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Hydrophobic Carriers of Vanadyl Ions Augment the Insulinomimetic Actions of Vanadyl Ions in Rat Adipocytes

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ABSTRACT: A novel family of vanadyl ion (VO^{2+}, oxidation state +4) carriers is introduced. These carriers possess C_2 symmetry, utilize two hydroxamate groups as ion binding sites, and optionally possess asymmetric carbons. Binding efficiencies and hydrophobicities are regulated by the use of a modular assembly. When applied to rat adipocytes, these carriers augment the potency of vanadyl ions to stimulate glucose metabolism. The complexes shift the dose–response curve to the left. Also, the maximal effect of vanadyl ions which is in the order of 20–30% of that of insulin is shifted toward maximal (100–115%) stimulation. Among several chelators studied, the order of synergistic potency was RL-252 ≥ RL-262 > 1367. RL-239, RL-280, and RL-261 had smaller effects, whereas RL-282 had a negligible effect. The synergistic action of RL-252 (and other chelators as well) on VO^{2+} was already observed at a molar ratio of 1:0.01 of VO^{2+} to RL-252, respectively, and maximal augmentation occurred at a molar ratio of 1:0.1. The superiority of the hydrophobic chelators relative to the hydrophilic ones, together with the low molar ratio of chelator to VO^{2+} to achieve maximal effect, strongly suggests that these chelators act as vanadyl ionophores. This notion was confirmed by carrier-facilitated extraction of VO^{2+} from water into $CHCl_3$ with the following order of decreasing efficacy: RL-262 > RL-252 > 1367 > RL-261. The chelators' potentiating effect may therefore be related to facilitated transport of VO^{2+} ions into the cells' interiors. The potency of vanadate ions (VO_3^-, oxidation state +5) was not increased by RL-252, although RL-252 proved to extract vanadate effectively from water into chloroform. This observation is in line with earlier findings that vanadyl ions, rather than vanadate ions, are the activating principle, and suggests that the effectiveness of vanadate is dependent on the cells' capability to reduce it to vanadyl ions. Vanadyl ions, on the other hand, do not require intracellular reduction events. Their limited solubility at neutral pH value and low permeability is now full overcome by the ionophores which facilitate their permeation at low concentrations of the cation. Moreover, the substantially lower potentiating effect of the D-isomer, RL-262 (D), than of the L-isomer, RL-262 (L), suggests that the vanadyl ions exert their function by interactions with chiral recognition sites. The clinical significance of this study is also discussed.

Intensive research carried out in the last decade has demonstrated that vanadate ions, (VO_3^-, oxidation state +5) and also vanadyl ions (VO^{2+}, oxidation-state +4) (Shechter & Karlish, 1980), mimic nearly all of the various actions attributed to insulin in a large variety of in vitro (cellular) systems (Tamura et al., 1984; Shechter, 1990). Thus, VO_3^- and VO^{2+} may be considered as wide-range insulin-mimicking agents. A new interest in vanadium emerged in 1985 when Heyliger et al. observed that vanadate ions administered orally (in drinking water) to streptozotocin-treated hyperglycemic rats reduced the high levels of circulating glucose down to normal values and ameliorated many of the aberrations induced by hyperglycemia (Heyliger et al., 1985; Meyerovitch et al., 1987). In addition, disorders not directly related to

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hyperglycemia were also partially cured by oral vanadate therapy in this diabetic experimental model (Rossetti & Laughlin, 1989). Recently, it was demonstrated that vanadate therapy is also effective in experimental models of non-insulin-dependent diabetes mellitus (NIDDM).[1] In db/db and ob/ob mice, oral vanadate therapy induces long and persistent states of normoglycemia, while subcutaneous injections of insulin fail to do so (Meyerovitch et al., 1991). Since oral administration of insulin in mammals is ineffective (Hirosova & Koldovsk, 1969), diabetic patients need to receive insulin by subcutaneous injection. The availability of orally active insulin substitutes could be of importance in the treatment of diabetics.

Although the stimulatory modes of vanadium in producing insulinomimetic effects are still unknown (Degani et al., 1981; Macara, 1980; Simons, 1979), it necessitates permeation across the membrane and most likely initiates insulin-like activities at a point downstream to the insulin receptor kinase (Shechter, 1990). Thus, vanadate is equipotent in stimulating glucose uptake and metabolism in cells, which have lost 50–60% of their insulin receptors by downregulation and are less responsive to insulin (Green, 1986). The latter fact has pronounced clinical and physiological advantages, since insulin resistance can occur on the level of the insulin receptor kinase itself as well as at points downstream to the insulin receptor (Becker & Roth, 1990; Reddy & Kahn, 1988).

