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Effect of vanadium(IV) compounds in the treatment of diabetes: in vivo and in vitro studies with vanadyl sulfate and bis(maltolato)oxovanadium(IV)

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Abstract

Vanadyl sulfate (VOSO_4) was given orally to 16 subjects with type 2 diabetes mellitus for 6 weeks at a dose of 25, 50, or 100 mg vanadium (V) daily [Goldfine et al., *Metabolism* 49 (2000) 1–12]. Elemental V was determined by graphite furnace atomic absorption spectrometry (GFAAS). There was no correlation of V in serum with clinical response, determined by reduction of mean fasting blood glucose or increased insulin sensitivity during euglycemic clamp. To investigate the effect of administering a coordinated V, plasma glucose levels were determined in streptozotocin (STZ)-induced diabetic rats treated with the salt (VOSO_4) or the coordinated V compound bis(maltolato)oxovanadium(IV) (abbreviated as VO(malto)_2) administered by intraperitoneal (i.p.) injection. There was no relationship of blood V concentration with plasma glucose levels in the animals treated with VOSO_4 , similar to our human diabetic patients. However, with VO(malto)_2 treatment, animals with low plasma glucose tended to have high blood V. To determine if V binding to serum proteins could diminish biologically active serum V, binding of both VOSO_4 and VO(malto)_2 to human serum albumin (HSA), human apoTransferrin (apoHTf) and pig immunoglobulin (IgG) was studied with EPR spectroscopy. Both VOSO_4 and VO(malto)_2 bound to HSA and apoHTf forming different V-protein complexes, while neither V compound bound to the IgG. VOSO_4 and VO(malto)_2 showed differences when levels of plasma glucose and blood V in diabetic rodents were compared, and in the formation of V-protein complexes with abundant serum proteins. These data suggest that binding of V compounds to ligands in blood, such as proteins, may affect the available pool of V for biological effects. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Recently, short clinical studies of 2–4 weeks duration have been done by Goldfine and Kahn, and others, administering vanadyl or vanadate at doses of 33–50 mg elemental V daily. These studies demonstrated V tolerability and improved glycemia with decreased fasting blood glucose and glycohemoglobin [2–4], and improved insulin sensitivity during euglycemic hyperinsulinemic clamp studies [2,5]. The insulin-like activity and therapeutic use of vanadium in diabetes has been recently reviewed [6,7]. To better explore the potential role of V salts in the treatment of type 2 diabetes mellitus, 25, 50, or 100 mg

elemental V daily were administered orally to 16 subjects with type 2 diabetes mellitus. The clinical portion of this study has been described [1]. Serum and urine V levels were measured to determine the pharmacokinetics, including absorption of V into the body at steady state and time course of disappearance of V from serum, and to correlate the serum V with clinical response.

Evidence suggests that most metals do not exist free in physiological fluids, as recently reported for copper in yeast [8,9]. Thus, when V salts are administered orally, they could bind rapidly to different physiological components on their way from the gastrointestinal tract to the intravascular space (blood). Its effectiveness could then be a function of the relative affinities of the V for the ligand(s) as compared to its biological target and the different physical chemical properties of various V complexes. The administration of V as a coordinated complex should

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provide a more uniform response and greater correlation of clinical response with total serum V levels. Indeed, recent animal studies with the $\text{VO}(\text{malto})_2$ complex suggest that this compound lowers elevated glucose levels in diabetic animals [10,11]. The structures of VOSO_4 and the V(IV) coordinated derivative $\text{VO}(\text{malto})_2$ are shown in Fig. 1. $\text{VO}(\text{malto})_2$ and other coordinated V compounds with insulin-like properties are superior to the simple salts [12–16]. To examine the hypothesis that administration of a coordinated V is more effective; a comparison of plasma glucose levels with V levels was done in STZ-induced diabetic rats which were either administered the V salt VOSO_4 or the coordinated $\text{VO}(\text{malto})_2$.

