	1.950	163
	$[(VO)_2(citrH_{-1})_2]^{4-}$ [e]	1.952	77[f]
VO^{2+}-HSA-oxalate	$(VO)_x$[m]HSA	1.947	165
VO^{2+}-HSA-phosphate	$(VO)_x$[m]HSA	1.947	165
VO^{2+}-HSA-glycine	$(VO)_x$[m]HSA	1.947	165
VO^{2+}-HSA-histidine	$(VO)_x$[m]HSA	1.947	165

[a] Concentrations: VO^{2+}, 1.0×10^{-3} M; HSA, 2.5×10^{-4} M; lactate, 6.0×10^{-4} M; citrate, 3.9×10^{-5} M; oxalate, 3.7×10^{-6} M; phosphate, 4.4×10^{-4} M; glycine, 9.1×10^{-4} M; histidine, 3.1×10^{-5} M. [b] Values measured in 10^{-4} cm^{-1}. [c] Ternary complex formed by VO^{2+}, hTf, and bL. [d] Species present in low amounts. [e] Zero-field splitting of 666×10^{-4} cm^{-1}. [f] For a dinuclear species, the coupling constant along the z axis is one-half of the value which would be observed for the corresponding mononuclear complex species having the same equatorial coordination mode.

of two equatorial nitrogens of two histidine residues by the (COO^-, O^-) couple belonging to citrate, with the possibility of an axial interaction of another COO^- group.

The four other systems VO^{2+}-HSA-bL (bL = oxalate, phosphate, glycine, and histidine) were measured at the ratios of 4:1:0.015 (oxalate), 4:1:1.8 (phosphate), 4:1:3.6 (glycine), and 4:1:0.12 (histidine). The results indicate that these bioligands are not able to complete with albumin for VO^{2+} coordination, even when their concentration is rather high (phosphate and glycine). The two ligands characterized by a greater affinity toward VO^{2+} (oxalate and histidine), instead, are present in too low of a concentration to bind vanadium. The spectra obtained with histidine and glycine are reported in Figure 9d,e and are superimposable to that of the binary system VO^{2+}-HSA.

Therefore, also in the ternary system with albumin, only the two lmm bioligands lactate and citrate are able to coordinate the VO^{2+} ion, forming mixed species VO^{2+}−HSA−lactate or VO^{2+}−HSA−citrate, in which the donor set (COO^-, O^-) presumably replaces two donors of the albumin chain.

The Quaternary Systems VO^{2+}-Transferrin-Albumin-Lactate/Citrate. We demonstrated above that, among the lmm components of the blood serum, only lactate and citrate can effectively compete with transferrin and albumin for vanadium binding; they form ternary complexes by replacing (at least, partially) with transferrin the synergistic anion bicarbonate and with albumin two equatorial donors groups (probably, two imidazole nitrogens) with the (COO^-, O^-) couple.

The next step is to examine the quaternary systems in which the VO^{2+} ion, transferrin, and albumin are present and the fourth component is lactate or citrate; they could well model physiological conditions. The following conditions were used: VO^{2+}/hTf/HSA/lactate 2:1:17:40.8 and VO^{2+}/hTf/HSA/citrate 2:1:17:2.68, where the VO^{2+} concentration is 8.8×10^{-5} M in both cases. The ratio hTf/HSA/lactate or hTf/HSA/citrate is the same as that present in blood serum. The ternary systems VO^{2+}-hTf-HSA and VO^{2+}-hTf-bL (bL = lactate or citrate), previously discussed, were considered for comparison.

