Interaction of V(V) complexes formed by picolinic and pyrazinecarboxylic acid derivatives with red blood cells

Daniele Sanna[a], Péter Buglyó[b], Sándor Nagy[b], Franc Perdih[c], Jessica Palomba[d], Valeria Ugone*[a], Eugenio Garribba[e]

[a] Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Trav. La Crucca 3, I-07100 Sassari, Italy.

[b] Department of Inorganic and Analytical Chemistry, University of Debrecen, Egyetem tér 1, H-4032 Debrecen, Hungary

[c] Faculty of Chemistry and Chemical Technology, University of Ljubljana, Večna pot 113, SI-1000 Ljubljana, Slovenia

[d] Dipartimento di Chimica e Farmacia, Università di Sassari, Via Vienna 2, I-07100 Sassari, Italy.

[e] Dipartimento di Scienze Mediche, Chirurgiche e Sperimentali, Università di Sassari, Viale San Pietro, I-07100 Sassari, Italy.

* Corresponding author. E-mail address: valeria.ugone@cnr.it (V. Ugone)

Abstract

The interaction with red blood cells (RBC) of vanadium(V) complexes formed by 3,5-difluoropicolinic acid (HpicFF), 3-hydroxypicolinic acid (H_2hypic) and pyrazinecarboxylic acid (Hprz) has been examined. Electron Paramagnetic Resonance (EPR) spectroscopy results suggest that V(V) is reduced in all cases to V(IV) inside the erythrocytes. The thermodynamic stability of V(V) and corresponding V(IV) complexes has been related with the species detected in the cellular environment; when the ligands form unstable complexes with both $V^{V}O_2^{+}$ and $V^{IV}O^{2+}$ ions (picFF and prz), the species formed inside RBC are similar to those observed when starting with vanadate(V) alone, that is $V^{IV}O^{2+}$ –formed upon V(V) reduction– distributing among the cellular bioligands forming different types of complexes. When in the ligand molecule other groups able to form chelated complexes are present (hypic), more stable species are formed inside the RBC. The amount of complex able to enter the RBC depends on the ligand structure which could influence the metal uptake. The interaction of different $V^{IV}O^{2+}$ complexes formed by picolinate (picFF, pic = picolinate, picCN = 5-cyanopicolinate) and pyrazinecarboxylate (prz and $przNH_2$ = 3-aminopyrazine-2-carboxylate) derivatives with hemoglobin (Hb), which is the main candidate to bind the $V^{IV}O^{2+}$ ion in the cytosol, was also investigated to rationalize the results. The ligands which form at physiological pH relatively stable complexes with $V^{IV}O^{2+}$ ($przNH_2$ and pic) can give inside the erythrocytes mixed species after the replacement of an equatorial water molecule by an imidazole nitrogen of a histidine residue of the protein, while the ligands which form with $V^{IV}O^{2+}$ unstable complexes (picCN, picFF, prz) yield the same species observed in the binary system $V^{IV}O^{2+}$–Hb.

Keywords

Vanadium complexes
Red blood cells
Hemoglobin
Electron Paramagnetic Resonance

1. Introduction

One of the most interesting application of vanadium compounds (VCs) is their development as promising metallodrugs in the treatment of several diseases, such as diabetes and cancer . In particular, the compound bis(ethylmaltolato)oxidovanadium(IV), BEOV, completed phases IIa of the clinical trials, and became the benchmark compound for the new VCs with anti-diabetic activity, even if these have provisionally been abandoned due to the expiry of the patent and to renal problems arising with several patients .

Despite their promising pharmacological properties, the mechanism of action of such compounds and the active species are not fully known. For a rational design of next generation metallodrugs, thermodynamic stability, behavior in aqueous solution, and speciation in biological fluids and cellular environment must be considered .

One of the main steps which the metallodrug undergoes once administrated is the transport in the bloodstream where ligand exchange, variation in coordination number and geometry, redox reactions at the metal center, hydrolysis and metabolic transformations can occur . The interaction of several vanadium compounds with the main bioligands present in the blood serum has been investigated . It has been also demonstrated that a significant fraction of metal drug in blood can interact with erythrocytes and can be taken up through different transport mechanisms . This important aspect, still little studied, could have two opposite effects: to store the metal species, so that the erythrocytes could be considered as a reserve, or to deactivate it, hindering the uptake of the metal drug by the target cells. The transport across the erythrocyte membrane can take place by passive diffusion (demonstrated for neutral $V^{IV}OL_2$ complexes) which occurs only if there is a difference of concentration between the extracellular fluid and the erythrocytes, or by transport across the anion channels (proved for vanadate(V)).

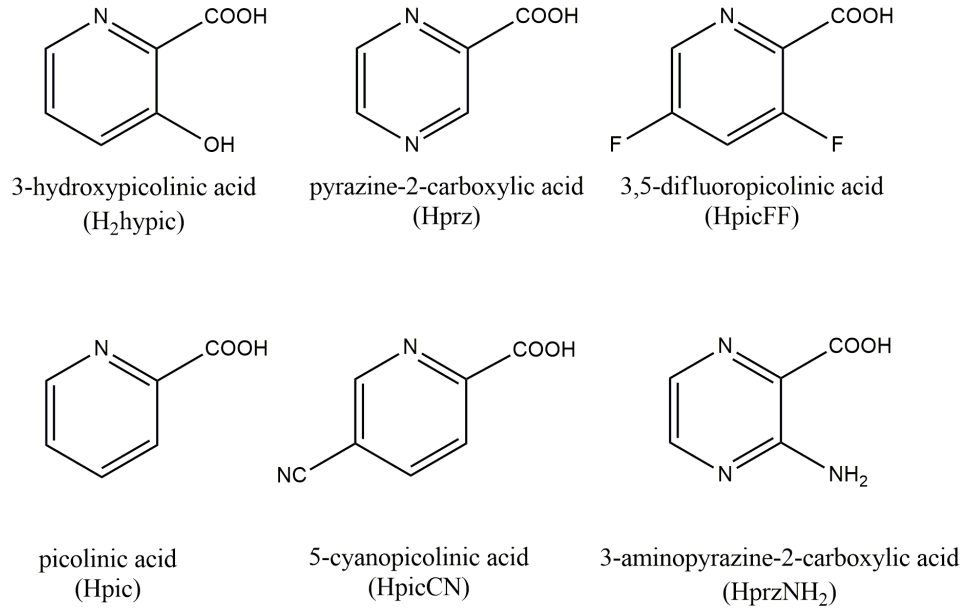
The interaction of V(V) with red blood cells has been reported in the literature and the results suggest that it crosses the red blood cell membrane, using the AE1 (anion exchanger 1) channels, more quickly than V(IV) . In fact the transport of vanadate(V) is inhibited by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), a specific inhibitor of the AE1 channels . Once inside the RBC, V(V) could be reduced to V(IV) by cellular reductants (like GSH) ; this was confirmed by the detection of EPR signals once the complex enters the cells . In particular, the signals of $V^{IV}O^{2+}$ bound to the sites β and γ of hemoglobin (Hb) were observed, proving that part of the metal is present in the RBC as a Hb complex. Additional species, indicated as $V^{IV}O(L^1,L^2)$ and $V^{IV}O(L^3,L^4)$, were proposed, in which the unidentified L^1, L^2, L^3 and L^4 ligands could be cytosol or membrane proteins or low molecular mass compounds .

