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Speciation in the aqueous $H^+/H_2VO_4^-/H_2O_2$/L-(+)-lactate system †

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A detailed study of the quaternary aqueous $H^+/H_2VO_4^-/H_2O_2$/L-(+)-lactate (Lac^-) system has been performed at 25 °C in 0.150 M Na(Cl) medium, representing the ionic strength of human blood, using quantitative ^{51}V NMR and potentiometric data (glass electrode). Data were evaluated with the computer program LAKE, which is able to treat combined EMF and NMR data. The pK_a-value for lactic acid was determined as 3.653 ± 0.002. The error given is 3σ. In the ternary $H^+/H_2VO_4^-/Lac^-$ system, eight complexes were found in the pH region 1.1–10.9; only half of them are mononuclear. Owing to the fast reduction of vanadium(V) in acidic solutions and to the slow equilibria of the inorganic vanadates under certain conditions, the final model of this ternary system presented in the study is limited to pH > 3.1. Solutions, in which reduction occurred to any extent, were excluded from all calculations, hence the study is limited to vanadium(V). In the quaternary $H^+/H_2VO_4^-/H_2O_2/Lac^-$ system, seven complexes could be found in addition to all binary and ternary complexes over the pH region 2.1–10.0, only two of which were mononuclear. Equilibrium is fast, but significant decomposition of peroxide occurs in acidic solutions over very short time, limiting the final model to pH > 4. Chemical shifts, compositions and formation constants are given, and equilibrium conditions are illustrated in distribution diagrams as well as the fit of the model to the experimental data. Simple biological tests were carried out to check the resistance of different peroxovanadate complexes (including the ones with Lac^-) against human catalase and the results are being presented here.

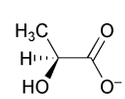
Introduction

Diabetes with its increasing incidence worldwide and many global and societal implications[1] is one of the most threatening and costly epidemics of the 21st century. Although insulin is widely used in the treatment of diabetes, the inconvenience accompanying its daily application makes orally administrable drugs desired. Furthermore, diabetes type II (insulin intolerance) cannot effectively be treated with insulin. Among other classes of compounds, vanadium complexes are of potential interest here.

The decrease in glucosuria upon treatment by sodium metavanadate was reported already in 1899.[2] By using vanadium together with hydrogen peroxide, the insulin enhancing effect is further improved due to the formation of peroxovanadates.[3,4] These discoveries boosted the research regarding the therapeutic properties of vanadium compounds[5–8] both with respect to a potential future oral drug and towards the understanding of the mechanisms of the biological actions of vanadium compounds. Speciation studies can provide key information in both fields. In the first case, different ligands must be tested to obtain a (peroxo)vanadate complex of maximum insulin enhancing potency and minimum toxicity. This, in theory, can be achieved by a compound of high selectivity and good absorption. In the second case, the ligands tested with vanadium should be molecules with which vanadium can interact in humans. These comprise among others dipeptides, representing for instance the active sites of enzymes, and low molecular mass blood serum ligands, elucidating the fate of vanadium compounds entering the blood stream.

Owing to their importance, vanadium–dipeptide systems have been extensively studied in the last two decades by ^{51}V NMR, both with[9,10] and without hydrogen peroxide.[11,12] For the quaternary systems $H^+/H_2VO_4^-/H_2O_2$/L-α-alanyl-L-histidine (Alahis)[13] and $H^+/H_2VO_4^-/H_2O_2$/L-α-alanyl-L-serine (Alaser),[14] the speciation has recently been established in 0.150 M Na(Cl) medium, which represents the ionic strength of human blood, and the results have been compared with those of other nitrogen and oxygen donor ligands.[15]

Research considering vanadium complexes with low molecular mass blood serum ligands, such as citrate and lactate, are widescale, involving both vanadium(IV) and vanadium(V). In this study, we have chosen L-(+)-lactate (Lac^-, Scheme 1) as ligand because of its biological significance and abundance. To avoid its oligomerisation, the sodium salt has been used instead of pure lactic acid, and the medium concentration has been kept constant with respect to sodium ions, letting chloride concentration vary somewhat, hence the notation Na(Cl). This way the medium concentration has been set to 0.150 M, to provide conditions similar to those in human blood. With this study, we have set double aims. The first was to test peroxovanadate–lactate complexes with respect to their stability and consequently their potential use as an oral drug in the treatment of diabetes. Lactate could be an ideal pharmaceutical candidate, owing to the fact that it is naturally occurring, hence not toxic in small scale, abundant, and cheap to produce. Secondly, this work can be considered as part of a series of speciation studies, trying to reveal vanadium interactions in human blood. Once vanadium speciation is established with all the major components in blood, including mixed ligand species, modelling can be used to determine the fate of any particular vanadium complex under physiological conditions, provided that the speciation with the given ligand in the complex is also known.



Scheme 1 L-(+)-Lactate (Lac^-).

In the literature, there are numerous speciation studies considering vanadium(V) complexation with α-hydroxycarboxylic acids, including lactic acid,[16–18] and recently the crystal structure of the main dimeric complex in this system has been solved.[19] Peroxovanadate–lactic acid complexes have been studied as well, both by NMR[20] and by crystallography.[21,22] The existence of mixed ligand complexes with lactic acid and picolinic and 6-methylpicolinic acid have been shown in the case of vanadium(IV).[23] To our knowledge, there are no studies prior to this work describing the full speciation in both the vanadium(V)–lactic acid and vanadium(V)–hydrogen peroxide–

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Table 1 Species, notation, formation constant and ^{51}V NMR chemical shifts in the $H^+/H_2VO_4^-/Lac^-$ system [0.150 M Na(Cl), 25 °C], including the H^+/Lac^- subsystem

p, *q*, *r*, *s*	Notation	logβ (3σ)	pK_a	Symbol	^{51}V NMR shift/ppm
0, 0, 0, 1	Lac^-	0		–	–
1, 0, 0, 1	Lac	3.653 (0.002)	3.653		
2, 2, 0, 2	$V_2Lac_2^{2-}$	19.68 (0.07)		**a**	−533
3, 2, 0, 2	$V_2Lac_2^{-}$ [a]	21.61 (0.27) [a]	1.93 [a]	**a**	−533
2, 3, 0, 2	$V_3Lac_2^{3-}$	22.58 (0.09)	–	**b**	−551 [central V]
	$V_3Lac_2^{3-}$			**b′**	−532
2, 4, 0, 2	$V_4Lac_2^{4-}$	24.59 (0.13)	–	**c**	−525
	$V_4Lac_2^{4-}$			**c′**	−540
−1, 1, 0, 1	$VLac^{3-}$ [b]	[b]	[b]	**d**	(< −525) [b]
0, 1, 0, 1	$VLac^{2-}$	0.88 (0.06)		**d**	−519
1, 1, 0, 1	$VLac^{-}$	6.92 (0.22)	6.04	**d**	−508
2, 1, 0, 1	VLac [a]	10.65 (0.06) [a]	(3.73) [a]	**e**	−535

[a] Not included in the final model. [b] No calculations were made on the species, its existence is concluded from ^{51}V NMR chemical shift changes in alkaline solutions (Fig. 2).

lactic acid systems over a wide pH range in 0.150 M Na(Cl) medium.

