Chapter 24

Vanadium Schiff Base Complexes: Chemistry, Properties, and Concerns about Possible Therapeutic Applications

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We discuss aspects related to the speciation of vanadium compounds (VCs) with salen-type ligands, as well as $V^{IV}O(acac)_2$ and $V^{IV}O(phen)_2(SO_4)$, namely the stability of their V^{IV} and V^{V} complexes in aqueous aerobic solutions at pH~7, and consequences on the study of toxicity, insulin mimetic and nuclease activity studies. We show that in these aerobic aqueous solutions V^{IV} Schiff-base (SB) complexes of the salen-type are normally not stable to oxidation to V^{V} and to hydrolysis of the ligand. $V^{IV}O(phen)_2(SO_4)$ and $V^{IV}O(acac)_2$ and are also not stable to oxidation, and a significant decomposition of these complexes occurs within the first 30 minutes after their dissolution. Therefore, when these VCs are used for *in vitro* or *in vivo* studies the active species is not known. Reduction of the salen SB to give amine compounds yields salan ligands which form much more stable complexes than the parent SB. When dissolved in non-degassed aqueous solutions at pH~7 the V^{IV}-salan compounds oxidise to V^{V}-salan complexes, but no hydrolysis is detected. At least with the cell lines tested these VCs are not toxic possibly because they do not enter the cells

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significantly. We emphasize that to understand if the VCs enter the cells or not is an important point to sort out in insulin-mimetic studies. In fact we also report some studies of nuclease activity of several salen and salan VCs, as well as of $V^{IV}O(acac)_2$ and $V^{IV}O(phen)_2(SO_4)$ with plasmid DNA. Many of these VCs show nuclease activity even in the absence of activating agents, so toxicity resulting from this may occur. We also study several parameters relevant for the nuclease activity of VCs, namely the nature and concentration of the buffer used.

Introduction

The presence of vanadium in biological systems and its insulin-enhancing action (*1*) and anticancer activity (*2*) has driven a considerable amount of research. Particular interest has been given to the study of the potential benefits of VCs as oral insulin substitutes for the treatment of diabetes. Coordinated ligands are said to be able to improve the absorption of vanadium, reducing the dose necessary for producing equivalent effects. However, the molecular mechanisms by which the VCs exert their insulin enhancing effects have not been clarified. In fact, in *in vitro* studies the nature of the vanadium species acting is often not known, and additionally in *in vivo* studies the role of serum proteins (e.g. albumin, transferrin) and that of insulin, which may act synergistically, is also not yet understood.

Several VCs with the tetradentate SB salen-type ligands have been proposed for use as insulin enhancing agents. The ability of $V^{IV}O$(salen) to reverse the hyperglycemic condition of alloxan-induced diabetic rats to near normal has been reported (*3*). However, the rats tended to become hypoglycemic, and withdrawal of treatment brought an immediate return to hyperglycemia.

To evaluate the use of a particular VC for oral treatment it must be understood:

1. How efficiently and in what form is the compound absorbed in the gastro-intestinal tract? The evaluation of the lipophilic-hydrophilic balance, and its speciation as a function of pH are among the important aspects to clarify.
2. How is vanadium transported in the bloodstream? The understanding of the binding of vanadium or the VC to serum proteins (e.g. serum albumin and transferrin) is important. Inorganic V^{IV} possibly binds stronger to transferrin, but other VCs may form protein-VC ternary complexes with albumin, and this preference may be changed.
3. How is vanadium delivered to cells? Protein-VC ternary or quaternary complexes may be more efficient in this respect than simple transport of inorganic V^{IV} or V^{V}.

4. Mechanism of action of the VC? For example in *in vitro* studies with 3T3-L1 adipocytes, $VO(acac)_2$ has been reported to exert its action by directly potentiating the tyrosine phosphorylation of the insulin receptor (*4*).
5. To exert its insulin-enhancing properties is it necessary or not that the VC enters into the cells? If it enters the cells then its toxic effects must be evaluated, and taken into account in order to use non-toxic effective doses. The possibility of its interaction with DNA should also be evaluated.