In another study, Garner et al. studied the interaction of vanadate(V) with intact human erythrocytes and with hemolysates by ^{1}H and ^{51}V NMR. They demonstrated that vanadium is transported through the membrane in the form of vanadate(V) within anion transporters, reduced in the cytosol by glutathione and complexed in the form of $V^{IV}O^{2+}$ by an array of intracellular coordination sites . Yang and coworkers

, in their study on the transport of vanadium complexes and their interaction with erythrocyte membrane, demonstrated that neutral VCs such as bis(maltolate)oxidovanadium(IV), [VO(ma)$_2$], and bis(acetylacetonate)oxidovanadium(IV), [VO(acac)$_2$], enter inside the cells by passive diffusion and have a faster absorption kinetic in comparison with $NaVO_3$. However, the two species have different diffusion capability: [VO(acac)$_2$] exhibits a faster absorption kinetics in comparison with [VO(ma)$_2$]. These results are consistent with the hypothesis that the different insulin-mimetic, and pharmacological in general, effectiveness of vanadium complexes arises from different transport mechanisms. Moreover, Yang *et al*. ascertained that the interaction of VCs with cellular membrane can have an important role in the stabilization of these species; they also noticed that this interaction induces a conformational change of the membrane proteins as well as modifications of the membrane structure. These changes could influence the mechanism of action and the toxicity of pharmacologically active VCs.

In a previous study, the interaction with RBC of some V(V) complexes with different stability was examined by some of us . The results showed that stable V(V) complexes (as that formed by 1,2-dimethyl-3-hydroxy-4(1H)-pyridinonate, dhp) cross the erythrocyte membrane without using the AE1 channel; once inside the erythrocytes, they are reduced and form the same complexes which were observed when starting from the analogous V(IV) complex, [$V^{IV}O(dhp)_2$], that is a species in which the water molecule of *cis*-[$V^{IV}O(dhp)_2(H_2O)$] has been replaced by an imidazole-N of a His residue of a protein, presumably Hb . With unstable V(V) complexes (as that formed by picolinic acid, Hpic), the uptake takes place by using the anionic channel, but inside the erythrocytes the same complexes observed when starting from [$V^{IV}O(pic)_2$] are detected. In particular, the species $V^{IV}O(L^3,L^4)$ observed with vanadate(V) was identified together with two mixed complexes, *cis*-$V^{IV}O(pic)_2$(N-His) and *cis*-$V^{IV}O(pic)_2(R\text{-}S^-)$, where a His-N donor of a protein, presumably Hb, or a thiolate donor belonging to GSH or to a membrane protein occupies the fourth equatorial site. This means that even ligands which form unstable complexes in the extracellular medium with V(V) can be transported, independently from vanadate(V), inside the erythrocytes and form relatively stable complexes with the reduced form of the metal ion, *i.e.* $V^{IV}O^{2+}$. When considering V(V) complexes with intermediate stability between those formed by dhp and pic, it is possible to hypothesize that the transport takes place with mechanisms which involve both anion channels and passive diffusion across the membrane, as in the case of 3-aminopyrazine-2-carboxylic acid .

In this work the interaction of vanadium(V) complexes formed by picolinic and pyrazinecarboxylic acid derivatives (HpicFF, H_2hypic, and Hprz, see Scheme 1 and S1) with RBC has been studied to verify if they are able to cross the erythrocyte membrane and in which form they can exist in the cellular environment. Furthermore, the interaction of the corresponding V(IV) complexes with Hb, which is the protein contained in greater amount in RBC, has been also investigated and the results compared with the systems formed by other pic/prz derivatives (Hpic, HpicCN and HprzNH_2, see Scheme 1).



Scheme 1 Ligands studied in this work.

2. EXPERIMENTAL SECTION

2.1. Materials

The compounds used, 3,5-difluoropicolinic acid (HpicFF), 3-hydroxypicolinic acid (H_2hypic), pyrazine-2-carboxylic acid (Hprz), picolinic acid (Hpic), 5-cyanopicolinic acid (HpicCN), 3-aminopyrazine-2-carboxylic acid ($HprzNH_2$), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 4-(2-pyridylazo)resorcinol (PAR), sodium chloride (NaCl), oxidovanadium(IV) sulfate trihydrate ($VOSO_4 \cdot 3H_2O$), ammonium vanadate(V) (NH_4VO_3), disodium hydrogenphosphate (Na_2HPO_4), and glucose are high purity Aldrich or Fluka products. Human hemoglobin (Hb) is a Sigma product (H7379). None of the products was further purified. The V(V) complexes, $NH_4[V^VO_2(picFF)_2] \cdot 1.6H_2O$, $NH_4[V^VO_2(Hhypic)_2] \cdot H_2O$, and $NH_4[V^VO_2(prz)_2]$, were prepared as described elsewhere .