Differential compartmentalization of V salts and coordinated V derivatives in blood may be explained by differential affinities to serum proteins. Differential binding to serum proteins could result in the formation of an additional compartment in the blood for V compounds. Interactions of VO^{2+} with proteins have frequently been used as a spin probe for studies of metal ion–protein interactions [17]. To examine the binding of VOSO_4 and $\text{VO}(\text{malto})_2$ to the abundant serum proteins transferrin, albumin and immunoglobulin (apoHTf, HSA, and IgG), EPR spectroscopy was performed to determine whether the vanadyl cation and $\text{VO}(\text{malto})_2$ formed a complex with these proteins, whether complexes formed by the different vanadium species with the same protein were similar or different. Detailed studies have previously been reported of interactions of VOSO_4 with transferrin and albumin [18–23]; however, little information is available on the interactions of $\text{VO}(\text{malto})_2$ with either of these proteins (Orvig/Thompson, personal communication). Furthermore, investigation of the interaction of either VOSO_4 or $\text{VO}(\text{malto})_2$ with immunoglobulin (IgG) has not previously been reported. Although most reported studies concerned low temperature EPR spectra [18,19,22,23, including personal communication from Orvig/Thompson], we also chose to carry out these studies at ambient room temperature, because such conditions better mimic those observed in vivo.

In summary, this paper combines studies in human type 2 diabetic patients dosed with VOSO_4 , in STZ-induced diabetic rats administered VOSO_4 and $\text{VO}(\text{malto})_2$, and also in vitro spectroscopic studies of the interactions of VOSO_4 and $\text{VO}(\text{malto})_2$ with abundant serum proteins.

The objective of these studies is to address the question of whether serum proteins can effectively form a unique biological compartment in blood for V, which may affect bio-availability.

2. Materials and methods

2.1. Materials

VOSO_4 , human serum albumin (HSA), human apotransferrin (apoHTf, T2252) and pig immunoglobulin (IgG, G2512) were purchased from Sigma–Aldrich. HSA and IgG were used directly. Apotransferrin could not be used directly as an isotropic EPR spectrum was observed between a chelator used in the isolation of the apoprotein and the V complexes (data not shown). Thus, to obtain the anisotropic spectra of the apoHTf protein–V complex it was necessary to exhaustively dialyze apoHTf against 100 mM Hepes and 0.1 M NaCl at pH 7.4 before adding the V compounds. $\text{VO}(\text{malto})_2$ was prepared as reported previously [24].

2.2. Patient protocol

Sixteen subjects with type 2 diabetes were studied for 12 weeks in a single blind placebo lead trial design as previously published [1]. Briefly, after baseline laboratory testing was performed subjects were started on placebo. At the end of placebo treatment subjects were admitted to the General Clinical Research Center of Brigham and Women's Hospital for a two-step euglycemic–hyperinsulinemic clamp study to quantify insulin sensitivity. Subjects were then switched from placebo to 25, 50 or 100 mg V/day administered orally in tablets with meals (three per day), as VOSO_4 (75, 150, 300 mg, divided daily, respectively) for 6 weeks. After the sixth week of VOSO_4 the euglycemic insulin clamp studies were repeated and VOSO_4 was discontinued. Subjects were monitored for an additional 2 weeks. Throughout the study patients were instructed to monitor their blood glucose four times daily prior to meals and at bedtime with a One Touch™ glucose monitor and weekly values were reported as averages for each of the four time points.

2.3. Insulin sensitivity

Two-step euglycemic–hyperinsulinemic clamp studies were done pre-treatment and post-treatment to assess insulin sensitivity as described [1]. Briefly, variable rate low-dose insulin infusion was administered overnight prior to the clamp to normalize blood glucose. After collection of baseline samples for hormones and substrates, a primed-continuous infusion of insulin at 0.5 mU insulin/kg per min (low dose) was started for a 2 h period, followed by a second 2-h infusion at 1.0 mU insulin/kg per min (high

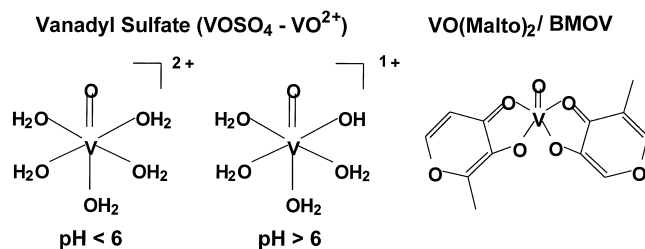


Fig. 1. VOSO_4 and $\text{VO}(\text{malto})_2$ structures.

dose). Throughout insulin administration, glucose was determined at 5-min intervals and a variable rate glucose infusion was adjusted to maintain euglycemia. Insulin sensitivity is reported for the two steady-state hyperinsulinemic levels as the metabolic rate (M) of glucose uptake, normalized for the mass in kilograms of the subject.