In the present work we mainly discuss aspects related to points 1 and 5. The complexes studied are: $Cs_2[V^{IV}O(SO_3\text{-sal})en]$ **1**, $V^{IV}O(salan)$ **2**, $V^{IV}O(pyren)$ **3**, $V^{IV}O(pyran)$ **4**, $Na[V^{IV}O(salDPA)]$ **5**, $V^{IV}O(NEt_3\text{-sal})en$ **6**, $V^{IV}O(NEt_3\text{-sal})chen$ **7**, $V^{IV}O(pOH\text{-sal})en$ **8**, $V^{IV}O(acac)_2$ **9** and $V^{IV}O(phen)_2(SO_4)$ **10**. Figure 1 shows the structure of some of the ligands.

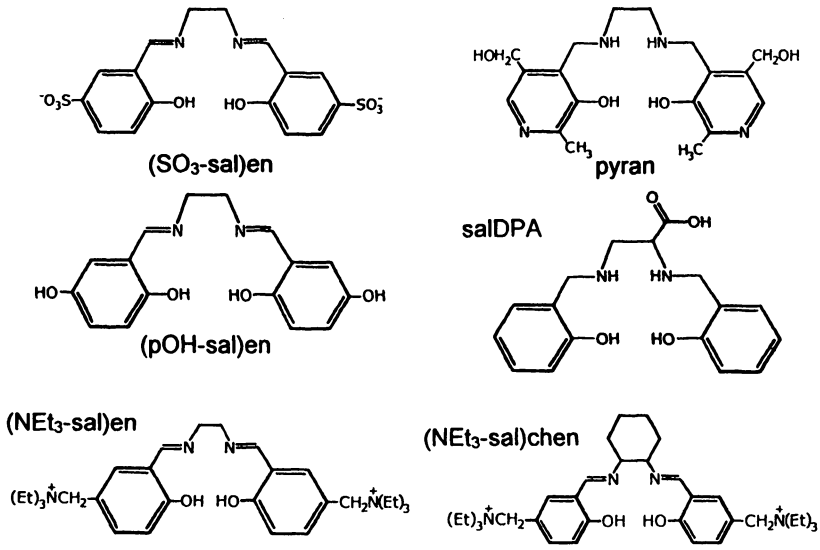

Figure 1. Molecular structures of some of the ligands of the VCs studied.

The stability of several of their V^{IV} and V^{V} complexes in aqueous solutions at pH ~7, and consequences on the study of toxicity, insulin mimetic and nuclease activity studies are discussed.

Results and Discussion

Salen SB V^{IV}-complexes, with general molecular formula shown in Figure 2, have the disadvantage of often not being soluble in water. Once in solution, there are often problems of oxidation to V^{V} and/or hydrolysis of the complex and/or of the SB ligand.

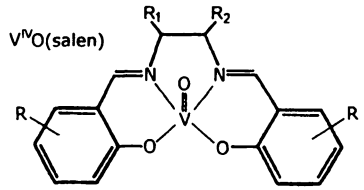

Figure 2. General molecular formula of salen-type oxovanadium(IV) complexes. One of the possible isomers is represented.

For example, the SB obtained by the condensation of pyridoxal and ethylenediamine (pyren) is moderately soluble in water and the complex formation with $V^{IV}O^{2+}$ could be studied in anaerobic conditions (speciation in Figure 3). $V^{IV}O$-complexes with pyren acting as a tetradentate ligand form for pH > ca. 4, but for pH > 5 the pyren ligand hydrolyses. In aerobic conditions the V^{IV} also oxidizes. It is clear that in *in vitro* or in *in vivo* studies the VO-pyren complexes, with pyren acting as a tetradentate ligand, will never be the predominant species present. We anticipate that this conclusion may be extrapolated for most $V^{IV}O$-salen systems.

This instability can often be overcome by reduction of the SB to give an amine compound (hereafter designated by salan or pyran, depending on the aromatic aldehyde involved). This presents interesting possibilities, as salan ligands will be more flexible and not restrained to remain planar when coordinated. We have previously reported the preparation of several new salen- and salan-type compounds, and of their V^{IV}- and V^{V}-complexes, namely compounds derived either from pyridoxal (pyr), salicylaldehyde or from salicylaldehyde-5-sulphonate (SO_3-sal) with ethylenediamine (*5,6*). The salen-type ligands and their VCs prepared using pyr and SO_3-sal are moderately soluble in water; therefore they may be particularly useful for therapeutic use.