2.2. Interaction of the vanadium(V) compounds with RBC

The solutions of the V(V) complexes were prepared by dissolving the solid compounds $NH_4[V^VO_2(picFF)_2]$, $NH_4[V^VO_2(Hhypic)_2]$, and $NH_4[V^VO_2(prz)_2]$ in PBS (phosphate-buffered saline, 140 mM NaCl, 5 mM Na_2HPO_4, 11 mM glucose, pH 7.40), to have a V concentration of 10 mM. After the solubilization of the complexes, the solutions undergo an acidification; the pH was brought to *ca.* 7.40 (physiological pH) with a diluted NaOH solution and later they were heated (*ca.* 70 °C) to decompose the decavanadate eventually formed.

The blood samples used in this work, containing EDTA as anticoagulant, were provided by the Servizio Trasfusionale Aziendale (Azienda Ospedaliero-Universitaria di Sassari). These samples were centrifuged for 10 min at 3000 rpm, to separate erythrocytes from plasma and leucocytes. The RBC were re-suspended in a volume of PBS equal to that of the separated plasma in order to have the same hematocrit value; later, the suspensions were centrifuged and washed two times with a PBS solution.

Erythrocytes in PBS were incubated at 37 °C with the solution containing the V(V) complexes to have a final V concentration in the extracellular medium of 9.09×10^{-4} M. After 1, 2 and 3 hours, the suspensions were centrifuged at 3000 rpm for 10 minutes to separate the extracellular medium from the erythrocytes, and these latter were washed for two times with a PBS solution.

RBC were transferred into quartz tubes, frozen at 120 K, and the EPR spectra were recorded. Even if the freezing produces erythrocyte lysates, the measured EPR spectra coincide with those of intact red blood cells. The electronic absorption spectra were measured on the extracellular fluid for the determination of the vanadium amount remained in solution and to establish, by difference, the amount of the vanadium complexes entered in the red blood cells, as described in detail the following section.

2.3. Determination of vanadium uptake by RBC

To establish the amount of vanadium that crosses the erythrocyte membrane, the V concentration in the extracellular medium was determined by a selective and sensitive spectrophotometric method based on the reaction with 4-(2-pyridylazo)-resorcinol (PAR) . PAR (L) forms with vanadium(V) an anionic complex $[V^{V}O_2L]^-$ in the pH range 4.5−8.0 with an absorption maximum at λ_{max} = 542 nm and ε_{max} = $(3.6-3.7) \times 10^4$ L mol^{-1} cm^{-1} . The limit of detection of the method is 0.0028 μg/mL .

Calibration curves were plotted by preparing standard solutions with final concentrations of each V(V) complex of 1, 2, 3, 4, 5×10^{-5} M and measuring the absorption at 542 nm in presence of PAR. The calibration curves allowed the determination of vanadium amount remained in solution in the examined systems where the V(V) complexes were incubated for 1, 2 and 3 hours with erythrocytes and, by difference, the amount of VCs taken up by the red blood cells.

2.4. Interaction of the vanadium(IV) compounds with hemoglobin

The solutions were prepared by dissolving in ultrapure water $VOSO_4 \cdot 3H_2O$ and the ligand (H_2hypic, Hprz, HpicFF, Hpic, HpicCN, and HprzNH_2) to obtain a $V^{IV}O^{2+}$ concentration of 6.2×10^{-4} M and a ligand-to-metal molar ratio of 2:1. HEPES was added to obtain a concentration of 0.1 M and the pH was raised to *ca.* 5.5. To 1 mL of this solution, Hb was added to obtain a protein concentration of 3.1×10^{-4} M. Subsequently, pH was adjusted to ca. 7.4 and EPR spectra were immediately recorded. Argon was bubbled through the solutions to ensure the absence of oxygen and avoid the oxidation of $V^{IV}O^{2+}$ ion.

2.5. Spectroscopic Measurements

Anisotropic EPR spectra were recorded on frozen solutions (120 K) with an X-band (9.4 GHz) Bruker EMX spectrometer equipped with a HP 53150A microwave frequency counter. To increase the signal-to noise ratio, signal averaging was used . EPR spectra were simulated with the software Bruker WinEPR SimFonia (version 1.26 (beta), Bruker Analytik GmbH, 1997).

As usual for the analysis of the EPR spectra , in all of the figures reported in this paper, only the high field region is shown, the part more sensitive to the identity and to the amount of the several species in solution. Spectrophotometric measurements were carried out with a Perkin-Elmer Lambda 35 spectrophotometer.

2.6. Potentiometric Measurements

The protonation constant of 3-hydroxypicolinic acid and the stability constants of its $V^{V}O_2^+$ complex were determined by pH-potentiometric titrations on 15.00 mL of samples at 25.0 ± 0.1 °C and at a constant ionic strength of 0.20 M (KCl), and $V^{V}O_2^+$ concentration in the range of (0.5−2.0) × 10^{-3} M. The molar ratios explored were 1:1, 1:2, and 1:4. The titrations were performed over the pH range 1.9−11.0, or until precipitation occurred with a carbonate-free KOH of known concentration (*ca*. 0.2 M) under a purified argon atmosphere. A Mettler Toledo T50 titrator equipped with a combined glass electrode (type 6.0233.100) was used for the pH-metric measurements. The electrode system was calibrated according to Irving *et al.* , and the pH-metric readings were converted into hydrogen ion concentration. The water ionization constant, pK_w, was 13.76 ± 0.01 under the conditions employed. The reproducibility of the points included in the evaluation was within the 0.005 pH unit in the whole pH range measured. The stability of the complexes, reported as the logarithm of the overall formation constant $\beta_{pqr} = [(HVO_4)_pL_qH_r]/[HVO_4]_p[L]_q[H]_r$, where HVO_4 stands for HVO_4^{2-} ion and L is the deprotonated form of the ligand. The log β values were calculated with the aid of the PSEQUAD program . The uncertainties (3σ values) of the stability constants are given in parentheses in Table 1. During the calculations, hydrolysis of the metal ion was assumed, and the appropriate values for various hydrolytic species are summarized in Table S1.

3. Results and discussion

3.1. Interaction of $NH_4[V^{V}O_2(picFF)_2]$ complex with RBC

The crystal structure of the V(V) complex formed by HpicFF, $NH_4[V^{V}O_2(picFF)_2]\cdot 1.6H_2O$, has been previously reported ; in this complex, the metal ion is hexacoordinated with distorted octahedral geometry with the two pyridine-N donors in *trans* position to the two oxido of the $V^{V}O_2^+$ unit, which have a relative *cis* disposition.

