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The Structural Basis of Action of Vanadyl (VO²⁺) Chelates in Cells

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Abstract

Much emphasis has been given to vanadium compounds as potential therapeutic reagents for the treatment of diabetes mellitus. Thus far, no vanadium compound has proven efficacious for long-term treatment of this disease in humans. Therefore, in review of the research literature, our goal has been to identify properties of vanadium compounds that are likely to favor physiological and biochemical compatibility for further development as therapeutic reagents. We have, therefore, limited our review to those vanadium compounds that have been used in both *in vivo* experiments with small, laboratory animals and in *in vitro* studies with primary or cultured cell systems and for which pharmacokinetic and pharmacodynamics results have been reported, including vanadium tissue content, vanadium and ligand lifetime in the bloodstream, structure in solution, and interaction with serum transport proteins. Only vanadyl (VO²⁺) chelates fulfill these requirements despite the large variety of vanadium compounds of different oxidation states, ligand structure, and coordination geometry synthesized as potential therapeutic agents. Extensive review of research results obtained with use of organic VO²⁺-chelates shows that the vanadyl chelate *bis*(acetylacetonato)oxidovanadium(IV) [hereafter abbreviated as VO(acac)₂], exhibits the greatest capacity to enhance insulin receptor kinase activity in cells compared to other organic VO²⁺-chelates, is associated with a dose-dependent capacity to lower plasma glucose in diabetic laboratory animals, and exhibits a sufficiently long lifetime in the blood stream to allow correlation of its dose-dependent action with blood vanadium content. The properties underlying this behavior appear to be its high stability and capacity to remain intact upon binding to serum albumin. We relate the capacity to remain intact upon binding to serum albumin to the requirement to undergo transcytosis through the vascular endothelium to gain access to target tissues in the extravascular space. Serum albumin, as the most abundant transport protein in the blood stream, serves commonly as the carrier protein for small molecules, and transcytosis of albumin through capillary endothelium is regulated by a *Src* protein tyrosine kinase system. In this respect it is of interest to note that inorganic VO²⁺ has the capacity to enhance insulin receptor kinase activity of intact 3T3-L1 adipocytes in the presence of albumin, albeit weak; however, in

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the presence of transferrin no activation is observed. In addition to facilitating glucose uptake, the capacity of VO^{2+} -chelates for insulin-like, antilipolytic action in primary adipocytes has also been reviewed. We conclude that measurement of inhibition of release of only free fatty acids from adipocytes stimulated by epinephrine is not a sufficient basis to ascribe the observations to purely insulin-mimetic, antilipolytic action. Adipocytes are known to contain both phosphodiesterase-3 and phosphodiesterase-4 (PDE3 and PDE4) isozymes, of which insulin antagonizes lipolysis only through PDE3B. It is not known whether the other isozyme in adipocytes is influenced directly by VO^{2+} -chelates.

In efforts to promote improved development of VO^{2+} -chelates for therapeutic purposes, we propose *synergism* of a reagent with insulin as a criterion for evaluating physiological and biochemical specificity of action. We highlight two organic compounds that exhibit synergism with insulin in cellular assays. Interestingly, the only VO^{2+} -chelate for which this property has been demonstrated, thus far, is $\text{VO}(\text{acac})_2$.

Keywords

vanadium; oxidovanadium(IV); chelate; acetylacetonate; insulin signaling; diabetes mellitus; synergism

1. Introduction

Vanadium, a group V trace element that belongs to the first transition metal series, was previously considered an element in search of a biological function [1]. However, its essential role is now well established in a number of organisms, for instance, the vanadium-iron nitrogenase of *Azotobacter vinelandii* [2] and vanadium haloperoxidases in marine algae and diatomaceous organisms forming short-lived brominated or iodinated compounds [3]. In addition, vanadium is concentrated from sea water into blood cells by tunicates, *e.g.*, *Ascidia nigra* and *Ascidia ceratodes*, in which at least 90% of the element is found endogenously as vanadium(III) sulfate [4, 5]. Although the status of vanadium as an essential trace element in humans is uncertain because no specific function for it has been identified [6–8], widespread interest has been directed for many years at the biological chemistry of vanadium because of its perceived potential for development as a pharmacologic agent for the treatment of diabetes. In this respect, the literature is rich with reports of investigations evaluating the capacity of vanadium compounds to facilitate cellular uptake of glucose, to suppress lipolysis of triacylglycerols in adipocytes, and to influence the plasma concentration of glucose in small laboratory animals and in humans. These studies have included inorganic vanadium [9–13] as well as chelated forms of vanadium in oxidation states III [14], IV [15–17], and V [18, 19]. Because no compound has proven efficacious as a pharmacologic agent for treatment of chronic disease, our objective in this review has been to identify from the literature those characteristics that vanadium compounds likely should possess to be suitable for long-term intake.

The interest in vanadium chelates, particularly as therapeutic agents for the treatment of diabetes, was sharply potentiated by the reports of Posner and coworkers, who showed that vanadate reacted with H_2O_2 stimulated the phosphorylation of the insulin receptor (IR)[§] in

endosomes [18, 20] and intact rat adipocytes [21] with efficacy comparable to that of insulin. In addition, they showed that the peroxovanadium compound caused irreversible oxidation of the catalytically required cysteine residue in the active site of a protein tyrosine phosphatase anchored to the endosomal membrane presumed to regulate the autophosphorylative activity of the IR [22]. Because autophosphorylation of the β -subunit was known to result in phosphorylation of specific tyrosine residues upon insulin binding [23, 24], a protein tyrosine phosphatase enzyme had to be involved in regulating the phosphorylation state of the IR. Subsequently Kennedy and coworkers demonstrated that knockout mice, in which the gene for PTP-1B bound to the endoplasmic reticulum was disrupted, exhibited increased insulin sensitivity in glucose and insulin tolerance tests [25]. The PTP-1B(−/−) mice showed increased phosphorylation of the IR in liver and muscle tissue after insulin injection in comparison to PTP-1B(+ / +) mice. Their results demonstrated that PTP-1B has a major role in modulating both insulin sensitivity and fuel metabolism in liver and muscle tissue.

Shortly after the initial reports from Posner's laboratory, Orvig, McNeill, and coworkers using STZ diabetic rats demonstrated that the vanadium compound *bis*(2-methyl-3-hydroxy-4-pyronato)oxidovanadium(IV), known as *bis*(maltolato)oxidovanadium(IV) [VO(malto)₂], exhibited by several-fold a significantly more potent plasma glucose lowering effect than VOSO₄ [15, 26]. The effect was independent of the route of administration of the vanadium compounds, *i.e.*, oral gavage or ip injection, and was observed for chronic administration as well as for single dose, acute administration. Furthermore, with respect to equivalents of VO²⁺ administered, the organic VO²⁺-chelate was decisively more effective, and acute administration of VO(malto)₂, whether by oral or ip routes, resulted in a sustained euglycemic effect lasting in some cases up to 14 weeks. In comparison VOSO₄-treated diabetic rats reverted to hyperglycemia within 12 to 24 h. Because the organic VO²⁺-chelate was more efficacious than inorganic VO²⁺, whether administered by oral or ip routes, its greater potency could not be attributed to only enhanced gastrointestinal absorption of the organic VO²⁺-chelate over that of inorganic VO²⁺. The results also demonstrated convincingly that chelation of VO²⁺ with organic ligands facilitated cellular and tissue uptake of vanadium. These results, thus, provided a strong impetus in multiple laboratories worldwide to investigate the role of the organic chelating ligand to optimize efficacy of vanadium uptake [17, 27–39]. The pursuit to develop a VO²⁺-chelate as a pharmacologic

§Abbreviations: acac, acetylacetonate, as in *bis*(acetylacetonato)oxidovanadium(IV) or VO(acac)₂; alx, allixin; ASP, acylation-stimulating protein; CHO, Chinese hamster ovary; ENDOR, electron nuclear double resonance; EGFR, epidermal growth factor receptor; EPR, electron paramagnetic resonance; ERK, extracellular-signal-regulated kinase, a member of the MAPK (mitogen activated protein kinase)/ERK signaling family; ESEEM, electron spin echo envelope modulation; FDG, 2-(fluorine-18)-2-deoxy-D-glucose; FFA, free fatty acids; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, glucose transporter (protein); GLUT4 is the glucose transporter protein that is insulin-sensitive; GSK-3 β , glycogen synthase kinase-3 β ; HSL, hormone sensitive lipase; ip, intraperitoneal; 5Ipa, 5-iodo-picolinate *vs.* VO(5Ipa)₂ stands for *bis*(5-Iodo-2-picolinato)oxido-vanadium(IV) or VO(5-Iodo-2-picolinate)₂; IR, insulin receptor; IRS-1, insulin receptor substrate protein-1; iv, intravenous; MEK, mitogen activated protein kinase; MRI, magnetic resonance imaging; OPT, 1-*N*-oxide-pyridine-2-thiolate, as in *bis*(1-*N*-oxide-pyridine-2-thiolato)oxidovanadium(IV) or VO(OPT)₂; pa, 2-picolinic acid (correspondingly, VO(pa)₂ represents *bis*(2-picolinato)oxidovanadium(IV) or VO(2-picolinate)₂; 3mpa, 3-methyl-2-picolinic acid, and 6mpa, 6-methyl-2-picolinic acid have analogous definitions); PDE, phosphodiesterase. (there are 11 different members of this family of enzymes, of which PDE3B is insulin-responsive in adipocytes. Adipocytes also contain a member of the subfamily 4D of phosphodiesterases, PDE4D; PP1, phosphoprotein phosphatase-1; PDK1, phosphoinositide-dependent kinase-1; PET, positron emission tomography; PIP₂, PIP₃, phosphatidylinositol-4,5-*bis*-phosphate, phosphatidylinositol-3,4,5-*tris*-phosphate; PI3K, phosphatidylinositol-3 kinase; Plin, perilipin A; PKA, PKC, protein kinase A, protein kinase B (often known as Akt), protein kinase C; PTP-1B, protein tyrosine phosphatase-1B; SCID, severe compromised immunodeficient; STZ, streptozotocin.

agent for treatment of diabetes mellitus culminated in introduction of the ethyl analog of $\text{VO}(\text{malto})_2$, *bis*(2-ethyl-3-hydroxy-4-pyronato)oxidovanadium(IV) [$\text{VO}(\text{Etmalto})_2$], into preclinical, in-human Phase I [40] and Phase II [41] studies. Despite the extensive, preclinical investigations carried out with small laboratory animals prior to in-human studies suggesting potential therapeutic efficacy, the Phase II investigations were terminated after three months because of renal complications [42].

In this review we have emphasized investigations where experiments with cell systems and laboratory animals can be correlated on the basis of experimental results. Most important in our analysis of the literature have been measurements of vanadium and ligand content in tissues, physical studies of interactions of vanadium chelates with serum transport proteins, determination of their structure in solution, and observed lifetime of vanadium compounds in the bloodstream of laboratory animals, experimental variables that are essential for evaluating pharmacologic effects. Only VO^{2+} -chelates, *i.e.*, vanadium in its V^{4+} oxidation state, satisfy all of these requirements: They have been employed in investigations of both small laboratory animals and intact cell systems. In addition, of the large variety of vanadium compounds that have been employed in biological systems, quantitative results with respect to tissue content, interaction with serum proteins, and lifetime in the bloodstream have been reported more consistently with use of VO^{2+} -chelates than with other types of vanadium compounds. Also, a variety of physical studies have been carried out to define their three-dimensional structures in solution and interactions with serum transport proteins. Although Posner and coworkers in their pioneering work investigated the capacity of chelated peroxovanadium compounds to influence plasma glucose levels of both Sprague-Daley and BB rats and showed through use of intact cell systems that they enhanced the IR kinase activity [22, 43–45], there are no reports of vanadium and ligand tissue content, lifetime in the bloodstream, or interaction of the chelated species with serum proteins through which pharmacokinetic and other pharmacologic effects of the peroxovanadium compounds could be evaluated.

Because VO^{2+} -compounds facilitate a short-term, euglycemic condition in diabetic humans [46–51], we have tried to identify experimental parameters that are likely to favor further design of pharmacologic reagents for long-term treatment of disease. In this respect, specificity of action is always a critical objective, but how can specificity be best demonstrated through investigations using intact cells and laboratory animals? We suggest at least one criterion as a sensitive indication of specificity that can be applied: *Synergism*. If we apply this concept to reactions underlying insulin signaling, synergism is defined by the observation that *the simultaneous, combined action of the pharmacologic reagent with insulin is greater than the sum of their individual actions*. Observation of an insulin-like effect generated by some pharmacologic reagent does not imply or require synergism with insulin; also, synergism is not a requirement for use of a pharmacologic reagent for therapeutic purposes. However, if the condition of synergism is observed through experiment, the reagent is likely to exhibit physiological and biochemical specificity with decreased, undesirable side-reactions because it indicates that the reagent acts on an enzyme signaling pathway that is physiologically compatible with its regulation by insulin. Two examples of synergism of small molecule activators of the classical insulin signaling

pathway in the cytosol are (1) the identification of hydroxychalcone, the compound in cinnamon that enhances cellular glucose uptake [52], and (2) a non-peptide analog of a fungal metabolite that not only activates IR kinase but also increases selectivity of the IR over other, related tyrosine kinase receptors, *e.g.*, the epidermal growth factor receptor (EGFR) [53]. Synergism can also be demonstrated through *in vivo* experiments. Posner and coworkers demonstrated synergism of a chelated peroxovanadium compound with insulin in lowering the plasma glucose of diabetic BB rats [45]. The results in all of these reports clearly indicate that the combined action of each compound with insulin results in a greater effect than would be produced by the sum of their individual actions.

For detailed information about the use of vanadium as a chemical synthesis reagent, we refer the reader to reviews by Hirao [54, 55]. Also, there are extensive reviews on the properties of vanadium compounds in biological and chemical reactions [56–59].