2.4. Determination of vanadium concentrations in patient serum and urine

Serum and urine levels of elemental V were determined by GFAAS using a Perkin-Elmer (Norwalk, CT) 4110Z (Zeeman) spectrophotometer [25]. Standard curves were prepared in serum and urine from a commercially available atomic absorption standard, and a quality-control sample was analyzed every 10 samples. Human serum and urine samples were obtained for V analysis during placebo days 2 and 7; during treatment at days 2, 7, 14, 28 and 42; and at days 2, 7 and 14 after treatment was discontinued. Serum V kinetics was modeled for each patient using a first-order kinetic model of the type $C_t = C_o e^{-kt}$ where C_t is the serum concentration at any time (t). C_o is the initial concentration, and k is the first-order rate constant for elimination [26]. Due to the variation in the individual patient response, k will vary from patient to patient and is actually an experimental parameter calculated for each patient.

2.5. Rat protocol

Male Wistar rats weighing between 190 and 220 g were obtained from the Animal Care Center, South Campus UBC. Animals were housed two per cage, and acclimatized for a period of 4–7 days after arrival. Diabetes was induced by a single intravenous tail vein injection of STZ 60 mg/kg in 0.9% NaCl under light halothane anesthesia. On day 3 following STZ administration animals with a plasma glucose level >13 mM were considered diabetic. On day 7, diabetic animals were divided into three groups: diabetic receiving only saline (0.9%), $n=6$; diabetic receiving $\text{VO}(\text{malto})_2$, $n=8$; and diabetic receiving $\text{VO}(\text{malto})_2$, $n=8$. Drugs were prepared to deliver 0.1 mmol/kg (32 mg/kg) $\text{VO}(\text{malto})_2$ or 22 mg/kg $\text{VO}(\text{malto})_2$ to administer similar doses of elemental V, or saline placebo in a volume of 10 ml/kg by i.p. injection. Samples were collected by tail nick and expression of blood into heparinized capillary tubes prior to and 2, 12, 18, 24, 48 and 72 h post V or placebo administration and stored as whole blood at -70°C before being sent for V analysis. V levels were determined by atomic absorption analysis by Elemental Research (North Vancouver, Canada) after digestion in 1:1 nitric acid. Blood for glucose analysis was centrifuged at $10\,000\times g$ for 15 min and the plasma glucose levels were

determined immediately using a Beckman II Glucose Analyzer.

2.6. EPR studies

The ambient temperature EPR spectra were recorded on a Bruker ESP 300 spectrometer using 1-mm quartz capillary tubes that were placed in 4-mm quartz tubes at 298 K. The spectra reported here were recorded at 9.507 GHz (X-band) and at 40–40.1 mW microwave power. A modulation frequency of 100 kHz, modulation amplitude of 5.00 G, time constant of 164 ms, sweep width of 2000 G and sweep time of 168 s were used. Typically four scans with 4096 points per scan were recorded with a center field of 3350 G. The low temperature EPR spectra were recorded at 122 K. The parameters used to record the low temperature spectra shown here were identical to those used at 298 K. The spectrometer center field was calibrated using a powder sample of 2,2-bis(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH, $g=2.0037+0.00020$). ^{51}V NMR spectroscopy was used to document the oxidation of the V as the EPR signal of the solution disappeared. The EPR spectroscopic data were processed using Bruker WINEPR System Software Version 2.11. Spectra are shown using Jandel Corporation's Sigma Plot Version 2.01.

2.7. Statistics

Data are expressed as the mean \pm S.D. unless otherwise noted. Data significance was determined using a two tailed students *t*-test (significance was P values ≤ 0.05). Pierson product-moment correlation coefficients and their associated P values were computed using Minitab.

3. Results

3.1. Vanadium in serum and urine of treated type 2 diabetic patients

The total elemental V content in serum and the urinary clearance is shown in Fig. 2 for patients receiving 100 mg V per day (300 mg $\text{VO}(\text{malto})_2$). Individual response to the drug was variable for this patient population, similar to the responses seen in diabetic subjects receiving 25 and 50 mg V per day [27]. However, there was linear correlation of peak V in serum with the dose administered ($R^2=0.993$, [1]). Patients who had higher levels of V in serum also had higher V in their urine. The detailed pharmacokinetics of the absorption of V into serum, urine and blood is part of a manuscript in preparation (P. Kostyniak et al.). The summary of data concerning serum V levels for all patients is shown in Table 1. The average of the individual $t_{1/2}$ values for elimination of V from serum was significantly higher ($P\leq 0.05$) in the 50-mg V/day compared to the 100-mg V/day group.

