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In vitro study of the insulin-mimetic behaviour of vanadium(IV, V) coordination compounds

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Abstract A representative set of vanadium(IV and V) compounds in varying coordination environments has been tested in the concentration range 1 to 10^{-6} mM, using transformed mice fibroblasts (cell line SV 3T3), with respect to their short-term cell toxicity (up to 36 hours) and their ability to stimulate glucose uptake by cells. These insulin-mimetic tests have also been carried out with non-transformed human fibroblasts (cell line F26). The compounds under investigation comprise established insulin-mimetic species such as vanadate ($[H_2VO_4]^-$), $[VO(acetylacetonate)_2]$, $[VO_2(dipicolinate)]^-$ and $[VO(maltolate)_2]$, and new systems and coordination compounds containing *OO*, *ON*, *OS*, *NS* and *ONS* donor atom sets. A vitality test assay, measuring the reduction equivalents released in the mitochondrial respiratory chain by intracellular glucose degradation, is introduced and the results are counter-checked with ^{3}H-labelled glucose. Most compounds are toxic at the 1 mM concentration level, and most compounds are essentially non-toxic and about as effective as or more potent than insulin at concentrations of 0.01 mM and below. V^V compounds tend to be less toxic than V^{IV} compounds, and complexes containing thio functional ligands are somewhat more toxic than others. Generally, *ON* ligation is superior in insulin-mimetic efficacy to *OO* or *O*/*NS* coordination, irrespective of the vanadium oxidation state. There is, however, no striking correlation between the nature of the ligand systems and the insulin-mimetic potency in these cell culture tests, encompassing 41 vanadium compounds, the results on 22 of which are reported in detail here. The syntheses and characteristics of various new compounds are provided together with selected speciation results. The crystal and molecular structures of $\{[VO(naph\text{-}tris)]_2\}$ [where naph-tris is the Schiff base formed between *o*-hydroxynaphthaldehyde and tris(hydroxymethyl)amine] are reported. Electronic supplementary material to this paper can be obtained

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Keywords Vanadium compounds · Toxicity · Insulin mimesis · Cell culture tests · Solution speciation

Introduction

Among the manifold of actions pertinent to the regulatory peptide hormone insulin, several functions have been shown to be mimicked by simple "inorganic" vanadium compounds as well as by oxovanadium(IV and V) centres coordinated to organic ligands. This insulin-mimetic behaviour encompasses the stimulation of glucose intake into and subsequent degradation within glucose-metabolizing cells, the inhibition of gluconeogenesis, and glycogenolysis, and the inhibition of lipolysis (stimulation of lipogenesis) [1, 2]. In the case of lacking insulin supply (diabetes mellitus of type I) or insulin tolerance (diabetes mellitus of type II), disorders in the metabolism of glucose and fatty acids occur. An unbalanced glucose metabolism leads to unphysiologically high blood glucose levels (hyperglycemea); uncontrolled degradation of fatty acids produces acetyl-CoA in amounts which can no longer be metabolized in the citric acid cycle and thus give rise to an accumulation of harmful ketonic bodies. Among the inferences of diabetes are malfunctions of the peripheral blood circulation and thus damage of the peripheral tissues and the risk of developing vision disorder by damage of the small blood vessels in the retina; these disorders can be counteracted – in principle – by vanadium compounds. The great adantage of vanadium compounds as compared to insulin is the oral applicability of the former, and their positive effects also in the case of type II diabetes; a disadvantage is the toxicity especially of vanadate but also, to a certain extent, of other vanadium compounds [3, 4]. Vanadyl (VO^{2+}) compositions (such as vanadyl sulfate) are approved in dietetic products in several countries.

In a less broad sense, the insulin-mimetic potency of vanadium compounds is usually referred to in terms of the ability to lower the blood glucose level by activating the glucose transport into the cells. The present study is restricted to this latter approach. In a simplified manner, the action of insulin as a regulator of the glucose level can be understood in terms of a signal transduction to the membrane-bound glucose carrier, allowing glucose to be transported into the cell. This signal transduction comes about as insulin docks to the outer side of the transmembrane insulin receptor, a tyrosine kinase, initiating tyrosine phosphorylation at the inner side. Insufficient insulin supply, or lacking response to insulin, diminishes or prevents glucose intake. The insulin-mimetic vanadate [5, 6] (at physiological pH and concentrations mainly $H_2VO_4^-$; $pK_a = 8.1$) – if present – can enter cells through phosphate and sulfate channels. Vanadate is a well-established inhibitor of phosphatases [7], and a possible mechanistic path of its action is the inhibition of a protein tyrosine phosphatase, thus preventing dephosphorylation of the autophosphorylated insulin receptor. Alternatively, the insulin receptor kinase and/or glucose carrier may be activated either indirectly (through the stimulation of a non-receptor kinase) or directly by vanadylation of receptor and/or glucose carrier tyrosines, thus giving rise to glucose intake. In vitro investigations with peroxovanadium complexes have shown that activation of the receptor kinase in hepatoma cells can possibly be traced back to the inhibition of a tyrosine phosphatase [4].

Another inorganic vanadium compound, vanadyl sulfate, has also been shown to exhibit insulin-mimetic potential in animal models, viz. streptozotozin-induced diabetic rats [8, 9]. As far as toxicity is concerned, the vanadyl ion $[VO(H_2O)_4OH]^+$ is superior to vanadate in that it is less toxic. At pH values >4.5, however, i.e. as soon as the vanadyl sulfate leaves the stomach, sparingly soluble oxovanadium hydroxides are formed. The absorption thus depends on the formation of secondary compounds with ligands provided by the intestinal medium, a process which reduces the absorption rate to about 2% [10]. Once absorbed, vanadium is coordinated to a considerable extent to the phosphate groups in bones, thus providing a long-term effect [10, 11], an additional advantage over insulin. The low intestinal absorption of vanadyl and the toxicity of vanadate have initiated a search for alternative vanadium compounds containing organic ligands. The importance of a proper balance of hydro- and lipophilicity as imparted by the coordination sphere has been pointed out [12]. Promising vanadium compounds with respect to insulin mimesis are $[VO(maltolate)_2]$ (under stomach conditions $[VO(maltolate)(X)(H_2O)_y]$, where X can be OH^- or some other endogenous or exogenous small vanadium binder molecule or ion) [13, 14, 15], which is about to go into phase II of clinical tests with humans, and $[NH_4][VO_2(dipicolinate)]$, which has been successfully applied orally to diabetic cats [16]. Other compounds of varying efficacy comprise $[VOL_2]$ (L = acetylacetonate [17], picolinate [18], dithiocarbamate [19, 20], biguanide [21], bis(salicylidene)ethylenediamine [22] and pyridinones [23]). Also noteworthy is this context is the V^V hydroxylamido complex $[VO(OH)(Me_2NO)_2]$, an excellent reversible inhibitor of protein tyrosine phosphatase [24]. In order to be effective [25], a vanadium compound should fulfill a number of preconditions: hydrophilicity and lipophilicity should be balanced by an appropriate design of the ligand system in order to allow absorption and transport in the blood stream; the complex should be stable, at least to the extent where it partially survives the acidic conditions in the stomach (although this

[1] For compound **8**, the following information is available: details of the structure determination and refinement, description of the structure, and ORTEP view of the molecule (Fig. S1). Also available is the schematic presentation of the MTT test (Fig. S2), as well as additional compounds for which toxicity and insulin-mimetic tests have been carried out.

problem might well be overcome by encapsulation techniques); the ligand sphere should contain a site for bio-recognition in order to facilitate the transmembrane transport; the complex should contain an empty or easily accessible (by ligand exchange) site for coordination to the target molecule. An additional demand is, of course, minimized toxicity. In the light of these requirements, we have carried out first screening tests, using fibroblast cell cultures, with respect to short-term toxicity and insulin-mimetic efficacy. Vanadate and several of the compounds for which insulin-mimetic potency has been established earlier have been included for comparison.

Materials and methods

Preparation and characterization of vanadium complexes

Figure 1 summarizes the 22 compounds for which test results are presented in this paper. The solvents used are indicated.