The ligand HpicFF has two electron withdrawing groups on pyridine ring resulting in higher acidity than that of the unsubstituted derivative. The pK_a of the carboxylic group (1.60) is similar to that of pic (*ca.* 1), while that of the group $N_{pyr}H^+$ is considerably lower (2.83) in comparison with the value measured for pic (5.19). Even if data about the stability of the V(V) species are not available in the literature, it is possible to hypothesize – based on the acidity of the potential coordinating groups – that the complexes are even less stable compared to those formed by pic. It is therefore expected that the species distribution at pH 7.40 is not significantly different from that of vanadate(V) alone, with the difference that in solution is present, in the completely deprotonated form, also the ligand picFF.

After incubation at 37 °C of a solution containing $NH_4[V^VO_2(picFF)_2]$ for 1, 2 and 3 h with red blood cells, the EPR spectra depicted in Figure 1a-c are obtained.

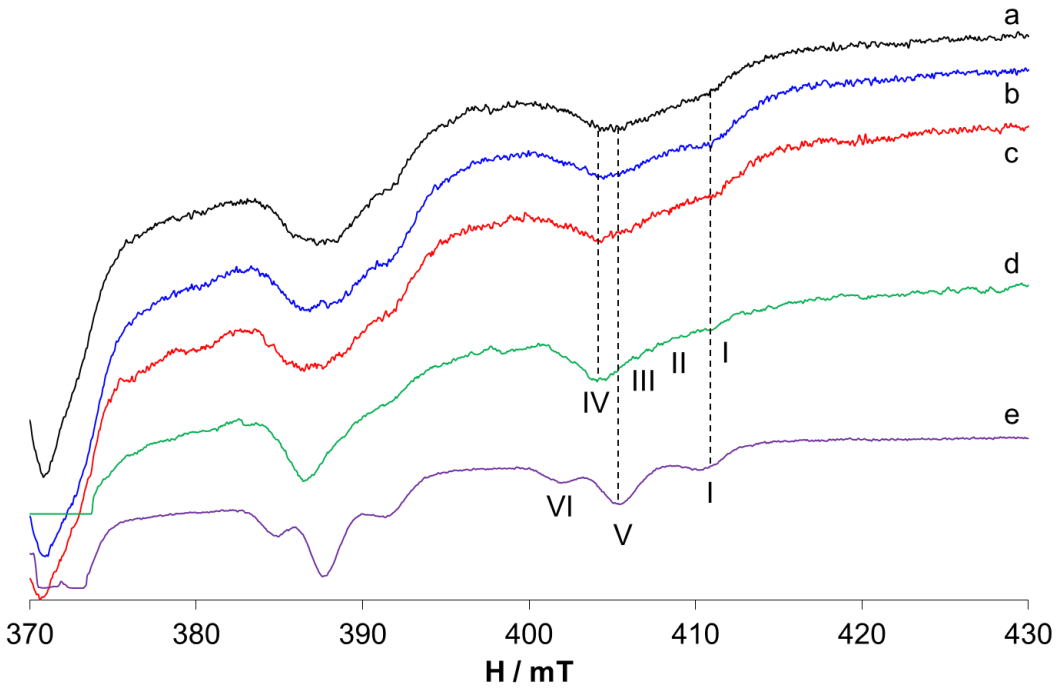


Figure 1 High field region of the anisotropic EPR spectra recorded at 120 K on: sample containing erythrocyte lysates incubated with $NH_4[V^VO_2(picFF)_2]$ (V concentration 9.09×10^{-4} M) for 1 h (a); for 2 h (b); for 3 h (c); d) sample containing erythrocyte lysates incubated with vanadate(V) for 3 h; e) sample containing erythrocyte lysates incubated with $NH_4[V^VO_2(pic)_2]$ for 3 h. With **I**, **II**, **III**, **IV**, **V**, and **VI** are indicated the M_I = 7/2 resonances of $V^{IV}O(L^3,L^4)$, $(V^{IV}O)Hb^{\beta}$, $(V^{IV}O)Hb^{\gamma}$, $V^{IV}O(L^1,L^2)$, *cis*-$V^{IV}O(pic)_2(Hb)$, and cis-$V^{IV}O(pic)_2(R\text{-}S^-)$, respectively.

The results obtained in this system resemble to some extent what was obtained with the V(V)-pic system (Figure 1e) , but the spectra are less resolved, as if species with intermediate A_z values were also present. A more careful examination shows that in this case the situation is intermediate between that observed in the system V(V)-pic and that containing vanadate(V) alone (Figure 1d). Since, HpicFF is more acidic than Hpic, the completely deprotonated ligand is less basic than pic and therefore it forms less stable

complexes with $V^{IV}O^{2+}$. Then, even assuming that this ligand can cross the erythrocyte membrane, once inside the red blood cells it would be not able to form sufficiently stable complexes with $V^{IV}O^{2+}$, which – in its turn – can interact with cellular bioligands forming the same species previously observed with vanadate(V) (**I**, **II**, **III** and **IV** in Figure 1) . However, it can be hypothesized that a small amount of *cis*-$V^{IV}O(picFF)_2(H_2O)$ is formed in the cytosol which transforms in the mixed species $V^{IV}O(picFF)_2$(N-His), analogously to that observed with pic (**V** in Figure 1), resulting in the detection of broad bands (Figure 1a-c). In Table S2 the experimental spin Hamiltonian parameters previously reported for **I-V** species are listed . It is also possible that the differences between picFF and pic are partly due to the lower tendency of picFF to cross the erythrocyte membrane. The formation of unstable complexes with $V^{IV}O^{2+}$ at pH 7.4 with picFF, can be confirmed by potentiometric data . In fact, at pH 7.4 and with L/M = 2/1 and $V^{IV}O^{2+}$ 9.09×10^{-4} M, the % of $V^{IV}O^{2+}$ present as complexes is negligible (< 1%), while it is almost entirely present as $(VO)_2(OH)_5$ (*ca*. 99%) (Figure S1).