2. Structure of vanadyl (VO^{2+}) chelates in solution

2.1 Molecular structure of VO^{2+} -chelates

Although the insulin enhancing properties of a variety of VO^{2+} -chelate systems have received much attention through animal and cell studies, the three-dimensional structures of only *bis*(acetylacetonato)oxidovanadium(IV) $[\text{VO}(\text{acac})_2]$ and $\text{VO}(\text{malto})_2$ have been determined in both *crystals and in solution*. The structural formulas of $\text{VO}(\text{malto})_2$ and $\text{VO}(\text{acac})_2$ are illustrated in Figure 1 and their molecular structures in solution are shown in Figures 2 and 3, respectively [60]. In both cases the chelates are synthesized in aqueous solution under refluxing conditions and crystallized upon cooling the reaction mixture [30, 61]. The molecular structure of $\text{VO}(\text{malto})_2$ in crystals displays C_2 symmetry having only an internal molecular dyad coincident with the $\text{V}=\text{O}$ bond while $\text{VO}(\text{acac})_2$ displays C_{2v} symmetry [30, 62, 63]. In both chelates, the vanadium atom lies approximately 0.3 – 0.6 Å above the plane defined by the four oxygen-donor atoms with the $\text{V}=\text{O}$ bond perpendicular to the plane. Although the vanadyl group is usually pictured as doubly bonded, the short vanadium – oxygen distance of 1.59 – 1.63 Å found in VO^{2+} -chelates is indicative of a triply bonded interaction, as originally described by Ballhausen and Gray [64]. For both chelates the immediate environment of the vanadyl group in crystals provides no indication of structural changes induced through their introduction into aqueous solvents.

The molecular structures of $\text{VO}(\text{malto})_2$ and $\text{VO}(\text{acac})_2$ in solution, as illustrated in Figures 2 and 3, respectively, were determined by application of ENDOR spectroscopy [60]. Hanson *et al.* suggested that $\text{VO}(\text{malto})_2$ undergoes a change in structure in solution from the *trans* complex observed in crystals to a predominantly *cis*-structure (*cf.*, Figure 1) in which a solvent molecule occupies the equatorial site previously filled by the maltolato keto oxygen atom [65]. The only structure detected through ENDOR studies, as seen in Figure 2, was a *cis*-conformation with a solvent molecule occupying an equatorial site [60]. ENDOR spectroscopy also revealed a solvent molecule hydrogen-bonded to the axial vanadyl oxygen atom. In comparison, $\text{VO}(\text{acac})_2$ undergoes essentially no conformational alteration but acquires a solvent molecule hydrogen-bonded to the axial vanadyl oxygen atom, as for $\text{VO}(\text{malto})_2$, and an axial solvent molecule with its oxygen atom in the inner coordination sphere opposite the vanadyl oxygen atom.

The coordination structure of $\text{VO}(\text{malto})_2$ in solution illustrated in Figure 2 indicates that incorporation of an amino acid side chain as an equatorial ligand is likely to occur upon binding of $\text{VO}(\text{malto})_2$ to proteins. On the basis of EPR spectra, Buglyo *et al.* [66] and Liboiron *et al.* [67] have concluded that $\text{VO}(\text{malto})_2$ in aqueous solution acquires a *cis*-conformation in the presence of *N*-methyl-imidazole with the imidazole N ϵ atom coordinated to vanadium as an equatorial donor-atom. Garibba and coworkers have made comparable interpretations using the *N*-methyl pyridinone analog of $\text{VO}(\text{malto})_2$ [68].

While X-ray crystallography and ENDOR spectroscopy of $\text{VO}(\text{malto})_2$ and $\text{VO}(\text{acac})_2$ reveal in each case only one conformation, both chelates in solution display spectral features that have been interpreted as reflecting conformational changes as a function of pH [30, 69] or temperature [65]. Figure 4 displays first- derivative, EPR absorption spectra of $\text{VO}(\text{acac})_2$ in H_2O and methanol as a function of $[\text{H}^+]$. The spectral features for species A in H_2O , *viz.*, A' in methanol, are readily identified at high pH with species B, *viz.* B', appearing more prominently as the pH is lowered. Finally species C, *viz.* C' becomes detectable as the pH is lowered further. Interestingly the EPR spectroscopic parameters of the three species obtained at liquid helium temperatures for each solvent system, summarized in Table 1, shows that species A, B, and C in H_2O are structurally equivalent, correspondingly, to A', B', and C' in methanol. Figure 4 also indicates that at very low pH a fourth species labeled D is detected but only in H_2O . The spectroscopic parameters of this species listed in Table 1 are identical to those of the *penta-aquo* VO^{2+} ion, *i.e.*, $[\text{VO}(\text{H}_2\text{O})_5]^{2+}$ [70–73].

Figure 5 illustrates titration curves constructed from EPR spectra of $\text{VO}(\text{acac})_2$ in H_2O identifying ionizations that control the reversible interconversion of species A(+ B), C, and D as a function of pH. (Species A and B are not separable due to overlapping resonance features.) The ionization with $\text{pK}_a \sim 1.5$ can be unambiguously interpreted because it controls release of the VO^{2+} moiety as the *penta-aquo* VO^{2+} ion under conditions of pH 2.5. However, the origin of the ionization with $\text{pK}_a \sim 3.1$ has not been assigned unambiguously. Others have attributed this ionization to dissociation of a ligand forming a $[\text{VO}(\text{acac})]^+$ hemi-chelate or change to a *cis*-conformation with an equatorially coordinated water molecule [30, 69]. Figure 6 compares the ENDOR proton spectra of $\text{VO}(\text{acac})_2$ species A' and C' showing that they are essentially identical. This observation rules against any change in conformation or stoichiometry of the coordinating ligands as a function of pH because the ENDOR spectra are consistent only with two acetylacetonate ligands coordinated equatorially to the VO^{2+} moiety [32, 60, 73]. Because the proton ENDOR features of an equatorially coordinated solvent molecule are readily identified, as for $\text{VO}(\text{malto})_2$ in methanol (*cf.*, Figure 2), the spectra rule against any change in the chemical origins of the oxygen-donor ligands. The structural differences of the three species, A, B, and C, *viz.*, A', B', and C' in methanol, must be very subtle because they are not resolvable within the linewidths of ligand proton ENDOR features on the basis of which vanadium – ^1H distances were calculated (to within $\pm 0.10 \text{ \AA}$) to define the structure in Figure 3 [60].

The EPR spectra of $\text{VO}(\text{malto})_2$ in aqueous solutions similarly show changes that have been interpreted as reflecting multiple conformations or changes in VO^{2+} -ligand stoichiometry as a function of temperature or pH [65]. Three vanadyl-maltolate species were identified on the basis of the temperature dependence of EPR spectra at pH 6 over the 296 – 352 K range

with the appearance of a fourth species identified as $[\text{VO}(\text{H}_2\text{O})_5]^{2+}$. The EPR spectra were interpreted on the basis of simulated spectra of a mixture of *trans* and *cis* isomers with the *cis* isomer dominant; spectral features identified at low pH were attributed to a mixture of a $\text{VO}(\text{malto})^+$ complex and the *penta-aquo* VO^{2+} [65]. Similarly, speciation studies of $\text{VO}(\text{malto})_2$ on the basis of pH-potentiometric titrations, as illustrated in Figure 7, have been interpreted as due to multiple species over the pH 2 – 8 range [66]. A second vanadyl-maltolate spectral species appears in the pH 2.0 – 4.5 range, and there is evidence for formation of $[\text{VO}(\text{H}_2\text{O})_5]^{2+}$ at pH < 3.

The reported analysis of the pH-potentiometric titrations *assumes* 1:1 metal-maltolate stoichiometry for the species formed in the pH 2 – 4.5 range [66]. As noted for the $\text{VO}(\text{acac})_2$ complex earlier, there is no structural evidence for the loss of a chelating ligand as the pH is decreased. The amplitudes of the ENDOR features of $\text{VO}(\text{acac})_2$ at neutral and low pH in Figure 6 indicate no change in ligand stoichiometry or coordination geometry in contradiction to the interpretation of speciation diagrams of $\text{VO}(\text{acac})_2$ made by others [30, 69]. Because the stability constants for formation of $\text{VO}(\text{malto})_2$ [61, 66] are comparable to those for the $\text{VO}(\text{acac})_2$ complex [70], it is unlikely that the vanadyl-maltolate species formed in the pH 2 – 4.5 range reflects a change in metal-ligand stoichiometry from that at neutral pH. We must conclude that the chemical origin of the ionization of both $\text{VO}(\text{malto})_2$ and $\text{VO}(\text{acac})_2$ with pK_a in the 3 – 4 range has yet to be identified. Assignment of this ionization based on structural data remains an important objective for complete understanding of the chemistry of these organic VO^{2+} -chelates.

2.2 Structure of VO^{2+} -chelates in the presence of serum transport proteins

The most important question in investigating the pharmacology of a chemical reagent is What is the biologically active species? This question is particularly important with respect to organic VO^{2+} -chelates because of their pH dependent ionizations and dissociable ligands. In addition, VO^{2+} is subject not only to ligand exchange but also to oxidation. For these reasons the VO^{2+} -chelate introduced into a living laboratory animal or human by injection for transport to target organs through the bloodstream may not be the same chemical species that enters cells and is responsible for its biological effects. Because serum albumin and transferrin are known to bind a large variety of organic molecules and metal ions [74, 75] as the major transport proteins in the bloodstream approaching concentrations of 0.5 mM and 0.04 mM in the serum [76], respectively, it is expected that VO^{2+} -chelates may interact with these serum proteins in the bloodstream.

Of the dipositive transition metal ions, not only does VO^{2+} form the most stable complex with the acetylacetonate ligand, but also $\text{VO}(\text{acac})_2$ is one of the most stable vanadyl chelates known with stability constants [$\log K_1$ and $\log K_2$] of 8.4 and 15.4, respectively [70]. In the presence of serum albumin, $\text{VO}(\text{acac})_2$ forms an adduct of 1:1 stoichiometry, as illustrated in Figure 8. Analysis of the ENDOR features belonging to the methyl and C(3) protons of the acetylacetonate ligands in Figure 8 and isothermal calorimetry titrations show that $\text{VO}(\text{acac})_2$ binds as an intact complex with serum albumin with a dissociation constant $K_d \approx 2 \times 10^{-7}$ M [32, 77]. Undoubtedly the high stability of $\text{VO}(\text{acac})_2$ inhibits dissociation of the chelating ligands, for binding of VO^{2+} to serum albumin is significantly weaker ($\log K$

≈ 5) [78, 79]. Garibba and coworkers have concluded that VO(acac)₂ is not bound by albumin [68]. Their conclusion is based on the lack of superhyperfine broadening of the EPR spectral features of VO(acac)₂ in frozen solutions (120 K) in the presence of serum albumin. Because superhyperfine broadening of the first-derivative EPR spectral components of VO²⁺ complexes is observed only for equatorially coordinated ligands [80], the presence of acetylacetonate ligands equatorially coordinated to the central VO²⁺ ion precludes any change in EPR linewidths. Observation and analysis of proton ENDOR absorptions not attributable to acetylacetonate protons, as shown in Figure 8, provides conclusive evidence that VO(acac)₂ forms an adduct of 1:1 stoichiometry with serum albumin.

Of considerable importance is that VO(acac)₂ remains essentially intact in the bloodstream and enters cells as an intact complex [77]. This conclusion, made on the basis of non-invasive MRI technology and ENDOR spectroscopic investigations, is the only study hitherto that indicates that the VO²⁺-chelate injected into the bloodstream of a living, laboratory animal is likely the same species that is responsible for its biological effect. Figure 9 compares the time dependent accumulation of VO(acac)₂ by a xenograft, prostate cancer tumor in the hind leg of a rat with that of a clinically used Gd³⁺ complex. Because the electron spin-lattice relaxation rate of VO²⁺ is comparable to that of Gd³⁺, the time-dependent uptake into and washout from muscle and tumor tissue could be monitored by MRI for both metallo-chelates. Both complexes were injected in separate experiments into the tail vein. Because VO²⁺ forms a polymeric, EPR-silent precipitate at neutral pH [70], the paramagnetism of the vanadium species entering tumor tissue had to have its origin either in the VO(acac)₂ complex itself or in a vanadyl-protein species that prevented polymerization of the VO²⁺ ion. ENDOR spectra of the vanadyl species that had entered prostate cancer cells obtained from excised xenograft tumor tissue and separated from the surrounding muscle tissue were identical to those in Figure 6 [77]. Analysis of the ENDOR spectra according to the stratagem described earlier [60, 73] showed that the proton resonances belonged to acetylacetonate ligands coordinated to VO²⁺ in the equatorial plane. While we cannot exclude the possibility that some VO(acac)₂ dissociates in the bloodstream and possibly also in tumor tissue, a significant fraction of VO(acac)₂ injected into the tail vein of the rat has to have remained intact upon entering tumor cells to be detectable by ENDOR. On this basis, we conclude that the intact chelate is likely the chemical species that interacts with its target enzyme in the cytosol. Through kinetic studies we have identified the target enzyme as PTP-1B [81].

The interaction of VO(malto)₂ with serum albumin and transferrin has been investigated by Liboiron *et al.* [67]. Through these investigations it is apparent that the stability of VO(malto)₂ in the presence of serum transport proteins is much less than that of VO(acac)₂. By application of EPR spectroscopy, Liboiron and coworkers concluded that the VO²⁺ moiety was stripped of its chelating ligands upon addition of apotransferrin to solutions of VO(malto)₂, the resulting EPR spectra being identical to those obtained by addition of VOSO₄ to apotransferrin. Serum albumin apparently forms two different adducts with VO(malto)₂, namely one in which the VO²⁺-chelate is intact, the VO²⁺ being axially

coordinated by an amino acid residue, and one in which a maltolate ligand is lost resulting in a (maltolato)-VO²⁺-serum albumin complex [67].