The synthesis, physical properties and in some cases also the structure of the following vanadium complexes have been reported previously: $K_2[VO(O_2)(cmaa)(H_2O)]$ [cmaa = carboxymethylaspartate(3–)] (**1**) (from KVO_3, H_2O_2 and H_3cmaa) [26], $NH_4[VO_2(dipic)]$ (**6**) [27], **7a** and **7b** [28], $[VO(pydone)_2]$ (**9**) [29] (for additional details, see below), $Cs_2[VO(SO_3salen)]$ (**11**) [30], $[VO(acac)_2]$ (**12**) [31], [VOCl(van-thiosemicarbazone)] (**19**) [28], $[VO(thiopyox)_2]$ (**20**) [32] and $[VO(mal)_2]$ (**21**) [4]. [VO(UDPglu)] (**2**) is the dominant species in an aqueous solution of ionic strength 0.15 M (NaCl) containing vanadyl sulfate (1 mM) and uridine-5'-diphosphoglucose (2 mM), adjusted to pH 7.3. An aqueous solution containing vanadate (**5**) was prepared by dissolving ammonium metavanadate in water and adjusting the solution to pH 7.

{VO[bis[1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylate]]}·H_2SO_4·10H_2O, [VO(cf)], **3**

Vanadyl sulfate hydrate (54.7 mg, 0.253 mmol) was dissolved in 30 mL of water and ciprofloxacin (cf) (200 mg, 0.604 mmol) was added during stirring. To the green, slightly turbid solution a few drops of 2 M sulfuric acid were added to obtain a clear green solution of pH 3.5. Green crystals of the product were grown by evaporation in air. Attempts to determine a well-defined crystal structure failed. The complex is formulated in accordance with corresponding copper complexes [33, 34, 35]. The terminal piperazinyl-*N* are protonated; the counter charge is provided by sulfate. Analysis: calc. for $C_{34}H_{56}F_2N_6O_{21}SV$ ($M = 1005.9$ g mol^{-1}): C 40.59, H 5.61, N 8.36; found: C 40.74, H 5.51, N 8.46%. IR (NaCl): 1615 [$\nu(CO)_{ring}$], 951 [$\nu(V=O)$] cm^{-1}. EPR (H_2O): $g_0 = 1.992$, $A_0 = 107\times10^{-4}$ cm^{-1}.

[VO{pyridylidene-tris(methoxy)methylamine}], [VO(py-tris)], **4**

Tris(hydroxymethyl)aminomethane (121 mg, 1 mmol) and pyridine-2-carbaldehyde (123 mg, 1 mmol) were dissolved in 50 mL of ethanol and refluxed for 2 h, and the solvent removed in vacuo. The residue (the Schiff-base ligand thus formed) was dissolved in 50 mL of CH_2Cl_2. To this solution, $VO(acac)_2$ (265 mg, 1 mmol)

►

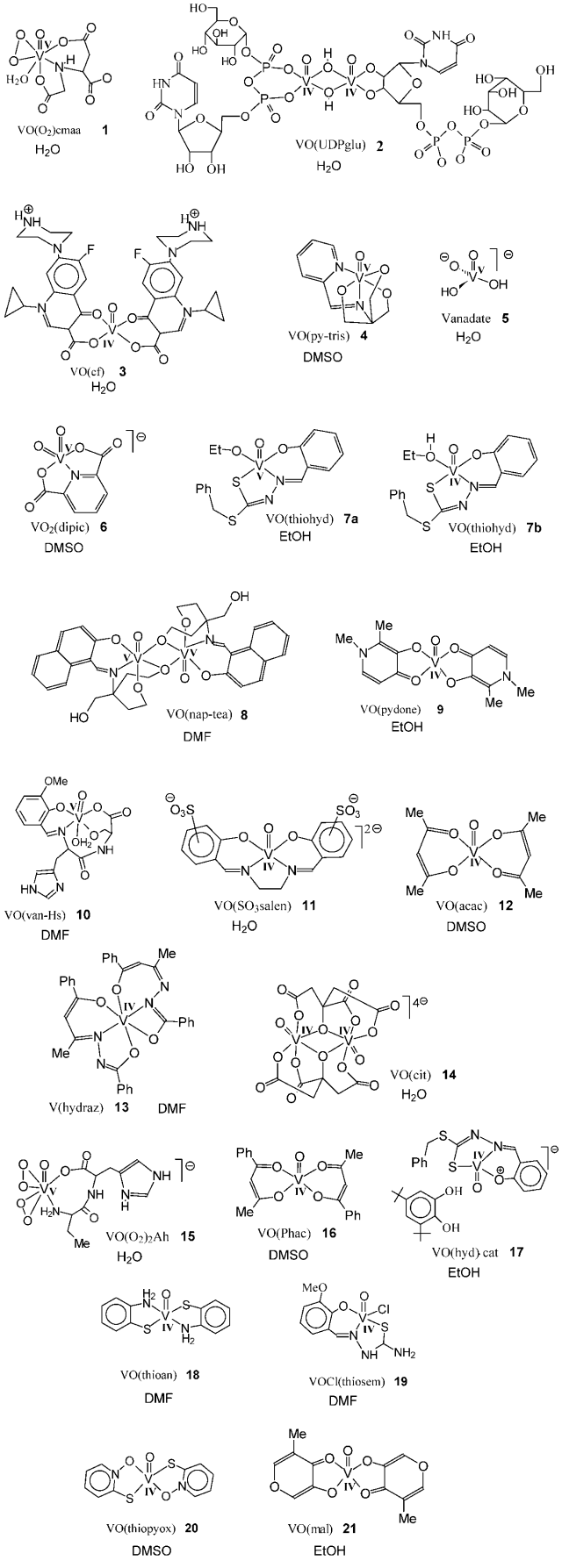


Fig. 1 Structural formulae of vanadium complexes tested in the present paper. The oxidation state of vanadium is indicated, as is the solvent used in the in vitro tests

was added. After 4 h of stirring at room temperature, a black-green precipitate of **4** was filtered off, washed with pentane and dried under vacuum. Yield: 13 mg (50%). Analysis: calc. for $C_{10}H_{11}N_2O_4V{\cdot}4H_2O$ ($M = 226.23$ g mol^{-1}): C 34.69, H 5.53, N 8.09, V 14.71; found: C 34.22, H 5.55, N 8.03, V 14.41%. IR: 1606 [ν(C=N)], 941 [ν(V=O)] cm^{-1}.

[V_2O_2{naphthalylidene[hydroxymethyl-bis(oxymethyl)] aminomethane}$_2$], [VO(nap-tris)], **8**

VO(acac)$_2$ (1.31 g, 5 mmol) and naphthalylidene-tris(hydroxymethyl)aminomethane [1.35 g, 5 mmol; prepared from 2-hydroxynaphthalene-1-carbaldehyde and tris (hydroxymethyl)aminomethane in refluxing ethanol/toluene 1/1] were dissolved in 30 mL of absolute ethanol and the mixture heated for 4 h. The resulting pale green precipitate was filtered off, washed twice with ethanol and then with ether, and dried in vacuo. Recrystallization was carried out from DMF. Yield: 60%. Analysis: calc. for ½**8**·DMF, $C_{15}H_{14}NO_5V{\cdot}$DMF ($M = 412.32$ g mol^{-1}): C 52.44, H 5.13, N 6.71; found: C 52.22, H 5.14, N 6.71%. IR: 1618 [ν(C=N); compare 1636 cm^{-1} for the free ligand], 952 [ν(V=O)] cm^{-1}. Crystals of **8**·4DMF suitable for X-ray diffraction analysis were grown in a DMF solution kept at room temperature for two weeks. Structure data have been deposited with the Cambridge Crystallographic Centre, deposition no. CCDC 161515.

[VO{bis(1,2-dimethyl-3-hydroxy-4-pyridinone)}], [VO(pydone)$_2$], **9**

According to MS, IR, electronic, EPR and EXAFS [29], **9** attains a square-pyramidal structure. The complex was also characterized in MeOH, DMSO, DMF, $CHCl_3$, pyridine and H_2O, under aerobic and anaerobic conditions, by electronic, EPR and ^{51}V NMR spectroscopies and by potentiometry and cyclic voltammetry [23, 36, 37, 38]. In aqueous solution and under anaerobic conditions the compound is not oxidized; the electronic and EPR spectra are very similar to those observed in the solid state. According to speciation studies using potentiometry and EPR, the dominant species at physiological pH is [VO(pydone)$_2$], with less than 5% of the 1:1 hydroxy complex [VO(pydone)OH]. Under aerobic conditions, however, aqueous solutions of the V^{IV} complex are oxidized to V^{V} complexes with stoichiometries and structures dependent on pH and metal-to-ligand ratio [36, 37]. At physiological pH the predominant oxidized species, as determined by ^{51}V NMR, are the 1:2 complex [VO_2(pydone)$_2$]$^-$, $\delta(^{51}V)$ –476, and the 1:1 complex [VO_2(pydone)OH(H_2O)], $\delta(^{51}V)$ –402 relative to $VOCl_3$ [38].