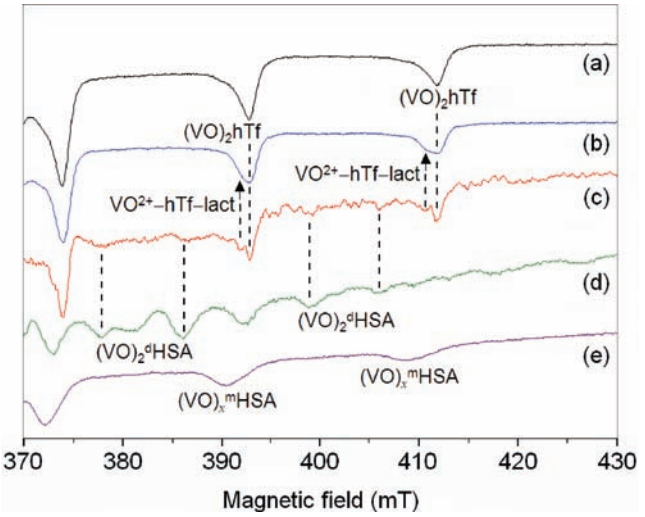


Figure 10. High-field region of the X-band anisotropic EPR spectra recorded at a pH of 7.4 in frozen solutions (120 K) containing (a) 2:1 VO^{2+}/hTf (VO^{2+}, 5.0×10^{-4} M), (b) 2:1:40.8 VO^{2+}/hTf/lactate (VO^{2+}, 5.0×10^{-4} M), (c) 2:1:17:40.8 VO^{2+}/hTf/HSA/lactate (VO^{2+}, 8.8×10^{-5} M), (d) 1:1 VO^{2+}/HSA (VO^{2+}, 7.5×10^{-4} M), and (e) 4:1 VO^{2+}/HSA (VO^{2+}, 1.0×10^{-3} M). HEPES (0.1 M) and HCO_3^- (2.5×10^{-2} M) in all cases.

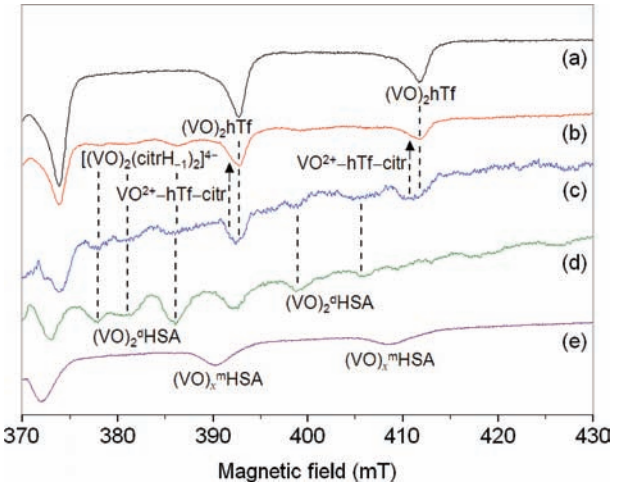
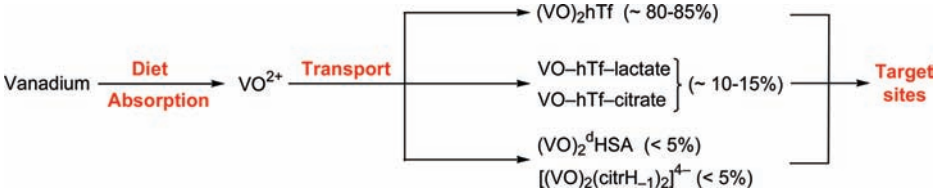


Figure 11. High-field region of the X-band anisotropic EPR spectra recorded at a pH of 7.4 in frozen solutions (120 K) containing (a) 2:1 VO^{2+}/hTf (VO^{2+}, 5.0×10^{-4} M), (b) 2:1:2.68 VO^{2+}/hTf/citrate (VO^{2+}, 5.0×10^{-4} M), (c) 2:1:17:2.67 VO^{2+}/hTf/HSA/citrate (VO^{2+}, 8.8×10^{-5} M), (d) 1:1 VO^{2+}/HSA (VO^{2+}, 7.5×10^{-4} M), and (e) 4:1 VO^{2+}/HSA (VO^{2+}, 1.0×10^{-3} M). HEPES (0.1 M) and HCO_3^- (2.5×10^{-2} M) in all cases.