The interactions of VO(acac)₂ and VO(malto)₂ with serum albumin and transferrin are directly reflected by the capacity of each chelate to influence insulin signaling in cultured 3T3-L1 adipocytes measured on the basis of Western immunoblots illustrated in Figure 10 [82]. The capacity of these two chelates to enhance insulin signaling is compared, furthermore, to that of VOSO₄ and VO(OPT)₂. The experiment essentially illustrates what the consequence of the action of each VO²⁺-compound at the cell surface would be in the presence of either albumin or transferrin. The influence of each VO²⁺-compound on insulin signaling is best evaluated by comparing the intensity of tyrosine phosphorylation of the β -strand of the IR (marked IR β) and of the IRS-1 protein, the signaling intermediary protein that binds to tyrosinephosphorylated β -strands of the IR (*vide infra*). In the presence of serum albumin, VOSO₄ generates a slight increase in the intensity of IR β and IRS-1 bands over the control lanes for corresponding identical concentrations of added insulin. In the presence of transferrin, however, addition of VOSO₄ results in *no increase* in the intensity of the IR β and IRS-1 bands over that observed for corresponding controls. Under the conditions of the experiment [82], we can expect that essentially all of the inorganic VO²⁺ was bound by transferrin. Because transferrin receptors are found in all tissues, including 3T3-L1 adipocytes [83], the lack of phosphorylation of IR β and the IRS-1 protein in this case means that either the VO²⁺-transferrin complex is not bound by the transferrin receptor or dissociation of VO²⁺ from transferrin is not compatible with its mobilization to enzymes of the insulin signaling pathway upon internalization of the VO²⁺-transferrin complex into the cytosol. There are no studies defining the pathway for processing the VO²⁺-transferrin complex in cells, as has been demonstrated for iron-transferrin complexes [84, 85]. The results in Figure 10 contradict the suggestions of Orvig and coworkers [33, 67] that the insulin-like influence of VO(malto)₂ or VO(Etmalto)₂ is due to binding of VO²⁺ by serum transferrin in the bloodstream.

In comparison to VOSO₄, VO(malto)₂, and VO(OPT)₂, the bands enhanced by VO(acac)₂ in Figure 10 are the most prominent in the presence of either serum albumin or serum transferrin. Makinen and Brady demonstrated that glucose uptake by 3T3-L1 adipocytes was maximally increased by VO(acac)₂ in the presence of serum albumin when the albumin : VO(acac)₂ ratio = 1.0, and, as illustrated through Figure 8, VO(acac)₂ forms a 1:1 adduct with serum albumin [32]. From these results, we conclude that the albumin: VO(acac)₂ adduct is internalized into the cytosol allowing the VO²⁺-chelate to interact with enzymes of the insulin-signaling pathway. It is apparent from Figure 10 that this protein:VO²⁺-chelate complex exhibits the greatest capacity to facilitate tyrosine phosphorylation of IR β and IRS-1 proteins. Also, on the basis of the intensity of the bands for tyrosine phosphorylated IR β and IRS-1 in the presence of each of the four vanadium compounds, we conclude that albumin is the optimal carrier protein for their entry into cells.

Figure 10 compares the insulin enhancing capacity of VOSO₄ with three VO²⁺-chelates in the presence of serum albumin and serum transferrin on the basis of the intensity of tyrosine phosphorylated IR β and IRS-1 bands in Western blots. The bands were identified, respectively, as belonging to the β -strand of the IR and the IRS-1 protein by application of

antibodies specific for their tyrosine-phosphorylated form [82]. Phosphorylation of specific tyrosine residues in these two proteins constitutes the initiating point of insulin signaling. A detailed comparison of the bands for each chelate in Figure 10 shows that only for $\text{VO}(\text{acac})_2$ is the intensity of the bands, when generated by the combined action of $\text{VO}(\text{acac})_2$ and insulin together, more visibly greater than the sum of that of the chelate alone and of insulin alone in the presence of albumin. We ascribed this observation to *synergism*, meaning, as explained above, that the combined action of the chelate with insulin is greater than the sum of their individual effects [32, 82]. In other words, $\text{VO}(\text{acac})_2$ acts *specifically* via an enzyme-signaling pathway in a manner that is physiologically compatible with the action of insulin. A variety of adducts containing VO^{2+} and formed with serum transport proteins or small, molecule species such as citrate or lactate have been characterized with the implicit assumption that all of the adducts have the capacity to enhance glucose uptake [68, 79, 86]. Except for $\text{VO}(\text{acac})_2$ and VO^{2+} in the presence of albumin, as discussed above, whether any of the adducts characterized in those studies possess specific, physiologically relevant, insulin-mimetic activity has yet to be demonstrated on the basis of experiment.

The experimental results in Figure 10 can be viewed as representing the action of the vanadium compounds at the surface of insulin sensitive cells after leaving the interstitial fluid. The entry of solute molecules into interstitial fluid from the blood stream occurs through intravascular endothelial cells, known as the blood-tissue barrier [87, 88]. Albumin is the most important carrier protein, regulating the transport of steroids, thyroxine, amino acids, and drugs into the extravascular space. This process known as *transcytosis* is mediated by an endothelial, vesicular transport system involving albumin-specific binding proteins on the endothelial cell surface and activated by a *Src* protein tyrosine kinase-dependent pathway [89–92]. No investigations have been conducted with respect to the transcytotic process by which VO^{2+} -chelates gain access into the extravascular space. It is to be expected that their transcytosis is not mediated by simple diffusion in the absence of a carrier protein. We believe, therefore, with respect to development of a VO^{2+} -chelate as a therapeutic reagent, that preferential binding to albumin with retention of its structure intact will be important because of the ten-fold higher concentration of albumin in circulating blood over that of transferrin and the demonstrated role of albumin as a carrier protein for transcytosis of a variety of small molecules, amino acids, and drugs [88–91].

3. Biological action of VO^{2+} -chelates

3.1 *In vivo* studies in diabetic laboratory animal models

Table 2 summarizes results of studies involving the use of VO^{2+} -chelates to investigate the capacity of vanadyl chelates to lower blood glucose levels in diabetic laboratory animals, to enhance glucose uptake by intact cells, or to exhibit antilipolytic effects. Because lowering of serum glucose levels induced by vanadium treatment has not been observed in normal, *i.e.*, non-diabetic, animals, a diabetic condition is necessarily generated in animals either by injection of a reagent such as STZ or by genetic manipulation.

Several methods of vanadium administration have been described, *e.g.*, acute and chronic oral or gastrointestinal gavage, acute and chronic ip injection, iv injection, or dissolving the

vanadium compound in drinking water. We have excluded in this review results obtained through investigations in which the vanadyl compounds were administered through the drinking water because of likely time-dependent oxidative changes of vanadyl compounds with no control over the composition of vanadium compounds ingested and lack of control of the quantity of vanadium ingested by individual animals over time. Furthermore, we have also excluded an instance of iv administration [26] because it was reported that the VO^{2+} -chelate, in this case $\text{VO}(\text{malto})_2$, was associated with a sustained glucose-lowering effect only when administered by continuous iv infusion.

Because the method of administration of a pharmacological reagent to laboratory animals is important in comparing results, we have focused on results based on the same method of administering the vanadyl chelate, selecting results obtained by acute oral gavage or acute ip injection. The differences between acute and chronic treatment of laboratory animals can be dramatic: For instance, Thompson and coworkers reported that the bis-liganded vanadyl complex formed with the natural product curcumin, *i.e.*, 1,7-*bis*(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, a compound known for its neuroprotective activity [93], were ineffective in reducing plasma glucose levels in STZ-diabetic rats [94]. On the other hand, the literature contains numerous reports on the capacity of curcumin to facilitate lowering of plasma glucose, to decrease insulin resistance, or to treat metabolic syndrome. The difference is that the experiments by Thompson and coworkers were carried out by acute oral administration of curcumin and its VO^{2+} -chelate [94] while results reported by other investigators are based on oral gavage of curcumin to STZ rats [95] or *db/db* diabetic mice [96] on a daily basis for 6–7 weeks.

The VO^{2+} -chelates characterized by ligands with four, equatorial oxygen-donor atoms belonging to the 3-hydroxypyronato core have received the most attention in investigations using small laboratory animals. They include $\text{VO}(\text{malto})_2$, $\text{VO}(\text{Etmalto})_2$, $\text{VO}(\text{isopropylmalto})_2$, $\text{VO}(\text{n-butylmalto})_2$ [33] and *bis*(2-*n*-pentyl-3-hydroxy-5-methoxy-6-methyl-4-pyronato)-oxidovanadium(IV), more commonly known as *bis*(allixinato)oxidovanadium(IV) [$\text{VO}(\text{alx})_2$] because the ligand *allixin* is found in garlic as a natural product [37]. Although the inner coordination shell is identical for all of these VO^{2+} -chelates, we can expect that $\text{VO}(\text{alx})_2$ should show some increased stability in solution due to its more pronounced hydrophobic character [97]. The most detailed studies on the plasma glucose lowering capacity of this family of VO^{2+} -chelates in laboratory animal models following acute oral gavage or acute ip injection have been reported for $\text{VO}(\text{malto})_2$ and $\text{VO}(\text{Etmalto})_2$ [26, 33, 61, 98]. Whether administration was by acute oral gavage or by acute ip injection, significant glucose lowering to levels associated with non-diabetic animals was observed within 24 h following administration of the compound to STZ-diabetic rats, as illustrated in Figure 11. Comparable observations have been reported following a single ip injection of $\text{VO}(\text{alx})_2$ to STZ-diabetic mice [37]. While the extent of the glucose lowering effect STZ-diabetic rats was dose-dependent [33], there was no correlation between the glucose lowering effect with the blood vanadium concentration. However, with respect to Figure 11, doses of either $\text{VO}(\text{malto})_2$ or $\text{VO}(\text{Etmalto})_2$ 0.8 – 1.0 mmol vanadium/kg body weight resulted in significant mortality due to hypoglycemia. The results of these investigations demonstrate clearly that chelation of VO^{2+} significantly

improved uptake of the vanadyl moiety over that for inorganic VO^{2+} and sustained a euglycemic effect for up to 72 h.

Separate radio-labeling of vanadium [98] and the organic ligand [33] has shown that hydroxy-pyrone-type VO^{2+} -chelates exhibit significant lability and are subject to dissociation in the blood stream, as illustrated in Figure 12. Comparable investigations with use of other VO^{2+} -chelates have not been carried out although clearly such data are needed for drug development. While the results in Figure 12 show that the ligands of the VO^{2+} -chelate, in this case $\text{VO}(\text{Etmaltol})_2$, quickly dissociate when introduced into the animal through oral gavage, they illustrate only time-dependent changes in the total plasma vanadium and plasma ligand concentrations but provide neither indication of the oxidation state of the vanadium nor relative distribution as VO^{2+} , a VO^{2+} -protein complex, or the intact complex. Dissociation of the organic ligand from the VO^{2+} moiety, thus, greatly complicates identification of the pharmacologically active species responsible for glucose lowering in the intact animal. One can make only the qualitative conclusion that increased stability of the VO^{2+} -chelate may provide a path to this objective: Thompson and coworkers [33] point out that oxidation of $\text{VO}(\text{isopropylmaltol})_2$ in solution was noticeably slower than for $\text{VO}(\text{malto})_2$, $\text{VO}(\text{Etmaltol})_2$, and $\text{VO}(n\text{-butylmaltol})_2$ in their investigations and that the capacity of the isopropyl analog for plasma glucose lowering was achieved at a significantly lower blood vanadium level than for the other VO^{2+} -chelates. Also, based on monitoring the amplitude of the central component of the eight-line EPR absorption spectrum of the paramagnetic VO^{2+} moiety, Sakurai and coworkers [97] have demonstrated that the mean residence time of $\text{VO}(\text{alx})_2$ in the circulating bloodstream of a rat is 2.0- and 1.8-fold longer than that of $\text{VO}(\text{malto})_2$ and $\text{VO}(\text{Etmaltol})_2$, respectively. These observations, particularly in view of the need to terminate Phase II clinical trials for $\text{VO}(\text{Etmaltol})_2$ because of renal complications [42], indicate that very high stability of a VO^{2+} -chelate in aqueous solution is a prerequisite for design of an effective pharmaceutical reagent if the objective is treatment of diabetes mellitus.

Reul, Brichard, Crans, and coworkers [17, 30] have employed $\text{VO}(\text{acac})_2$ together with 3-alkyl-acetylacetonate analogs as probes of glucose metabolism. Although most of their studies were carried out with VO^{2+} -chelates dissolved in drinking water, they have compared the influence of the chelates on blood glucose levels of STZ-diabetic Wistar rats after a single, acute ip injection [17]. The results are illustrated in Figure 13. Of particular interest is that $\text{VO}(\text{acac})_2$ was the only chelate associated with a significant decrease in plasma glucose concentration that was sustained for up to 5 days, as shown in Figure 13 and that it resulted in the highest levels of plasma vanadium after a single ip injection. Although Reul and coworkers discussed the multiple factors possibly underlying the greater efficacy of $\text{VO}(\text{acac})_2$ with respect to its 3-alkylacetonate analogs and its high blood vanadium level, there was no apparent, satisfactory explanation [17]. It is likely, in view of the discussion above in “Section 2.2 *Structure of VO^{2+} -chelates in the presence of serum transport proteins*”, that the greater capacity of $\text{VO}(\text{acac})_2$ to counteract hyperglycemia is attributable simply to its high stability and capacity to form a 1:1 adduct with serum albumin, both factors increasing its residence time in the bloodstream.