The % of intracellular V, measured after 1, 2 and 3 h, are the following: 32.8%, 50.0% and 58.3% (see Figure 2). These values are lower than those measured in the system V(V)-pic (53.3%, 81.0%, and 92.9%, respectively). A possible explanation of this difference, since vanadate(V) and the ligands should independently cross the erythrocyte membrane, could be due to the larger steric hindrance of picFF compared to pic or to some kind of interaction between the substituted ligand and the anion channels which hinders, in part, the crossing of vanadate(V). This hypothesis is in accordance with the EPR results.

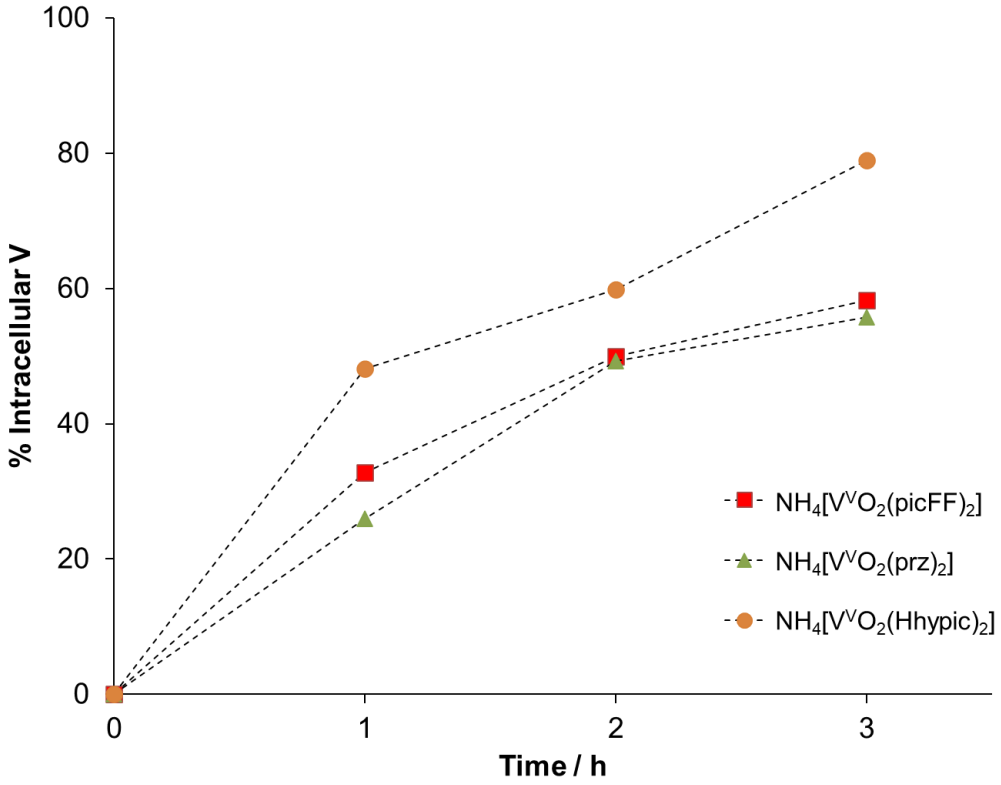


Figure 2 Time dependence of the percent concentration of vanadium inside the red blood cells after the incubation with solutions containing $NH_4[V^VO_2(picFF)_2]$, $NH_4[V^VO_2(Hhypic)_2]$ and $NH_4[V^VO_2(prz)_2]$ at 37 °C.

3.2. Interaction of $NH_4[V^VO_2(prz)_2]$ complex with RBC

The X-ray structure of $NH_4[V^VO_2(prz)_2]$ is reported in literature and it is similar to that of the picFF complex where the two pyridine(pyrazine)-N are *trans* to two oxido of the $V^VO_2^+$ unit, which have a relative *cis* disposition. The presence of two nitrogen atoms in the aromatic ring of pyrazine-2-carboxylic acid (Hprz) considerably decreases the basicity of the nitrogen adjacent to the carboxylate group ($pK_a < 1$); the other nitrogen has a pK_a of 1.83, while the carboxylic group has $pK_a = 2.73$. Overall, the basicity of the donors (N_{prz}, COO^-) considerably decreases in comparison with the same donors of picolinic acid. Considering this fact, as pointed out for the system with picFF, it is expected that the species distribution at physiological pH in a system containing $NH_4[V^VO_2(prz)_2]$, is practically indistinguishable from that containing vanadate(V) alone, while the ligand is present in solution, not coordinated to the metal, in the completely deprotonated form.

After incubation at 37 °C of a solution containing $NH_4[V^VO_2(prz)_2]$ for 1, 2 and 3 h with red blood cells, the EPR spectra depicted in Figure 3 are obtained.