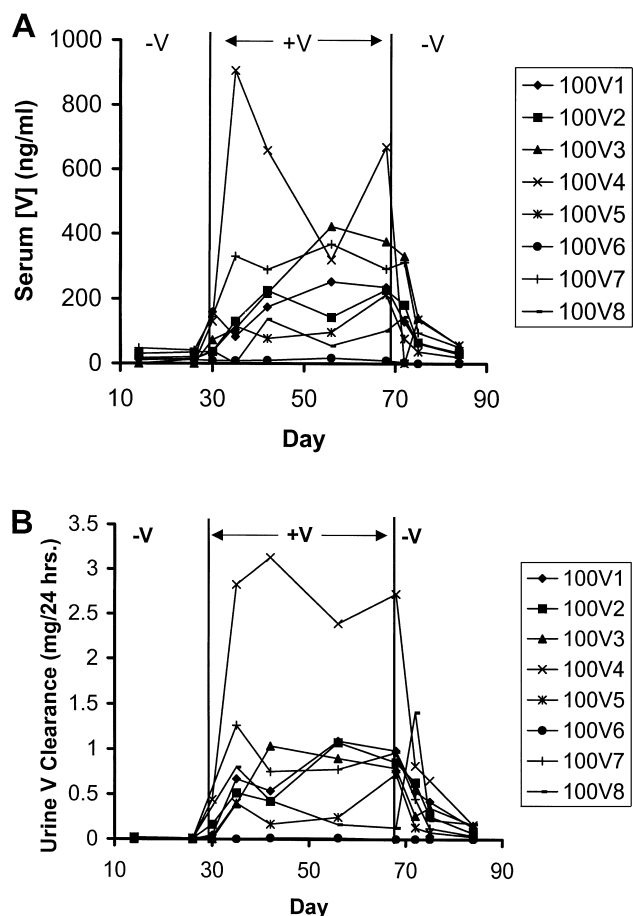


Fig. 2. Time course of serum and urinary vanadium clearance in type 2 diabetic patients orally taking 100 mg elemental vanadium daily as VOSO_4 . Symbols representing each patient are shown on the graph. (A) V in serum; (B) V clearance in urine. V concentrations were determined by GFAAS as described in Section 2.

3.2. Comparison of clinical response of type 2 diabetic patients with serum V levels

We (Goldfine and Willsky) have previously shown that treatment of type 2 diabetic patients with VOSO_4 improved insulin sensitivity in three out of five patients dosed with 50 mg V/day and four of eight patients dosed with 100 mg V/day. However, the positive response of those patients was not sufficient to produce a statistically significant change for the mean glucose utilization of either group. The mean fasting blood glucose was significantly reduced by VOSO_4 (50 and 100 mg V/day) treatment [1]. In this report we correlated the individual clinical responses with peak serum vanadium levels.

The clinical responses of all patients for mean fasting blood glucose and euglycemic clamp M at low and at high insulin levels (clamp low and high) are given in Fig. 3. Clinical responses were treated as a fractional response (Final Value – Original Value)/Original Value. Thus, if V treatment improved the mean fasting glucose the fractional change would be negative, while the fractional clamp

Table 1

Summary of the serum V data from all patients

Patient	Peak V (ng/ml)	Steady state V (ng/ml)	$t_{1/2}$ (days)
25V-1	16.8	16.8	3.8
25V-2	8.30	^a	^a
25V-3	21.1	13.8	12
Ave. \pm S.D.	15.4 \pm 6.5	15.3 \pm 2.1	7.7 \pm 5.5
50V-1	114	110	5.8
50V-2	30.2	30.2	9.8
50V-3	51.7	16.4	7.5
50V-4	125	95.7	6.3
50V-5	87.0	67.2	9.4
Ave. \pm S.D.	81.7 \pm 40.5	63.8 \pm 40.3	7.8 \pm 1.8
100V-1	253	244	3.6
100V-2	228	185	5.3
100V-3	423	400	5.6
100V-4	906	549	4.6
100V-5	213	155	5.0
100V-6	16.6	^a	^a
100V-7	368	330	5.8
100V-8	145	78.4	7.6
Ave. \pm S.D.	319 \pm 268	278 \pm 161	5.4 \pm 1.2