{VO(H_2O)[N-2-oxido-3-methoxysalicylidene)-histidyl-serine (2–)]}, [VO(van-Hs)], **10**

L-Histidyl-L-serine (60.5 mg, 0.25 mmol) and sodium acetate trihydrate (68 mg, 0,5 mmol) dissolved in 1 mL of water were treated with a solution of *o*-vanillin (38.05 mg, 0.25 mmol) in 1.25 mL of ethanol. To this mixture, $VOSO_4{\cdot}5H_2O$ (54 mg, 0.25 mmol) dissolved in 0.4 mL of water was slowly added. After 2 h of stirring, a light green precipitate had formed, which was filtered off and washed with cold ethanol and ether, and dried in high vacuum. Yield 68.5 mg (60%). Analysis: calc. for $C_{17}H_{17}N_4O_7V{\cdot}H_2O$ ($M = 459.31$): C 44.46, H 4.39, N 12.20; found: C 44.34, H 4.43, N 12.10%. IR (KBr): 1676 [ν(CONH)], 1618 [ν(C=N), 969 [ν(V=O)] cm^{-1}; ^{51}V NMR (DMF): δ –529 (relative to $VOCl_3$).

{V[bis(phenylacetylacetonato-benzoylhydrazone)]}, [V(hydraz)$_2$], **13**

VO(Phacac)$_2$ (PhacacH$_2$ = phenylacetylacetone) (390 mg, 1 mmol) and benzoylhydrazine (270 mg, 2 mmol) were dissolved in 40 mL of absolute methanol and refluxed for 4 h. The mixture was then cooled to room temperature, the precipitate filtered off, washed with methanol and ether, and dried under high vacuum. Yield 630 mg (80%). Analysis: calc. for $C_{34}H_{28}N_4O_4V$ ($M = 607.53$ g mol^{-1}): C 67.22, H 4.65, N 9.22, V 8.38; found: C 66.85, H 4.76, N 9.14, V 8.68%. EPR (THF): g_0 1.9177, g_z 2.0033; A_0 69.9, A_z 138.5×10^{-4} cm^{-1}; perpendicular (xy) components not resolved; the small coupling constants may account for the non-oxo character of this V^{IV} complex.

K_4[V_2O_2(μ-citrate)$_2$]·6H_2O, [VO(cit)], **14**

VCl_3 (0.09 g , 0.58 mmol) and 0.11 g of anhydrous citric acid (0.58 mmol) were mixed in water (5 mL). The pH of the reaction mixture was adjusted to ca. 8 with an aqueous solution of 0.4 M KOH, and stirring continued overnight. The blue solution was then taken to dryness. The residue was redissolved in 2 mL of nano-pure H_2O, and 2-propanol was added. Within 2 days, blue crystals formed at 4 °C, which were isolated by filtration and dried in vacuo. The yield was 0.20 g (~89%). Analysis: calc. for $C_{12}H_{20}K_4O_{22}V_2$ ($M = 774.58$ g mol^{-1}): C 18.60, H 2.58, K 20.14; found: C 18.67, H 2.53, K 19.90%. IR (KBr): 1636–1600 [ν_{as}(COO$^-$)], 1425–1330 [ν_s(COO$^-$)] cm^{-1}. UV/Vis (water at pH≈8): 217 (ϵ 6000), 262 (ϵ 3600), 320 (sh) (ϵ≈720), 560 (ϵ 27), 700 nm (sh) (ϵ≈18).

In a similar reaction, 0.16 g of $VOSO_4$ (1.04 mmol) and 0.20 g of citric acid (1.04 mmol) when reacted in water (5 mL) and at pH ca. 8 led, upon addition of 2-propanol, led to the isolation of large blue crystals. The yield of the reaction was ~50%. IR spectroscopy and X-ray unit cell determination provided positive identification of the crystalline product as $K_4[V_2O_2(C_6H_4O_7)_2]{\cdot}6H_2O$.

The biological tests carried out with **14** gave the same results as those which were carried out with the related complex $K_3[V_2O_2(C_6H_4O_7)(C_6H_5O_7)]{\cdot}7H_2O$, prepared from VCl_3 and citric acid (0.58 mmol) in water at a pH of ca. 6. The structures of the complex anions $[\{VO(C_6H_4O_7)\}_2]^{4-}$ and $[\{V_2O_2(C_6H_4O_7)(C_6H_5O_7)\}]^{3-}$ in the presence of different cations (K^+, Na^+, NH_4^+, Hneo$^+$) have been reported [39, 40, 41, 42].

[VO(O_2)$_2$(alanyl-histidine)]$^-$, [VO(O_2)$_2$*Ah*]*,* **15**

Aqueous solutions (1 mM) containing sodium vanadate, L-alanyl-L-histidine and H_2O_2 in a molar ratio of 1:1:2 at an ionic strength of 0.15 M (NaCl) and adjusted to pH 7.3 were employed. According to detailed speciation studies [43], the dominating compound in these solutions is the anionic bis(peroxo) complex **15**, with the alanyl-histidine presumably coordinated through the imine nitrogen of the imidazole moiety, as shown in Fig. 1. This mode of coordination has previously been established for the bis(peroxo)vanadates formed with imidazole, *N*-methylimidazole, histidine and glycylhistidine in aqueous solutions [44, 45, 46], as well as for the structurally characterized [VO(O_2)$_2$(imidazole)]$^-$ [47]. This compound decays slowly within weeks. After 10 days, 45% of the overall vanadium is incorporated in this bis(peroxo) complex; after 17 days, the amount goes down to 37%.

[VO(benzoylacetonate)$_2$], [VO(Phac)], **16**

The compound was synthesized following, in part, a published procedure [48]. Vanadyl sulfate pentahydrate (2.5 g, 10.54 mmol), dissolved in 50 mL of water, was added to benzoylacetone (3.42 g, 21.08 mmol), dissolved in 100 mL of ethanol. After stirring for 15 min, the pH (1.6) was adjusted to 7 with NH_3/water, the green precipitate thus obtained was filtered, washed with water and ether, and dried. Yield 3.55 g (86.5%). Analysis: calc. for $C_{20}H_{18}O_5V$ ($M = 389.31$ g mol^{-1}): C 61.79, H 4.66; found: C 61.69, H 4.86%. IR (KBr): 998 [ν(V=O)] cm^{-1}. EPR (CH_2Cl_2): g_0 2.009; A_0 106.7×10^{-4} cm^{-1}.

*{VO[salicylaldehyde (benzylthio(thiocarbonyl) hydrazonate)]}·bis(*tert-*butyl)catecholate, [VO(hyd)·cat],* **17**

Compound **7a** [28] (302 mg, 0.73 mmol) and 3,5-*t*-Bu_2-catechol (145 mg, 0.73 mmol) were dissolved in absolute THF and stirred for 21 h under an inert gas atmosphere. The blue-grey precipitate was filtered off and washed twice with 5 mL portions of pentane. The combined black-blue filtrates were treated with additional 20 mL of pentane and kept at –20 °C for 2 days to yield a black-blue, crystalline powder which, according to elemental analysis, contained one THF of crystallization per formula unit. Analysis: calc. for $C_{29}H_{33}N_2O_4S_2V{\cdot}C_4H_8O$ ($M = 660.76$ g mol^{-1}): C 59.99, H 6.25, N 4.24, V 7.71; found: C 60.00, H 6.27, N 4.25, V 7.55%. IR (KBr): 1584 [ν(C=N)], 1543 [ν(Ph-C=N)], 987 and 975 [ν(V=O)] cm^{-1}. The presence of catechol and THF has also been confirmed by ^{1}H NMR ($CDCl_3$). Further evidence for a strong CT interaction between the vanadium complex and the catechol arises from the effective deshielding of the ^{51}V nucleus [49, 50]. The two resonances, $\delta(^{51}V)$ –237 and –264 (in $CDCl_3$ relative to $VOCl_3$), also indicate the presence of isomers in solution.