As a paramagnetic reagent to increase contrast intensity for MRI, we noted earlier that the VO^{2+} -chelate was accumulated into xenograft tumor tissue 4 h post iv injection and that it still exhibited proton ENDOR spectra attributable to intact $\text{VO}(\text{acac})_2$, as in Figure 6 [77]. Comparable stability has not been demonstrated for any of the other vanadium compounds discussed hitherto. The far greater stability of $\text{VO}(\text{acac})_2$ compared with two other VO^{2+} -chelates used widely as probes of glucose metabolism is illustrated in Figure 14. The diagram compares the time-dependent decrease in the intensity of EPR absorption spectra and the intensity of the ENDOR proton absorptions of covalent hydrogen atoms of the ligands of $\text{VO}(\text{acac})_2$, $\text{VO}(\text{malto})_2$, and $\text{VO}(\text{OPT})_2$ under solution conditions, including serum albumin, when added to cells [82]. The loss of paramagnetism and of the ENDOR features of covalent hydrogen atoms of the chelating ligands indicates dissociation of the organic ligands from the VO^{2+} moiety. In the presence of serum albumin, only $\text{VO}(\text{acac})_2$ remained stable at room temperature for the 8-day experiment.

Sakurai and coworkers [99–102] have employed derivatives of picolinic acid (pa) as the chelating ligand to identify factors that optimize the glucose lowering effect and the transfer of vanadium into the blood stream from different anatomical regions of the alimentary canal. As one important factor, they found that intra-ileal administration of vanadyl compounds resulted in higher levels of blood vanadium than observed for oral and jejunal administration [99].

3.2 Organ distribution of vanadium following acute and chronic administration

Orvig, McNeill, and coworkers provided analytical data on the accumulation of vanadium into tissues following acute oral or ip administration of VOSO_4 and $\text{VO}(\text{malto})_2$ into normal male Wistar rats [98] and following acute oral gavage of $\text{VO}(\text{Etmalto})_2$ into female Sprague-Dawley rats [33]. While experiments using ^{14}C -labeled $\text{VO}(\text{Etmalto})_2$ [33] essentially confirmed earlier results [98], the use of radioactive ligand allowed differentiation of the pharmacokinetics of vanadium and the ethylmaltol ligand separately. The maximum tissue accumulation of vanadium for acute administration followed the order: small intestine >> kidney > bone $\approx$ liver. The order for the ^{14}C -ligand was kidney >> small intestine > liver > bone. This difference undoubtedly reflects not only the separate concentration profiles in Figure 12 but also greater accumulation of the ^{14}C -ligand in kidney tissue because of its excretion into urine, the ligand having become dissociated from VO^{2+} in the bloodstream. Also, while the time to reach maximum concentration of the ^{14}C -ligand into soft tissues was of the order of 6 – 9 min, the time to reach maximum concentration of vanadium in bone was ~ 7.0 h. This pronounced time differential to achieve maximum uptake sharply underlines the lability of $\text{VO}(\text{Etmalto})_2$ in the bloodstream.

Chronic administration of a VO^{2+} -chelate leads to bone tissue accumulating the highest concentration of vanadium, as demonstrated for $\text{VO}(\text{malto})_2$ or $\text{VO}(\text{alx})_2$ administered to STZ-diabetic mice [37], $\text{VO}(\text{5Ipa})_2$ in STZ rats [100], and $\text{VO}(\text{alx})_2$ administered to KKA ^{y} diabetic mice [37]. The histogram presentation of vanadium content of different tissues in Figure 15 illustrates the vanadium tissue profile of STZ diabetic Wistar rats treated chronically with $\text{VO}(\text{5Ipa})_2$ by ip injection for 14 consecutive days followed by 14 days of no vanadium treatment prior to sacrificing. While vanadium is possibly in the form of

vanadate substituting for phosphate in the mineralized hydroxyapatite structure of bone [103], the paramagnetism of the vanadium species indicates that VO^{2+} is present in bone tissue [104]. Of considerable interest is that Dikanov and coworkers have analyzed the superhyperfine couplings of ^1H and ^{31}P nuclei in the vicinity of VO^{2+} sites measured by application of ESEEM spectroscopy. They have suggested that the vanadyl (VO^{2+}) ion is coordinated in a tridentate, structural motif by a triphosphate group providing both equatorial and axial oxygen-donor atoms [105, 106]. Figure 16 illustrates the coordination environment of VO^{2+} incorporated into the hydroxyapatite matrix of mineralized bone tissue compatible with the results of ESEEM studies [105, 106]. Examples of phosphate groups axially coordinated to VO^{2+} have not been previously observed. The VO^{2+} ion, as pointed out by Williams, substitutes readily for Ca^{2+} and Mg^{2+} because its ionic radius, surface charge density, and tendency to prefer oxygen-donor ligands are intermediate between those for Ca^{2+} and Mg^{2+} [107]. On this basis, the incorporation of VO^{2+} into mineralized bone tissue can be viewed essentially as an isostructural probe of Ca^{2+} sites in the crystalline structure of hydroxyapatite.

One aspect in which the incorporation of VO^{2+} into bone tissue may be important is in relation to diabetic osteopathy. Diabetic individuals are known to have a higher incidence of bone fractures than non-diabetic individuals, particularly with respect to middle-aged and elderly patients. The pathological changes associated with diabetic osteopathy differ from those associated with senile or post-menopausal osteoporosis. Zhang and coworkers have investigated specifically the effect of $\text{VO}(\text{acac})_2$ on bone structure after chronic daily treatment of alloxan-induced diabetic Sprague-Dawley rats for 35 consecutive days [108]. In addition to looking for toxic effects such as weight loss and gastrointestinal irritation, they specifically measured parameters of bone formation and resorption, bone mineral density, biomechanics, histomorphology, and plasma biochemical changes. While the plasma glucose levels of diabetic rats treated with $\text{VO}(\text{acac})_2$ were sustained after day 1 at the same level as that of euglycemic, normal rats, as expected, there were no differences in the plasma levels of Ca^{2+} , phosphate, alkaline phosphatase, and tartrate-resistant acid phosphatase in non-diabetic rats treated with $\text{VO}(\text{acac})_2$ compared to the corresponding levels in normal rats that did not receive vanadium. Significantly, they noted that the plasma level of osteocalcin, a protein secreted by osteoblasts and used as a marker of osteoblast activity, was decreased in diabetic rats (7.5 ± 1.92 ng/mL) but was increased by $\text{VO}(\text{acac})_2$ treatment; however, the level of osteocalcin after $\text{VO}(\text{acac})_2$ treatment (12.61 ± 1.08 ng/mL) still remained lower than the baseline level in non-diabetic rats (15.45 ± 4.45 ng/mL) and in non-diabetic rats treated with vanadium (15.11 ± 1.83 ng/mL). Osteocalcin has been shown to mediate insulin secretion from the pancreas and adiponectin expression in adipocytes [109]. In addition, the levels of creatinine, cholesterol, and triglycerides were brought to essentially normal values in diabetic rats treated with $\text{VO}(\text{acac})_2$. Although treatment of diabetic rats with $\text{VO}(\text{acac})_2$ did not alter bone mineral density, treatment with this VO^{2+} -chelate did improve or normalize the ultimate strength, trabecular thickness, and the mineral apposition rate in diabetic rats [108]. In addition, there were no kidney or liver changes and no signs of gastrointestinal irritation as a result of the chronic treatment with $\text{VO}(\text{acac})_2$.

In a very different type of study, Paglia *et al.* investigated the influence of local administration of $\text{VO}(\text{acac})_2$ at the fracture site on bone healing in healthy, diabetic-resistant, male BB Wistar rats [110]. The results are illustrated in Figure 17 contrasting the progression of healing from 10 to 21 days post-fracture for saline-treated and $\text{VO}(\text{acac})_2$ treated rats. Testing different levels of vanadium treatment, they found that local delivery of 1.5 mg $\text{VO}(\text{acac})_2/\text{kg}$ into the femoral canal prior to fracture (without change in plasma glucose levels) resulted in 88% higher collagen content at 10 days post-fracture and 31% more mineralized tissue after 21 days post-fracture than in saline controls. Furthermore, at 4 weeks post-fracture, the vanadium treated femora exhibited significantly improved mechanical properties, *e.g.*, maximum torque to failure, torsional rigidity, and effective shear modulus, compared with saline controls. Also, histochemical microscopic examination of the fracture site of femora of vanadium treated rats showed a significantly increased number of blood vessels and vascular endothelial growth factor-C (VEGF-C) positive cells, indicating increased vascularization compared to that in saline controls. Ten percent of the locally administered vanadium remained bound to the femora at day 1 post-fracture, this value decreasing to approximately 5% at day 7. The results indicated that local administration of $\text{VO}(\text{acac})_2$ significantly improved the rate of healing of the fracture site. Further studies to determine optimal delivery of the chelate with respect to time following the fracture and evaluation of delivery methods of the chelate are needed to better evaluate potential therapeutic use of a VO^{2+} -chelate in instances of bone fracture.

3.3 Insulin signaling pathways

3.3.1 Glucose uptake by muscle and adipose tissue—The potential of organic VO^{2+} -chelates as pharmacologic reagents has been characterized according to their capacity to enhance cellular uptake of glucose, reduce the blood glucose level of diabetic laboratory animals, or to suppress epinephrine stimulated release of free fatty acids by adipocytes. On this basis the chelates have been described as *insulin-mimetic*, *insulin-enhancing*, or *insulin-like*. Therefore, to evaluate and compare the extent to which organic VO^{2+} -chelates *simulate* the action of insulin in cells, we briefly review first the present state of understanding of the molecular mechanisms and signaling pathways underlying insulin action that have been determined on the basis of experiment.

As illustrated in Figure 18, insulin, released in its mature form as a 51-amino acid polypeptide from pancreatic β -cells in response to high blood glucose, does not enter cells directly but binds first to a specific plasma membrane receptor to initiate a cellular signal that is transmitted along a branched pathway to specific enzymes in the cytosol and to the nucleus of the cell. In the cytosol insulin signaling regulates glucose uptake and metabolism or suppresses lipolysis and promotes synthesis of triacylglycerides and glycogen, depending on the insulinsensitive tissue. In the nucleus it activates transcription of specific genes not only to increase the cellular content of enzymes responsible for processing glucose and suppressing lipolysis but also to slow the transcription of enzymes that are regulated by epinephrine and glucagon. While some research has been focused on the influence of vanadium compounds on protein transcriptional activity [34, 111–113], in this review we have focused only on the influence of VO^{2+} -chelates on insulin signaling in the cytosol. Therefore, in Figure 18 the signaling pathways in the cytosol involving enzymes responsible

for only glucose uptake, glycogen synthesis, and the suppression of lipolysis are schematically illustrated.

Insulin binding to the α -subunits of the IR triggers a conformational change that is transmitted to the β -subunits extending through the cell membrane into the cytosol. The result is activation of the autophosphorylative kinase activity of the β -subunits whereby three tyrosine residues in each β -subunit are phosphorylated [23, 24]. Signaling intermediates known as IRS proteins are next recruited to the β -subunits of the IR upon tyrosine phosphorylation. The IRS proteins, of approximate 185,000 Daltons, act as cell signaling intermediates and undergo phosphorylation of specific tyrosine residues via the kinase activity of the β -subunits of the IR [114, 115]. The IRS-proteins function as IR-specific docking proteins to transmit the signaling cascade to multiple downstream molecules. IRS-1 is the signaling intermediate involved in glucose uptake and glycogen formation.

The phosphorylated tyrosine residues of the IRS-1 protein create docking sites for the p85 regulatory subunit of PI3K [116, 117]. Activated PI3K then catalyzes phosphorylation of PIP₂ to PIP₃ at the plasma membrane. In turn, PIP₃ serves as a docking site for PDK1 and Akt (also known as protein kinase B or PKB). PDK1 phosphorylates Akt/PKB at Thr308, activating the serine/threonine kinase domain of Akt/PKB [118–121]. Ultimately, Akt/PKB is the key enzyme functioning as a central point regulating both glucose uptake and inhibition of lipolysis.

The entry of glucose into cells is facilitated by glucose transporter (GLUT) proteins. While all cells contain GLUT1, insulin sensitive cells, *i.e.*, muscle and adipose tissue, in addition contain GLUT4 that is stored in cytoplasmic membranous vesicles and is translocated to the plasma membrane of the cell upon insulin binding to its receptor. In contrast, liver and pancreatic β -cells contain GLUT2 that facilitates essentially immediate equilibrium of the glucose concentration of the bloodstream with that of the cytoplasm. Thus, although glucose entry into hepatocytes is independent of insulin binding to its receptor, insulin binding increases the cellular content of key enzymes in liver tissue that are responsible for processing glucose, namely, hexokinase IV (glucokinase), glycogen synthase, phosphofructokinase-1, and pyruvate kinase. It also slows the transcription activity of antagonist enzymes regulated by glucagon, *e.g.*, glucose-6-phosphatase, fructose-1,6-bisphosphatase-1, pyruvate carboxylase, and phosphoenolpyruvate carboxykinase.

The translocation of GLUT4 from cytoplasmic storage vesicles to the cell membrane occurs through both a PI3K-dependent pathway and a PI3K-independent pathway [122]. Upon activation, Akt/PKB promotes phosphorylation of AS160, a protein that contains a GTPase-activating domain required for membrane trafficking of small G-proteins [123]. Glucose uptake is then initiated by Akt/PKB catalyzing phosphorylation of GSK-3 β rendering it of low activity. (GSK-3 β downregulates glycogen synthase activity by phosphorylating specific serine residues.) In addition, PP1, released upon insulin binding, catalyzes the dephosphorylation of glycogen synthase. Rendering glycogen synthase active by hydrolytic removal of its phosphoryl groups promotes glucose storage as glycogen.

The PI3K independent pathway functions in parallel to the PI3K dependent pathway and uses the adaptor protein APS to recruit GLUT4 storage vesicles to the plasma membrane. Once insulin binds and activates the IR, the adaptor protein APS binds to activated IR and recruits c-Cbl (*Casitas B-lineage lymphoma oncogene*) and a c-Cbl-associated protein [124]. Phosphorylated c-Cbl is translocated to lipid rafts recruiting the CrkII-C3G complex through interaction with the SH2 domain of CrkII. Then, C3G activates the small GTP-binding protein, TC10, by causing GTP/GDP exchange. Activation of TC10 is essential for insulin-stimulated glucose uptake and GLUT4 translocation. On this basis, TC10 can be considered as a second signal for translocation of GLUT4 to the plasma membrane.