Vanadium salts are thus seriously considered as therapeutic agents in the case of human diabetes. In in vitro systems vanadate is more effective than vanadyl and the rates of stimulation of vanadate approach those of insulin. Addition of vanadyl leads to partial stimulation due to its lower membrane permeation properties and lower solubility at neutral pH (Degani et al., 1981; Heyliger et al., 1985; Meyerovitch et al., 1987; Pederson et al., 1989; Shechter & Karlish, 1980) which limit applicable concentrations to 100–200 nM. Vanadate, on the other hand, has the disadvantage of being 6- to 10-fold more toxic than vanadyl (Ramanadham et al., 1989; Waters, 1977). Identifying means to enhance permeability of the less toxic vanadyl ion at low vanadyl concentrations would therefore open new possibilities for vanadium-based treatment of diabetes.

This study focuses on the design and synthesis of chiral ion carriers that effectively bind and transport vanadyl ions across lipid membranes. The structures of these carriers rely on a dipodal topology which allows a modular assembly of the binders. The modular assembly enables systematic modifications until optimal performance is achieved. In this work we demonstrate that these chelators exhibit a potentiating effect in stimulating glucose metabolism in rat adipocytes when administered together with vanadyl ions. We further show that the effectiveness of the carriers depends on the position of their ion binding cavity, on their hydrophobicity, and on their chiral sense, the hydrophobic derivatives and L-amino acid derivatives being the more potent agents.

Experimental Procedures

Materials. Insulin was purchased from Sigma, and D-[U-^{14}C]glucose (4–7 mCi/mol) was from New England Nuclear. Collagenase type 1 (134 units/mg) was obtained from Worthington.

Preparation of Ionophores. RL-252, RL-262, RL-239, RL-280, and RL-282 were synthesized according to the procedures described in detail in Shanzer et al. (1991).[2] The synthesis of the dipodal chelators involved the following (see Figure 2): (i) preparation of the bis-carboxylates **1** and **2**, (ii) preparation of hydroxamates **4**, and (iii) coupling of the bis-carboxylates with the hydroxylamines **3** or hydroxamates **4** to provide the carrier. The tripodal chelator 1367 was prepared in an analogous fashion, using tris-carboxylate instead of bis-carboxylate as anchor (Tor et al., 1987). All amino acid residues used were of the natural L-configuration except for RL-262, which was prepared from either L-Leu or D-Leu to provide enantiomeric carriers. The experimental details for the preparation of RL-252 are as follows:

(*A*) *Preparation of Active Ester* **2**. Diol (9.36 g) was treated dropwise with 0.6 mL of 40% aqueous NaOH, and then 13.2 mL of acrylonitrile (freshly purified by passing through neutral alumina) was added. Reaction temperature did not exceed 30 °C. The mixture was then stirred overnight at room temperature, neutralized with diluted aqueous HCl, dissolved in 500 mL of ethyl acetate, washed with water, dried, and concentrated to give 18.45 g of material. The crude product was hydrolyzed by treating 2.40 g with 2.7 mL of concentrated HCl in an oil bath of 95–100 °C for 6 h. After cooling to room temperature, the residue was suspended in ethyl acetate, washed with water, dried, and concentrated to give 2.1 g of diacid. The crude diacid (3.7 g, 0.015 mol) was dissolved in 200 mL of acetonitrile (dried over alumina). Pentachlorophenol (8.79 g) and (dimethylamino)pyridine (400 mg) were then added, and the mixture was cooled in an ice bath and treated with 5.0 mL of diisopropylcarbodiimide. The mixture was then stirred at room temperature for 1–2 days, concentrated in vacuo, and chromatographed on silica gel, yielding 2.83 g of the active ester **2** (mp 88–90 °C).