[VO-(o-aminothiophenolate)$_2$], [VO(thioan)], **18**

$VO(acac)_2$ (265 mg, 1 mmol) was dissolved in 20 mL of absolute ethanol and treated with a solution of *o*-aminothiophenol (250 mg, 2 mmol) dissolved in 20 mL of ethanol. The mixture was refluxed for 4 h, filtered, and kept at room temperature for 2 days. A silvery precipitate formed, which was filtered off, washed with ether and dried under high vacuum. Yield: 90 mg (30%). Analysis: calc. for $C_{12}H_{12}N_2O_2S_2V$ ($M = 315.19$ g mol^{-1}): C 45.71, H 3.84, N 8.88; found: C 45.72, H 3.97, N 8.82%. IR (KBr): 984 [ν(V=O)] cm^{-1}. EPR (THF): g_0 1.9259; A_0 88.9×10^{-4} cm^{-1}.

Cell cultures and biological tests

Tests were performed on Simian virus transformed Swiss 3T3 mice fibroblasts (cell line SV 3T3), and on human skin fibroblasts of a healthy donor (F26), obtained by explantate culture. The SV 3T3 and F26 fibroblasts were maintained in monolayer cultures in T80 plastic tissue culture bottles at 37 °C under a humidified atmosphere containing 5% CO_2. Dulbecco's modification of Eagle's medium (DMEM, Sigma) was employed, containing 4.5 g/L of glucose and L-glutamine, supplemented with 10% of fetal calf serum (Cytogen), 50 units of penicillin (Sigma) and 50 μg of streptomycin (Sigma) per mL. For further treatment, cells were removed with 0.05% trypsin (Sigma) in saline solution. See also [51].

In all tests, an insulin group (cells incubated with insulin instead of the vanadium compound) and a control group (neither insulin nor vanadium compound present) were included, and all tests were carried out in three-fold.

Vanadium complexes were dissolved in the solvents indicated in Fig. 1, followed by sterile filtration in the case of water.

For toxicity tests, cells were grown in 96 multi-well plates to sub-confluency, and the cells were incubated with the vanadium complexes for 12, 24 and 36 h in DMEM. The supernatant medium was removed. Trypan blue (0.2% w/v) in phosphate buffered saline solution was added to the cells and the ratio of stained to non-stained cells was determined after 5 min of incubation time. The counts were related to the overall amount of cells present (=100%). The mean error was ca. 10%.

Tests for insulin-mimetic activity were based on the MTT-reduction essay. The yellow, soluble MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) is reduced by dehydrogenases of living cells in the mitochondrial electron transfer chain to the insoluble purple formazan blue. Cells with an increased level of glucose attain a higher reduction rate in comparison to a control group. The amount of formazan blue was measured at 570 nm in a multi-well reader (SLT 340 ATC).

Cells were grown to sub-confluency on 96 multi-well plates. The supernatant medium was removed and exchanged for serum-free DMEM without phenol red, supplemented with selenium (5 μg/L), glucose (3.5 g/L), transferrin (5 mg/L), hydrocortisone (0.4 mg/L), glutamine (200 mg/L), streptomycin (50 μg/L) and 50 units of penicillin per mL (all Sigma grade chemicals). The cells were incubated in this (insulin-free) medium for 24 and 72 h. The supernatant medium was then removed, and DMEM (without phenol red, as specified) supplemented with MTT (0.5 g/L) and the solution of the vanadium complex were added to the cells. The cells were incubated at 37 °C in a 5% CO_2 atmosphere. After 4 h the supernatant was poured off, and the reaction stopped with 0.5 M HCl in 2-propanol. Photometric measurements were carried out before and after extraction of the dye with HCl/2-propanol. In most cases, the two data sets essentially paralleled each other. The data for the 2-propanol extracts have been used in this presentation, because they are more reliable since they are free of possible inferences caused by coloured vanadium compounds. Mean errors for the absorbances are around 0.005 (0.001–0.012).

For a selected number of compounds (see Results), glucose uptake by SV 3T3 cells was also determined by incubating the cells after 24 h of insulin depletion with the vanadium compound and D-[C6-^{3}H]glucose (Amersham Pharmacia; applied activity 9.6×10^4 Bq/mL). After 7 h of incubation time, the supernatant and the cells were separated, the cells washed three times, and the activity measured after trypsination and addition of a scintillation cocktail (Ready Safe, Beckmann, USA; 5 mL per sample, measuring time 5 min per sample) with a Tricarb liquid scintillation analyser, model 2700TR (Packard). Triplet tests for four concentrations of vanadium compounds (200, 20, 2 and 0.2 μM) plus an insulin and a control group were carried out. The activities, after correction for the background, are presented as excess (in %) over the control group. Mean error 4–9 Bq.

Results

Of the 41 vanadium compounds subjected to the present screening, 22 have been chosen for this presentation. Criteria for this choice were a broad variability of the ligand sphere, sufficient solubility, and stability of the solutions over several months. Data on the remaining 19 vanadium compounds (for a listing see Supplementary material) are available from the authors on request. The 22 complexes discussed in this paper are presented in Fig. 1 by structural formulae (based on structure determination or deduced on the basis of known structures of related complexes, elemental and spectroscopic analyses), a running number, an abbreviated text formula which allows for a rapid identification, and the solvent used in the biological tests. The crystal and molecular structures of **8** have been determined (Supplementary material). The dinuclear compound contains the rhomboidal *anti* [O=V(μ-O)]$_2$ core typical for many other oxovanadium(V) complexes [52, 53, 54, 55]. Some of the compounds for which insulin-mimetic behaviour has been established earlier, for animal models or patients, have been included. These are vanadate (**5**, mainly $H_2VO_4^-$ under physiological conditions; for the speciation of vanadate as a function of pH and concentration see [56]), [VO(pydone)$_2$] (**9**) [23], [VO(acac)$_2$] (**12**) [17], [VO_2(dipic)]$^-$ (**6**, [16]; for speciation see [57]), [VO(thiopyox)$_2$] (**20**, [32]; for speciation see [58]) and [VO(mal)$_2$] (**21**, [13, 14]; for speciation see [15]). In the solid state, [VOL_2]^0 type neutral complexes are five

coordinate with tetragonal pyramidal geometry. Upon dissolution, depending on the donor strength of the solvent, $V^{IV}O$ may coordinate a solvent (solv) molecule in the sixth vacant site, resulting in a six-coordinated complex $[VOL_2(solv)]$ [4, 59]. This complex may exist in two isomeric forms, namely with the incoming ligand *trans* or *cis* to the oxo group. Although the literature is not in complete agreement [59], the consensus strongly favours an equilibrium between the two forms and the position of this equilibrium seems to be strongly dependent on the solvent, the nature of the coordinating donors and the chelate ring size [4, 59]. Such a situation has been established for, inter alia, complexes **9** [60], **12** [17], **20** [58] and **21** [14, 59]. Aqueous solutions of **9** are oxidized to $V^{V}O_2$(pydone) species in the presence of air ([38]; see also Materials and methods). Compound **7b** forms a *trans* complex in THF [28] and possibly in other polar solvents as well. Aqueous solutions of **12** contain *cis*-$[VO(acac)_2H_2O]$, *trans*-$[VO(acac)_2H_2O]$ and $[VO(acac)(H_2O)_3]^+$ [17]. A similar situation has been reported for the maltolato complex **21** [14], and it was also found that these (VOL_2) type complexes transform into mixed ligand complexes in the presence of low molecular mass bioligands, e.g. phosphate or citrate [15, 58, 60]. $[VO(O_2)_2Ah]$ (**15**), the predominant pH 7 species in solutions containing vanadate, peroxide and alanylhistidine, is in equilibrium with the ternary monoperoxo complex and binary peroxovanadates [43, 44, 45, 46]; cf. also Materials and methods. $[VO_2(dipic)]^-$ (**6**) is stable up to pH values of ca. 6; it is in equilibrium with its hydrolysis products (vanadates and free ligand) at pH 7 [57]. The compounds VO(acac) (**12**), VO(cit) (**14**; for speciation see [61]) and VO(pydone) (**9**) were double-checked with samples from different sources (**9**, **12**) or preparations (**14**). The results on these samples confirmed the reliability of both the toxicity and the insulin-mimetic tests described below.

Toxicity tests

Toxicity tests were carried out by incubating transformed fibroblasts from mice (cell line SV 3T3) with solutions of the vanadium complexes for 12, 24 and 36 h, followed by addition of trypan blue. This dye penetrates the membrane of dead cells only, and hence only dead cells adopt a bluish colour. SV transformed fibroblasts attain the physiological features of adipocytes, which effectively metabolize glucose. At vanadium concentrations, $c(V)$, below 10 μM, practically none of the vanadium compounds was toxic. The presentation of results (Fig. 2) is hence restricted to $c(V) = 1000$, 100 and 10 μM for the following five categories: (A) vanadium(V) compounds, (B) vanadium(IV) compounds, (C) vanadium compounds containing oxygen-functional ligands only, (D) vanadium compounds with mixed *O*/*N* ligand sets, and (E) vanadium complexes with *S*-functions in addition to *O*- and/or *N*-functions.