^a Not determined.

responses would be positive, indicating increased insulin sensitivity. Of the three patients receiving 25 mg V/day, one showed improved clinical responses for two variables. Of the five patients receiving 50 mg V/day, two showed an improved response for two variables, while two showed improved responses for all three variables. Of the eight patients receiving 100 mg V/day four responded well for two variables and three responded well for all three.

To correlate peak serum V to these three clinical responses Pearson product–moment correlation coefficients and their associated P values were calculated for the natural log of peak serum V and the three fractional clinical responses for all patients. The results showed correlation and P value of the natural log of peak serum V with mean fasting blood glucose to be 0.26, $P=0.34$, respectively, with low insulin clamp to be -0.17 , $P=0.53$, and with high insulin clamp to be 0.08, $P=0.77$. There was clearly no correlation at the individual level of peak serum V with any of the three clinical responses in these patients treated with VOSO_4 .

3.3. Comparison of non-fasting plasma glucose and blood vanadium in rats with STZ-induced diabetes treated with VOSO_4 and $\text{VO}(\text{malto})_2$

When $\text{VO}(\text{malto})_2$ and VOSO_4 were administered to rodents in a single i.p. injection, VOSO_4 did not statistically significantly lower plasma blood glucose in any of the eight animals tested. In contrast, $\text{VO}(\text{malto})_2$ substantially reduced plasma glucose in six of the eight animals tested. For example, at the 24 h time point the mean plasma glucose level for all the animals treated with $\text{VO}(\text{malto})_2$

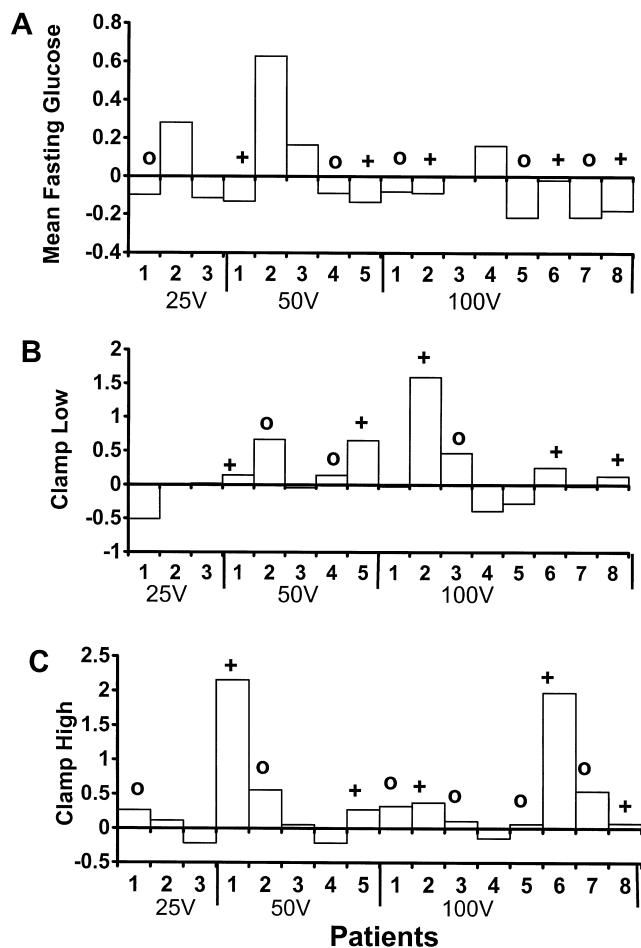


Fig. 3. Response of type 2 diabetic patients orally dosed with VOSO_4 for mean fasting blood glucose and euglycemic clamp at two insulin levels. (A) Mean fasting blood glucose. (B) Euglycemic clamp at high insulin concentration. (C) Euglycemic clamp at low insulin concentration. (○) Indicates the five patients who showed an improved response for two of the three variables and is located on each graph for which the response improved for that patient (25V-1, 50V-4, 100V-1, 5 and 7). (+) Indicates the five patients who showed an improved response for all three clinical variables and is located on all three graphs for that patient (50V-1 and 5, 100V-2, 6, and 8). The clinical responses in (A–C) were measured as described in Section 2, and are given as fractional responses as described in the text.