(*B*) *Preparation of Amine* **4** ($n = 1$, $R^{I} = iBu$, $R^{II} = Me$). Cbz-L-leucine [5.3 g (0.02 mol)] was dissolved in 150 mL of acetonitrile (dried over basic alumina). Pentachlorophenol (5.8 g, 0.022 mol) was then added, followed by addition of diisopropylcarbodiimide (0.025 mol). The mixture was stirred for 1 day at room temperature, concentrated, chromatographed on silica gel, and then filtered though neutral alumina to provide 10.41 g of pure pentachlorophenolate (mp 125–126 °C, intermediate not shown in Figure 2). The phenolate (5.2 g, 0.01 mol) was dissolved in 50 mL of dry methylene chloride and treated with a solution containing 1.04 g (0.0125 mol) of methylhydroxylamine hydrochloride, 1.21 g (0.0125 mol) of triethylamine, and 50 mg of *N*-hydroxysuccinimide in 100 mL of methylene chloride. The mixture was stirred overnight and concentrated in vacuo, and the residue was chromatographed on silica gel to provide 1.30 g of pure Cbz-leucine hydroxamate (mp 71–73 °C, intermediate not shown in Figure 2). The latter (960 mg, 0.003 mol) was dissolved in 100 mL of ethanol and hydrogenated at atmospheric pressure in the presence of 500 mg of Pd/C, 10%. Filtration, concentration, and chromatography on silica gel yielded 472 mg of pure hydroxamate amine **4**.

(*C*) *Coupling of Active Ester* **2** *with Amine* **4**. Bis-carboxylate **2** (1.13 g) was dissolved in 50 mL of dry methylene chloride and treated with a solution containing 675 mg of amine **4**, *N*-hydroxysuccinimide (50 mg), and imidazole (300 mg) for 4 days. Chromatography of the crude reaction product on silica gel yielded 542 mg of the bis-hydroxamate RL-252 (mp 44–46 °C): IR ($CDCl_3$) ν 3260, 1636, 1114 cm^{-1}; NMR ($CDCl_3$) δ 5.13 (m, 2 H, CHiBu), 3.65 (m, 4 H, OCH_2), 3.20

[1] Abbreviations: NIDDM, non-insulin-dependent diabetes mellitus; KRB, Krebs–Ringer–bicarbonate; BSA, bovine serum albumin.

[2] Shanzer, A., Libman, J., & Lifson, S. U.S. Patent approved October 1991.

Table I: Extraction Efficiencies and Potencies of the Various Chelators in Augmenting Vanadyl-Dependent Stimulation of Lipogenesis

chelator designation[a]	% of V extracted[b]	% of V released[c]	concentration to produce half-maximal potentiation (μM)	relative potentiation ability (%)
RL-252	17	29	1.50 ± 0.05	100
RL-262	18.4	49	1.60 ± 0.05	90
RL-262 (D)			3.50 ± 0.1	43
RL-261	9.0	<2	8.33 ± 0.4	18
RL-239			4.17 ± 0.1	36
RL-280			5.00 ± 0.2	30
RL-282			25.0 ± 1.5	
1367	10.0	40	2.63 ± 0.1	57

[a] All amino acid residues used were of L-configuration, unless otherwise state. [b] % of VO^{2+} extracted from water into chloroform. [c] % of VO^{2+} released from complex in chloroform to water.

(s, 3 H, NCH_3), 3.18 [ABq, 4 H, $C(CH_2O)$], 2.45 ppm (m, CH_2C O).

Extraction experiments were performed by equilibrating equal volumes of 1 mM vanadyl sulfate in 1 N aqueous H_2SO_4 with 1 mM chelator in chloroform for 3 days. Then the two phases were separated and the organic phase equilibrated with equal volumes of 10 mM glutathione in 0.1 N aqueous H_2SO_4. Similarly, 1 mM sodium vanadate in 1 N aqueous H_2SO_4 was equilibrated overnight with an equal volume of 1 mM chelator RL-252 in chloroform. The phases were then separated, and the organic layer was equilibrated with an equal volume of 10 mM glutathione in 0.1 N aqueous H_2SO_4. Vanadium contents of all the aqueous layers were determined by inductive-coupled plasma atomic absorption (Perkin-Elmer 5500). The results are summarized in Table I.

Cell Preparation. Rat adipocytes were prepared essentially by the method of Rodbell (1964). The fat pads of male Wistar rats were cut into small pieces with scissors and suspended in 3 mL of KRB buffer containing NaCl, 110 mM; $NaHCO_3$, 25 mM; KCl, 5 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 1.3 mM; $MgSO_4$, 1.3 mM; and 0.7% BSA (pH 7.4). The digestion was performed with collagenase (1 mg/mL) in a 25-mL flexible plastic bottle under an atmosphere of carbogen (95% O_2, 5% CO_2) for 40 min at 37 °C with vigorous shaking. Five milliliters of buffer was then added, and the cells were passed through a mesh screen. The cells were then allowed to stand for several minutes in a 15-mL plastic test tube at room temperature, floating, and the buffer underneath was removed. This procedure (suspension, floating, and removal of buffer underneath) was repeated three times.