In this study, potentiometry was used together with ^{51}V NMR spectroscopy to collect data, which were evaluated by the least squares program LAKE,[24] designed to handle multi-method data simultaneously. ^{13}C NMR was used to confirm the stability of lactate in the presence of hydrogen peroxide and EPR spectroscopy to check for possible reduction of vanadium(V) to vanadium(IV). Simple biological tests with human blood have been carried out to investigate the stability of the formed peroxovanadate complexes *vs*. human catalase in comparison with other peroxovanadate–ligand systems.

Results and discussion

The subsystems

Without the accurate knowledge of the speciation in the subsystems $H^+/H_2VO_4^-$, H^+/L-(+)-lactate (Lac^-), and $H^+/H_2VO_4^-/H_2O_2$ under the same experimental conditions, complete equilibrium analyses of the ternary $H^+/H_2VO_4^-/Lac^-$ and the quaternary $H^+/H_2VO_4^-/H_2O_2/Lac^-$ systems are impossible. Formation constants in the binary $H^+/H_2VO_4^-$,[13] and the ternary $H^+/H_2VO_4^-/H_2O_2$ system,[25] have already been described. The pK_a value of lactic acid in 0.150 M Na(Cl) medium was found to be 3.653 ± 0.002 (Table 1), determined from eight automated titrations. Experimental details are given in ref. 26 and in the Experimental section.

The $H^+/H_2VO_4^-/Lac^-$ system

Besides the already known ones from the binary $H^+/H_2VO_4^-$ system, eight new resonances were observed in this system over the pH range 1.1–10.9, belonging to seven species. Complex formation is favoured only in the acidic region (Fig. 1), being very weak at the physiological pH of 7.4, and only marginal in more alkaline solutions.

As can be seen from Fig. 1, all vanadium is bound to lactate at pH < 4.5 at $[V]_{tot}$ = 10 mM and $[Lac]_{tot}$ = 15 mM. A relatively broad peak appears at about −540 ppm in the spectra of the most acidic solutions (peak **e** in Fig. 1), which disappears rapidly as the pH increases. LAKE calculations showed that the species giving rise to this resonance is an uncharged mononuclear monoligand complex, VLac. There is a change in the chemical shift of this peak, from −535 ppm at pH *ca*. 3 to −544 ppm at pH = 1.1 (Fig. 2). This can be attributable to either protonation or an exchange process, which is fast on the NMR timescale. Were the latter to apply, the chemical shift of the resulting peak should lie between the "true" chemical shifts of the two species taking part in the exchange, the exact position being dependent on the relative amount of these two species to each other. This has been verified in a series of

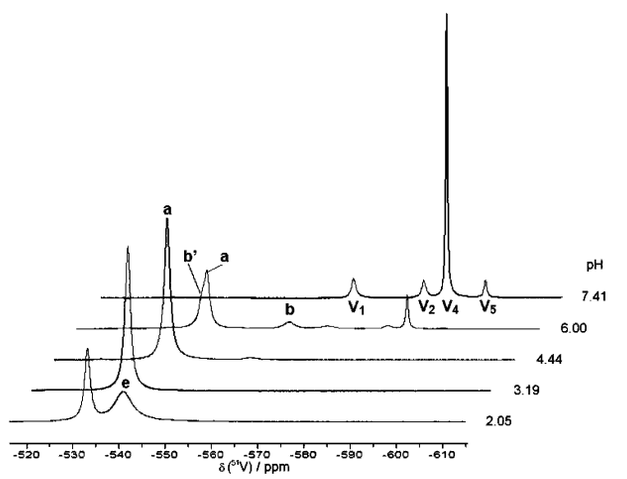

Fig. 1 Spectra over the pH range in the ternary $H^+/H_2VO_4^-/Lac^-$ system ($[V]_{tot}$ = 10 mM, $[Lac]_{tot}$ = 15 mM). Peaks **c**, **c′** and **d** are not marked, owing to their extremely small amount under the conditions applied here. Letters represent resonances of the following species: **a** $V_2Lac_2^{2-}$; **b** $V_3Lac_2^{3-}$ [central vanadium atom], **b′** $V_3Lac_2^{3-}$ [two VLac parts]; **e** VLac.

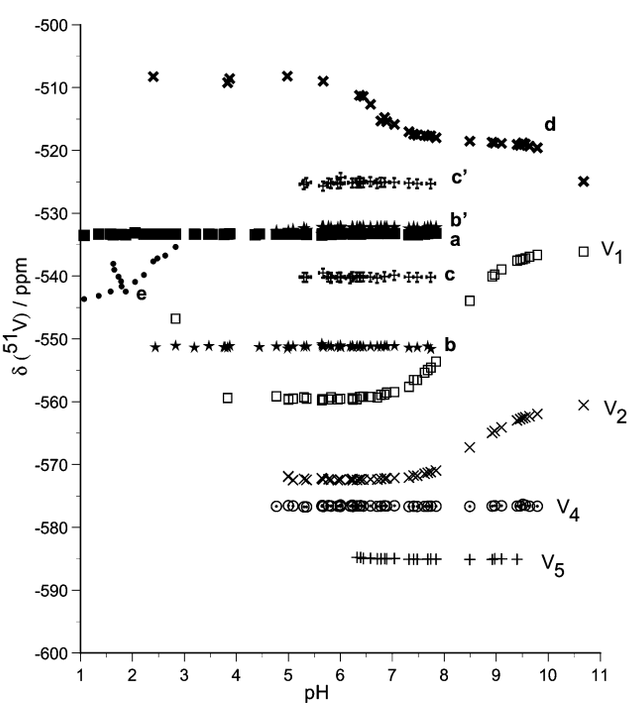

Fig. 2 ^{51}V NMR chemical shifts in the ternary $H^+/H_2VO_4^-/Lac^-$ system as a function of pH.

experiments, in which the pH and vanadium total concentration were kept constant but the amount of added lactate was increased stepwise. The higher the total lactate concentration was, the closer the peak moved to its original −535 ppm chemical shift position, hence the strange steep feature in the chemical shift curve of resonance e in Fig. 2 at pH about 2. This means that the chemical shift of the complex VLac is −535 ppm, but it is in exchange with another species, which has a chemical shift value of about −545 ppm. This latter species is dominant when pH < 2, and VLac is more pronounced between pH 2 and 3. Given the chemical shift of the species and the very acidic nature of the solutions, the species giving rise to the −545 ppm resonance is certainly VO_2^+, the chemical shift of which was found to be −544.6 ppm. In addition, this monomeric species seems to have a different structure than that of the other monomeric species in the system (peak **d** in Fig. 2). In Table 1, this structural difference is illustrated by a pK_a value put in parentheses for VLac, since the difference between VLac and $VLac^-$ is not just a simple protonation as the notation would falsely indicate, and therefore the pK_a value can be considered as a "pseudo" one.