The obtained results are presented in Figures 10 and 11 and show that most of the metal ion is coordinated to transferrin in the form of $(VO)_2$hTf, while only a small fraction is available for albumin binding. Under these experimental conditions (VO^{2+}/HSA ≪ 1), albumin can form with VO^{2+} only the dinuclear species $(VO)_2$[d]HSA, which is detected in small amounts in the VO^{2+}-hTf-HSA system (Figure 2c) and which is characterized, as previously discussed, by a very low intensity EPR signal.[26] The two lmm bioligands, lactate and citrate, could form ternary complexes with hTf or HSA.

Scheme 1. Possible Transformations and Distribution of Vanadium in Blood Serum



In the quaternary system with lactate, it is possible to notice the presence of $(VO)_2$hTf as the main component, and of the mixed species VO^{2+}−hTf−lactate (Figure 10b) as a minor component. However, the presence of a small amount of $(VO)_2{}^{d}$HSA is observed, whereas no transitions attributable to $(VO)_x{}^{m}$HSA can be detected (cf. Figure 10c and d). On the basis of the EPR measurements, the existence of the mixed complex VO^{2+}−HSA−lactate can be also discarded (expected A_z value of 163×10^{-4} cm^{-1} vs 167×10^{-4} cm^{-1} measured).

The results obtained in the quaternary system VO^{2+}-hTf-HSA-citrate are comparable, and the species are the same as those detected with lactate (Figure 11): the binary complex $(VO)_2$hTf and the ternary VO^{2+}−hTf−citrate, with the presence of a minor amount of $(VO)_2{}^{d}$HSA or $[(VO)_2(citrH_{-1})_2]^{4-}$. The fact that signals of the dinuclear species formed by albumin and citrate with VO^{2+} cannot be easily distinguished must be considered in the interpretation of the spectra: this depends on the very similar values of D, 631 and 666×10^{-4} cm^{-1}, respectively.[26,35] Therefore, it is not possible to understand whether in aqueous solution only $(VO)_2{}^{d}$HSA or $[(VO)_2(citrH_{-1})_2]^{4-}$ exists or both of them. By comparison with the quaternary system containing lactate, we believe that $(VO)_2{}^{d}$HSA is surely present and that a minor participation of $[(VO)_2(citrH_{-1})_2]^{4-}$ is possible. For this system too, the existence of $(VO)_x{}^{m}$HSA and of the ternary complex VO^{2+}−HSA−citrate can be ruled out; thus, albumin has a minor participation in the coordination of the VO^{2+} ion with the formation of $(VO)_2{}^{d}$HSA.

The results of the EPR measurements on the quaternary systems VO^{2+}-hTf-HSA-lactate and VO^{2+}-hTf-HSA-citrate can be summarized, affirming that, under "pseudo-physiological" conditions, most of VO^{2+} ion interacts with transferrin as $(VO)_2$hTf and, secondarily, as ternary VO^{2+}−hTf−lactate and VO^{2+}−hTf−citrate complexes. Albumin seems, instead, to play a marginal role, and only the formation of $(VO)_2{}^{d}$HSA should be possible.

Conclusions and Outlook

With this study, we provide further insights into the transport of the biologically essential vanadium element. EPR spectroscopy, used through the repeated acquisition of weak signals, confirms this to be one of the most powerful techniques available to study systems where the concentration of the components is very low, such as those ternary or quaternary ones formed by VO^{2+}, human serum apo-transferrin, albumin, and a lmm component of blood serum (lactate or citrate).

EPR data of the ternary system VO^{2+}-hTf-HSA recorded at the physiological ratio between albumin and transferrin (17:1) seem to confirm the previous results in the literature[14,22,26] and indicate that transferrin is stronger than albumin. Therefore, under physiological conditions, in which the VO^{2+} concentration (~200 nM)[12] is much lower than that of transferrin (37 μM),[22,24] this latter could bind all vanadium present in the blood serum with the coordination sites non-saturated by the Fe^{3+} ion (about 70% of the total sites of transferrin).[56] However, the conclusion that transferrin quantitatively binds VO^{2+} must be considered carefully, because our data prove that a small amount of vanadium is present in solution, when high ratios of HSA/VO^{2+} are used, as dinuclear complex $(VO)_2{}^{d}$HSA.[50]