Lipogenic Assay. Vanadyl–chelator complexes were prepared in plastic tubes by mixing $VOSO_4$ (500 nmol) and 5–500 nmol of the various chelators (dissolved in ethanol) for 2–5 h at 22 °C and at pH 4.5. Unless otherwise stated, the chelators were in their L form. For measurement of glucose uptake and its incorporation into lipids (lipogenesis) the adipocyte suspensions (3×10^5 cells/mL) were divided into plastic vials (0.5 mL per vial) and incubated for 60 min at 37 °C under an atmospheric of 95% O_2/5% CO_2 with 0.2 mM [U-^{14}C]glucose, in either the absence or presence of insulin (100 ng/mL), vanadate, vanadyl, and vanadyl–chelator complex (prepared as described above). No change in the pH value upon addition of between 1 and 10 μL of either vanadyl or vanadyl–chelator complexes to the buffered cell suspensions was observed. Lipogenesis was terminated by adding toluene-based scintillation fluid (1.0 mL per vial), and the radioactivity in extracted lipids was counted (Moody et al., 1974). Control experiments were conducted with either ethanol alone (0.04–0.2%) or RL-252 (0.1–0.3 mM). In all experiments insulin-stimulated lipogenesis was 4- to 5-fold higher than basal.

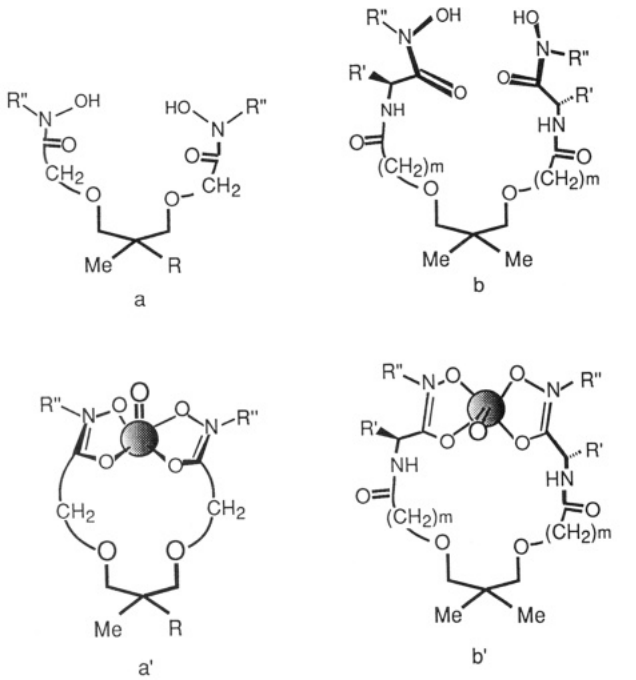


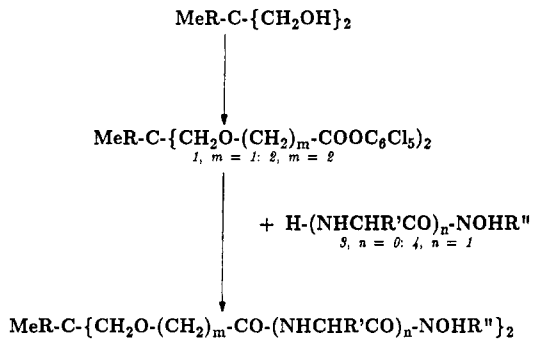
FIGURE 1: Schematic representation of dipodal chelators and vanadyl ion complexes without amino acid bridges (a and a') and with amino acid bridges (b and b').

The results shown in tables and figures are presented as means ± SE. The number of experiments ranged between 3 and 7. The assays were performed in duplicate or triplicate.