All salan ligands proved to be efficient binders of V^{IV} and V^{V}, namely pyran, salDPA and (SO_3-sal)an. We prepared several $V^{IV}O$- and $V^{V}O_2$-pyran complexes and studied their properties (*5,6*). The solution speciation revealed that pyran formed much more stable complexes with both $V^{IV}O^{2+}$ and $V^{V}O_2^{+}$ than the corresponding SB (*5*). Either with pyran or with salDPA, in 1:1 M:L solutions, at pH 7, ~100% of V^{IV} or V^{V} are in the form of 1:1 complexes and no free vanadium is detected (see for example in Figure 4 speciation diagrams for the V-salDPA systems).

Stability of V-salen and V-salan Type Complexes in Aqueous Solution

From what was mentioned above about Figure 3 it is clear that at pH ~7 the SB complexes such as the $V^{IV}O$(salen) are not the predominant vanadium

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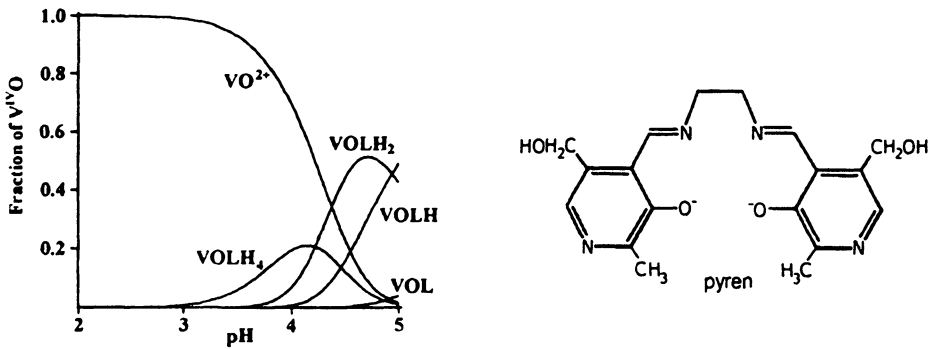

Figure 3. Speciation in the $V^{IV}O$-pyren system for C_V=2 mM and L:M = 2, calculated using data from (5). The ligand is L = pyren^{2-}. The $VOLH_2$ species corresponds to $V^{IV}O$-complexes protonated at the pyridine N-atoms.

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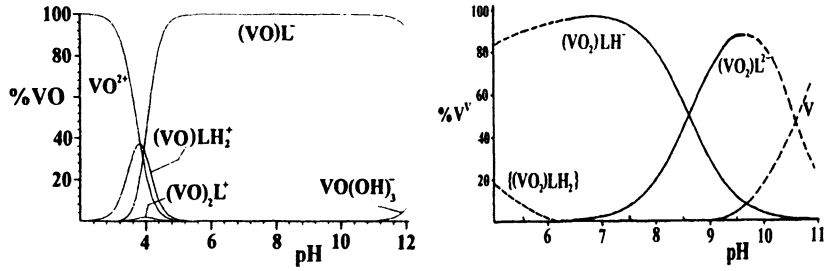

Figure 4. Speciation in the systems (A) $V^{IV}O$-salDPA (C_V = 1 mM, anaerobic conditions) and (B) $V^{V}O_2$-salDPA (C_V = 3 mM), for a L:M ratio of 1, calculated using data from (7); [L = salDPA^{3-}]. At pH = 7, ~100% of V is in the form of V-ligand complexes.

species in aqueous solution. Moreover, we measured UV-Vis spectra with time of 100 μM aqueous solutions of [V^{IV}O(pOH-sal)en] **8** at pH 7.0 in several buffers: phosphate, TRIS and HEPES (all 10mM, non-degassed and with stirring). Figure 5 shows the results for the phosphate buffer; those with TRIS and HEPES buffers are almost identical. It is clear that after 2 h the nature of the V species in solution changed very significantly, the V^{IV} partly oxidized to V^{V} and the SB ligand hydrolysed. After 24h the vanadium speciation is totally different from that immediately after dissolving the complex. If similar conditions are used in *in vitro* studies with cellular systems, and they often are, not much more than the overall effect observed can be stated, and any proposal for the mechanism of action of the V^{IV}O(salen) complexes on the cells under study will be mostly speculation.

With the V-salan complexes, their V^{IV}- and V^{V}-stability constants are much higher. The oxidation of the V^{IV}-centre may occur in aerobic conditions at pH~7, but only the corresponding V^{V}-complexes form, and in the final solution no detectable amounts of free vanadates are present, even in cell culture medium, as was shown by ^{51}V NMR studies [e.g. see refs. (*5-8*) and Figure 6B].