General trends can be summarized as follows.

1. Toxic effects increase with increasing exposure time of the cells to the vanadium compounds, suggesting that the compounds retain – at least to a certain extent – their identity.
2. Toxicity decreases with decreasing concentration.
3. Most of the vanadium compounds are toxic at $c(V) = 1$ mM. The only non-toxic compound at $c(V) = 1$ mM, even after 36 h of incubation, is VO (van-Hs) (**10**), a V^V complex containing a Schiff base ligand composed of *o*-vanillin and the dipeptide histidyl-serine. Low toxicity is also observed for the dipicolinat complex **6** with V^V, and the V^{IV} complexes with quinolone (**3**) and citrate (**14**).
4. Most complexes are negligibly toxic or non-toxic at $c(V) = 0.01$ mM and below, i.e. at concentrations of physiological and pharmacological relevance. A complex with high toxicity even at the comparatively low concentration of 0.01 mM is compound **20**, a V^{IV} complex of 2-mecaptopyridine *N*-oxide. Other complexes exhibiting relatively high cell toxicity include vanadate (**5**) and VO(acac) (**12**), $VO(O_2)$cmaa (**1**), the hydrazone complexes $V^{IV}O$(thiohyd) (**7b**) and VO (hyd)·cat (**17**) and the pyridinone complex **9**. Given the fact that vanadate is a very good inhibitor of phosphatases, its toxicity is not unexpected. The reasons for the toxicity of the other compounds remain to be elucidated.
5. There are no striking differences in cell toxicity between the five categories of vanadium compounds. However, in disagreement with what is generally believed, V^V complexes (category A) tend to be less toxic than V^{IV} complexes (category B). On the other hand, the thiohydrazone complexes **7a** (V^V) and **7b** (V^{IV}), with the only difference being ethanolate versus ethanol coordination, are an example for the relative unimportance of the vanadium oxidation state. Further, complexes containing *S*-functionalities (category E) appear to damage cells somewhat more effectively than those where thio ligation does not occur (C and D). It should already be emphasized at this point that complexes with low toxicity do not necessarily belong to the family of more active compounds as this relates to the ability to induce glucose translocation into the cells.

Insulin-mimetic tests

The tests for the ability of vanadium compounds to trigger glucose intake into cells was carried out with transformed SV 3T3 fibroblasts from mice and with non-transformed human fibroblasts (cell line F26). Cells were grown to sub-confluency. The culture medium was then depleted of insulin for 24 h (SV 3T3) and 72 h (SV 3T3 and F26), and thereafter incubated for 4 h with the dissolved vanadium compound. The glucose intake was determined by a vitality test based on MTT (cf. Fig. S2),

i.e. addition of yellow MTT, which is reduced to MTT-formazan blue by reduction equivalents stemming from glucose in the mitochondrial respiratory chain. The amount of MTT-fomazan blue was measured photometrically, the absorbance being related to the amount of glucose incorporated by the cell. The data are presented graphically in Fig. 3 (SV 3T3, 24 h, categories C, D and E; 72 h, categories A and B) and Fig. 4 (F26, 72 h, categories A through E). The diagrams also contain the data for a control group (neither insulin nor vanadium compound added) and for a group where insulin was employed instead of vanadium.

In order to ascertain the validity of the vitality test, glucose intake was also measured directly for a selected number of compounds. For this purpose, SV 3T3 cell cultures were treated with [^{3}H]glucose and the respective vanadium compound (c(V) = 200, 20, 2 and 0.2 M), and the ^{3}H activity measured after 7 h of incubation time. No significant c(V) dependence of the glucose intake was observed. The results are compared in Fig. 5 with those of the SV 3T3 vitality tests. Except for vanadate (**5**), there is correspondence.

With respect to the insulin-mimetic activity, we shall again summarize significant trends:

1. SV 3T3 (transformed mice fibroblasts; Fig. 3).

   (a) At c(V) = 1 mM, i.e. at a concentration where most vanadium compounds are toxic, there is no insulin-mimetic effect for most of the vanadium species, that is, the absorbance is below (24 h tests) or close to (72 h tests) the control group.

   (b) Maximum activity is found in the concentration range 0.1 to 0.001 mM. In the cell cultures which were treated with the vanadium compounds after

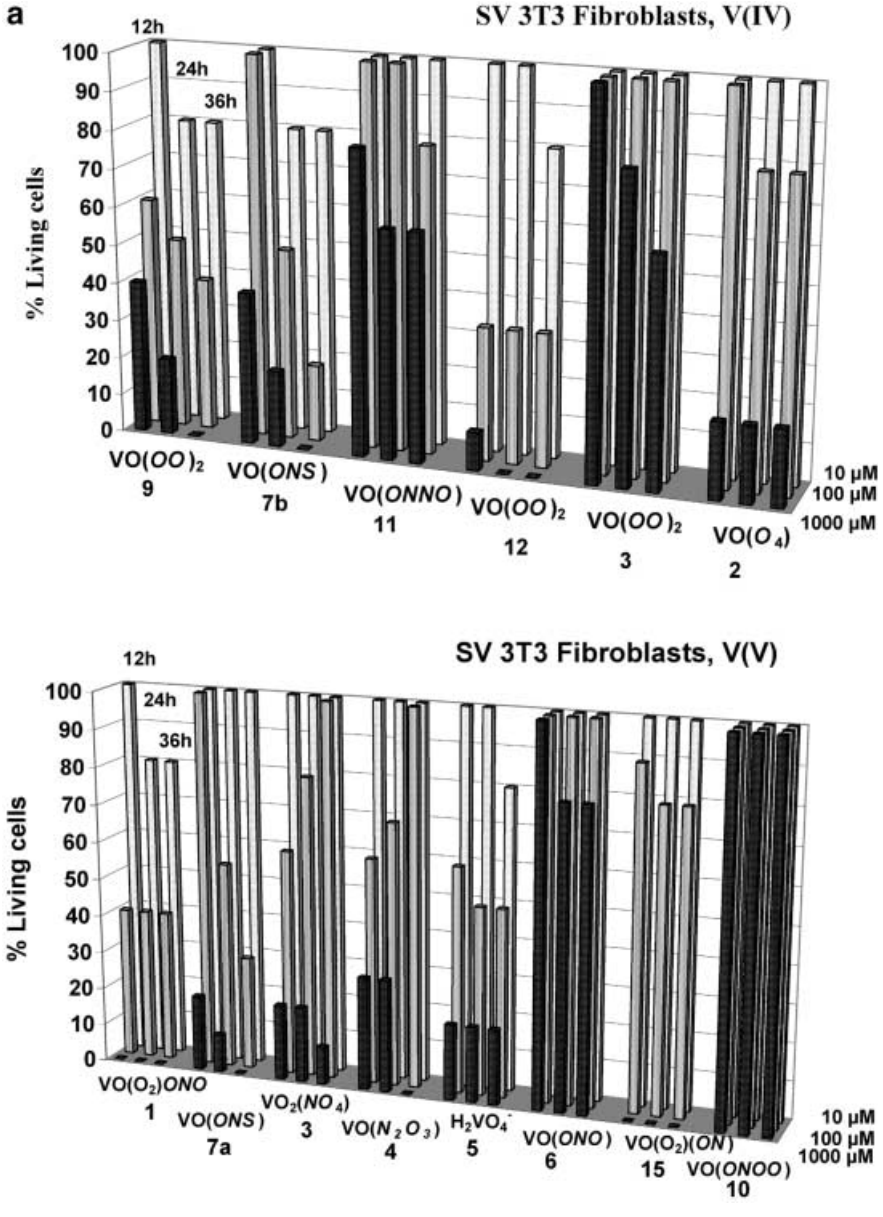
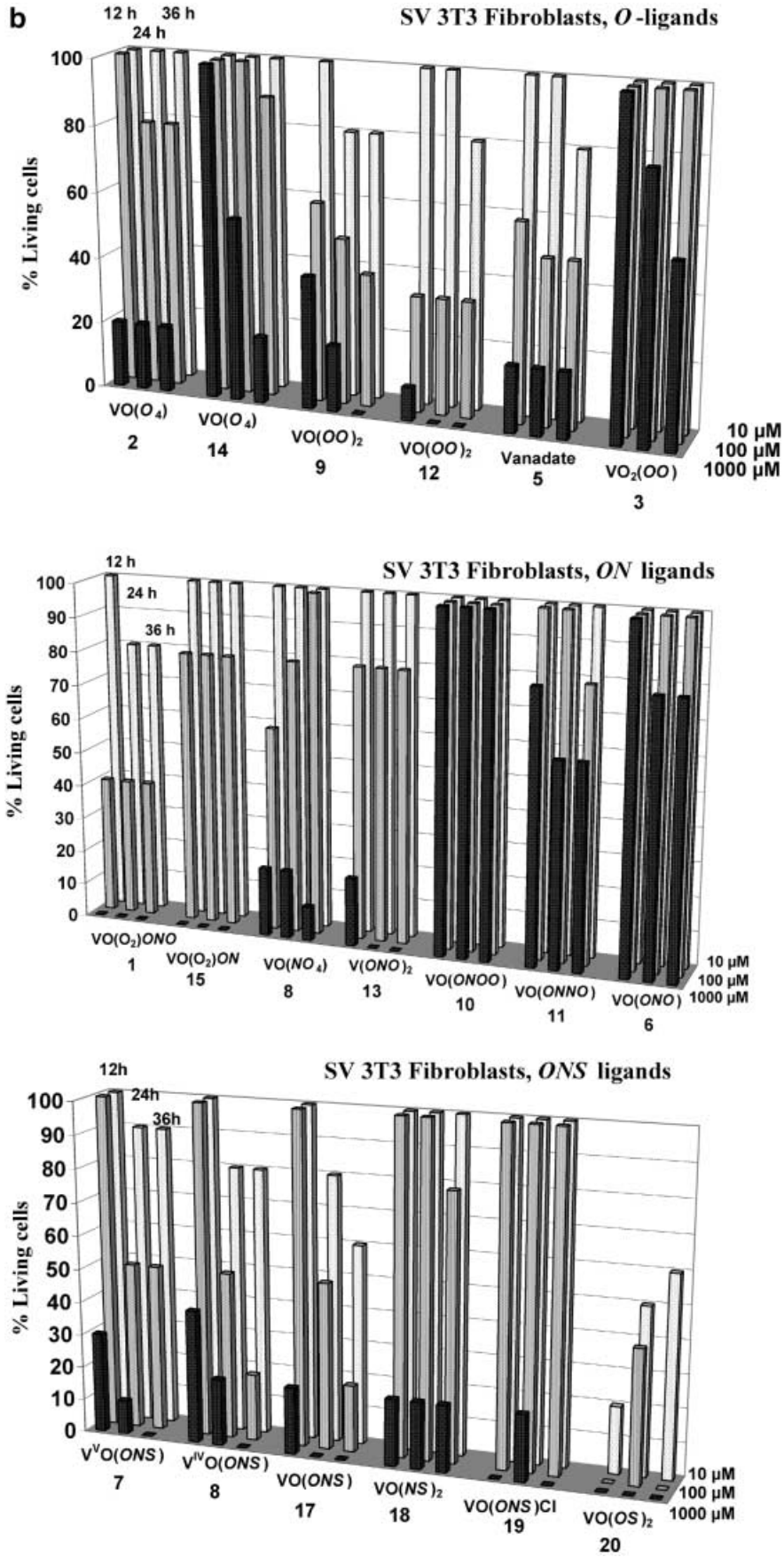