Results

Design, Synthesis and Physicochemical Characterization of Vanadyl Ionophores. In order for a binder to facilitate transmembranal ion uptake, it has to meet several stringent requirements (Morf et al., 1979). These involve selective metal ion binding in the aqueous phase, effective penetration of the metal complex into the biological membrane, and ready release of the guest ion in the cytoplasma or transfer to the biological recognition site. Obtaining these characteristics requires balanced properties: moderate (rather than maximal) complex stability and moderate partition coefficients between aqueous and lipid media. The design of vanadyl ionophores is further complicated by the rather limited information on the relationship between structure and stability in vanadyl complexes (Boas & Pessoa, 1987), although the square pyramidal geometry seems to prevail (Boas & Pessoa, 1987; Dodge et al., 1961; Butler, 1990).

On the basis of these considerations dipodal (C_2-symmetric) dihydroxamates were selected as candidate molecules (Figure 1). Hydroxamate groups were to form electrically neutral, lipid-soluble complexes with VO^{2+} (Fisher et al., 1989), and the dipodal backbones were to adjust to the desired square pyramidal ion binding cavities. Optional introduction of amino acid bridges between the anchor and the binding sites (Figure 1b) was to vary the geometry and strain of these complexes and thereby their stability. In addition, the use of optically active amino acids was to provide chiral probes which allow us to trace possible chiral discrimination by the biological VO^{2+} recognition sites. The extended versions of these structures were conceived to allow for any square pyramidal geometry (both cisoid and transoid) with the V=O bond along the molecules main axis (Figure 1b'). The shorter analogues,



code	m	n	R	R'	R"
RL-239	1	0	Me	-	Me
RL-280	1	0	Pr	-	Me
RL-282	1	0	Me	-	CH_2CH_2COOEt
RL-262	1	1	Me	iBu	Me
RL-261	1	1	Me	Me	Me
RL-252	2	1	Me	iBu	Me

FIGURE 2: Representation of the steps involved in the synthesis of the C_2-symmetric chelators. The active esters **1** and **2** are reacted with the amines **3** or **4** to provide the chelators. The amines **4** are amino acid derivatives (R^I = iBu, Leu; R^I = Me, Ala) obtained by amidating the respective amino acids with *N*-methylhydroxylamine.

devoid of the amino acid spacers (Figure 1a), were anticipated to impose more constraint and to accommodate merely cisoid square pyramidal geometries (Figure 1a'). Both types of binders, the extended and shorter variants, were assembled in a modular fashion to enable adjustment of the hydrophobicity profiles. In the former (n = 1, in Figure 2) this was achieved by varying the nature of the amino acid bridges (R^I = iBu or Me), and in the latter (n = 0, in Figure 2), by modifying the substituents of the anchor (R = Me or Pr groups) or termini (R^{II} = Me or CH_2CH_2COOEt), respectively (Figure 2).

A series of C_2-symmetric chelators were prepared. The synthetic strategy involved (i) preparation of the bis-carboxylate anchors **1** and **2** in Figure 2, (ii) preparation of hydroxamate residues, **3** and **4** in Figure 2, and (iii) coupling of the hydroxylamine or hydroxamate residues with the anchors to provide the binders. The tripodal tris-hydroxamate $EtC[CH_2OCH_2CH_2C(O)NHCHiBuC(O)N(OH)CH_3]_3$ was also synthesized as a reference compound for comparison. The reaction scheme followed is illustrated (Figure 2).

The chelators were found to extract vanadyl ions, VO^{2+}, from aqueous into lipid media (chloroform), and subsequently to release the bound metal when treated with aqueous glutathione solutions. The extraction efficacies are summarized in columns 2 and 3 of Table I. As seen from the data, the efficacies of the chelators to extract VO^{2+} from water into chloroform decrease in the order RL-262 > RL-252 > 1367 > RL-261 (Table I, column 2). Under analogous conditions RL-252 was found to extract 31% vanadate from water into chloroform, and to release 81% of the bound ion, when treated with aqueous glutathione.

Effect of Vanadate and Vanadyl in Stimulating Lipogenesis in Rat Adipocytes. Initially, we evaluated the dose–response curve and extent of stimulation of lipogenesis by both vanadate and vanadyl (Figure 3). Vanadate stimulated lipogenesis to the same extent as insulin. The ED_{50} value for vanadate amounted to 180 ± 25 μM. Vanadyl also stimulated lipogenesis, but, unlike vanadate, stimulation is incomplete and amounts to about 20–30% of the maximal effects of the hormone or of vanadate (Figure 3). The ED_{50} value for vanadyl was calculated to be 100 ± 20 μM in stimulating lipogenesis. In a previous study (Shechter & Karlish, 1980), we observed almost the same pattern of stimulation of glucose