3.3.2 Inhibition of lipolysis in adipose tissue—To understand insulin-mediated inhibition of lipolysis, it is critical to understand cAMP-dependent activation of lipolysis. Cyclic-AMP-dependent activation of lipolysis in adipocytes is initiated by epinephrine binding to its membrane receptor. The conformational change in the receptor causes activation of the stimulatory heterotrimeric G-protein complex and replacement of GDP by GTP bound to the G α subunit. The G α subunit moves in the plane of the plasma membrane to interact with a nearby molecule of membrane bound adenylyl cyclase. Adenylyl cyclase is thereby activated to catalyze conversion of ATP into cAMP. Cyclic-AMP activates PKA, a serine/threonine kinase, that in turn catalyzes phosphorylation of HSL and Plin A. HSL is an enzyme of broad specificity that catalyzes the hydrolysis of tri-, di-, and mono-acylglycerides. Plin proteins encapsulate lipid droplets in adipocytes and protect them from lipases. Polyphosphorylation of Plin A proteins leads to dissociation of Plin A and Plin A bound Abhd5, aka CGI-58 (originally named AB-hydrolase Containing 5), from lipid droplets. The stored lipids are, thus, exposed to lipases [125–127].

Insulin mediated inhibition of lipolysis occurs through Akt/PKB catalyzing phosphorylation of membrane bound PDE3B, activating it to degrade cAMP into 5'-AMP [128–130]. The resultant decrease in cAMP content reduces PKA activity leading to an overall decrease in the release of free fatty acids and glycerol. Decreased activity of PKA also leads to decreased phosphorylation of the inhibitor-1 protein (I-1) from the glycogen granule, releasing PP1 to catalyze the dephosphorylation of HSL and Plin A [131, 132]. These activities lead to further suppression of lipolysis mediated by insulin.

3.4 In vitro studies of intact cell systems

3.4.1 VO²⁺-chelates facilitating glucose uptake—Different intact cell systems have been used to investigate the basis of the influence of VO²⁺-chelates on metabolic pathways regulated by insulin. They are primary adipocytes obtained from surgically resected adipose tissue of rats, cultured 3T3-L1 adipocytes generated by inducing differentiation of 3T3-L1 fibroblasts, and Chinese hamster ovary cells genetically engineered to overexpress the human insulin receptor, abbreviated as CHO-HIR. Although there are differences in the fluxes of the metabolic pathways regulated by insulin and although the cell systems differ from each other, the results are in essential agreement, as evident through the summary of results in Table 2.

The Western immunoblot in Figure 19 compares the influence of three organic VO^{2+} -chelates on tyrosine phosphorylation in 3T3-L1 adipocytes in the absence and presence of added insulin [82]. In this case, the incubation medium for the serum-starved cells contained 0.25 mM chelate in the presence of 0.25 mM serum albumin. Albumin was included because of the earlier observation [32] that it enhanced the capacity of $\text{VO}(\text{acac})_2$ to facilitate glucose uptake. Immunoprecipitation of the tyrosine phosphorylated forms of $\text{IR}\beta$ and IRS-1 in Panels C and B, respectively, confirms that these two phosphorylated proteins, constituting the initiating point of insulin signaling, are contained within the bands labeled as $\text{IR}\beta$ and IRS1. Their known masses are $\sim 90,000$ Daltons for the β -subunit and $\sim 185,000$ Daltons for the IRS1 protein. Inspection of the intensity of the bands for each chelate with corresponding control basal lanes in the absence and presence of insulin shows that the order of activity is $\text{VO}(\text{acac})_2 > \text{VO}(\text{OPT})_2 > \text{VO}(\text{malto})_2$. The intensities of the bands show that $\text{VO}(\text{acac})_2$ exhibits prominent synergism with insulin. Synergism with $\text{VO}(\text{OPT})_2$ and with $\text{VO}(\text{malto})_2$ is equivocal when evaluated on the basis of intensities for both the $\text{IR}\beta$ and the IRS-1 bands. Bazuki *et al.* have made similar observations with the Zn^{2+} analog of $\text{VO}(\text{OPT})_2$ but did not test for synergism [133].

Reference to Figure 10 in which the tyrosine phosphorylative activity of the three chelates are compared with that of inorganic VO^{2+} in the presence of albumin and transferrin shows essentially the same rank order of chelate activity as in Figure 19 but also indicates that inorganic VO^{2+} weakly facilitates tyrosine phosphorylation of $\text{IR}\beta$ in addition to IRS-1 in the presence of albumin. This observation stands in apparent contradiction to the results of Pandey and coworkers, using CHO-HIR cells. They reported that the autophosphorylative activity of $\text{IR}\beta$ was not detected upon addition of inorganic VO^{2+} [13, 134]. The differentiating factor from that in Figure 19 is that the assay medium for CHO-HIR cells apparently did not include albumin according to their description of experimental conditions. The result of Srivastava and coworkers is explained by the observations of Shechter and coworkers [135, 136], who showed that VOSO_4 inhibited the insulin receptor tyrosine autophosphorylative kinase activity. According to their description of experimental methods, the latter investigators also did not add albumin to the assay medium. The observation in Figure 10 that inorganic VO^{2+} facilitates autophosphorylation of $\text{IR}\beta$, albeit weak, means that the presence of albumin enables entry of VO^{2+} into the cell with access to cytosolic enzymes constituting the insulin signaling pathway. Albumin receptors on the adipocyte cell surface [137] are the likely vehicle by which VO^{2+} -albumin complexes [68, 138] are internalized into the cytosol.

As schematically illustrated in Figure 18, tyrosine phosphorylated IRS-1 creates docking sites for recruitment of PI3K, resulting in the serine/threonine phosphorylation of Akt/PKB. Mehdi and Srivastava working with CHO-HIR cells showed with Western immunoblots, as illustrated in Figure 20, that $\text{VO}(\text{acac})_2$ facilitated phosphorylation of Akt/PKB more strongly than $\text{VO}(\text{malto})_2$ and VOSO_4 and that this activity was inhibited by wortmannin, a classical inhibitor of PI3K [139]. Furthermore, Figure 20 also shows correspondingly that inhibition of the catalytic activity of PI3K by wortmannin prevents phosphorylation of Akt/PKB and phosphorylation of GSK-3 β . Comparable results were reported for $\text{VO}(\text{acac})_2$ [82] and $\text{VO}(\text{pa})_2$ [140] added to 3T3-L1 adipocytes. Also, Sakurai and coworkers showed

that VO(3mpa)₂ [140] and VO(alx)₂ [141] promoted recruitment of GLUT4 to the plasma membrane of 3T3-L1 adipocytes.

As diagrammatically illustrated in Figure 18, these enzymes constitute the classical insulin signaling pathway in the cytosol regulating the uptake of glucose and its conversion into glycogen. The influence of VO(acac)₂ facilitating conversion of glucose into glycogen through its influence on the insulin signaling pathway is shown in Figure 21 confirming that the down regulation of GSK-3 β results in generation of glycogen catalyzed by glycogen synthase. Panel C of Figure 21 compares the influence of VOSO₄, VO(acac)₂, and insulin on tyrosine phosphorylation of 3T3-L1 proteins in the presence and absence of wortmannin. The intensities of the bands for tyrosine phosphorylated IR β and IRS-1 are identical in the presence and absence of wortmannin. Because wortmannin inhibits PI3K and thereby inhibits the production of glycogen, as seen in Panel B of Figure 21, the Western blot demonstrates that VO(acac)₂ does not activate Akt/PKB or glycogen synthase directly. In short, the target enzyme of VO(acac)₂ regulates the tyrosine phosphorylation level of the IR and IRS-1. The enzyme that has a major role in regulating the phosphorylative state of IR β and IRS-1 is PTP-1B [25]. Through kinetic studies we have shown that VO(acac)₂ acts as an *uncompetitive* inhibitor of PTP-1B in the presence of a physiologically relevant substrate containing a phosphotyrosinyl residue simulating residues 988 – 998 of the EGFR [81].

Shechter and coworkers have shown that VOSO₄ and VO(acac)₂ activate a purified cytosolic protein tyrosine kinase (cytPTK) in rat adipocytes that they have described as unrelated to the autophosphorylative kinase activity of IR β but that nonetheless participates in insulin-independent regulation of glucose metabolism [142, 143]. No more specific assignment of the metabolic action of cytPTK has been given. They have attributed all VO²⁺-mediated, insulin-like effects to this cytosolic enzyme. These interpretations are in direct conflict with the results illustrated through Figures 10 and 19 – 21 obtained with either intact 3T3-L1 or CHO-HIR cells. While assays of the partially purified, cytosolic PTK were carried out with the enzyme in a cell-free, 40,000g supernatant fraction with use of an artificial substrate polyGlu₄ Tyr [11, 135, 136], the lipogenesis assays were carried out with *intact adipocytes* suspended in Krebs-Ringer buffer containing *albumin* prepared according to the protocol of Moody *et al.* [144]. The assay consists of adding D-[2-³H]glucose to intact adipocytes and measuring the VO²⁺-stimulated radioactivity incorporated into lipid extracted into toluene after the prescribed incubation period. Figure 22 reproduced from Li *et al.* [136] compares VOSO₄ stimulated lipogenesis to that stimulated by VO(acac)₂ in *intact*, primary rat adipocytes. Although Shechter and coworkers attribute the vanadyl stimulated lipogenesis activity to the cytPTK enzyme [136], Figures 10 and 19 show that VO(acac)₂ and VOSO₄ are both associated with the capacity to facilitate tyrosine autophosphorylation of the IR β of intact cells in the presence of albumin, and that VO(acac)₂ activity is significantly greater than that by VOSO₄, as is reflected by the results in Figure 23. On the basis of results presented above from investigations carried out with intact CHO-HIR cells [139] and intact 3T3-L1 adipocytes [32, 82], the results in Figure 22 are ascribable only to the classical insulin signaling pathway outlined in Figure 18.

3.4.2 Inhibition of lipolysis by VO²⁺-chelates—The release of free fatty acids (FFA) from adipocytes and its inhibition by insulin has an important role in energy homeostasis of

the body. This is essentially the result of two simultaneously occurring processes: lipolysis and FFA re-esterification. In lipolysis, triglycerides are hydrolyzed to FFA and glycerol that are released from fat cells. While some of the FFA can be re-esterified to triglycerides, little of the glycerol is taken up and re-utilized by fat cells. Insulin promotes conversion of glucose into FFA and synthesis of triglycerides. It also inhibits lipolysis, as pointed out in Figure 18, through activation of PI3K and PDE3B, stimulating fractional FFA re-esterification and inhibition of release of FFA.

Over the last 20 – 25 years, an additional factor has been recognized as important in promoting glucose uptake and triglyceride synthesis—the acylation-stimulating protein (ASP)—that is generated in and secreted from adipocytes into the bloodstream [145–147]. This 76-amino acid protein promotes translocation of glucose transporter proteins to the cell membrane surface for glucose uptake in both muscle cells [146] and in adipocytes [148]. ASP exhibits antilipolytic activity [147] and promotes fractional FFA re-esterification by increasing the activity of diacylglycerol acyltransferase, the final enzyme in synthesis of triglycerides [149]. While ASP signaling involves the protein kinase C (PKC) pathway [150], its post-receptor action involves phospholipase C (PLC), PKC, and calcium-dependent phospholipase A₂, in addition to PI3K, Akt/PKB, and release of intracellular Ca²⁺, the latter three components participating also in signaling activated by insulin [148]. Because insulin stimulation of glucose transport in muscle cells [151] and in both rat and 3T3-L1 adipocytes [152] can be partially inhibited by specific inhibitors of PLC and PKC, components of ASP signaling, it is possible that overlap of ASP and insulin signaling activity occurs. With respect to the biological activity of VO²⁺-chelates, overlapping involvement of ASP signaling has never been considered. Although both ASP and insulin inhibit lipolysis via the action of PDE3B, it is not established whether there is involvement of the PDE4D isozyme also as part of the action of ASP [147]. Insulin activated hydrolysis of cAMP occurs only through the action of PDE3B. Activating insulin antilipolysis in adipocytes could, thus, invoke some participation by PDE4D because of the partial overlap of insulin and ASP signaling pathways.

Sakurai and coworkers have carried out a variety of studies demonstrating that VO²⁺-chelates inhibit the release of FFA from rat adipocytes stimulated upon addition of epinephrine [16, 31, 102, 140, 153–155]. As described with respect to Figure 18, epinephrine activates phosphorylation of a series of enzymes through PKA by stimulating production of cAMP. The antilipolytic action of insulin counters the production of cAMP by activating signaling through PI3K and Akt/PKB to activate PDE3B. The most complete studies from the Sakurai laboratory have been focused on a series of VO²⁺-chelates formed with analogs of picolinic acid. Figure 23 compares Western blots on the basis of which the dose-dependent capacity of VOSO₄, VO(pa)₂, VO(3mpa)₂, and VO(6mpa)₂ is illustrated resulting in the phosphorylation of Akt/PKB in 3T3-L1 adipocytes [140]. The extent of phosphorylation promoted by VOSO₄ is distinctly lower than that by VO²⁺-picolinate chelates. Figure 24, correspondingly, compares the capacity of the VOSO₄, VO(pa)₂, and VO(6mpa)₂ to inhibit epinephrine-stimulated release of FFA from rat adipocytes [154]. Only through careful examination of Figure 24, can one conclude that inhibition of FFA release follows the order VO(6mpa)₂ > VO(pa)₂ > VOSO₄, but the differences are very small. In the same study, differences in the capacities of each of the VO²⁺-compounds to

promote glucose uptake were greater and more marked [154]. The differences in the capacity to inhibit FFA release and to promote glucose uptake may reflect variations in the efficiency of coupling of signaling between pAkt/pPKB and GLUT4 translocation for glucose uptake and between pAkt/pPKB and activation of PDE3B for inhibition of FFA release. The differences may also reflect variations in the relative contributions of each VO^{2+} - compound to antagonize lipolysis via the insulin-signaling pathway or some another mechanism.