Several other types of VCs also oxidize/decompose slowly in solution. For example, in aqueous aerobic solutions at pH~7.4, the V^{IV} of $V^{IV}O(acac)_2$ **9** and $V^{IV}O(phen)_2(SO_4)$ **10** oxidizes (**9** slower than **10**), but with **9**, the only V^{V} product detected by ^{51}V NMR after ~4h is the monovanadate (V1) - Figure 6A.

Visible spectra (400-1000 nm) of non-degassed aqueous solutions of **9** simulating cell incubation (containing 132 mM NaCl, 4 mM KCl, 1.2 mM NaH_2PO_4, 1.4 mM $MgCl_2$, 6mM glucose, 10 mM HEPES, at pH~7.4) also change with time. After a small initial increase in absorption (up to ~35 min.), the absorption decreases ca. 25% after 4 h, and ~60% after 25h. At least part of the decrease in the absorption in the visible range is due to V^{IV} oxidation, the intensity of the EPR spectra also decreases, but no V^{V} complex was detected in

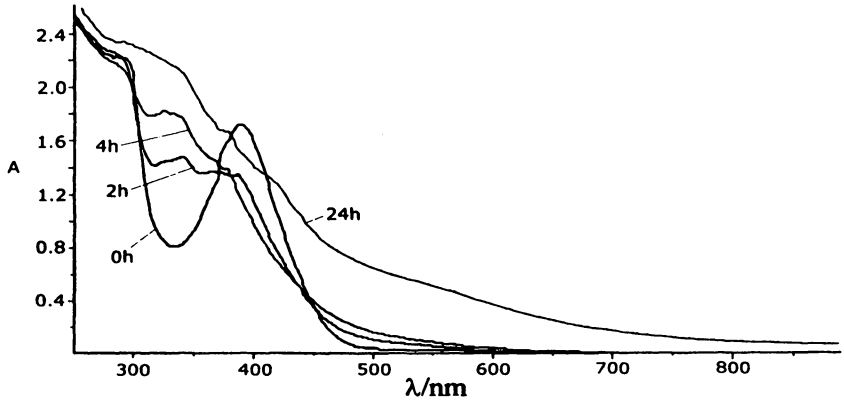

*Figure 5. UV-Vis spectra measured at different time intervals of 100 μM aqueous aerobic solutions of [$V^{IV}O$(pOH-sal)en] **8** at pH 7.0 in 10 mM phosphate buffer. t=0 h corresponds to the moment of the addition of the complex dissolved in DMSO to the buffer (DMSO: ~4%, buffer ~96%).*

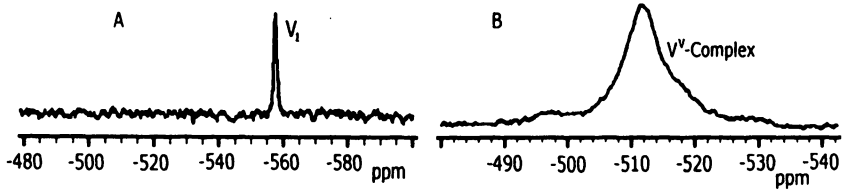

*Figure 6. ^{51}V NMR spectra of the oxidation products of ~3 mM of (A) $V^{IV}O(acac)_2$ **9** and of (B) Na[$V^{IV}O$(salDPA)] **5** at pH~7.4 in an aqueous aerobic solution containing the Dulbecco's Modified Eagle's Medium – High Glucose.*

the corresponding ^{51}V NMR spectra (Figure 6A). Similar results were obtained with $V^{IV}O(phen)_2(SO_4)$.

While no significant difference in the UV spectra of $V^{IV}O$(pOHsal)en in different buffers were found (see above), distinct changes with time in the UV spectra of 100 μM $VO(acac)_2$ solutions were detected, at least comparing the phosphate and the TRIS buffers. The intensity of the UV spectra decreased faster in TRIS buffer aerobic solutions.