Fig. 2 Data on in vitro toxicity tests of vanadium coordination compounds (cf. Fig. 1) at three different concentrations [c(V) = 1000 µM, *black*; 100 µM, *grey*; 10 µM, *dotted*]. Cells were counted after 12 h (*left bar* of each triplet), 24 h (*middle*) and 36 h (*right*) incubation times

Fig. 2 (Contd.)


b
SV 3T3 Fibroblasts, O-ligands
12 h
24 h
36 h
% Living cells
VO(O4) 2
VO(O4) 14
VO(OO)2 9
VO(OO)2 12
Vanadate 5
VO2(OO) 3
10 µM
100 µM
1000 µM
SV 3T3 Fibroblasts, ON ligands
VO(O2)ONO 1
VO(O2)ON 15
VO(NO4) 8
V(ONO)2 13
VO(ONOO) 10
VO(ONNO) 11
VO(ONO) 6
SV 3T3 Fibroblasts, ONS ligands
12h
24h
36h
V^V O(ONS) 7
V^IV O(ONS) 8
VO(ONS) 17
VO(NS)2 18
VO(ONS)Cl 19
VO(OS)2 20

24 h cultivation in culture media devoid of insulin, most of the vanadium complexes appear to be more effective than insulin itself. This may, however, be due to an additive effect (residual insulin + vanadium). In the cell samples which were kept in insulin-free media for 72 h and incubated with vanadium thereafter, most of the compounds are clearly less effective than insulin. The increase of the absorbance by a factor of 2–3 on going from the 24 h to the 72 h samples reflects an increase of the number of cells per well.

(c) Except for the low efficacy at higher concentrations, there is no apparent correlation between toxicity and the extent of insulin-mimetic action. Thus, VO(py-tris) (**4**) and VO(thiohyd) (**7a** and **7b**) are quite effective although comparatively toxic, and the moderately toxic VO_2(dipic) (**6**) and VO(SO_3salen) (**11**) are effective, while the non-toxic VO(van-Hs) (**10**) is only moderately effective.

(d) Similarly, V^{IV} complexes (which are generally more toxic than V^{V}) tend to be more effective insulin-mimetics than V^{V} complexes; cf. Fig. 3.