Measurement of inhibition of either epinephrine stimulated FFA release or glycerol release from adipocytes by a pharmacological reagent is not a sufficient criterion to claim that the reagent acts similarly to only insulin. The antilipolytic action of insulin involves both inhibition of lipolysis and re-esterification of FFA released from adipocytes. For this reason quantitative determination of both FFA and glycerol as the end products of lipolysis released into the incubation medium has been traditionally considered as a more accurate measure of cellular lipolysis [147, 156–158]. On this basis, increasing the insulin concentration should both reduce lipolysis and increase FFA re-esterification, reflected by a decrease in the FFA/ glycerol ratio.

Of interest is that Sakurai and coworkers have demonstrated that partial reversal of the antilipolytic action of $\text{VO}(\text{pa})_2$, $\text{VO}(\text{malto})_2$, and VOSO_4 is observed in the presence of wortmannin and cilostamide [145]. Although cilostamide is a specific inhibitor of PDE3B, that is activated by insulin (*cf.*, Figure 18), adipocytes also contain PDE4D. This latter isozyme is inhibited specifically by rolipram and is not sensitive to cilostamide. Only by testing for reversal of antilipolytic action with both inhibitors, cilostamide and rolipram, is it possible to distinguish between insulin-like antilipolysis and antilipolysis through a different pathway [159]. For instance, use of cilostamide and rolipram separately to test for reversal of inhibition of lipolysis facilitated by ginseng extract showed that its antilipolytic effect is mediated via PDE4D rather than through the insulin-signaling pathway [160]. In addition, the possible direct influence of VO^{2+} -chelates on PKA, PDE3B, and PDE4D has not been investigated. PKA is inhibited by VOSO_4 [161]; inhibition of PKA by VO^{2+} -chelates would result in an antilipolytic effect but not via an insulin signaling pathway. Microsomal, rabbit liver PDE4D is directly *activated* by vanadylglutathione and vanadyl-cysteamine complexes [162]. Other VO^{2+} -chelates may have a similar stimulatory effect not only on the PDE4D isozyme in adipocytes but also on PDE3B, resulting in antilipolysis not dependent on the insulin signaling pathway.

Liu and coworkers have compared the antilipolytic capacity of $\text{VO}(\text{acac})_2$, $\text{VO}(\text{malto})_2$, and sodium metavanadate (Na_3VO_4) on the basis of Western immunoblots together with glycerol release from 3T3-L1 adipocytes in the presence of glucose [163]. In contrast to the investigations from the Sakurai laboratory, epinephrine was not used to stimulate lipolysis in these experiments. Therefore, observations of Liu and coworkers relate only to basal antilipolytic activity in the cell. Liu and coworkers observed that the antilipolytic capacity of the vanadium compounds followed the order $\text{VO}(\text{acac})_2 > \text{VO}(\text{malto})_2 \gg \text{VO}_4^{3-}$. Western immunoblot analysis of the influence of $\text{VO}(\text{acac})_2$ to counteract enzymes underlying lipolysis is illustrated in Figure 25. The results show that potentiation of the insulin signaling pathway by $\text{VO}(\text{acac})_2$ to result in phosphorylation of Akt/PKB caused decreased intensity

of the bands for phosphorylated HSL and phosphorylated perilipin (labeled pHSL^{ser660} and p-Perilipin, respectively in Figure 25A), as expected. As explained through Figure 18, the insulin signaling pathway for antilipolysis results in phosphorylation of PDE3B catalyzed by phosphorylated Akt/PKB causing conversion of cAMP to 5'-AMP to reduce the activity of PKA. Decreased generation of p-HSL and p-Perilipin potentiated by VO(acac)₂ is also evident in Figure 25B. The action of wortmannin or of the Akt/PKB inhibitor labeled *Akti* in the absence of VO(acac)₂ abolishes the antilipolytic effect via the insulin signaling pathway. Therefore, inhibition of the insulin signaling pathway by wortmannin or Akti in the presence of VO(acac)₂ would be expected similarly to inhibit antilipolytic action if the VO²⁺-chelate acted purely via the insulin signaling pathway. While it is possible that inhibitor concentrations should be higher in the presence of VO²⁺-chelates, there is considerable loss of intensity of the band for phosphorylated perilipin that is difficult to rationalize. This observation suggests that VO(acac)₂ may also exert a direct, activating influence on one of the phosphodiesterase isozymes. The results in Figures 23 – 25 indicate that the antilipolytic action of VO²⁺-chelates in adipocytes occurs at least in part through the insulin signaling pathway. However, clarification of the mechanism of action of VO²⁺-chelates through use of cilostamide and rolipram separately is needed to test for the influence of chelates on both phosphodiesterase isozymes. Also, as in the case of 3T3-L1 adipocytes in Figures 10 and 19, testing for synergism of chelate action on antilipolysis in adipocytes would help to establish specificity of chelate action.

3.4.3 Influence of VO(acac)₂ in the absence of GLUT4—The most frequently used tracer molecule in positron emission tomography (PET) imaging for detection and characterization of cancer lesions is 2-(fluorine-18)-2-deoxy-D-glucose (FDG). However, because glucose competes with FDG for entry into cells via the same glucose transporter systems [164], imaging based on FDG relies not only on tumor size but also on the local metabolic characteristics of the type of cancer. FDG PET imaging relies on the differential uptake of FDG by the malignant tumor and the surrounding normal tissue; however, some types of breast cancer are characterized by low glucose uptake and give rise to false negatives [165–167]. Because we have found that VO(acac)₂ displays the greatest capacity to enhance cellular glucose uptake and exhibits far greater stability in solution compared to VO(malto)₂ and VO(OPT)₂ [32, 82], we tested whether VO(acac)₂ exhibits the capacity to enhance FDG uptake by malignant cells.

The upper panel of Figure 26 compares coronal PET images of FDG uptake by MDA-MB-231 cultured human breast carcinoma cells in 9 wells of a 12-well plate [168]. Quantification of PET signal intensity due to FDG uptake, as described earlier, is displayed in the lower panel showing an approximate linear dependence of enhanced FDG uptake over the 1 – 8 μ M range of VO(acac)₂. The uptake is significant because the experiments were carried out in the presence of a background 5 mM glucose concentration, equivalent to the fasting level of blood glucose in a normal adult. Enhanced FDG uptake was also observed in the presence of a 10 mM background glucose concentration although the signal intensity was more attenuated.

Although the results in Figure 26 show that VO(acac)₂ promotes increased FDG uptake by cultured cancer cells, the *in vivo* physiological factors regulating delivery of an agent to a

specific site and tissue differ greatly from *in vitro* conditions of cultured cells. Therefore, to extend these experiments further, we investigated whether treatment of a small laboratory animal such as severe, compromised, immunodeficient (SCID) mice with VO(acac)₂ prior to FDG PET imaging would enhance the intensity contrast between cancer tissue of a xenograft tumor and the normal surrounding tissue. Because, as illustrated in Figure 9, MRI imaging experiments showed that there was slow, gradual accumulation of VO(acac)₂ into the xenograft tumor over a period of up to 3.5 – 4 hours after injection, we tested whether enhanced FDG uptake depended on the time interval between ip injection of VO(acac)₂ and the onset of FDG PET imaging. An early result from these studies is illustrated in Figure 26, whereby maximum FDG uptake was observed approximately 4 hours post-VO(acac)₂ injection, closely correlated with the time of 3.5 – 4 hours in Figure 9 to reach a plateau in the accumulated VO(acac)₂ content of tumor tissue. As seen in Figure 27, longer intervals between the time of ip injection of VO(acac)₂ and the onset of FDG PET imaging resulted in decreased enhancement of FDG uptake by xenograft tumor tissue. While the maximum uptake is greater than 2-fold in Figure 26, we have continued these experiments and have observed up to 6-fold enhanced FDG uptake compared to controls. Clearly optimal conditions to establish dose dependence and exact time interval of chelate injection prior to imaging need to be established. Nevertheless, these preliminary results indicate that use of VO(acac)₂ as a pharmacologic agent to enhance FDG uptake by malignant tissue has the potential to improve detection of both small tumors and tumors of low metabolic activity.

The results shown in Figures 26 and 27 were not predictable because MDA-MB-231 cells are not insulin sensitive, and use of VO²⁺-chelates to augment insulin-like activity has been directed hitherto primarily to insulin-sensitive tissues and cells. In the case of cancer cells, glucose uptake occurs through the GLUT1 protein, the expression and translocation of which is under very different regulation and control than that of GLUT4 in insulin sensitive tissues. The observation of FDG uptake facilitated by VO(acac)₂ in cancer tissue elicits the questions What is the likely mechanism of its action? and Does the mechanism differ from that in insulin sensitive cells? We have shown in earlier studies that VO(acac)₂ acts as an uncompetitive inhibitor against PTP-1B in the presence of a physiologically relevant substrate such as the phosphotyrosine-containing undecapeptide simulating residues 988 – 998 of the EGFR [81]. PTP-1B is critical to the regulation of cell signaling processes in normal, diabetic, and cancer cells. Also, IRS proteins act as important signaling intermediaries in normal and cancer cells (for instance, see ref. [169]). Inhibition of PTP-1B prolongs the phosphorylative lifetime of IRS proteins, thereby increasing the cellular content of phosphorylated Akt, a constitutive enzyme that triggers a cascade of responses in cancer cells including increasing the GLUT1 content of the plasma membrane. On this basis, the molecular basis of the capacity of VO(acac)₂ to facilitate glucose uptake by cancer cells is consistent with its action in insulin-sensitive cells, namely inhibition of PTP-1B prolonging the cellular lifetime of phosphorylated signaling intermediates responsible for regulating glucose uptake.

4. Conclusions

A large number of vanadium compounds has been synthesized as potential therapeutic agents for treatment of diabetes mellitus. Hitherto none has proven efficacious for long-term

use in humans. One may well, therefore, ask the question in view of this vast quantity of experimental results and expended effort What have we learned that may contribute for improved development of vanadium compounds for therapeutic purposes? To this end we have restricted our review to experimental results in which vanadyl (VO^{2+}) chelates were employed because only this class of vanadium compounds has been tested in primary or cultured cell systems and in small, laboratory animals through which investigations pharmacologic and pharmacokinetic properties of the vanadium compounds have been evaluated.

Analysis of published results showed that the likely most important property of a VO^{2+} -chelate, in addition to activating insulin signaling, is high stability in solution and in interaction with serum transport proteins. Small molecules such as VO^{2+} -chelates cannot be expected to simply diffuse through membranes. They must undergo translocation from the bloodstream through the endothelium of capillaries into the interstitial fluid. Because it is important that a VO^{2+} -chelate survive these interactions with macromolecules for cell entry, further research must address the mechanisms by which these processes occur. Our analysis of the literature suggests that stability and binding to serum albumin will play an important role in further development of VO^{2+} -chelates as therapeutic agents.

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Highlights

Organic vanadyl (VO^{2+}) chelates used as probes of insulin mimesis have been reviewed.

Properties needed for potential therapeutic application have been identified.

Long-term stability and retention of structure when bound to albumin are necessary.

Synergism of a chelate with insulin is suggested as a criterion for drug development.

Only *bis*(acetylacetonato)oxovanadium(IV), thus far, fulfills these requirements.

Bis(acetylacetonato)oxovanadium(IV)

Bis(maltolato)oxovanadium(IV)

Figure 1.

Illustration of the chemical bonding structures of $\text{VO}(\text{acac})_2$ (upper) and $\text{VO}(\text{malto})_2$ (lower) shown as square pyramidal complexes each with four, equatorial oxygen-donor atoms from the two chelating ligand molecules. The atomic numbering of the hydrogen atoms in the maltolate ligands illustrates the standard nomenclature for its designation as 3-hydroxy-2-methyl-4-pyrone. The lower part of the diagram compares *cis* and *trans* conformations of the coordinating ligands. *S* represents a solvent molecule in the *cis* conformation. Both complexes exhibit *trans* conformations in crystals, as determined

through X-ray crystallographic studies [30, 62, 63]. In solution, $\text{VO}(\text{acac})_2$ remains in a *trans* conformation while $\text{VO}(\text{malto})_2$ acquires an equatorially coordinated solvent molecule and one of the maltolato ligands switches an oxygen-donor atom to the axial site opposite the vanadyl oxygen atom. Adapted from ref. 60.

Figure 2.

Stereo graphics model of the structure of VO(malto)_2 as found in methanol constructed according to ENDOR determined vanadium-proton distances [60] and rendered in stick-diagram form. The stick diagram rendering allows illustration of the methanol molecule more clearly that is acquired as an equatorial ligand whereby the 4-keto group (*cf.*, Figure 1) in one of the maltolate ligands changes from an equatorial position to occupy the axial coordination site. A methanol molecule hydrogen-bonded to the vanadyl oxygen atom is also shown identified spectroscopically [60]. Atomic coloring scheme: C, green; H, white; O, red; V, cyan. From ref. 60 with permission of the copyright holder.

Figure 3.

Stereo graphics model of the structure of $\text{VO}(\text{acac})_2$ in methanol constructed according to ENDOR determined vanadium-proton distances, as described in ref. 60. The upper structure illustrated in stick-diagram form shows the planar, *trans* conformation of the equatorially coordinated acetylacetonate ligands with two methanol molecules, one coordinated axially and the second hydrogen-bonded to the vanadyl oxygen atom. The lower structure shows the complex with space-filling atomic rendering in the same projection as for the upper structure. From ref. 60 with permission of the copyright holder. Atomic coloring scheme as in Figure 2.