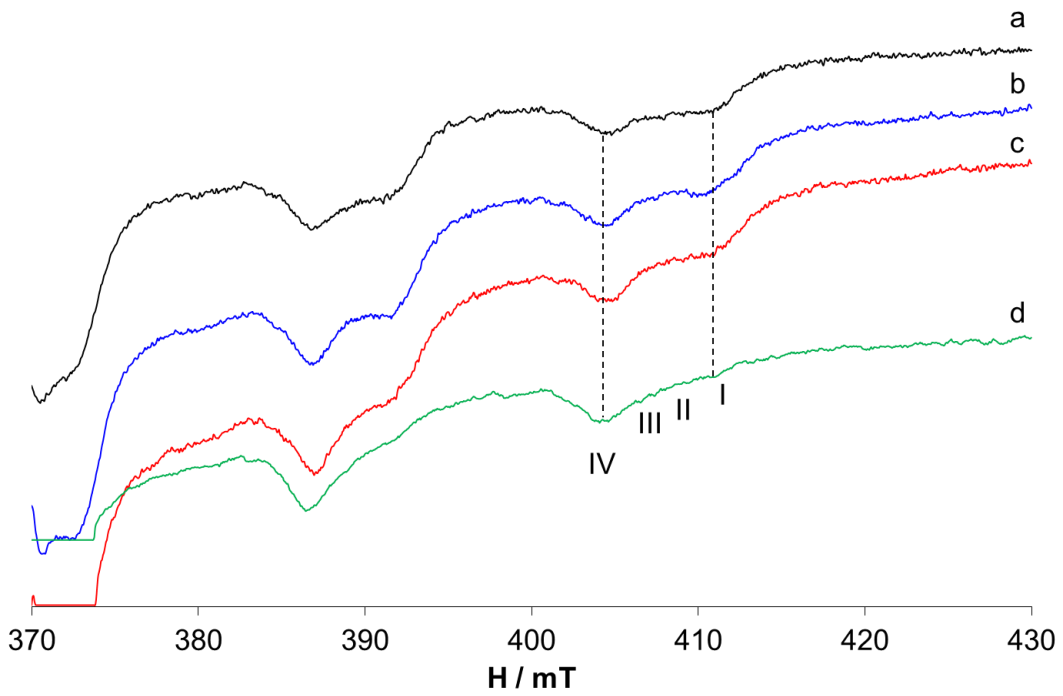


Figure 3 High field region of the anisotropic EPR spectra recorded at 120 K on the systems: sample containing erythrocyte lysates incubated with $NH_4[V^VO_2(prz)_2]$ (V concentration 9.09×10^{-4} M) for 1 h (a); for 2 h (b); for 3 h (c); d) sample containing erythrocyte lysates incubated with vanadate(V) for 3 h. With **I**, **II**, **III** and **IV** are indicated the $M_I = 7/2$ resonances of $V^{IV}O(L^3,L^4)$, $(VO)Hb^{\beta}$, $(VO)Hb^{\gamma}$ and $V^{IV}O(L^1,L^2)$, respectively.

These EPR spectra, which suggest the presence of V(IV) species inside RBC, are rather similar to those obtained with vanadate(V) (Figure 3d); this is in agreement with the fact that prz is a ligand which forms not particularly stable complexes with $V^{IV}O^{2+}$ due to the low basicity of its donor groups. Then at physiological pH, $V^{IV}O^{2+}$ exists as hydrolyzed species and the situation is the same as that observed when starting from vanadate(V), whether or not the prz ligands is able to cross the erythrocyte membrane.

The % of intracellular V are the following: 26.0% after 1 h, 49.3% after 2 h and 55.8% after 3 h (see Figure 2). These values are very similar to those measured in the system V(V)-picFF.

3.3. Interaction of $NH_4[V^VO_2(Hhypic)_2]$ complex with RBC

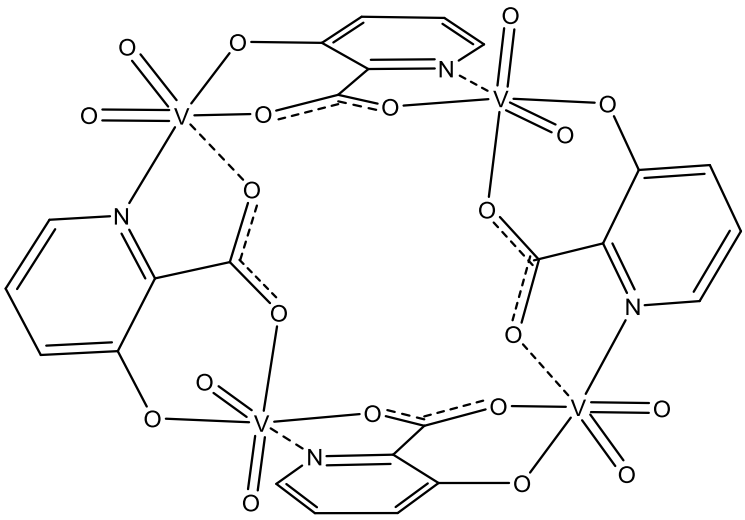
3-Hydroxypicolininc acid (H_2hypic), besides forming complexes with picolinate-like coordination, using the pyridine nitrogen and the adjacent carboxylate, can form $V^{IV}O^{2+}$ complexes with salicylate-like coordination, via the deprotonated phenolate and adjacent carboxylate [41]. The structure of the V(V) complex, $NH_4[V^VO_2(Hhypic)_2]\cdot H_2O$, is reported in the literature and consists of a hexacoordinated

species with distorted octahedral geometry where the two carboxylate-O are *trans* to two oxido of the $V^{V}O_2^+$ unit, which have a relative *cis* disposition [33].

The ligand has two pK_a values corresponding to the deprotonation of the carboxylic and phenolic groups, and the stability constants of its V(V) complexes are determined here for the first time. Based on the stability constants reported in Table 1, it is expected that this ligand forms with V(V) more stable complexes than those formed by picFF, and that a significant amount (55.2%) of the metal is present in complexed form at physiological pH when the vanadium concentration is 9.09×10^{-4} M; in fact, at pH 7.4 the species distribution is the following: $H_2VO_4^-$ 28.4%, HVO_4^{2-} 4.8%, $HV_2O_7^{3-}$ 1.3%, $H_2V_2O_7^{2-}$ 6.6%, $V_4O_{12}^{4-}$ 3.4%, $[VO_2(Hhypic)(hypic)]^{2-}$ 14.8%, $[VO_2(hypic)(OH)]^{2-}$ 38.7%, and $[(VO_2)_4(hypic)_4]^{4-}$ 1.7%. The distribution diagram of the species as a function of pH is shown in Figure S2.

Table 1 Protonation constants of the ligand and stability constants for the V(V) complexes of 3-hydroxypicolinic acid (H_2L) at 25.0 ± 0.1 °C and I = 0.20 mol dm^{-3} (KCl)

Complex	Logβ
HL	10.85(3)
H_2L	15.89(5)
VO_2LH	33.35(4)
VO_2LH_{-1}	22.09(9)
$VO_2L_2H_2$	49.05(6)
VO_2L_2H	42.9(2)
$(VO_2L)_4$	126.4(2)
Fitting	8.59×10^{-3}
No. Points	404



Scheme 2 Structure of the tetranuclear species $[(V^{V}O_2)_4(hypic)_4]^{4-}$.

The distribution observed in the system V(V)-H_2hypic is similar to that observed in the corresponding V(IV) system (Figure S3), where, besides the mononuclear complexes, the existence of a tetranuclear species $[(V^{IV}O)_4(hypic)_4(H_2O)_4]$ was hypothesized which also exists in solution at physiological pH . Its formation has been confirmed by a crystallographic study , where it was shown that the structure is cyclic with the ligands bridging two $V^{IV}O^{2+}$ ions with mixed coordination (N_{pyr}, COO^-) on one side and (O^-, COO^-) on the other. In the system with V^V, a comparable species $[(V^{V}O_2)_4(hypic)_4]^{4-}$ should be formed, as pH-potentiometric measurements suggest (Scheme 2). The similar behavior of the two V oxidation states can be explained considering that in the V(IV) structure a water molecule is in *cis* position respect to V=O and therefore the replacement with an oxido group upon the oxidation should take place without a structural rearrangement.