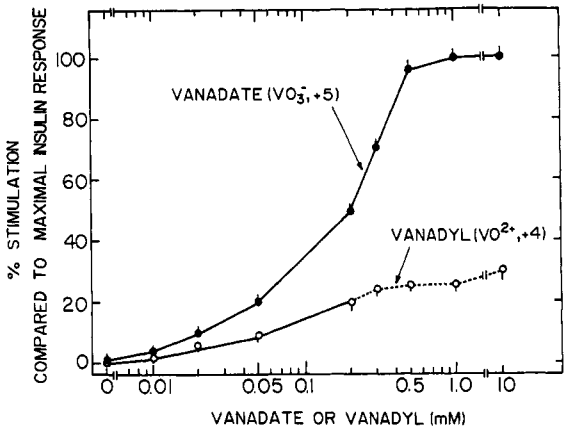


FIGURE 3: Effects of increasing concentrations of vanadate or vanadyl on rates of lipogenesis. Lipogenesis was carried out for 1 h at 37 °C in vials, containing about 1.5×10^5 cells suspended in KRB buffer, 0.2 mM [U-^{14}C]glucose, and the indicated concentrations of vanadate (●) or vanadyl (○) ions. Results are expressed as percentage of stimulation compared to a maximal insulin response. The dashed line represents data collected at concentrations where vanadyl salts precipitate.

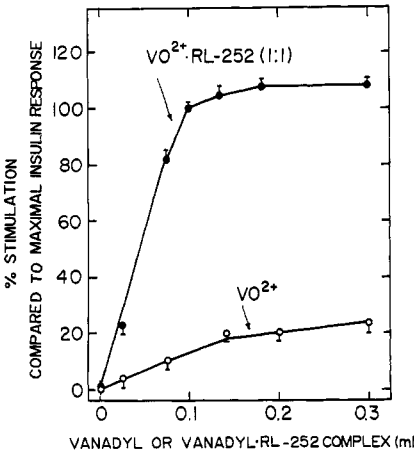


FIGURE 4: Synergistic effect of RL-252 on VO^{2+}-dependent stimulation of lipogenesis. Lipogenesis was carried out at pH 7.4 for 1 h at 37 °C under the experimental conditions described in the legend to Figure 3, with the indicated concentrations of vanadyl (VO^{2+}) or VO^{2+}·RL-252 complex (molar ratio 1:1.0). The complex was prepared as described under Experimental Procedures. Aliquots (1–7.5 μL) were then added to adipocyte suspensions (0.5 mL each, in KRB buffer, pH 7.4). Control experiments revealed no pH change at the highest concentration of VO^{2+}·RL-252 added here.

oxidation as well, by vanadate and vanadyl in rat adipocytes. The lack of full stimulation by VO^{2+} was attributed to its low solubility and low permeability at neutral pH values (Shechter & Karlish, 1980), the combination of which limits the attainable intracellular VO^{2+} concentrations.

Effect of RL-252 on VO^{2+}-Dependent Stimulation of Lipogenesis. In the experiments summarized in Figure 4 vanadyl was preincubated with equimolar concentrations of RL-252 (2–5 h at room temperature) before addition to the cells, which were then assayed for lipogenesis. Under these conditions, the dose–response curve was shifted to the left. Also, the extent of stimulation increased and even exceeded that of insulin itself. Thus, ED_{50} values of VO^{2+} alone or VO^{2+}·RL-252 complex were 100 ± 20 and 30 ± 4 μM, respectively, and the extent of stimulation was 25 ± 3% and 115 ± 5%, respectively. Altogether, the carriers increased the potency of VO^{2+} in stimulating lipogenesis 7- to 10-fold in various experiments. Ethanol (0.2%) or RL-252 alone (0.1–0.3 mM) did not show any effect on basal or insulin-stimulated lipogenesis (Table II).

Table II: Effect of RL-252 on VO^{2+}- and VO_3^--Stimulated Lipogenesis in Rat Adipocytes[a]

additions	[U-^{14}C]glucose incorporated into lipids [cpm/(3 × 10^{-5} cells·h)]	% of the maximal insulin response
none	2300 ± 70	0
insulin, 16.7 nM	10200 ± 100	100
RL-252, 200 μM	2340 ± 70	0
RL-252, 200 μM, + insulin, 16.7 nM	10150 ± 100	100
vanadyl, 60 μM	3500 ± 70	15
vanadyl, 60 μM, + RL-252, 20 μM	10700 ± 120	106
vanadate, 50 μM	4000 ± 50	21
vanadate, 50 μM, + RL-252, 10 μM	3800 ± 70	19
vanadate, 50 μM, + RL-252, 30 μM	4100 ± 70	23
vanadate, 50 μM, + RL-252, 50 μM	3650 ± 40	17

[a] Experimental details are as described in the legend to Figure 4. Vanadyl and vanadate were preincubated with the ionophores for 2–5 h at pH 4.5 before application to the cell.