The results obtained with the ternary systems VO^{2+}-hTf-bL or VO^{2+}-HSA-bL, where bL is lactate, citrate, oxalate, phosphate, glycine, or histidine, show that only citrate and lactate are able to compete with transferrin and albumin for the complexation of the VO^{2+} ion, forming mixed species, the other lmm components of blood serum being present in a too low of a concentration (oxalate and histidine) or having a too low affinity toward VO^{2+} (phosphate and glycine). The affinity of lactate is connected with its moderate strength as a ligand (possibility to chelate VO^{2+} through a deprotonated alcoholic group and a carboxylate) and with a high concentration (1.51×10^{-3} M),[22,24] whereas that of citrate mainly depends on its strength as a tridentate ligand provided with the (COO^-, O^-, COO^-) donor set rather than its concentration in blood serum (9.9×10^{-5} M).[22,24]

The measurements of the quaternary systems VO^{2+}-hTf-HSA-lactate and VO^{2+}-hTf-HSA-citrate allow for an understanding regarding in which form VO^{2+} is transported in blood serum and how it is distributed between the hmm and lmm components. The results indicate that most of it is present as $(VO)_2$hTf, a low amount as mixed complex VO^{2+}−hTf−lactate or VO^{2+}−hTf−citrate, and the very low remaining part as $(VO)_2{}^{d}$HSA; in the system with citrate, there is the further possibility of formation of the dinuclear species $[(VO)_2(citrH_{-1})_2]^{4-}$. Their percentage amounts can be quantified only approximately on the basis of the intensities of the EPR signals and of the spectra simulations (Figures 3 and 6 and Figure S23, Supporting Information), and the values reported in Scheme 1 can be assumed.

Therefore, we can conclude that vanadium is transported in the organism mainly by transferrin with a secondary participation of citrate and lactate as the mixed species with transferrin, while albumin forms only a small amount of the binary $(VO)_2{}^{d}$HSA complex.

Concerning the coordination modes of VO^{2+} to the two hmm serum proteins, we can conclude that only the binding to transferrin is specific; that is, it takes place at the two Fe^{3+} sites. Ternary complexes with the lmm bioligands can be

(56) Taylor, D. M. In *Perspectives on Bioinorganic Chemistry*; Hay, R. W., Dilworth, J. R., Nolan, K. B., Eds.; JAI Press: London, 1993; Vol. 2, pp 139−159.

formed only if these latter possess special steric and specific charge requirements in order to fit into the cavities in which the two VO^{2+} ions are coordinated.[49] Lactate is well-suited for this purpose and can replace, at least partly, the bicarbonate anion as the synergistic anion. The same is true for citrate, but the replacement takes place to a lesser extent, probably because of its higher negative charge. On the contrary, the binding of VO^{2+} to albumin is less specific: although it contains the strong binding site on the *N*-terminal side provided with one amino, two deprotonable amide, and one imidazole nitrogen donors,[51] the observation in the physiological pH range of only one EPR signal with a rather high A_z value indicates that the 5−6 equiv of VO^{2+} ions are coordinated elsewhere.[26] We believe that the coordination takes place probably at the surface of the protein, where the metal ions are more "exposed" to the interaction with the lmm bioligands eventually present; as a consequence, ternary complexes are more easily formed, with the probable replacement of the donors belonging to the amino acids of the protein chain with the set (COO^-, O^-). The only factor governing the formation of ternary complexes VO^{2+}−HSA−bL is the thermodynamic stability of the species, and there is no high selectivity for the bioligands.

Finally, we point out two future developments of this work: the recording of the EPR spectra with a still lower concentration of vanadium, to better model real physiological conditions,[12] and the application and extension of these results to the transport in the organism of the insulin-enhancing vanadium compounds.

Supporting Information Available: All the complete EPR spectra (Figures S1−S19), the comparison between the complete spectra recorded in the binary, ternary and quaternary systems (Figures S20−S22) and the simulation of the spectrum measured in the VO^{2+}-hTf-citrate system (Figure S23). This material is available free of charge via the Internet at http://pubs.acs.org.