In very acidic solutions (pH < 3), rapid reduction of vanadium(V) to vanadium(IV) was observed. This, together with the presence of initially forming tridecavandates[27] in the most acidic solutions, made a complete speciation study impossible at pH values below 3. In such acidic solutions, reduction occurs much faster than the decomposition of tridecavanadates, limiting our final equilibrium calculations to the pH region 3.1–10.9. In this region, the presence of the neutral VLac complex is negligible; hence, it is not included in the final model (see Table 1).

Problems with reduction occur even in weakly acidic solutions. Although reduction is substantially slower at elevated pH values than in the very acidic region, in weakly acidic solutions decavanadates can form, the decomposition of which can take up to 24 h. During that time, reduction can be considerable. The initial effort of minimizing reduction by keeping solutions in darkness failed. Therefore, the order of addition of the components had to be changed. Solutions were prepared in a way that neutral vanadium and lactate solutions were mixed with a $[Lac]_{tot}$ to $[V]_{tot}$ ratio higher than 1 (so that all vanadium is bound to lactate), allowed to equilibrate for approximately 2 h, then hydrochloric acid was added to set the pH. Whenever possible, cross-checks were performed by adding acidic lactate solutions to neutral vanadate samples. The results obtained were identical in both methods. Furthermore, in case reduction has occurred, the solution was excluded from all calculations.

The dominant species in the ternary $H^+/H_2VO_4^-/Lac^-$ system is without doubt the symmetrical $V_2Lac_2^{2-}$ dimer, giving rise to the −533 ppm resonance. It is present over a wide pH range, from acidic to neutral solutions, possessing a constant chemical shift (resonance **a** in Fig. 2). The stoichiometry of this complex is already known,[17,18] as well as its crystal structure.[19] We have also found that protonation occurs in very acidic solutions without changing the chemical shift, resulting in a $V_2Lac_2^-$ complex. This species, however, is not included in the final model, since its presence above pH 3 is negligible.

At pH above 5, a shoulder appears on the downfield side of the −533 ppm resonance. This shoulder was not observed by Hati *et al.*,[18] possibly owing to the 50 Hz line-broadening applied in their study. It was found, however, as a separate peak with 2-ethyl-2-hydroxybutyric acid, and therefore suspected to be present in the lactic acid case too. They assigned this resonance to a trinuclear bisligand complex, $V_3Lac_2^{3-}$, which gives rise to two peaks, one of them is the aforementioned −533 ppm resonance, and another one is at −550 ppm.[18] Our study affirmed this conclusion by finding the −532 ppm (peak **b′** in Figs. 1 and 2) resonance to be in a 2 : 1 integral ratio with the −551 ppm resonance (peak **b** in Fig. 1), and residing very close to the resonance of the symmetrical $V_2Lac_2^{2-}$ dimer at −533 ppm (peak **a** in Fig. 1), indicating very similar environments of the vanadium atoms in these two cases. Neither of the peaks shows changes in their chemical shifts, and LAKE calculations did not indicate protonation/deprotonation either. The small (1 ppm) difference between the chemical shifts reported here and those of Hati *et al.*[18] can be attributed to the fact that in their study, 1.0 M NaCl medium and HEPES buffer were used.

Apart from these species, another high nuclearity complex was found, clearly showing the preference of vanadium to form oligonuclear complexes with lactic acid, instead of mononuclear ones. This species is the $V_4Lac_2^{4-}$ tetramer, giving rise to two relatively broad peaks with an integral ratio of 1 : 1 at −525 and −540 ppm (peaks **c′** and **c** in Fig. 2), one on each side of the dominant −533 resonance (peak **a** in the figures). This complex has not been described earlier and therefore no structural data are available in the literature considering this species. Owing to its very small amount, we were unable to determine its structure.

Besides the acidic VLac species, other monomeric species were found in this ternary system as well ($VLac^{2-}$ and $VLac^-$, Table 1) being differently protonated forms of the same complex, and giving rise to a single peak that moves from −519 to −508 ppm upon protonation (peak **d** in Fig. 2). These species only occur at a low level, but exist over a wide pH range, from pH approximately 2.5 to about 10.5. Moreover, $VLac^{2-}$ is the only ternary complex found in alkaline solutions. There is an indication of further deprotonation of this species (see the curvature in the plot of its chemical shift in very alkaline solutions, Fig. 2) but this could not be firmly proved by calculations, owing to the extremely small amount of the species, hence its omission from the final model. Protonation of $VLac^{2-}$, yielding $VLac^-$, results in broadening of the resonance and loss of its integral intensity as well. Of the monomeric species, therefore, $VLac^{2-}$ is dominant. Fig. 3 shows the distribution of (a)

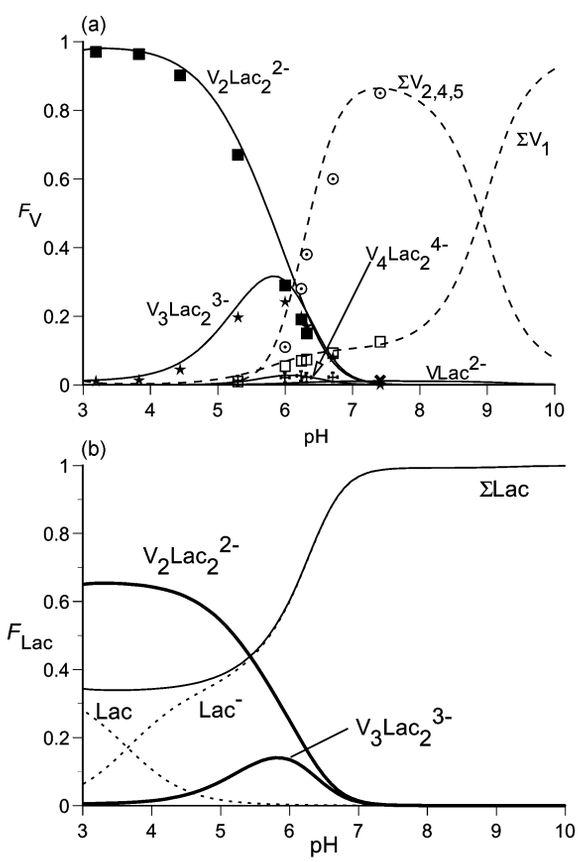

Fig. 3 (a) Distribution of vanadium at $[V]_{tot}$ = 10 mM, $[Lac]_{tot}$ = 15 mM. Symbols represent experimental points. (b) Distribution of lactate at $[V]_{tot}$ = 10 mM, $[Lac]_{tot}$ = 15 mM. The minor $V_4Lac_2^{4-}$ and $VLac^{2-}$ species are omitted for clarity.

vanadium and (b) lactate at $[V]_{tot}$ = 10 mM and $[Lac]_{tot}$ = 15 mM, *i.e.* under the same concentration conditions as in Fig. 1.

Complexation is clearly favoured at acidic pH and higher nuclearity species are dominant over the mononuclear ones under these conditions. Symbols represent experimental data points, and they provide a good fit to the calculated curves even in the case of minor complexes with sometimes overlapping peaks, demonstrating the validity of the model.