was 11.4 ± 3.3 mM while the corresponding values for the VOSO_4 treated animals were 22.0 ± 2.3 mM (data not shown). Fig. 4 illustrates the range of plasma glucose and blood V levels measured at 12 and 48 h for all animals. The blood V levels in the VOSO_4 treated animals during these time points had a range from 227 to 1229 ng/ml, while the range for the $\text{VO}(\text{malto})_2$ treated animals was very similar, from 80 to 1164 ng/ml. For the VOSO_4 treated animals high plasma glucose levels persisted in the presence of high levels of V in blood (as seen in the upper right quadrant of Fig. 4). Low blood glucose levels were not associated with low blood V (left portion of Fig. 4), a trend similar to that observed in our human type 2 diabetic patients. In contrast, at blood vanadium levels over 380

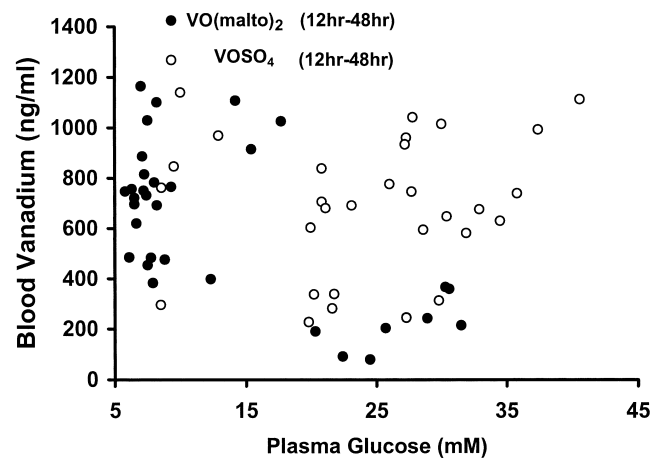


Fig. 4. Comparison of plasma glucose and blood vanadium in STZ diabetic rats treated with VOSO_4 and $\text{VO}(\text{malto})_2$. Open circles represent rats treated with VOSO_4 ; closed circles represent rats treated with $\text{VO}(\text{malto})_2$. Graph contains all values obtained at 12, 18, 24 and 48 h after injection. Experimental details are given in Section 2.

ng/ml, plasma glucose levels less than 16 mM were seen (upper left quadrant Fig. 4); while at blood V levels less than 370 ng/ml, plasma glucose levels remained high (lower right quadrant Fig. 4). Furthermore, the data in the lower right quadrant of the figure comes from the two animals that did not respond to $\text{VO}(\text{malto})_2$ treatment with lowered plasma glucose. These results show a trend in which the blood V values do not seem to be related to plasma glucose levels in the animals treated with the V salt VOSO_4 ; while blood V does seem to be related to plasma glucose levels in the animals treated with the coordinated $\text{VO}(\text{malto})_2$.

3.4. Interaction of VOSO_4 and $\text{VO}(\text{malto})_2$ with human serum albumin (HSA)

Spectra were recorded using ambient and low temperature EPR spectroscopy with a slight excess of metal compound for both bovine serum albumin (data not shown) and HSA. Selective spectra were also recorded at a greater excess of metal compound to protein. Solutions containing hydrated VO^{2+} at neutral pH form EPR invisible species and no signal is observed in solutions containing Hepes buffer and VOSO_4 [20] (data not shown). However, when solutions of HSA (0.29 mM), 0.1 mM VOSO_4 and 0.1 M Hepes (pH 7.4) are combined an EPR spectrum is observed similar to that reported previously for the albumin–vanadyl complex [20,21]. Since low temperature spectra have improved separation and the complexes under investigation have very similar parameters, only the low temperature EPR spectra of the HSA and VOSO_4 are illustrated here in Fig. 5.