Figure 4.

Comparison of EPR spectra of VO(acac)₂ in methanol (upper set) and in H₂O (lower set) as a function of [H⁺]. The vertical lines identify the high- and low-field components of the different species detected: A', B', and C' in methanol; A, B, C, and D in H₂O. See ref. 32 for description of conditions under which the spectra were collected and for explanation of the double integration of the spectra to estimate relative concentrations of each species. From ref. 32 with permission of the copyright holder.

Figure 5.

Titration of $\text{VO}(\text{acac})_2$ in H_2O by addition of small aliquots of aqueous HCl or NaOH . The data were obtained by double integration of EPR spectra of the type illustrated in Figure 4 collected as a function of pH. The symbols indicate the direction of titrations: *, from low pH to high pH; and $\circ$ from high pH to low pH, to indicate complete reversibility of the prototropic ionizations. The solid lines are theoretical curves calculated for single ionizations to represent the distribution of each species (A + B), C, and D and fitted by a non-linear, least-squares method to the data. The spectral features for species A and B could not be separated because of their overlapping contributions. f represents the fractional content of a spectroscopically defined VO^{2+} species such that $\sum f_i = 1.0$. See ref. 32 for further details of the fitting procedure. From ref. 32 with permission of the copyright holder.

Figure 6.

ENDOR spectra of $VO(acac)_3$ for species A' at low $[H^+]$, *i.e.*, high pH, (solid line) and species C' at high $[H^+]$, *i.e.*, low pH, (dotted line) in methanol. The pair of features seen in the $-7/2_{\parallel}$ spectrum is also seen in the $-3/2_{\perp}$ spectrum with an added pair of prominent ENDOR features having twice the splitting. These observations assign the features to the $A_{\parallel}$ and $A_{\perp}$ hyperfine couplings of the methyl hydrogen atoms and specify that they are located in the equatorial plane (*cf.*, refs. 32, 60, and 73). The weak features observed only in the $-3/2_{\perp}$ spectrum belong to the hydrogen atom covalently bonded to C(3) (confirmed by

selective deuteration). The identical spectral features of species C' and species A' according to amplitude and line width demonstrate that there is no change in ligand coordination geometry or vanadium : ligand stoichiometry as a function of $[H^+]$. Furthermore, the observation of only two pairs of spectral features for the methyl protons requires the complex to exhibit 2-fold rotational symmetry. The abscissa indicates the ENDOR shift where 0 MHz designates the proton Larmor frequency. See ref. 32 for further details. From ref. 32 with permission of the copyright holder.

Figure 7. Concentration distribution curves calculated for VO(malto)₂ from pH titration data. In ref. 66, the pH titration data were not supplied; only the theoretical curves are illustrated. The labels [VOA₂], [VOA]⁺, etc. represent the vanadyl species belonging to each curve according to the interpretations of Buglyo and co-workers [66]. The curves are calculated for [VO²⁺] = 0.002 M and vanadium:maltolate ratio of 1:2. Adapted from ref. 66.

Figure 8.

(Panel A) Comparison of the $-7/2_{\parallel}$ and $-3/2_{\perp}$ ENDOR spectra of $\text{VO}(\text{acac})_2$ as a function of increasing albumin concentration relative to vanadium. The blue spectra are of free $\text{VO}(\text{acac})_2$ in the absence of albumin. Albumin : VO^{2+} ratios are: yellow, 1.0; green, 2.0; red, 5.0; black, 10.0. The increasing amplitude of resonance absorptions seen in the frequency region immediately adjacent to the Larmor frequency (0 MHz) arise from protons associated with amino acid residues of the protein. The amplitude of these features upon subtraction of that of the free chelate is plotted in Panel B as a function of the BSA : VO^{2+} . In Panel B, the theoretical line is calculated for saturation of the protein with chelate for one binding site and the intersection of the asymptotes yields a 1:1 BSA: $\text{VO}(\text{acac})_2$ adduct. While the K_D of 1×10^{-6} M could be estimated from these data, a more precise evaluation by isothermal calorimetry of 2×10^{-7} M was made later [77]. The figure was reconstructed from diagrams in ref. 32.

Figure 9.

Time-series plots illustrating the accumulation of paramagnetic contrast agents in a xenograft prostate tumor generated in the hind leg of a rat. The intensities for both $\text{VO}(\text{acac})_2$ and the Gd^{3+} contrast agent were measured from high-resolution T_2^*/T_1 – weighted magnetic resonance images acquired with an echo planar spectroscopic imaging sequence at 4.7 Tesla. The plots were averaged over five *in vivo* experiments for each paramagnetic species. Time along the plot is indicated by the double-headed arrow beginning with injection of $\text{VO}(\text{acac})_2$ or the Gd^{3+} contrast agent into the tail vein of the rat. It is seen that there is a near-immediate accumulation and wash-out of both $\text{VO}(\text{acac})_2$ and the Gd^{3+} chelate from the region of the tumor followed by a slow, time-dependent uptake of the VO^{2+} -species into tumor tissue. There is only low uptake of the Gd^{3+} chelate in the region of the tumor followed by near immediate washout from both tumor and the surrounding muscle tissue. This type of uptake and washout profile is anticipated for Gd^{3+} contrast agents remaining in the vascular blood pool. On the other hand, there is slow accumulation of the paramagnetic VO^{2+} -species into tumor tissue reaching a plateau after 3.5 – 4 h. ENDOR spectra of tumor tissue separated surgically from the surrounding muscle tissue showed spectra comparable to those in Figure 6 confirming that the VO^{2+} -chelate providing the paramagnetism as a contrast agent was the intact $\text{VO}(\text{acac})_2$ chelate. Adapted from ref. 77.

Figure 10.

Comparison of the effect of albumin and transferrin on the capacity of VO²⁺-chelates to enhance tyrosine phosphorylation in 3T3-L1 adipocytes. Insulin in the indicated amounts and 0.25 mM vanadyl compounds were added to adipocytes in the presence of either 0.25 mM albumin or 0.25 mM transferrin. Adipocytes had been previously serum-starved as described [82] and were transferred to HEPES-buffered Krebs-Ringer solution prior to addition of albumin or transferrin and VO²⁺-chelates. See ref. 82 for further details. From ref. 82 with permission of the copyright holder.

Figure 11.

Change in plasma glucose levels of STZ treated male Wistar rats following oral gavage administration of VO(malto)₂ or VO(Etmalto)₂. Upper panel: Blood glucose levels over time following administration of (●) VO(malto)₂ or (○) VO(Etmalto)₂. The dose was 0.6 mmol/kg dissolved in water (*n* = 40 for each chelate). Lower panel: Plasma glucose levels measured after 20 hr following oral gavage administration at indicated dosage levels (for combined data sets *n* = 20). Means not sharing a common superscript are significantly different (*P* < 0.05). Adapted from ref. 33.

Figure 12.

Plasma vanadium ($\blacktriangle$) and plasma ^{14}C ($\bullet$) concentrations after a single bolus of $\text{VO}(\text{Etmalto})_2$ synthesized with [2-ethyl-3-hydroxy-[2- ^{14}C]pyrone (50 mg/kg in 1% carboxymethylcellulose in a volume of 10 mL/kg) and administered to STZ male Wistar rats. Data are obtained from nine groups of rats ($n = 4$) at time points 0.5, 1, 2, 4, 8, 16, 24, and 48 hr following vanadium chelate administration. Adapted from ref. 33.

Figure 13.

Comparison of the time dependent changes in plasma glucose levels of STZ treated male Wistar rats after a single ip injection (see downward arrow) of a vanadyl compound (1.275 mg V/mg or 0.025 mmol/kg): VO(malto)₂, ▼; VO(3Etacac)₂, ●; VO(acac)₂, ■; VOSO₄, ○. Values indicated by the symbols are the means of eight rats with standard errors < 10%. * $P < 0.05$ or less vs. plasma glucose level at day 0. Adapted from ref. 17.

Figure 14.

Time dependent decrease in the intensity of EPR absorptions and ENDOR absorptions of covalent hydrogen atoms specific for the ligand of each VO^{2+} -chelate in HEPES-buffered Krebs-Ringer solution at pH 7.4. Solutions were identical to those in Figure 10 for addition of albumin to simulate conditions under which vanadyl compounds were added to adipocytes. The data in each case adhered to a single exponential decay with half-lives estimated as 4, 6, and 4000 days for $\text{VO}(\text{malto})_2$ (□, ■); $\text{VO}(\text{OPT})_2$ (△, ▲); and $\text{VO}(\text{acac})_2$ (○, ●), respectively. Filled symbols represent ENDOR measurements. Adapted from ref. 82.

Figure 15.

Histogram comparison of organ distribution of total vanadium accumulated in STZ diabetic Wistar rats after 14 days of daily ip injections of $\text{VO}(\text{5Ipa})_2$ (*cf.*, ref. 100 for schedule of dosage changes over the 14 day period) followed by 14 days of no vanadium injections. Results are presented as mean $\pm$ s. d. for $n = 4$ rats. Adapted from ref. 100.

Figure 16.

Schematic illustration of the coordination environment of VO^{2+} incorporated into hydroxyapatite. The structure is based on the analysis and interpretation of superhyperfine couplings of nearby ^1H and ^{31}P nuclei measured by ESEEM spectroscopy. It should be noticed that the vanadium species is rightfully assigned as VO^{2+} because of paramagnetism. However, Dikanov and coworkers have indicated three mono-anionic phosphate oxygens coordinating the central VO^{2+} moiety. One of the phosphate-oxygens should have been indicated as the keto-like $\text{O}=\text{P}$; likely the axial oxygen would best be assigned to this neutral species. Adapted from ref. 105.

Figure 17.

Histological comparison of progression of healing of fracture site induced in the right femur of rats treated locally with saline (control), 1.5 mg VO(acac)₂/kg, or 3.0 mg VO(acac)₂/kg. The micrographs obtained with a stereomicroscope compare the changes in the intra-medullary region of the bone under $1.67 \times$ magnification from 10 to 21 days post-fracture. Adapted from ref. 110.

Figure 18.

Diagrammatic illustration of insulin-signaling pathways regulating glucose uptake and antilipolytic action. See text for discussion. Abbreviations: AC (adenylyl cyclase), Akt (Protein Kinase B or PKB), AS160 (Rab GTPase-activating protein), ATGL (adipose triglyceride lipase), cAMP (cyclic-adenosine monophosphate, i.e., 3',5'-cyclic adenosine monophosphate), CGI-58 (comparative gene identification 58 is a lipid droplet-associated protein), DAG (diacylglycerol), FFA (free fatty acid), GPCR (heterotrimeric- $\alpha\beta\gamma$ - G-protein coupled receptor), GSK-3 (glycogen synthase kinase-3 β), GSV (GLUT4 storage vesicle), HSL (hormone sensitive lipase), IR (insulin receptor), IRS (insulin receptor substrate protein), MAG (monoacylglycerol), P (phosphoryl group), P85 (regulatory subunit of phosphatidylinositol-3-kinase of molecular weight 85,000), PDK1 (phosphoinositide-dependent protein kinase-1), PDE3B (phosphodiesterase 3B), PI3K (phosphatidylinositol 3' kinase), PIP₂ (phosphatidylinositol-4,5-bisphosphate), PIP₃ (phosphatidylinositol-3,4,5-trisphosphate), PKA (protein kinase A), Plin A (perilipin A), PP2A (protein phosphatase-2A), R (regulatory subunit of PKA that binds cAMP), TAG (triacylglycerol),.

Figure 19.

Comparison of the capacity of VO²⁺-chelates to facilitate tyrosine phosphorylation in 3T3-L1 adipocytes in the presence and absence of insulin. Adipocytes were serum starved and suspended in HEPES-buffered Krebs-Ringer solution pH 7.4 containing 0.25 mM serum albumin and incubated with the indicated amount of insulin and 0.25 mM VO²⁺-chelate for 5 min. Panel **A**: cell lysates were used for immunoblotting with anti-phosphotyrosine antibody. In Panel **B** cell lysates were subjected to immunoprecipitation with anti-tyrosine phosphorylated insulin receptor substrate-1 antibody; in Panel **C** lysates were subjected to

immunoprecipitation with anti-tyrosine phosphorylated β -subunit of the insulin receptor antibody to verify that enhanced phosphotyrosine bands contained IRS-1 and IR β proteins of the expected mass (185,000 and 90,000 Daltons, respectively). Adapted from ref. 82.

Figure 20.

Western immunoblots demonstrating that phosphorylation of Akt/PKB and GSK-3β induced by vanadyl compounds is dependent on PI3K activity. In this case, CHO-HIR cells were used incubated in the presence or absence of 100 nM wortmannin (W) for 30 min followed by incubation with either 100 nM insulin or 1 mM VO(acac)₂, VO(malto)₂, or VOSO₄ for 10 min. Anti-phospho-antibodies were used specific for Ser-473 of Akt/PKB in Panel A or Ser-9 of GSK-3β in Panel B in their phosphorylated state and antibodies specific for each enzyme to measure total enzyme content. It is seen that the capacity to induce phosphorylation of Akt/PKB follows the order: VO(acac)₂ > VO(malto)₂ >> VOSO₄. The absence of phosphorylation of Akt/PKB in the presence of wortmannin demonstrates that when PI3K is inhibited, Akt/PKB is not activated and, correspondingly, GSK-3β is not down regulated by Akt/PKB catalyzed phosphorylation. Adapted from ref. 139.

Figure 21.