In calculations attempting to represent physiological conditions, the monomeric species are of special importance, owing to the extremely low vanadium concentrations applied. In addition, the very high excess of the ligand would favour a mononuclear complex with a V : Lac ratio equal to 2. Although we could not find any $VLac_2$ species under the experimental conditions used in this study (lowest $[V]_{tot}$ = 0.5 mM, highest $[Lac]_{tot}$ = 90 mM), their existence cannot be completely ruled out in real physiological circumstances. With all the limitations of our model in mind, we performed calculations with $[V]_{tot}$ = 1 μM and $[Lac]_{tot}$ = 20 mM in 150 mM Na(Cl) medium. The extraordinary dominance of the $V_2Lac_2^{2-}$ dimer is apparent even at such low total vanadium concentration in acidic solutions. In more neutral or alkaline solutions, binary inorganic vanadates, that is the hydrolysis products of vanadium(v), are the predominating species instead of the monomeric $VLac^{2-}$ complex, despite the 20 000-fold excess of the ligand.

The $H^+/H_2VO_4^-/H_2O_2/Lac^-$ System

There are nine new peaks arising in this system along with those of the subsystems (Fig. 4). Similarly to the ternary $H^+/H_2VO_4^-/Lac^-$ system, complexation is favoured in the acidic region. This indicates that, unlike with imidazole,[25] the presence of peroxo ligands does not enhance complex formation with lactate. Rather, all formed quaternary species can be described as one or more peroxo ligands being added to an already existing vanadate–lactate species.

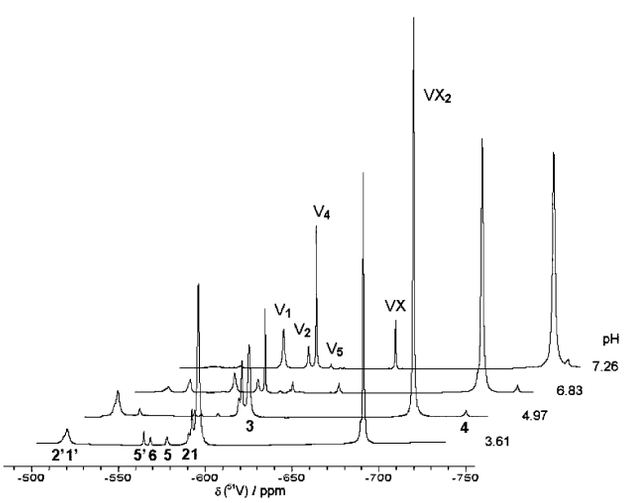

Fig. 4 Spectra over the pH range in the quaternary $H^+/H_2VO_4^-/H_2O_2/Lac^-$ system ($[V]_{tot}$ = 10 mM, $[H_2O_2]_{tot}$ = 14.9 mM, $[Lac]_{tot}$ = 20.1 mM).

The nine resonances found in this quaternary system belong to six species altogether (Table 2), and only one of them falls into the VX_2-region of the spectra (Fig. 5), otherwise dominated by inorganic diperoxovanadates (VX_2, Fig. 4). This region corresponds to species that contain one or more diperoxovanadium units, either with or without additional ligand(s). Comparing the chemical shift of peak **4** in Figs. 4 and 5 to shift values observed in other systems resulted in a preliminary assignment of the peak to a VX_2Lac complex. The existence of VX_2-ligand complexes seems to be a common feature of peroxovandate-ligand systems.[15] LAKE calculations confirmed the nuclearity of the species and revealed its 2− charge (Table 2).

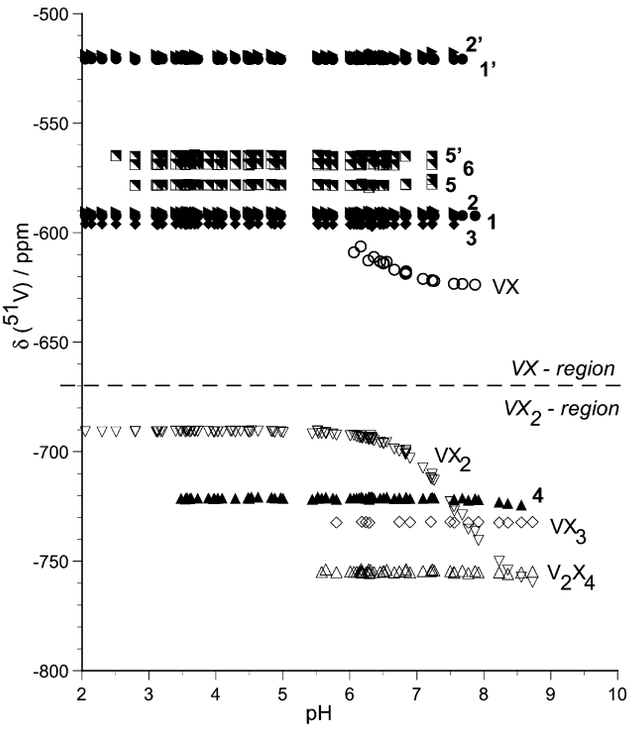

Fig. 5 ^{51}V NMR chemical shifts in the quaternary $H^+/H_2VO_4^-/H_2O_2/Lac^-$ system *vs.* pH. The chemical shifts from the ternary $H^+/H_2VO_4^-/H_2O_2$ are included for comparison, but those of the ternary $H^+/H_2VO_4^-/Lac^-$ and binary $H^+/H_2VO_4^-$ are omitted for clarity. The numbers represent resonances of the following species: **1** $V_2XLac_2^{2-}$ [VXLac part of the dimer], **1′** $V_2XLac_2^{2-}$ [VLac part of the same dimer]; **2** $*V_2XLac_2^{2-}$ [VXLac part], **2′** $*V_2XLac_2^{2-}$ [VLac part]; **3** $V_2X_2Lac_2^{2-}$; **4** VX_2Lac^{2-}; **5** $*V_2X_2Lac_2^{2-}$ [VXLac part], **5′** $*V_2X_2Lac_2^{2-}$ [VXLac part]; **6** $**V_2X_2Lac_2^{2-}$.

Although its chemical shift shows some curvature at the alkaline region, the extremely small amount of the compound at pH > 8 made calculations for a deprotonated species unreliable. The existence of such a species, however, cannot be ruled out, but its contribution can safely be neglected under the experimental conditions used in our study. There was another resonance observed in the spectra, which is excluded from the final model, being very broad and remaining extremely minor unless $[V]_{tot}$ > 20 mM and pH < 3 is applied. ‡

The main complexes in this quaternary system are denoted **1**, **2** and **3** in Figs. 4–6. Since complexes **1** and **2** give rise to two peaks each, primes were used in their notations. NMR spectra indicated that all these three complexes incorporated more than one vanadium atom, and LAKE calculations suggested dimeric species in the case of all three complexes (Table 2). Complex **3** is a symmetrical dimer, $V_2X_2Lac_2^{2-}$, as found in its crystal structure,[21,22] and hence gives rise to only one peak. Complexes **1** and **2**, on the other hand, are unsymmetrical, having the same composition of $V_2XLac_2^{2-}$ that is built up of a "VXLac" and a "VLac" part. The "VXLac" parts of the complexes (peaks **1** and **2** in Figs. 4–6) give rise to the resonances very close to that of $V_2X_2Lac_2^{2-}$ (peak **3** in Figs. 4–6) and the ternary inorganic peroxovanadate species VX^{2-}. These peaks lie in the "VX-region" of the spectra, while the other peaks (peaks **1′** and **2′** in Figs. 4–6) of the same complexes are found very close to, and sometimes even overlapping with the peaks from the ternary vanadate–lactate species. These peaks arise from the "VLac" part of the complexes. Since complex **1** and **2** have the same composition, we use asterisks to differentiate between them, in

‡ Its ^{51}V NMR chemical shift is about −502 ppm, and exists in acidic solutions only. Since high total vanadium concentration is needed for its formation, its biological importance is supposedly very limited. Therefore, no efforts were made to determine its composition, and it is omitted from Table 2 as well as from Fig. 5.