The spectra of 0.41 mM $\text{VO}(\text{malto})_2$ in 100 mM Hepes at both pH 7.4 and 9.1 are shown in Fig. 5 as background subtracted spectra before any data manipulation. The

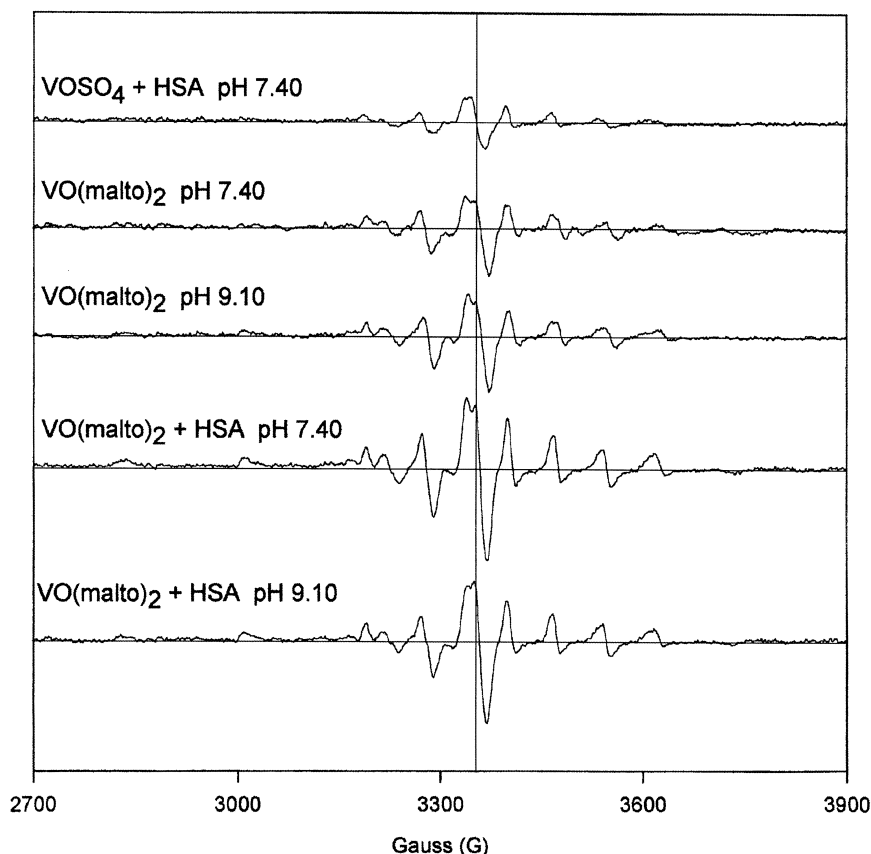


Fig. 5. EPR spectra of human serum albumin with VOSO_4 or $\text{VO}(\text{malto})_2$ at low temperature. Spectra listed from the top were obtained of solutions containing the following: 0.41 mM VOSO_4 and 0.29 mM HSA at pH 7.40; 0.41 mM $\text{VO}(\text{malto})_2$ at pH 7.40; 0.41 mM $\text{VO}(\text{malto})_2$ at pH 9.10; 0.41 mM $\text{VO}(\text{malto})_2$ and 0.28 mM HSA at pH 7.4; and 0.41 mM $\text{VO}(\text{malto})_2$ and 0.28 mM HSA at pH 9.10. Each solution also contained 100 mM Hepes. A perpendicular to the indicated baselines has been drawn intersecting zero for the top spectrum.

signal of $\text{VO}(\text{malto})_2$ in the absence of Hepes is weak at low temperature reflecting the difficulties in recording EPR spectra of frozen aqueous solutions (which form imperfect gels). Furthermore, more than one isomer is present in aqueous solution and can be observed in these spectra, as reported previously [28]. Since we did not examine the power saturation of the signals at low temperature in these spectra, the following results are qualitative. Combining solutions of HSA (0.28 mM), $\text{VO}(\text{malto})_2$ (0.41 mM) and Hepes (100 mM, pH 7.4) lead to EPR spectra in which the signals for both $\text{VO}(\text{malto})_2$ and the $\text{VO}(\text{malto})_2$ protein complex are observed. In order to convincingly see the new signal, we subtracted the signal due to $\text{VO}(\text{malto})_2$ using Sigma Plot (data not shown). The difference in the EPR spectra of the complexes formed by protein with VOSO_4 and $\text{VO}(\text{malto})_2$ is illustrated by the line drawn perpendicular to the baselines. The fact that this line intersects the VOSO_4 protein complex spectrum at zero (top spectrum), and the $\text{VO}(\text{malto})_2$ protein complex spectra above zero (bottom two spectra) demonstrates that these complexes are different.