Toxicity Tests

Some of the V^{IV}-complexes synthesized, namely **1-4**, have been tested *in vitro* for their toxicity and insulin-mimetic behaviour using transformed mice fibroblasts (*1*). Most of these complexes are toxic at C_V=1mM [except VO(pyran)], and negligibly toxic or non-toxic at C_V=0.01mM and below. $V^{IV}O$(pyran) showed no toxicity even after 36h of incubation with the fibroblasts in a 1 mM concentration. However, in aqueous aerobic solution these complexes either undergo hydrolysis and/or the V^{IV} oxidizes to V^V; therefore the studies do not really evaluate the toxicity of each of the $V^{IV}O$-complexes **1-4**, but of the mixture of V-species formed in the medium. For these four compounds, only in the case of $V^{IV}O$(pyran) we know that the complex does not hydrolyse, but forms V^VO_2(pyran) upon oxidation (*5*). The question raised here on the real composition of the incubating solutions is normally not considered or taken into account but is certainly relevant in most cases.

Viability tests were also made with Na_3VO_4 and Na[$V^{IV}O$(salDPA)] **5** with tumoral HeLa epithelial cells (for 72h) and with 3T3 L1 fibroblasts (for 48h) in the concentration range 1-200 μM. Similar results were found for both cell types. While the $IC_{50}(Na_3VO_4)$ = 32 μM for the HeLa cells, ~100% cellular viability was found with the oxidation products of **5** in all conditions used. Some of the results obtained are shown in Figure 7. These results suggest that while vanadate enters the cells and its toxicity increases with its concentration, the V^V-complexes of salDPA do not hydrolyse, the vanadium does not enter the cells significantly and the toxicity is much lower. Similar results were obtained with the V^V-pyran complexes (*8*).

Insulin Mimetic Tests

Some tests made with complexes **1-4** with transformed mice fibroblasts were described in (*1*). The glucose intake was determined by two different methods: (a) the vitality test based on MTT (*1*), and (b) glucose consumption and lactate production by enzymatic methods (*9*). Using test (a) for complexes **1-5**, maximum activity was found in the range C_V = 0.1 to 100 μM. After 24h of

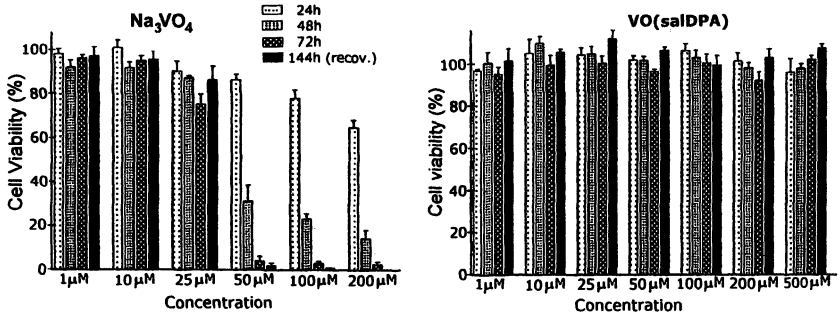

Figure 7. % cell viability of HeLa epithelial cells after exposure to sodium vanadate and to the oxidation products of Na[V^{IV}O(salDPA)], in the concentration range 1-200 μM, during different incubation periods.

incubation, in some cases some of the VCs appeared to be more effective than insulin itself. However, again the question about what was the real composition in V-species of the incubating solution may be raised. Only with pyran and with salDPA we may state that most of the vanadium is in fact coordinated by the original ligand. The effects observed could be due to free vanadates, which may enter the cells by the phosphate anion channel. With test (b), in the concentration range 1-5 μM, no significant glucose consumption and lactate production was found either with vanadate or with the oxidation products of **4** or **5**. Apparently, when the complexes do not decompose and vanadium does not significantly enter the cells, no insulin-mimetic effect is detected (*8*).

DNA Cleavage Reactions

If vanadium enters the cells, then its possible toxic effects should be evaluated at different levels, namely if it can interact with DNA and/or promote DNA cleavage. Studies of the effect of VCs on DNA have mainly concentrated on plasmid nicking caused by the VC itself or reactivity initiated by H_2O_2 or UV radiation. Namely some (hydroxy-sal)en vanadium complexes, particularly VO(pOH-sal)en **8**, have been reported to exhibit nuclease activity in the presence of an activating agent: mercaptopropionic (MPA) acid or Oxone, whereas in the absence of an activating agent no cleavage of DNA was induced. The reaction was reported to occur mainly at guanine residues (*10*).