Table 2 Species, notation, formation constant and ^{51}V NMR chemical shifts in the $H^+/H_2VO_4^-/H_2O_2/Lac^-$ system [0.150 M Na(Cl), 25 °C]. Asterisks denote complexes of the same nuclearity, in order of decreasing dominance with increasing number of asterisks

p, *q*, *r*, *s*	Notation	logβ (3σ)	pK_a	Symbol	^{51}V NMR shift/ppm
2, 2, 1, 2	$V_2XLac_2^{2-}$	24.96 (0.03)	–	**1**	−592 [VXLac]
	$V_2XLac_2^{2-}$			**1′**	−521 [VLac]
2, 2, 1, 2	$*V_2XLac_2^{2-}$	24.43 (0.06)	–	**2**	−590 [VXLac]
	$*V_2XLac_2^{2-}$			**2′**	−519 [VLac]
2, 2, 2, 2	$V_2X_2Lac_2^{2-}$	29.27 (0.03)	–	**3**	−596
−1, 1, 2, 1	VX_2Lac^{3-} [a]	[a]	[a]	**4**	(< −724) [a]
0, 1, 2, 1	VX_2Lac^{2-}	11.50 (0.04)	–	**4**	−721
2, 2, 2, 2	$*V_2X_2Lac_2^{2-}$	28.20 (0.07)	–	**5**	−578 [VXLac]
	$*V_2X_2Lac_2^{2-}$			**5′**	−565 [VXLac]
2, 2, 2, 2	$**V_2X_2Lac_2^{2-}$	27.68 (0.11)	–	**6**	−569

[a] No calculations were made on the species, its existence is concluded from ^{51}V NMR chemical shift changes in alkaline solutions (Fig. 5).

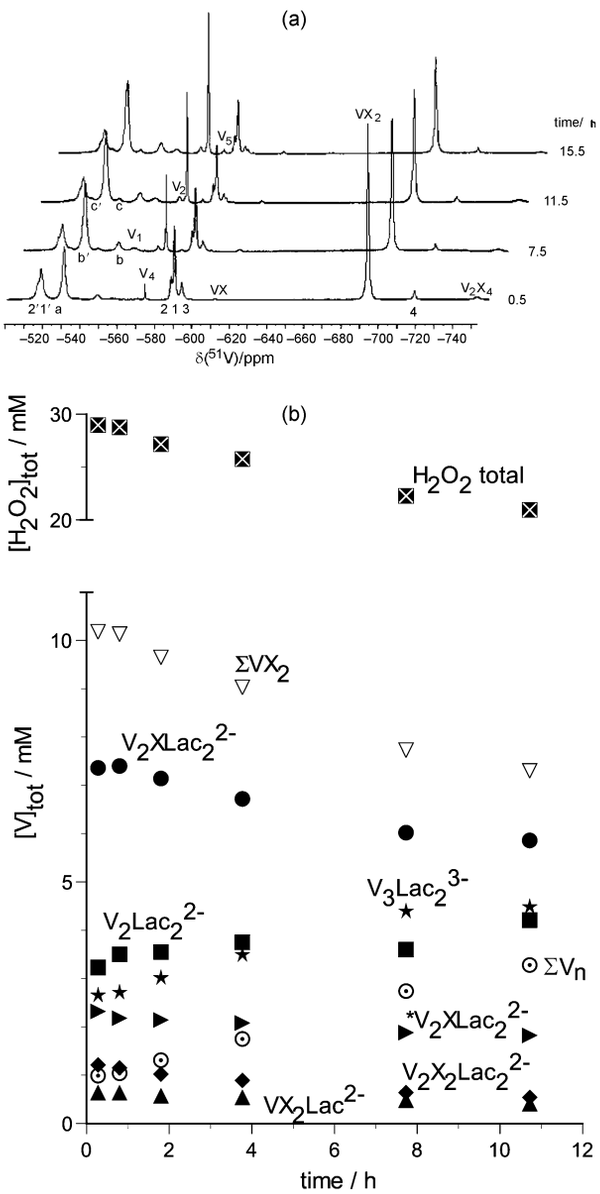

Fig. 6 Equilibrium times in the quaternary $H^+/H_2VO_4^-/H_2O_2/Lac^-$ system ($[V]_{tot}$ = 30 mM, $[H_2O_2]_{tot}$ = 30 mM, $[Lac]_{tot}$ = 60 mM) at pH = 6.5, illustrated by (a) spectra and (b) loss of H_2O_2. ΣV_n represents the sum of all inorganic binary vanadate species, the hydrolysis products of vanadium(V).

a way that the complex marked with the asterisk is the less dominant one ($V_2XLac_2^{2-}$ and $*V_2XLac_2^{2-}$ for complexes **1** and **2**, respectively). These findings, regarding the complexes $V_2XLac_2^{2-}$ and $*V_2XLac_2^{2-}$ as well as $V_2X_2Lac_2^{2-}$, are in complete agreement with the results published by Justino *et al.*[20]

Arguably, one of the most interesting features of this system is the existence of three, relatively small and sharp, distinct peaks with chemical shift values of −565, −569 and −578 ppm (peak **5′**, **6** and **5**, respectively, Figs. 4 and 5). As these three peaks always appear together with a reasonably constant integral ratio of 2 : 1 : 2, our preliminary suggestion was that they belong to one complex, which therefore has to contain at least five vanadium atoms. Initial LAKE calculations favoured a $V_5X_4Lac_5^{4-}$ composition of all possible pentameric nuclearities. The results of these calculations, however, were far from being convincing, especially when it came to predictions of speciation at high vanadium total concentrations. With carefully planned experiments, we could observe changes in the ratio between the two flanking peaks and the peak in the centre. Ratios between the peaks **5** and **5′** were found to be 1 : 1 within the experimental error range. This indicated that both peak **5** and **5′** belong to one species, while peak **6** belongs to another. These two species exist over the same pH range and are always minor compared to $V_2X_2Lac_2^{2-}$, $V_2XLac_2^{2-}$ and $*V_2XLac_2^{2-}$. LAKE calculations showed them to have the same composition as the aforementioned major dimeric compound $V_2X_2Lac_2^{2-}$, *i.e.* peaks **5** and **5′** belong to an asymmetrical $*V_2X_2Lac_2^{2-}$ compound, which probably consist of two non-identical "VXLac" parts, while peak **6** belongs to a symmetrical $**V_2X_2Lac_2^{2-}$ species containing two identical "VXLac" parts (Table 2). Again, asterisks denote compounds of the same composition in decreasing order of dominance with increasing number of asterisks. The results are of much better quality (much lower 3σ values) compared to the ones in the case of the pentameric compound, and predictions made by this model were found to be convincingly reliable.