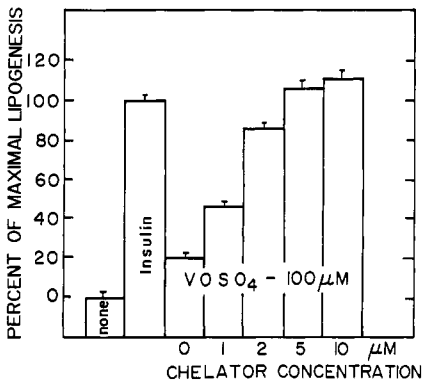


FIGURE 5: Stimulation of lipogenesis at varying molar ratios of RL-252 to vanadyl ions. VO^{2+}·RL-252 complexes with different molar ratios were prepared by preincubating the increasing concentrations of the chelator with $VOSO_4$ as described under Experimental Procedures. Aliquots were then added to adipocyte suspensions to achieve the indicated concentrations. Lipogenesis was carried out for 1 h at 37 °C.

Effective Molar Ratio of RL-252 to Vanadyl Ions. In the experiments summarized in Figure 5, a constant concentration of $VOSO_4$ with varying concentrations of RL-252 was administered to obtain molar ratios of VO^{2+} to RL-252 ranging from 1:0.01 to 1:0.1. A potentiating effect was already observed at a molar ratio of 100:1, and the effect was maximal at a molar ratio of 10:1 of VO^{2+} to RL-252, respectively (Figure 5). Thus, very low concentrations of RL-252 already exhibit a maximal potentiating effect. These results strongly suggest that RL-252 acts in this system as a vanadyl ionophore.

Lack of Effect of RL-252 on Vanadate. In contrast to the effect observed with vanadyl ions, RL-252 did not potentiate vanadate (VO_3^-) in stimulating glucose metabolism. This was true under several experimental conditions, namely, at varying molar ratios of VO_3^- to RL-252 and at short or prolonged preincubating conditions. Thus, the potentiating effect of RL-252 is restricted to vanadyl ions (Table II).

Activity of Related Chelators in Potentiating Vanadyl-Dependent Lipogenesis. The potentiating actions of several related chelators on vanadyl-stimulated lipogenesis were studied (Table I). RL-262 and RL-252 were found to have the strongest potentiating effects. The order of potency was found to be RL-252 ≥ RL-262 > 1367 > RL-239 > RL-280 > RL-261 > RL-282.

Activity of Enantiomeric Vanadyl Carriers, RL-262 (L) *versus RL-262* (D). The potentiating effects of the enantiomeric carriers, RL-262 (L) assembled from L-Leu and

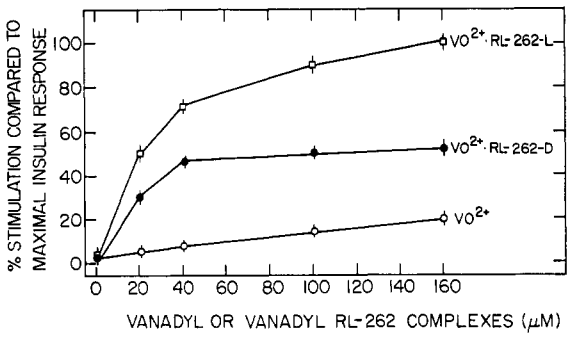


FIGURE 6: Differential synergistic effects of enantiomeric vanadyl carriers on VO^{2+}-dependent stimulation of lipogenesis. Lipogenesis (1 h; 37 °C) was carried out at increasing concentrations of VO^{2+} (○), VO^{2+}·RL-262 (L) (□), or VO^{2+}·RL-262 (D) (●) complexes. The complexes (molar ratio 1:1.0) were prepared as described under Experimental Procedures.