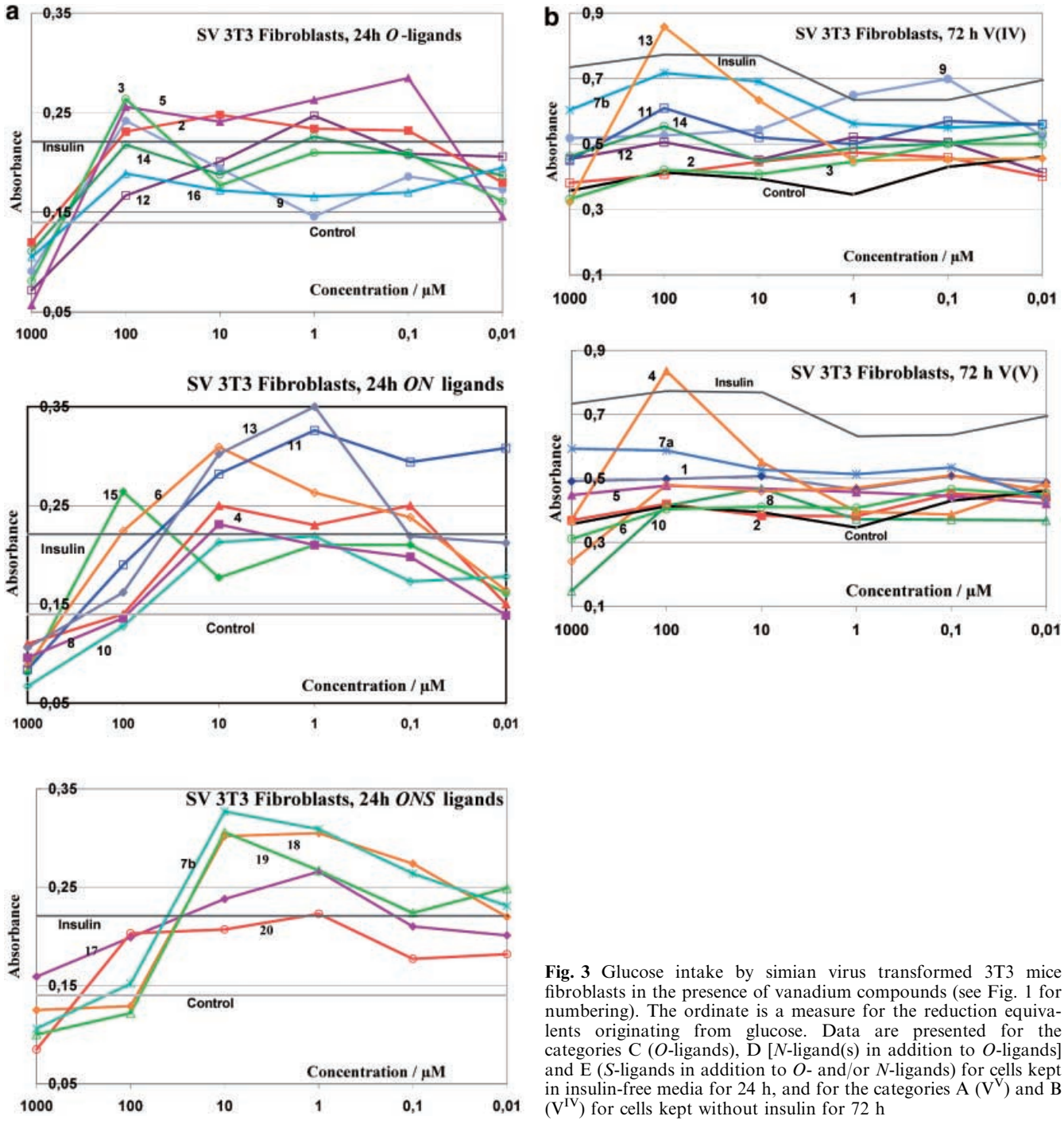


Fig. 3 Glucose intake by simian virus transformed 3T3 mice fibroblasts in the presence of vanadium compounds (see Fig. 1 for numbering). The ordinate is a measure for the reduction equivalents originating from glucose. Data are presented for the categories C (*O*-ligands), D [*N*-ligand(s) in addition to *O*-ligands] and E (*S*-ligands in addition to *O*- and/or *N*-ligands) for cells kept in insulin-free media for 24 h, and for the categories A (V^{V}) and B (V^{IV}) for cells kept without insulin for 72 h

These observations corroborate the assumption that the complexes undergo speciation within the cell, thus giving rise to active species different from those originally employed. This point will be accentuated below.

(e) As clearly revealed by Fig. 3 for the C, D and E categories, *ON* and *O*/*NS* ligands give rise to a generally substantially higher efficacy than observed with exclusive *O* coordination.

2. F26 (human fibroblasts; Fig. 4). The trends observed for the F26 cell line (Fig. 4, 72 h insulin depletion) are about the same as those observed for the SV 3T3 fibroblasts, although the differences, mainly as far as the concentration dependence is concerned, are smoothed out. Another obvious difference is the considerably higher efficacy – as compared to insulin – of the vanadium complexes in the F26 cell cultures. The following compounds are promising with respect to their insulin-mimetic behaviour and concomitant low toxicity in the physiological concentration range: $VO(O_2)$cmaa (**1**), VO(cf) (**3**), VO(van-Hs) (**10**), VO(thiohyd) (**7b**), $VO(SO_3$salen) (**11**), $VO(O_2)$Ah (**15**), VO(thioan) (**18**) and VO(mal) (**21**). In addition, V(hydraz) (**13**), VO(cit) (**14**) and VO(UDPglu) (**2**) exhibit high efficacy combined with low toxicity at *c*(V) of 200 to 20 μM. Again, complexes containing *N*- and/or *S*-functionalities in addition to *O*-functions are more effective. The efficient and well-established insulin-mimetic maltolato complex **21** is an exception. The dipicolinato complex **6**, which has successfully been applied to cure feline patients [13], is effective in

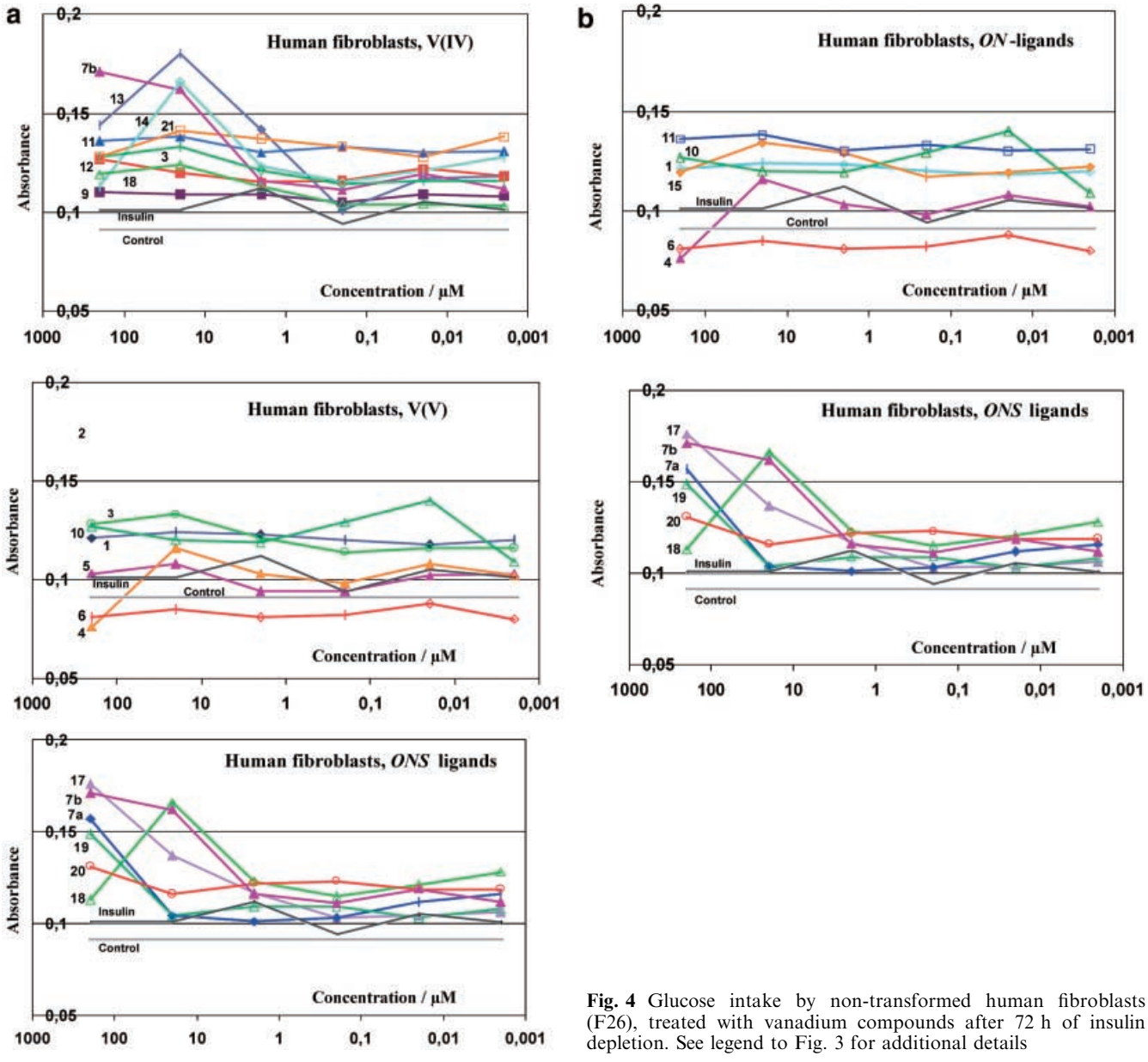


Fig. 4 Glucose intake by non-transformed human fibroblasts (F26), treated with vanadium compounds after 72 h of insulin depletion. See legend to Fig. 3 for additional details

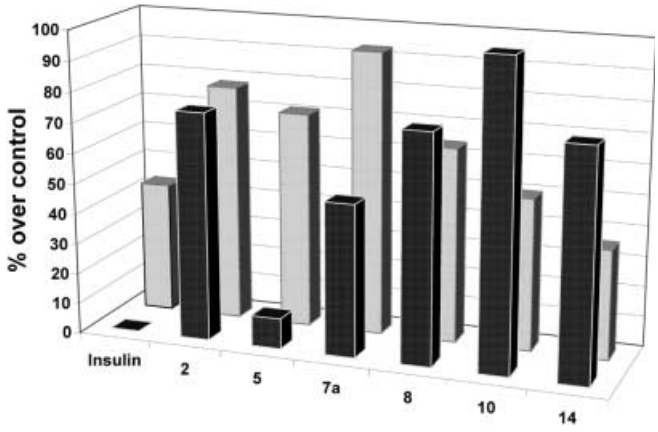


Fig. 5 Glucose intake as measured by intracellular ^3H activity after incubation of cells with ^3H-labelled glucose and vanadium compounds [*dark bars*; mean values for $c(V) = 200–0.2$ μM], and by the MTT vitality (Fig. S2) [*light bars*; $c(V) = 10$ μM]. In either case, the cell line SV 3T3 after 24 h of insulin depletion has been employed. The percentage glucose in excess of a control group is indicated on the ordinate

the SV 33T fibroblasts, while its efficacy is very low towards the F26 cell line, impressively stressing the different response to different cell lines.