The loss of hydrogen peroxide was found to be negligible in neutral and alkaline solutions over several hours, while severe decomposition of hydrogen peroxide occurred in acidic solutions within an hour. Equilibrium was attained within 15 min, and at pH about 6.5, the loss of peroxide is only marginal within the first hour (Fig. 6(a) and (b)). This, however, requires a certain order of addition of the components. If vanadate and lactate are first mixed, and then hydrogen peroxide is added to the equilibrated solution, very rapid decomposition of hydrogen peroxide occurs in acidic solutions. This is probably owing to the reduction of vanadium(V) to vanadium(IV) by lactate, as the generated vanadium(IV) may catalyse peroxide decomposition. Another problem with equilibration emerges in the pH range 4–7, where the decomposition of initially formed decavanadates (see above) takes up to 24 h, during which time substantial loss of peroxide can occur. The straightforward solution of the problem is the addition of hydrogen peroxide to vanadate solutions first, then lactate and finally acid or base to set the desired pH value. This method was used in all preparations of the solutions, but whenever possible, cross-checks were performed with different order of addition. They provided the same result.

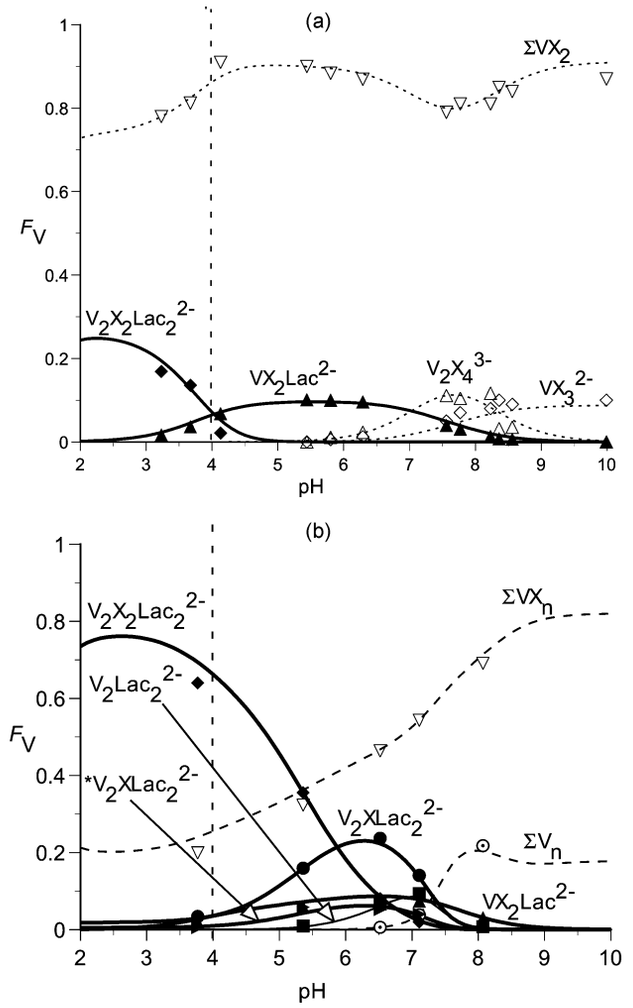

Fig. 7 (a) Distribution of vanadium at $[V]_{tot}$ = 9.1 mM, $[H_2O_2]_{tot}$ = 21.8 mM, $[Lac]_{tot}$ = 68.2 mM. (b) Distribution of vanadium at $[V]_{tot}$ = 15 mM, $[H_2O_2]_{tot}$ = 20 mM, $[Lac]_{tot}$ = 135 mM. Symbols represent experimental points in both cases. ΣVX_n stands for the sum of all inorganic peroxovanadates and ΣV_n for the sum of all inorganic binary vanadate species, the hydrolysis products of vanadium(v). The lower pH limit of the calculations is indicated by dashed lines at pH = 4.

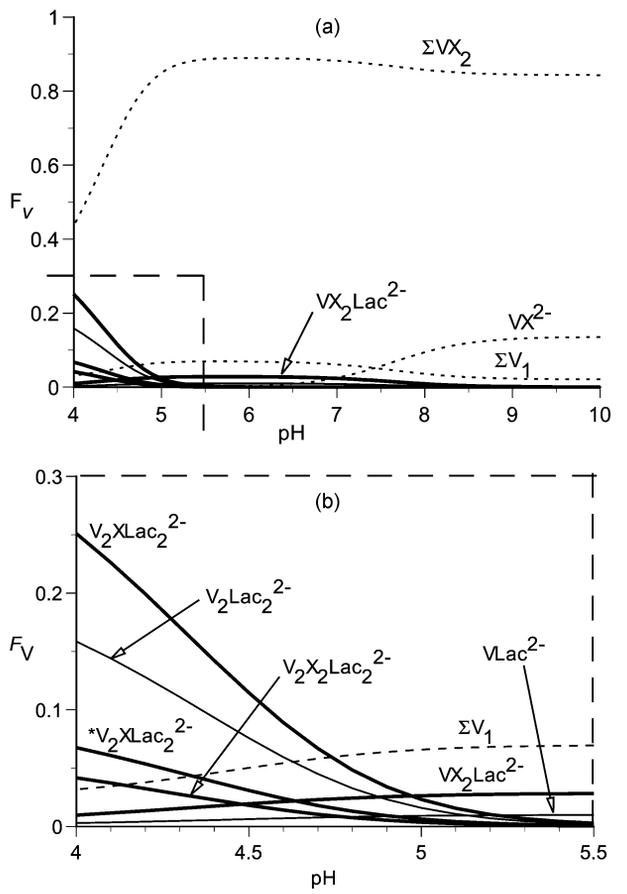

Fig. 8 Distribution of vanadium at $[V]_{tot}$ = 0.001 mM, $[H_2O_2]_{tot}$ = 0.01 mM, $[Lac]_{tot}$ = 20.0 mM, representing close to physiological ratios.

Fig. 7(a) and (b) illustrate how well the model (solid and dashed lines) describes the experimental data obtained by ^{51}V NMR (symbols), and demonstrates some of the key features of this system. As can be seen, the fit of the data points to the calculated curves is very good, even for minor species. It should be pointed out here again that quaternary peroxovandate–lactate species are relatively weak. Even if more than a seven-fold excess of lactate to vanadate is applied (Fig. 7(a)), most of the vanadium is bound in ternary inorganic peroxovanadates, predominantly in VX_2 species. To increase the amount of quaternary species, excess of lactate, and $[H_2O_2]_{tot}/[V]_{tot} < 2$ are needed (Fig. 7(b)). In this case, however, ternary vanadate–lactate ($V_2Lac_2^{2-}$) and inorganic vanadate species (denoted as ΣV_n) are present as well, and at pH values higher than 5.5, inorganic peroxovanadate species (ΣVX_n) become dominant again.