After incubation at 37 °C of a solution containing $NH_4[V^{V}O_2(Hhypic)_2]$ for 1, 2 and 3 h with red blood cells, the EPR spectra depicted in Figure 4 are obtained.

Observing Figure 4, it can be immediately noticed the existence, inside the red blood cells, of one V(IV) species, differently to what observed for the other complexes; it is also possible to notice that, besides the anisotropic signals of this monomeric species, it is superimposed a broad signal due to the existence of the previously discussed tetranuclear species $[(V^{IV}O)_4(hypic)_4(H_2O)_4]$. In fact, when vanadate(V) crosses the erythrocyte membrane, it is reduced to $V^{IV}O^{2+}$; this latter in the presence of the ligand hypic, which analogously to pic is able to cross the red blood cell membrane, forms the particularly stable tetranuclear species. For the monomeric species the following parameters are measured: g_z 1.948, A_z 163.4 × 10^{-4} cm^{-1}; these parameters are in agreement with the formation of the bis-chelated $V^{IV}O$ complex with 2 × (COO^-, O^-) coordination, $[V^{IV}O(hypic)_2]^{2-}$, previously characterized in the literature .

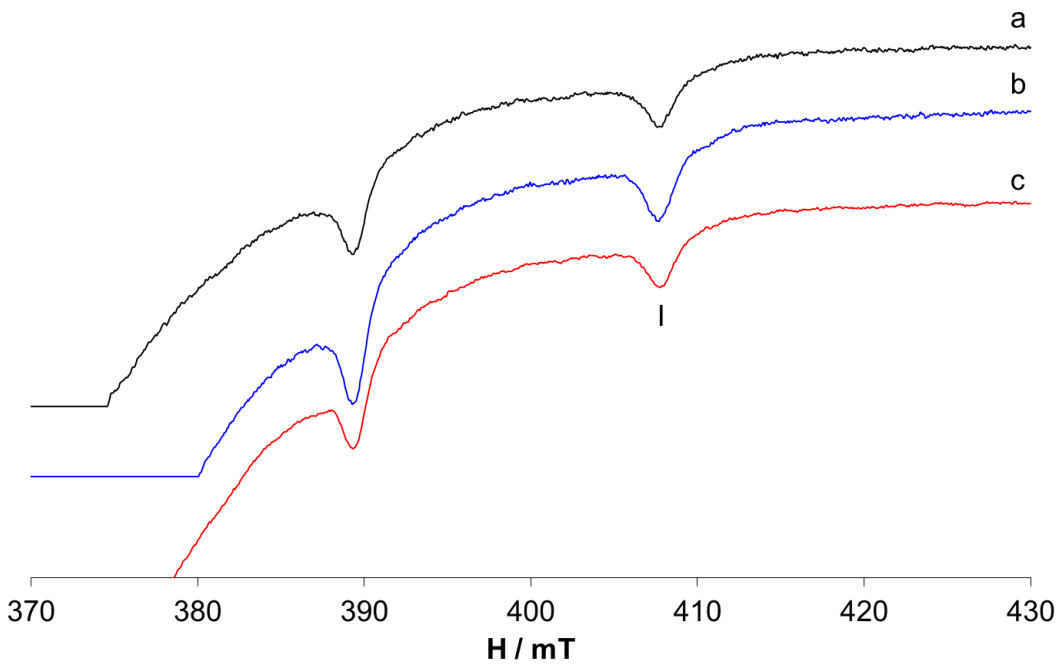


Figure 4 High field region of the anisotropic EPR spectra recorded at 120 K on samples containing erythrocyte lysates incubated with $NH_4[V^{V}O_2(Hhypic)_2]$ (V concentration 9.09×10^{-4} M) for 1 h (a); for 2 h (b); and for 3 h (c). With **I** are indicated the $M_I = 7/2$ resonances of $[V^{IV}O(hypic)_2]^{2-}$ with 2 × (COO^-, O^-) coordination.

The % of intracellular V can be quantified as follows: 48.1%, 59.8%, and 79.0% at 1, 2 and 3 h, respectively (see Figure 2). These values are intermediate between those measured for pic and the derivative picFF. Since vanadium(V) is present, mainly in the complexed form, the transport mechanism could be different from that of free vanadate(V) discussed for picFF and prz.

3.4. Interaction of the systems $V^{IV}O^{2+}$–(pic/prz derivatives) with Hb

In the systems previously examined by some of us and in those studied in this work, the reduction of $V^{V}O_2^{+}$ to $V^{IV}O^{2+}$ takes place, and therefore it is useful to verify which species are formed after the interaction of the corresponding V(IV) compounds with hemoglobin, since it is known that Hb is the main candidate to bind vanadium inside the erythrocytes. For this purpose, the systems $V^{IV}O^{2+}$/(pic/prz derivative)/Hb have been investigated using a molar ratio 2/4/1, analogously to other previously examined systems .

Previous studies showed that with ligands which form stable complexes with $V^{IV}O^{2+}$, the ternary species $VOL_2(Hb)$ (i.e. L = maltolate, dhp), with hemoglobin coordinating via a monodentate imidazole nitrogen of a histidine residue, is the only one existing in solution at pH 7.4 . Moreover, the adduct *cis*-$VO(pic)_2(Hb)$ was also identified in the system with picolinate .