DNA cleavage was analysed by monitoring the conversion of supercoiled plasmid DNA (Sc) to nicked circular DNA (Nck) and linear DNA (Lin). We made this study with several complexes in varying conditions of incubating medium, amount of complex or DNA, incubation time, temperature or under inert atmosphere or not. The complexes tested (all starting as V^{IV}-compounds) were **1-10,** as well as Cu(pOHsal)en, $VOSO_4$ and $NaVO_3$.

We found that the concentration and nature of buffer could be important, e.g. cases where the use of 0.01 M TRIS buffer could induce DNA cleavage, with 0.1 M this did not occur. The use of a few-months-old solution of some reagents (e.g. bromophenol blue) appeared also to induce some different results in some cases. An incubation time of 1 h at 37ºC normally gave results equivalent to ~12 h at room temperature (ca. 20ºC). Incubation under N_2 in some cases yielded less DNA cleavage than in the presence of air.

Some complexes induced DNA cleavage in the absence of activating agents, namely VO(pyran), $VO(acac)_2$ and $VO(phen)_2(SO_4)$, and more efficiently in the presence of oxone or MPA (see for example Figure 8). Others, e.g. **5** and **8**, only induced DNA cleavage in the presence of activating agents.

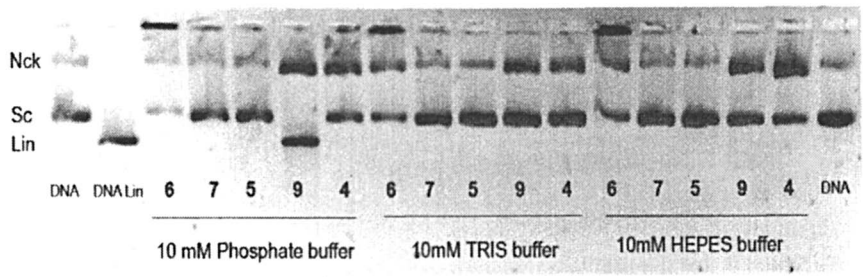

Figure 8. Cleavage of supercoiled plasmid DNA (Sc) by vanadium with no addition of activators. Nck and Lin refer to the nicked and linear DNA forms, respectively. The lanes marked DNA and DNALin refer to the plasmid DNA and to the plasmid linearized by enzymatic digestion.

Globally, for the set of complexes tested, the order of efficiency to cleave DNA is: $VO(phen)_2(SO_4)$ > $VO(acac)_2$ > Cu((pOHsal)en) ≥ VO(pyran) VO((NEt$_3$-sal)en) > VO(salDPA) ≈ VO((NEt$_3$-sal)chen) > VO((pOHsal)en)) > [VO(SO$_3$-sal)en] > $VOSO_4$ (no cleavage). The first five complexes caused DNA cleavage without the need of additional activating agents. The results were reproducible for the two amounts of DNA tested (0.1 μg and 0.5 μg). The extent of DNA cleavage observed after incubation is slightly higher if it is carried out in aerobic conditions. For example with **5**, some DNA linearization could be observed only after incubation for 1h at 37ºC in aerobic conditions, but not at room temperature nor under N_2. With complex **4** in similar conditions, DNA linearization is more extensive in aerobic conditions than under N_2 atmosphere.

As may be seen in Fig. 8, $VO(acac)_2$ behaved differently in the 3 buffers tested: in phosphate buffer it could linearize DNA, while no linear form is detected in TRIS or HEPES buffers. Addition of oxone or MPA increases the extent of DNA cleavage, but MPA much less than oxone.

As mentioned above, for VO(pOHsal)en in aqueous solution no significant difference was found in the UV-Vis spectra and their change with time when the pH is set at ~7 with the phosphate, TRIS or HEPES buffers. With $VO(acac)_2$ some distinct behaviour was found. However, we believe that the different DNA cleavage ability of $VO(acac)_2$ in solutions containing distinct buffers, and the observation that some the complexes studied show DNA cleavage ability in 0.01 M TRIS buffer, but not in its 0.1 M solutions, is probably due to the TRIS molecules acting as OH radical scavengers (assuming the DNA cleavage requires hydroxyl radicals).

Conclusions

Many VCs tested *in vitro* here and elsewhere revealed their potential insulin-enhancing properties. If we envisage VCs for oral treatment of diabetes, it is important to consider that oral application normally provides an intimate contact of VCs with oxygen. Moreover, oxidation and the acidic stomach conditions will convert most complexes to a partially hydrolysed V^V species. Most *in vivo* and *in vitro* studies concerning insulin-mimetic VCs do not take this into account properly, or do not recognize that the observations made may result from a very distinct species from the originally VC used. The synergistic effect of serum proteins and that of insulin only recently started to be evaluated, but is also far form being understood.