This feature is even more pronounced when modelling physiological conditions (Fig. 8(a) and (b)). Here, very low vanadium total concentration (1 μM for modelling, as the average physiological concentration of vanadium is 0.2 μM) is used with a 10-fold excess of hydrogen peroxide and an enormous, 20000-fold excess of lactate. Without doubt, the complexation of lactate to vanadate in the presence of excess H_2O_2 is favoured in the acidic region, and the presence of quaternary peroxovanadate–lactate complexes is almost negligible at the physiological pH of blood (7.4). Again, VX_2 species dominate over the whole pH range used in the model. Under these conditions, higher nuclearity (dimeric) species are minor, as expected. What is surprising is the fact that ternary vanadate–lactate species ($V_2Lac_2^{2-}$ and $VLac^{2-}$) are present although a ten-fold excess of hydrogen peroxide to vanadate is applied. The limitations of the modelling, however, should always be kept in mind. The validity of our speciation under these extreme conditions cannot be tested experimentally. Although the mononuclear species were found to be very minor in our study and we have not found any monovanadium–bislactate complexes, their existence or even dominance over the found dimeric or monovanadium–monolactate species cannot be ruled out under such extreme low $[V]_{tot}$ and excess of ligand as used in the model. According to our findings, however, lactate does not seem to be efficient enough to bind vanadate in human blood, despite its large excess. It is probably not a good candidate for a possible oral drug either, as it is likely to lose vanadium, which is then transported independently from lactate, by other naturally occurring carrier ligands in blood. Nonetheless, the knowledge of vanadium speciation in the presence of lactate is useful if not essential for comparison with other ligands and for understanding the fate of vanadium in human blood. In case of a future drug candidate ligand, speciation studies can be useful to elucidate possible interactions any particular vanadium compound can face when entering the blood stream.

Stability tests against catalase in human blood samples

Some simple tests were done to check the stability of the complexes against human catalase and compare them to other systems with different ligands. Solutions were prepared at pH about 7.4, with $[V]_{tot}$ = 20 mM, $[H_2O_2]_{tot}$ = 40 mM and 40 mM of the following ligands: imidazole, picolinic acid, L-α-alanyl-L-histidine, L-α-alanyl-L-serine, lactic acid, citric acid and in one case without any ligand, to include the inorganic

peroxovanadates for comparison. 2 ml of each solution was placed into an NMR tube, and its spectrum recorded. Then a few drops of freshly taken human blood were added. Spectra were recorded after different time intervals to monitor the changes and to follow the loss of peroxide over time. The purpose of the experiment was to investigate whether the enzyme catalase, which is responsible for the decomposition of hydrogen peroxide in human blood, would recognize the peroxo ligand bound to vanadium as peroxide or not. In other words: would the introduced peroxovanadate complexes reach their target, or would they decompose and thereby make the nature of the original compound irrelevant with respect to their actual insulin enhancing effect.

The results are shown in Fig. 9 and indicate that peroxovanadates "packed" into different complexes with various organic molecules are accessible to different extents for catalase. Therefore, the original form of the introduced vanadate compound seems to play an important role in its fate in human blood and in determining its final effectiveness. Fig. 9 shows that peroxovanadates "wrapped" into complexes with an imidazole moiety, such as in L-α-alanyl-L-histidine or imidazole itself, resist catalase the best. Considering the simplicity of the tests, the results with these two ligands are well within the experimental error. Picolinic acid species seem to be more vulnerable, which is an unexpected result considering the very strong and stable peroxovanadate–picolinate complexes formed in this system. Removing the imidazole part of the molecule will affect the results substantially, as is the case with L-α-alanyl-L-serine. This dipeptide does not contain any aromatic nitrogen donor atoms, and the system resists catalase to less extent. Even weaker resistance was found with lactate and citrate. These two ligands behave very similarly, and form only weak complexes at physiological pH. From the speciation studies performed with these two simple carboxylic acid ligands, most vanadium should exist in the form of inorganic peroxovanadates under the conditions present in the tests. However, their behaviour is slightly different from that of the pure inorganic peroxovanadate system, which has the weakest resistance towards hydrogen peroxide of all the investigated systems, although its rate of decomposition by catalase is still approximately two orders of magnitude slower than that of pure hydrogen peroxide (without vanadate) according to earlier studies.[28] These simple tests demonstrated that speciation alone cannot predict completely the fate of vanadium compounds in humans, but is able to give very useful guidelines. The stability of the compounds against human catalase for instance, is in very good accordance with the stability of complexes formed in these systems[15] with the exception of picolinic acid, which seems to form more "vulnerable" complexes than could have been concluded from its speciation only.

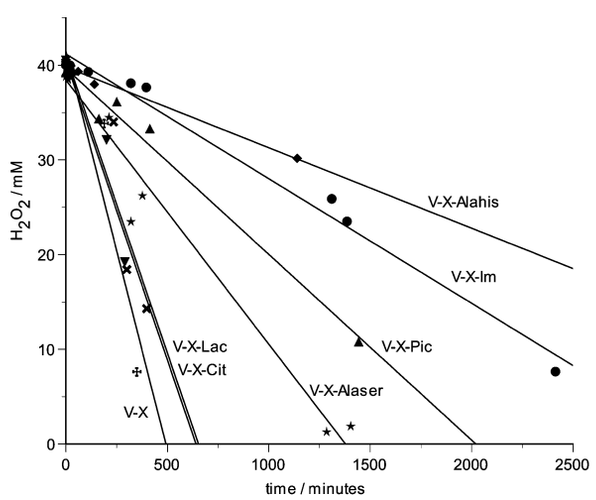

Fig. 9 The resistance of different peroxovanadate complexes against human catalase in fresh blood samples, as determined by plotting the H_2O_2 concentration in the solutions as a function of time. Inorganic peroxovanadates (V–X) are included for comparison. Abbreviations: Lac = L-(+)-lactic acid, Alahis = L-α-alanyl-L-histidine, Alaser = L-α-alanyl-L-serine, Im = imidazole, Pic = picolinic acid, Cit = citric acid. X represents the peroxo ligand in all cases.

Concluding remarks

The complete speciation has been established in the ternary $H^+/H_2VO_4^-$/L-(+)-lactate (Lac^-) and quaternary $H^+/H_2VO_4^-/H_2O_2/Lac^-$ systems in the physiological medium of 0.150 M Na(Cl) at 25 °C in the pH region 3–10 and 4–10, respectively, by potentiometry and ^{51}V NMR spectroscopy. In the ternary system altogether seven vanadate–lactate species were found, two of which are not included in the final model, because their existence is restricted to very acidic conditions (Table 1). Predominantly, higher nuclearity complexes are formed and complexation is favoured at acidic pH values. The exception is the monomeric species, $VLac^{2-}$, which is practically the only one present at the physiological pH of 7.4. These are such minor species, however, that even at extremely low total vanadium concentrations, the dimeric species $V_2Lac_2^{2-}$ is predicted to be more dominant in pH regions where they can coexist. In the quaternary system, six additional peroxovanadate–lactate species were identified (Table 2). Similarly to the ternary system, acidic conditions are favoured for complex formation, and higher nuclearity (dimeric) species were found to be predominant. Isomerism is frequent, and complexation is not enhanced by hydrogen peroxide. Instead, species are proposed to build up mainly by the addition of one or more peroxo units to the already existing vanadate–lactate complexes. When there is no H_2O_2 present, reduction is fast and substantial in acidic solutions, and negligible in neutral or alkaline solutions over the time needed for equilibration. Equilibrium is normally reached within 20 min; longer times are needed for the decomposition of originally formed decavanadates in the pH region 4–7. Over this time, however, reduction can be substantial, thus, the addition sequence of the components was found to be very important in these systems. Modelling physiological conditions with respect to the actual concentrations of vanadate, lactate and H^+, and simple biological tests suggest that lactate is probably not the ideal ligand for possible future vanadium containing oral drugs. Lactate, however, can be used as an additional component together with other ligands, as the formation of mixed ligand complexes is very likely to happen under certain circumstances, both with vanadium(V) and vanadium(IV),[23] or as a filling compound in drugs. In addition, weak complexation at physiological pH even with an extreme excess of lactate suggests that vanadium is transported in human blood by other means (probably by albumin or even more so by transferrin[29]) and not by lactate.