Discussion and conclusion

Speciation results on selected vanadium compounds {e.g. $[VO(maltolate)_2]$, $[VO(acac)_2]$, $[VO_2(dipicolinate)]^-$, $[VO(pydone)_2]$} in aqueous solution at varying pH, metal-to-ligand ratios, absolute concentrations, and in the presence of small biogenic ligands (vide supra) show that vanadium complexes are subject to reorientation patterns which include hydrolysis, ligand exchange and redox activity. Model calculations were carried out to describe the solution state of some $[VOL_2]$ type complexes in blood serum, taking into account both potential low molecular mass binders (such as citrate, phosphate, lactate and oxalate) and proteins like albumin and transferrin [62]. We shall consider the possible pathways of a vanadium compound, as illustrated schematically in Fig. 6. A typical starting compound may be a tetragonal-pyramidal oxovanadium(IV) complex, containing an oligo-functional *ONO* ligand, imparting thermodynamic and kinetic stability at least at physiological pH. Oral application provides intimate contact with oxygen; oxidation and the strongly acidic stomach conditions will convert the complex to a partially hydrolysed vanadium(V) species. This is absorbed by the intestinal mucosa, taken up by a transport protein (possibly albumin and/or transferrin; the transport capacity of both has been demonstrated for vanadate and vanadyl [63, 64, 65]) and finally translocated across the cell membrane. In the intracellular medium, reducing agents can redox-interact with the V^V complex. A frequently discussed candidate for reduction is glutathione (GSH). GSH does reduce V^V ([66, 67], see also [25] for a discussion), although it can be a rather slow and ineffectual reducing agent in vitro at pH 7.4 and 37 °C [68, 69]. To which extent (in a redox equilibrium) V^V is reduced to V^{IV} largely depends on the stabilization of V^V by complexation. Examples for stable V^V complexes with ligands containing thiolate functions are known [27, 70, 71]. The high intracellular excess of GSH, about three orders of magnitude more than vanadium, increases the possibility of reduction of V^V. As one of the possible pathways, we suggest reduction by GSH via formation of a short-lived glutathionato-vanadium complex, resulting in a V^{IV} species and a disulfide (GS-SG). Excess GSH coordinates readily to VO^{2+}, as has been demonstrated previously [66, 67, 72, 73], and GS-SG also is a potent ligand for VO^{2+}. Other effective reductants such as NADH or ascorbate will be used as alternative reducing agents, and further reduction to V^{III} cannot be excluded [25, 74, 75]. The V^{IV} (and perhaps V^{III}) complex thus formed may undergo ligand exchange with a variety of low and high molecular mass biogenic ligand systems available in the cell, symbolized by a carboxylato ligand in Fig. 6. Among the low molecular mass binders, ATP might be an important one, as it effectively binds VO^{2+} [76] and is present in cells in millimolar concentrations. Further hydrolytic degradation to "$VO(OH)_2(H_2O)$", which is insoluble around pH 7, and re-oxidation may yield vanadate. Re-oxidation of V^{IV} even in the presence of GSH has been shown to occur [68]. Potential oxidizing agents are oxygen, peroxide, superoxide (formed by autoxidation of vanadyl [77]) or OH radicals (generated in a [NADH-mediated] Fenton- or Haber-Weiss-like reaction from an appropriate vanadium species and peroxide/superoxide [78, 79]). Vanadate itself is subject to a complex speciation [56], in the course of which coordination to biogenic ligands, formation of mixed vanadate esters and anhydrides with alcoholic and carboxylic acid residues from various molecules, aggregation to oligovanadates and reduction may occur. Whether or not the active species triggering signal transduction for glucose intake is vanadate is unknown. The inhibitory action of vanadate towards phosphatases, as noted in the Introduction, is well established, as is the ability of vanadate to couple effectively to tyrosinate [80, 81]. Both actions may be responsible for the insulin-mimetic potency. On the other hand, an intact or partially intact vanadium complex may as well exert these properties, as long as a free site, or a site which is easily accessible, is available for substrate binding. This is the case in tetragonal-pyramidal vanadium compounds and in octahedral complexes with a labile group, presumably water, bound in the *trans* position of the oxo group [82]. Alternatively, the activity may mainly reside in the structural and electronic properties of the ligand system coordinated to and thus transported by vanadium, as suggested by the similarity between the highly active compound **11** and bis(*p*-phosphophenyl)methane, a high affinity substrate for human protein-tyrosine phosphatase 1B [83].

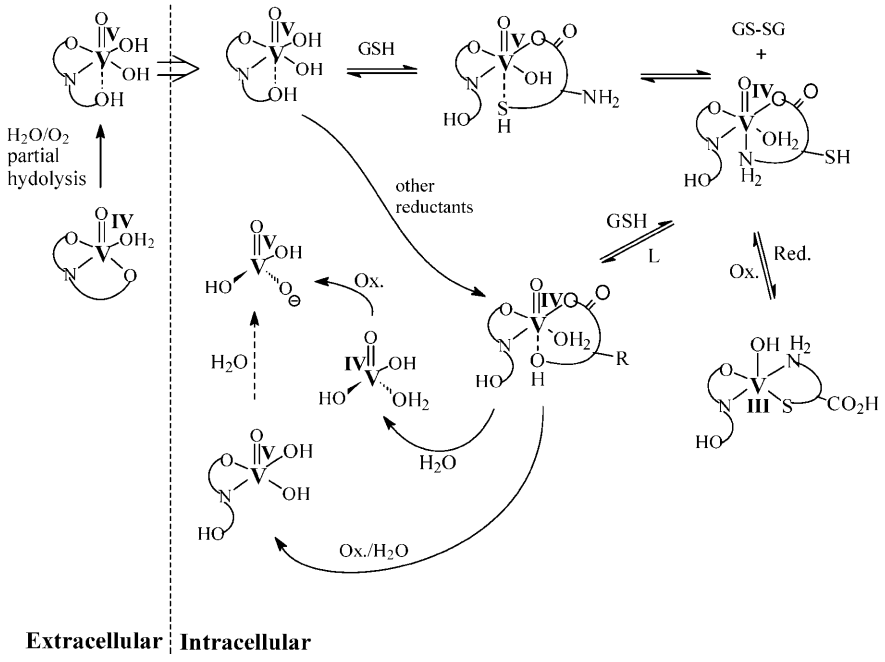


Fig. 6 Possible pathways of speciation of a vanadium compound

The significant differentiations in cell toxicity and insulin-mimetic action of the various vanadium compounds support the assumption that the complexes retain their identity, i.e. the ligands remain coordinated to the vanadium centre at least at the level of transport into the cell. We cannot exclude, however, that these differentiations might also be caused by interactions of the vanadium complex with the cell membrane. Similarly, differentiation in the insulin-mimetic effect might be traced back to discriminations in the capability of the cell membrane to transport a particular vanadium complex. Further studies directed towards an evaluation of the intake of vanadium compounds[2] into the cells and in vitro intracellular speciation to provide insight into this problem area are in progress.

At this point, we can exclude a significant correlation between insulin-mimetic efficacy and the vanadium oxidation state. This goes along with the ease of interconversion of vanadium redox states (vide supra) in cells, and with the observation that adipocytes contain distinct V^{IV} and V^{V} sensitive protein tyrosine phosphatases [66], which are possible targets for the insulin-mimetic action of vanadium compounds. Further, we can state, in general, a higher efficacy of vanadium centres coordinated to multifunctional ligand sets, i.e. *ON*, *OS*, *NS* and *ONS*, as compared to vanadium centres solely carrying oxygen functionalities. *S*-coordination tends to increase the toxicity, suggesting, in these specific cases, that the effective species differs from the one originally applied.

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[2] Preliminary studies of the uptake of vanadium compounds have been carried out with vanadate (**5**) and VO(thiohyd) (**7a**), confirming that these compounds are either incorporated into the cells or absorbed to the cell membrane. The following values have been found for SV 3T3 cells [vanadium by ASS, protein (cell material) by the BCA test]: **5** [external $c(V) = 200$ μM] 156 μg L^{-1} V/31 μg mL^{-1} protein; **7a** [external $c(V) = 20$ μM] 43 μg L^{-1} V/63 μg mL^{-1} protein

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