In this work the anisotropic EPR spectra for six different systems containing pic and prz derivatives as ligands (Scheme 1) were measured and they are depicted in Figure 5. After examining this figure, it is possible to identify two different behaviors: on the one hand there are the systems which behave similarly to the binary system $V^{IV}O^{2+}$/Hb (Figure 5, $\mathbf{V^{IV}O^{2+}}$), which form the species previously indicated with $(V^{IV}O)Hb^{\beta}$ and $(V^{IV}O)Hb^{\gamma}$, while on the other hand there are the systems where a component characterized by a value of A_z lower than that observed with $V^{IV}O^{2+}$ alone can clearly be distinguished. Therefore, with ligands that are not able to form stable complexes with $V^{IV}O^{2+}$ at pH 7.4 (Hprz, HpicFF and HpicCN), spectra similar to those measured in the binary system $V^{IV}O^{2+}$/Hb are obtained, while with ligands that form slightly more stable complexes (Hpic, $HprzNH_2$), the species *cis*-$VO(pic/prz\ derivative)_2(Hb)$ can be formed, in which hemoglobin coordinates with a monodentate imidazole nitrogen of a histidine residue.

In the case of Hprz and HpicFF, the detection of broader bands compared to those of $V^{IV}O^{2+}$/Hb suggests that a small amount of mixed complex *cis*-$VOL_2(Hb)$ could be present in solution, in agreement with what was observed in the experiments with RBC.

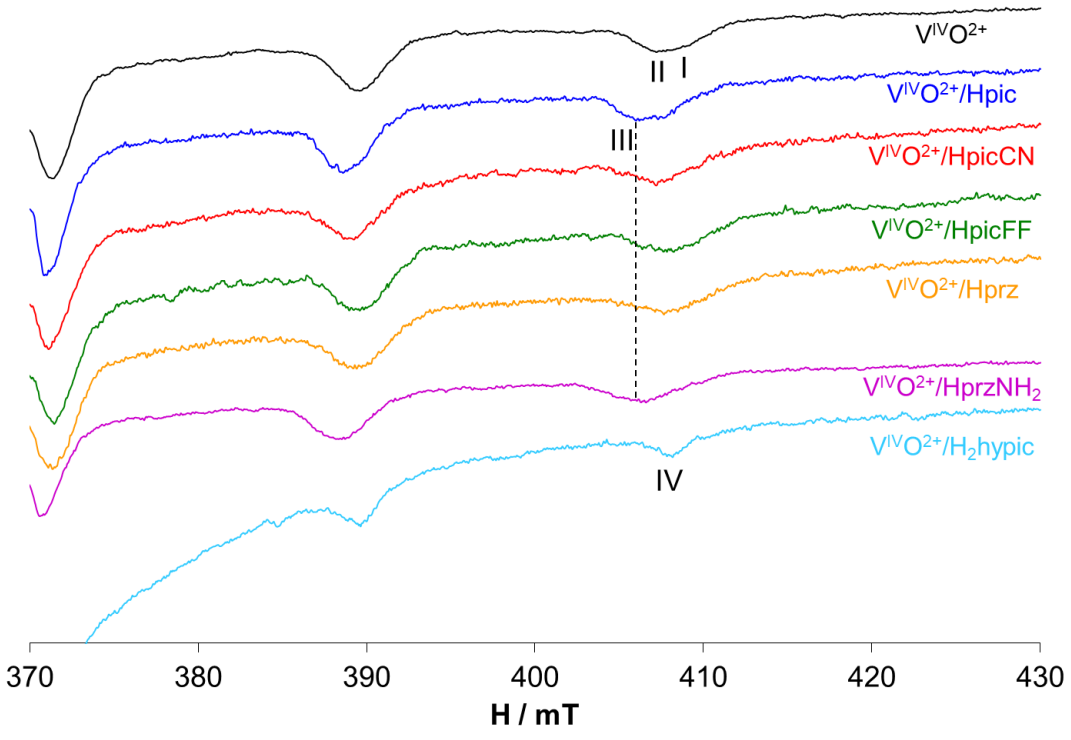


Figure 5 High field region of the anisotropic EPR spectra recorded at 120 K and at pH 7.4 on the systems containing Hb and different $V^{IV}O(pic/prz\ derivative)$ complexes. In all systems the $V^{IV}O^{2+}$ concentration is 6.2×10^{-4} M and the $V^{IV}O^{2+}$/(pic/prz derivative)/Hb molar ratio 2/4/1. With **I**, **II**, **III** and **IV** are indicated the $M_I = 7/2$ resonances of $(VO)Hb^{\beta}$, $(VO)Hb^{\gamma}$, *cis*-$VO(pic/prz\ derivative)_2(Hb)$ and $[V^{IV}O(hypic)_2]^{2-}$ respectively. The dotted

line indicates the component corresponding to the species *cis*-VO(pic/prz derivative)$_2$(Hb), with Hb coordinating via an imidazole-N of a His residue.

The situation is different in the case of H_2hypic because this ligand, as described above, has an alternative coordination mode, i. e. the salicylate-like with the (COO^-, O^-) donors.

The results can be summarized affirming that, depending on the stability of the V(IV) complexes, inside RBC binary or ternary species with Hb can be formed, the formers are detected when the binary complexes $V^{IV}O^{2+}$–L are unstable while the latter are observed when the $V^{IV}O^{2+}$–L species are stable enough to survive at physiological pH values.

4. Conclusions

The interaction with red blood cells of systems containing V(V) and picolinic or pyrazinecarboxylic acids derivatives confirms that V(V) is reduced in all cases to V(IV) inside the erythrocytes. However, the complexes formed inside the red blood cells not always coincide with the species previously observed with vanadate(V) .

When the ligands form unstable complexes with both V(V) and with $V^{IV}O^{2+}$ (picFF and prz) then the situation is very similar to that observed with vanadate(V); that is $V^{IV}O^{2+}$, once formed inside the red blood cells, distributes among the cellular bioligands forming different types of complexes. When in the ligand molecule other groups able to form chelated complexes are present (hypic), the situation is different and stable complexes can be formed with $V^{IV}O^{2+}$ in the cytosol with the starting ligand.

The interaction of V(V) complexes formed by picolinic or pyrazinecarboxylic acids derivatives previously examined by us and those examined in this work with hemoglobin makes possible to identify two different behaviors. The ligands which form at physiological pH relatively stable complexes with $V^{IV}O^{2+}$ (przNH$_2$ and pic) can form inside the erythrocytes mixed species after the replacement of a water molecule by an amino acid side-chain, presumably an imidazole of a histidine residue~~, presumably~~ of hemoglobin, while the ligands which form with $V^{IV}O^{2+}$ unstable complexes at pH 7.4 form the same species observed in the binary system $V^{IV}O^{2+}$–hemoglobin .

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