Experimental

Chemicals and analyses

Sodium L-(+)-lactate, $CH_3CH(OH)CO_2Na$ (Aldrich), has been used without further purification. To make sure there are no interactions between lactate and hydrogen peroxide, ^{13}C NMR spectra of 300 mM lactate (Lac^-) solution were recorded before and 69 h after the addition of peroxide (580 mM) with no changes observed. All other chemicals were used as described in ref. 13.

Notation

The equilibria studied are written with the components H^+, $H_2VO_4^-$, H_2O_2 and Lac^-. Thus, complexes are formed according to eqn. (1):

$$p\,H^+ + q\,H_2VO_4^- + r\,H_2O_2 + s\,Lac^- \rightleftharpoons (H^+)_p(H_2VO_4^-)_q(H_2O_2)_r(Lac^-)_s^{p-(q+s)} \quad (1)$$

Formation constants are denoted $\beta_{p,q,r,s}$, and complexes are given the notation (p, q, r, s) or $V_qX_rLac_s^{n-}$. X is used instead of the peroxo ligand to shorten the formulae. The total concentrations of vanadate, hydrogen peroxide and sodium L-(+)-lactate are denoted $[V]_{tot}$, $[H_2O_2]_{tot}$ and $[Lac]_{tot}$, respectively, and are given in mM in the figures.

Equilibration of the solutions

In the ternary $H^+/H_2VO_4^-/Lac^-$ system, solutions in general were allowed to stand approximately 2 h for equilibration before the measurements. In order to avoid the problems with the slowly decomposing decavanadates in weakly acidic solutions, lactate was added to vanadate solutions at neutral pH at $[Lac]_{tot}/[V]_{tot} > 1$, and the acid was added last to set the pH. Severe reduction during the 2 h of equilibration was observed only in solutions below pH 2.5. For calculations, NMR integral data have been used only above pH 3.1.

In the quaternary $H^+/H_2VO_4^-/H_2O_2/Lac^-$ system, the order of addition of the components was found to be very important. The general procedure would be to add hydrogen peroxide to the already equilibrated solutions with vanadate and lactate. This, however, was found to be reliable only in the neutral–alkaline pH range, where the reduction of vanadium(V) by lactate was negligible. In the acidic range, on the other hand, lactate reduces vanadium more rapidly, and the formed traces of vanadium(IV) seemed to decompose hydrogen peroxide very effectively, probably owing to catalytic processes. The straightforward method of adding lactate last to equilibrated peroxovanadate solutions has its own limitations as well. It is suitable only for solutions where the peroxide to vanadium ratio ≥2, otherwise in weakly acidic solutions decavanadates form, and their decomposition takes up to 24 h, during which time the peroxide loss is substantial, being even more so towards more acidic pH values. Therefore, samples in general were prepared in a neutral pH range, adding hydrogen peroxide to vanadate solutions, and adding lactate as the last component in the form of either an acidic or an alkaline solution to adjust the pH therewith, or adding the acid/base after the addition of lactate. When possible, cross-checks with different addition sequences were performed as well, confirming the established equilibrium.

The formation of the quaternary peroxo species is fast, requiring about 20 min for complete equilibrium. The decomposition of hydrogen peroxide was found to be substantial after 2 h only in acidic solutions. Therefore, solutions in general were allowed to equilibrate for 30–40 min before measurements. Owing to the rapid decomposition of peroxide in very acidic solutions, ^{51}V NMR integral data were used for calculations only in the pH range 4–10. Although the loss of peroxide was only marginal in this pH range, it was taken into account.

Potentiometric measurements

The EMF measurements in the binary H^+/Lac^- system have been performed as potentiometric titrations in 0.150 mM Na(Cl) medium at 25 °C with an automated potentiometric titrator as described in ref. 26. For the ternary $H^+/H_2VO_4^-/Lac^-$ and quaternary $H^+/H_2VO_4^-/H_2O_2/Lac^-$ systems, pH was measured as described in ref. 13.

NMR measurements

Spectra were recorded on a Bruker AMX500 spectrometer as described in refs. 13 and 25. ^{51}V NMR chemical shifts are given in ppm relative to $VOCl_3$.

EPR measurements

Spectra were recorded as described in ref. 30. No EPR data have been included in the calculations. EPR was used only to check for reduction of vanadium(V) to vanadium(IV), and no qualitative or quantitavie analysis of the spectra were performed.

Potentiometric data

The acidity constant for Lac^- has been determined from eight titrations with a total of 155 points. The pH range covered was 1.98–7.16 and the total concentration range $7.5 < [Lac]_{tot}/mM < 30$. Owing to the complexity of the ternary $H^+/H_2VO_4^-/Lac^-$ and the quaternary $H^+/H_2VO_4^-/H_2O_2/Lac^-$ systems and to the fast decomposition of hydrogen peroxide in acidic solutions, no titrations have been performed in these systems. Potentiometric data for them have been obtained by pH measurements with a combination electrode in each solution for which the NMR spectrum has been recorded ("point" solution).

NMR data

In the ternary $H^+/H_2VO_4^-/Lac^-$ system, 88 spectra and 2 serial measurements were recorded in the ranges $1.1 < pH < 10.9$, $0.5 < [V]_{tot}/mM < 40$ and $0.5 < [Lac]_{tot}/mM < 90$. In the quaternary $H^+/H_2VO_4^-/H_2O_2/Lac^-$ system, a total of 128 spectra and 6 serial measurements were recorded in the ranges $2.1 < pH < 10.0$, $5 < [V]_{tot}/mM < 40$, $5 < [H_2O_2]_{tot}/mM < 60$ and $5 < [Lac]_{tot}/mM < 135$. In the calculations, altogether 72 spectra were used, owing to limitations in pH, required equilibrium time, and reduction during the measurements.

Calculations

Data have been evaluated with the least squares program LAKE[24] as described in ref. 26. The LAKE program is capable of handling multimethod data simultaneously, which in the case of the present work means potentiometric and quantitative ^{51}V NMR integral data. Calculation and plotting of distribution diagrams were performed using WINSGW,[31] a program package based on the SOLGASWATER algorithm.[32]

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