RL-262 (D) assembled from D-Leu, were compared. As shown in Figure 6 and in Table I, the effectiveness of the D-enantiomer is around 40% lower than that of the L-enantiomer. The pronounced lower effectiveness of the D-enantiomer suggests specific interactions of the vanadyl carrier complexes with biological recognition sites.

Discussion

Lipophilic vanadyl carriers have been found to potentiate glucose metabolism when applied with vanadyl ions. The effectiveness of these chelators down to 100:1 vanadyl:carrier ratios suggests that they act by facilitating transport of vanadyl ion across cellular membranes. This notion was confirmed by carrier-facilitated extraction experiments of VO^{2+} from water into $CHCl_3$. By applying chelators of modular assembly, we also could identify the structural requirements for maximal extraction efficacy and potentiating effects. It thus became evident that the hydrophobicity of the amino acid bridge is of paramount importance: While the dipodal Leu derivative RL-262 is the most efficient extractor and, when loaded, VO^{2+} exceeds the potentiating effect of insulin, the corresponding Ala derivative RL-261 is 6-fold less efficient. Among carriers with identical amino acid bridges RL-252 and 1367, the dipodal structure proved superior to the tripodal one. This observation is attributed to the fact that the tetracoordinating ligand RL-252 suffices to saturate VO^{2+} ions, while the superfluous chain of the hexacoordinating ligand 1367 decreases the complex permeability. The inferiority of RL-239 and RL-280 which lack amino acid bridges may be due either to the unfavorable position of the binding groups, to reduced lipophilicity, or to both. Remarkable is the lack of potentiation of vanadate by carrier RL-252, in spite of its efficient extraction by RL-252. This apparent discrepancy supports early findings that vanadyl, rather than vanadate, is the insulin-mimetic active species (Shechter, 1990). It further suggests that the rate-limiting step in the activity of vanadate is its reduction to the tetravalent vanadyl ions by the cell. Vanadyl, as opposed to vanadate, does not require an intracellular reducing event upon cellular permeation. It neither inhibits phosphohydrolases (Macara, 1980; Simons, 1979) nor exhibits the high extent of mammalian toxicity (Ramanadham et al., 1989; Wates, 1977) as does vanadate. The fact that low (micromolar) concentrations of the lipophilic ionophores are sufficient to maximally potentiate the effect of vanadyl ions on glucose metabolism is believed to be of importance both for future studies in in vitro and in vivo experimental systems and for potential therapeutic applications. Most remarkable is the pronounced chiral discrimination observed when applying enantiomeric ionophores, RL-262 (L) and RL-262 (D), re-

spectively. This observation suggests interactions of the respective vanadyl carrier complexes with biological recognition sites with more effective release of vanadyl ions from the L-enantiomeric complexes. Experiments aimed at identifying the sites of action(s) of intracellular vanadyl are in progress as are in vivo studies of the most potent agents.

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^{1}H NMR Assignment and Secondary Structure of the Cell Adhesion Type III Module of Fibronectin†

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ABSTRACT: The secondary structure of the tenth type III module from human fibronectin has been determined using NMR. This type of module appears many times in a wide variety of proteins. The type III module described here contains an Arg-Gly-Asp sequence known to be involved in cell–cell adhesion. The module was expressed in yeast and characterized by amino acid sequencing and mass spectrometry. 2D and 3D NMR spectroscopy of ^{15}N-labeled protein was used to perform sequence-specific assignment of the spectrum. The secondary structure was defined by patterns of nuclear Overhauser effects, $^3J_{NH\text{-}\alpha CH}$ spin–spin coupling constants, and amide proton solvent exchange rates. The molecule consists of seven β-strands in two antiparallel β-sheets with an immunoglobulin-like fold similar to that predicted for homologous modules in the cytokine receptor super family [Bazan, J. F. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6934-6938]. The Arg-Gly-Asp sequence is located on a loop between the β-strands F and G.

Fibronectin, a large protein found in the extracellular matrix and the serum, is made up of three different kinds of structural units, usually called types I–III (Ruoslahti, 1988). In this respect it is similar to many proteins that have evolved by exon shuffling and duplication of a limited number of autonomously folding structural units, or modules (Patthy, 1991; Baron et al., 1991). The diverse biological roles of fibronectin have been extensively studied (Hynes, 1990). It is found in serum, where it binds to both fibrin blood clots and platelets, and is also a major component of the extracellular matrix which provides anchorage sites for cells and thus helps maintain the integrity of tissues. Fibromectin plays a key role in wound healing and embryonic development since it binds to other components of

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