The speciation of the V^{IV} and V^V with pyran and with salDPA is well understood (*5,7*), and it is known that at pH=7, ~100% of vanadium is in the form of the corresponding $V^{IV}O$-salan or V^VO_2-salan complexes. Both **4** and **5** were found to be non-toxic compounds. This may simply result from their low uptake from the cells, as found in human erythrocytes (*8*). However, while in the tests with mice fibroblasts (*1*) complexes **4** and **5** were found to be insulin-mimetic compounds, in the tests measuring the glucose intake rates by the hexokinase method (*9*) no significant stimulation was found (*8*). Moreover, ***in vitro* insulin-mimetic activity of 4 was not found in rat adipocytes (by inhibition of free fatty acids release experiments made by K. Kawabe and H. Sakurai).** The possibility of being an insulin-enhancing compound *in vivo* remains open.

As V-pyran and V-salDPA complexes were found to cause DNA cleavage, if they are significantly absorbed and keep their integrity inside the cells, then the possibility of DNA damage may be significant, and it would be toxic. At least with human erythrocytes it was found by EPR that the small amount of vanadium inside the cells is not in the form of $V^{IV}O$(pyran). This balance between the insulin mimetic effect of VCs and their possible DNA damage should be carefully evaluated before any compound could be considered for the treatment of diabetes. If the VC acts directly potentiating the tyrosine phosphorylation of the insulin receptor without entering the cell, problems of DNA damage could possibly be ruled out.

Acknowledgements

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References

1. Rehder, D.; Costa Pessoa, J.; Geraldes, C.F.G.C.; Castro, M.M.C.A.; Kabanos, T.; Kiss, T.; Meier, B.; Micera, G.; Pettersson, L.; Rangel, M.; Salifoglou, A.; Turel, I.; Wang, D. *J Biol Inorg Chem* **2002**, *7*, 384-396.
2. Evangelou, A.M. *Crit Rev Oncol/Hemat* **2002**, *42*, 249-265.
3. Durai, N.; Saminathan, G. *J Clin Biochem Nutr.* **1997**, *22*, 31-39.
4. Ou, H.; Yan, L.; Mustafi, D.; Makinen, M.W.; Brady, M.J. *J. Biol. Inorg. Chem.* **2005**, *10*, 874-886.
5. Correia, I.; Costa Pessoa, J.; Duarte, M.T.; Henriques, R.T.; Piedade, M.F.M.; Veiros, L.F.; Jakusch, T.; Dörnyei, A.; Kiss T.; Castro, M.M.C.A.; Geraldes, C.F.G.C.; Avecilla, F. *Chem. Eur. J.* **2004**, *10*, 2301-2317.
6. Correia, I.; Costa Pessoa, J.; Duarte, M.T.; Piedade, M.F.M.; Jackush, T.; Kiss, T.; Castro, M.M.C.A.; Geraldes, C.F.G.C.; Avecilla, F. *Eur. J. Inorg. Chem.*, 2005, 732-744.
7. Costa Pessoa, J.; Marcão, S.; Correia, I.; Gonçalves, G.; Dörnyei, A.; Kiss, T.; Jakusch, T.; Tomaz, I.; Castro, M.M.C.A.; Geraldes, C.F.G.C.; Avecilla, F. *Eur. J. Inorg. Chem.* **2006**, 3614-3621.
8. Delgado, T.C.; Tomaz, I.; Correia, I.; Costa Pessoa, J.; Jones, J.G.; Geraldes, C.F.G.C.; Castro, M.M.C.A. *J. Inorg. Biochem.*, **2005**, *99*, 2328-2339.
9. Delgado, T.C.; Castro, M.M.C.A.; Geraldes, C.F.G.C.; Jones, J.G. *Magn. Reson. Med.* **2004**, *51*, 1283-1286.
10. Verquin G.; Fontaine G.; Bria M.; Zhilinskaya E.; Abi-Aad E.; Aboukais A.; Baldeyrou B.; Bailly C.; Bernier J.L. *J. Biol. Inorg. Chem.* **2004**, *9*, 345-353.