Influence of VO(acac)₂ on glycogen synthesis induced through signal transduction. Serum starved 3T3-L1 adipocytes were treated in the absence or presence of 200 nM wortmannin for 15 min prior to addition of 10 nM insulin in the presence or absence of 0.25 mM VO(acac)₂ or VOSO₄, as indicated. Panel **A** : Lysates were analyzed with anti-phospho-GSK-3 β antibody. The immunoblots show that VO(acac)₂ in the absence and presence of insulin stimulates phosphorylation of GSK-3 β that is blocked by wortmannin. Panel **B** demonstrates that glycogen synthesis is stimulated by VO(acac)₂ in the absence and

presence of insulin and that this action is blocked by wortmannin (because of inhibition of PI3K as shown in Figure 20). The glycogen synthase (GS) activity ratio is measured with the substrate UDP- ^{14}C glucose as the activity of GS in the absence and presence of glucose-6-phosphate. While GS is maximally active in its totally dephosphorylated form, partially phosphorylated forms exhibit varying degrees of activity. Glucose-6-phosphate, as an allosteric activator, recruits all phosphorylated and dephosphorylated forms of GS, thus providing a means to measure the activity of the total cellular content of GS. Panel C compares anti-phospho-tyrosine immunoblots of adipocytes stimulated with $\text{VO}(\text{acac})_2$, or VOSO_4 in the absence or presence of insulin and in the absence or presence of wortmannin. The immunoblot demonstrates that the phosphorylation of IRS-1 and $\text{IR}\beta$ stimulated by $\text{VO}(\text{acac})_2$ (or $\text{VO}(\text{acac})_2$ in the presence of insulin) is not affected by wortmannin indicating that the site of $\text{VO}(\text{acac})_2$ action must lie proximal to PI3K. Thus, $\text{VO}(\text{acac})_2$ influences the enzyme that regulates the phosphorylation state of IRS-1 and $\text{IR}\beta$, namely PTP-1B [81]. The immunoblot in Panel C also demonstrates that the phosphorylation levels of IRS-1 and $\text{IR}\beta$ stimulated by $\text{VO}(\text{acac})_2$ in the presence of insulin is much greater than the sum of their individual effects. As pointed out earlier, this observation indicates synergism of $\text{VO}(\text{acac})_2$ action with that of insulin. Adapted from ref. 82.

Figure 22.

Comparison of the capacity of VO(acac)₂ and VOSO₄ to activate lipogenesis in *intact* rat adipocytes. Lipogenesis was assayed according to the protocol of Moody [144] for 1 hr at 37 °C with approximately 1.5×10^5 cells/vial suspended in Krebs-Ringer buffer supplemented with 0.2 mM D-[2-³H]glucose and VO(acac)₂ or VOSO₄. Although Shechter and coworkers [136, 142, 143] have identified a cytosolic protein tyrosine kinase in separate experiments that they ascribe to underlie the observed lipogenic action, the results in this diagram are compatible only with activation of lipogenesis through the classical insulin signaling pathway that both VO(acac)₂ and VOSO₄ influence, as shown through Figures 19 – 21 and as discussed in the text. Adapted from ref.136.

Figure 23.

Comparison of the dose-dependence of analogs of VO(pa)₂ to promote phosphorylation of Akt/PKB. **A** [VOSO₄]; **B** [VO(pa)₂]; **C** [VO(3mpa)₂]; **D** [VO(6mpa)₂]. Serum starved 3T3-L1 adipocytes were treated with 100 nM insulin or VO²⁺-chelate at the indicated concentrations for 5 min at 37 °C. Adapted from ref. 140.

Figure 24.

Histogram illustration of the dose-dependent inhibition of FFA release from intact rat adipocytes by analogs of VO(pa)₂. Adipocytes were incubated with insulin or vanadium compounds at the indicated concentrations for 30 min followed by addition of 10 μ M epinephrine and further incubated for 3 hr at 37 °C. Values represent the mean from 3 experiments $\pm$ s. d. VO²⁺-chelate concentrations are given in mM; insulin concentrations are given in nM. Adapted from ref. 154.

Figure 25.

Influence of VO(acac)₂ on antilipolysis in 3T3-L1 adipocytes. (A) Adipocytes were preincubated with or without wortmannin (0.1 μM) or Akti (2 μM) for 1 h and then treated with VO(acac)₂ for 1 h. The numbers indicating *Relative intensity* represent densitometric estimates of the intensity of the corresponding bands normalized to that of control. (B) Adipocytes were preincubated with or without the indicated concentrations of the MEK inhibitor (MEK1) at 0.1, 0.2, and 0.5 μM for 30 min and then treated with 0.1 mM VO(acac)₂ for 1 hr. Abbreviations: pHSL^{ser660}, hormone sensitive lipase phosphorylated at Ser660; p-Perilipin, phosphorylated Perilipin; pAkt, phosphorylated Akt/PKB; pERK, phosphorylated extracellular-signaling-kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Adapted from ref. 163.

Figure 26.

(Upper panel) Coronal images of FDG uptake by MDA-MB-231 cells cultured in 9 wells of a 12-well plate. VO(acac)₂ concentrations: Well 1 (0 μ M), Well 2 and 5 (5 μ M), Well 3 and 6 (10 μ M), Well 4 and 7 (15 μ M), Well 8 and 9 (25 μ M). Color is proportional to uptake of FDG (Blue, least intense; Red, most intense). (Lower panel) Scatter plot depicting the fractional population of voxels with intensity due to FDG uptake above the mean of cells in the control well (absence of VO(acac)₂) as a function of VO(acac)₂ concentration. Cf., ref. 168 for details of treatment of cells prior to addition of FDG. Adapted from ref. 168.

Figure 27.

Comparison of relative FDG uptake per unit volume of xenograft tumor tissue in female SCID mice as a function of the time interval between ip injection of VO(acac)₂ (approximately 0.0124 g VO(acac)₂/kg body weight dissolved in DMSO) and onset of FDG PET imaging. The relative FDG uptake was normalized according to body weight and the standardized FDG concentration in the liver. The control mouse received the equivalent volume of DMSO by ip injection. Each histogram bar represents the FDG uptake by the tumor tissue of a mouse measured at the indicated time post-VO(acac)₂ injection. There is an estimated $\pm 20\%$ uncertainty in measured FDG uptake values. Adapted from ref. 168.

Table 1

EPR Spectroscopic Parameters of Species of *bis*(acetylacetonato)oxidovanadium(IV) in Water and Methanol as a Function of pH or pH*, respectively.^{a,b,c}

Species	Solvent	$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}$	$A_{\perp}$	g_o	A_o
A	H ₂ O	1.946	1.976	182.0	59.0	1.966	99.5
A'	CH ₃ OH	1.946	1.978	185.5	60.0	1.967	101.5
B ^d	H ₂ O	1.945	1.974	185.0	64.0	1.964	104.0
B' ^d	CH ₃ OH	1.948	1.976	181.0	65.0	1.967	103.0
C	H ₂ O	1.939	1.982	192.5	67.0	1.968	109.0
C'	CH ₃ OH	1.940	1.979	194.0	68.0	1.966	111.0
D	H ₂ O	1.934	1.977	201.0	74.0	1.964	116.0

^a pH* in methanol solutions was measured as described by Bates [170].

^b Values of g are estimated to within ± 0.001 ; values of A are estimated to within ± 0.5 G. The microwave frequency was 9.432 GHz. Anisotropic spectroscopic parameters ($g_{\parallel}$, $g_{\perp}$, $A_{\parallel}$, $A_{\perp}$) were determined by spectral fitting of frozen solution spectra, and isotropic parameters (g_o , A_o) were determined from fluid solution spectra.

^c From ref. 32.

^d Overlapping resonance components of species A, A' allows only an approximate estimate

Table 2

Comparison of Influence of Vanadyl (VO^{2+}) Chelates on Cytosolic Insulin Signaling Pathways in Primary and Cultured Eukaryotic Cells and on Plasma Glucose Concentration in Small Laboratory Animals after Acute Oral or Intraperitoneal Administration.

Chelate	Test System	Results	Refs.
VO(acac)₂ VO(acac)₂	3T3-L1 adipocytes CHO-HIR cells male STZ Wistar rats	VO(acac) ₂ facilitated increased tyrosine phosphorylation (pY) of IRS1 and IRβ in 3T3-L1 adipocytes. VO(acac) ₂ facilitated significantly more pY than VO-(malto) ₂ , VO(OPT) ₂ and VOSO ₄ . VO(acac) ₂ also increased concentration of pAkt and pGSK-3β in treated cells that was inhibited by wortmannin. Wortmannin did not affect tyrosine phosphorylation of IRS1 and IRβ. Single ip injection (0.025 mmol/kg) decreased plasma glucose to near-normal levels for 5 days	[82] [139] [17]
VO(malto)₂ VO(malto)₂	3T3-L1 adipocytes CHO-HIR cells male STZ Wistar rats male STZ diabetic ddY mice	Tyrosine phosphorylation of IRS1 and IRβ facilitated although not as prominently as observed with VO(acac) ₂ . VO(malto) ₂ similarly promoted increased phosphorylation of Akt/PDB and GSK-3β. Similar results as for 3T3-L1 adipocytes; also inhibition of overexpressed PTP-1B in CHO cells showed VO(acac) ₂ > VO(malto) ₂ >> VOSO ₄ . Single oral (0.6 mmol/kg) dose or ip injection (0.1 mmol/kg) sustained decreased plasma glucose to near-normal levels for 2 days. Similar results obtained for 2-ethyl and 2-isopropyl analogs. Single ip injection (0.1 mmol/kg) resulted in decreased plasma glucose within 18 h that was sustained ~ 55 hr. Identical results with use of VO(Etmalto) ₂ .	[82] [139] [15, 33, 61, 98] [37]

Chelate	Test System	Results	Refs.
VO(alx)₂ VO(alx)₂	3T3-L1 adipocytes primary rat adipocytes male STZ diabetic ddY mice	VO(alx) ₂ facilitated enhanced pY of IRS1 and IRβ and increased phosphorylation of PI3K and GSK-3β that was sensitive to wortmannin. VO(alx) ₂ -promoted translocation of GLUT4 was inhibited by wortmannin. VO(malto) ₂ and VOSO ₄ enhanced phosphorylation of IRS1 and IRβ was not observed, in contrast to reports by others [82, 139], likely because of low concentrations (50 μM) and absence of albumin in the incubation medium. VO(alx) ₂ exhibited highest capacity (IC ₅₀ = 0.55 mM) to inhibit epinephrine stimulated FFA release compared to that of VO(malto) ₂ (IC ₅₀ = 0.67 mM) and VO(Etmalto) ₂ (IC ₅₀ = 0.63 mM). Single ip injection (0.1 mmol/kg) resulted in decreased plasma glucose within 18 hr that was sustained ~ 55 hr.	[141] [37] [37]
VO(OPT)₂ VO(OPT)₂	3T3-L1 adipocytes primary rat adipocytes normal male Wistar rats	VO(OPT) ₂ facilitated increased pY in IRS1 and IRβ with capacity greater than that of VO(malto) ₂ in the presence of either serum albumin or transferrin. (<i>cf.</i> , VO-acac) ₂ above. Inhibited epinephrine stimulated FFA release in a dose-dependent manner with an IC ₅₀ = 0.19 mM. Real-time <i>in vivo</i> measurement by EPR monitoring of paramagnetism of VO(OPT) ₂ and VOSO ₄ in the bloodstream after a single iv injection (~0.01 mmol/kg) showed a t _{1/2} of 14.6 and 5.2 min, respectively.	[82] [28] [28]

Chelate	Test System	Results	Refs.
VO(pa)₂ VO(pa)₂	3T3-L1 adipocytes primary rat adipocytes primary rat adipocytes primary rat adipocytes male STZ diabetic ddY mice	pY immunoblots of IRS and IRβ stimulated by VO(pa)₂ and its 3-methyl- and 6-methylpicolinate analogs showed VO(3mpa)₂ > VO(6mpa)₂ ≈ VO(pa)₂ > VOSO₄. The same relative ordering for generation of pAkt/pPKB was observed. Formation of pAkt/pPKB was not completely inhibited by wortmannin but inhibitor concentration was not indicated. Similar observations for chelate facilitated generation of pGSK-3β. Capacity to inhibit epinephrine stimulated FFA release showed VO(6mpa)₂ (IC₅₀ = 0.60 mM) > VO(pa)₂ (IC₅₀ = 0.73 mM) > VOSO₄ (IC₅₀ = 1.0 mM). VO(3mpa)₂ was not included in study. Capacity to facilitate glucose uptake by epinephrine treated cells followed the same rank ordering. Capacity of 6-ethyl-picolinate analog of VO(pa)₂ to inhibit epinephrine stimulated FFA release was essentially comparable to that of VOSO₄ on a concentration basis. No comparison was made to the parent chelate VO(pa)₂ in the study. Partial reversal of the inhibition of epinephrine stimulated FFA release by VO(pa)₂ and VO-(malto)₂ was observed with wortmannin (inhibitor of PI3K), cytochalasin B (inhibitor of GLUT4 translocation), and cilostamide (specific inhibitor of PDE3B). Single ip injection (0.02 mmol/kg) showed only VO(3mpa)₂ had capacity to lower plasma glucose. VO(pa)₂ and VOSO₄ produced no sustained lowering of plasma glucose concentration.	[140] 5 [154] [101] [155] [140]
VO(5Ipa)₂ VO(5Ipa)₂	primary rat adipocytes normal male Wistar rats	Capacity of VO(pa)₂, VO(6mpa)₂, and VO(5Ipa)₂ to inhibit epinephrine stimulated FFA release were compared. Results: VO(5Ipa)₂ (IC₅₀ = 0.45 mM) > VO(6mpa)₂ (IC₅₀ = 0.49 mM) > VO(pa)₂ (IC₅₀ = 0.59 mM) > VOSO₄ (IC₅₀ = 0.91 mM). Real-time <i>in vivo</i> measurement by EPR monitoring of paramagnetism showed mean residence times for VO(5Ipa)₂ (44.9 min), VO(3mpa)₂ (28.8 min), VO(6mpa)₂ (19.6 min), VO(pa)₂ (9.3 min) and VOSO₄ (4.4 min) in the bloodstream after a single iv injection (~0.01 mmol/kg).	[100] [100, 171]