The observation of a $\text{VO}(\text{malto})_2$ protein complex different from $\text{VO}(\text{malto})_2$ is best seen in Fig. 5 in the spectra of the $\text{VO}(\text{malto})_2$ protein complexes (bottom two

spectra) by viewing the asymmetric doublet directly left of the perpendicular line. In the bottom spectrum of $\text{VO}(\text{malto})_2$ and protein at pH 9.1 the right peak of the doublet is larger than the left peak, unlike the spectra for $\text{VO}(\text{malto})_2$ alone at either pH. Further support for the formation of a new signal for the $\text{VO}(\text{malto})_2$ protein complex was obtained from spectra containing excess protein to $\text{VO}(\text{malto})_2$ at different pH values in which as the protein concentration increased, the protein $\text{VO}(\text{malto})_2$ complex concentration increased, and the concentration of free maltol decreased (data not shown).

3.5. Interaction of VOSO_4 and $\text{VO}(\text{malto})_2$ with human apotransferrin (apoHTf)

Spectra were recorded using ambient and low temperature EPR spectroscopy with a slight excess of metal compound and dialyzed apoHTf. Given the interest in correlating these spectral studies with the data observed in animal and human studies, and the fact that the complexes observed in this system show very different parameters at ambient temperature, we show here only the spectra recorded at ambient temperature. The spectrum recorded for VOSO_4 confirmed that all V(IV) had formed EPR

invisible species with no signal [17]. However, when solutions of apoHTf (0.31 mM), VOSO_4 (0.46 mM) and Hepes (pH 7.5) are combined, an EPR spectrum is observed (Fig. 6, top). This anisotropic ambient temperature spectrum clearly shows that a protein complex is formed in these solutions and the spectrum corresponds to those reported previously [23].

In Fig. 6 (center), we also show the spectra of 0.45 mM $\text{VO}(\text{malto})_2$ in 100 mM Hepes at pH 7.5. Combining solutions of apoHTf (0.41 mM), $\text{VO}(\text{malto})_2$ (0.45 mM) and Hepes (100 mM, pH 7.5) gave an anisotropic EPR spectrum (Fig. 6, bottom). Since the isotropic signal for the $\text{VO}(\text{malto})_2$ is no longer observed, the anisotropic spectrum is due to the protein complex. Given the dramatic differences in A_0 values (9G) for the two protein complexes (Fig. 6, top and bottom) there can be no doubt that the observed complexes are different. To further characterize the nature of these complexes, additional experiments were conducted in which low temperature spectra confirmed the observation of two distinct protein complexes (data not shown). It is well known that NaHCO_3 enhances the observation of the HTf- VO^{2+} signal [17] and this was confirmed in our studies. However, there was no enhancement observed with the protein complex derived from the apoHTf and $\text{VO}(\text{malto})_2$ at either ambient or low temperature. In contrast, the presence of carbonate appeared to reduce the stability of $\text{VO}(\text{malto})_2$ and the protein derived spectrum from this species (data not shown).

3.6. Lack of interaction of VOSO_4 and $\text{VO}(\text{malto})_2$ with immunoglobulins at ambient temperature

In Fig. 7A (bottom left), the spectra is shown for a solution of VOSO_4 , and in the presence of 0.21 mM IgG (top left). Under these conditions, we were not able to observe any complex formation by VOSO_4 . Similarly, the spectrum of $\text{VO}(\text{malto})_2$ is shown in Fig. 7B (bottom right), and in the presence of IgG Fig. 7B (top right). These spectra show no evidence for protein complex formation in the presence of $\text{VO}(\text{malto})_2$. Note that combining solutions of pig IgG with $\text{VO}(\text{malto})_2$ gave an EPR spectrum identical to that of $\text{VO}(\text{malto})_2$ alone.

4. Discussion

The levels of vanadium achieved in serum and urine was highly variable when administered orally to type 2 diabetic subjects at doses of 25, 50 and 100 mg V daily as a simple salt (75, 150 and 300 mg VOSO_4). In contrast, the correlation of mean peak serum V with dose administered was high ($R^2=0.93$). At these doses, subjects reported symptoms of gastrointestinal distress, although none quit the study for these complaints, and symptoms were managed with either kaopectate or Immodium[®]. Adverse symptomology and toxicology issues [27,29] must be considered and minimized for vanadium compounds to be

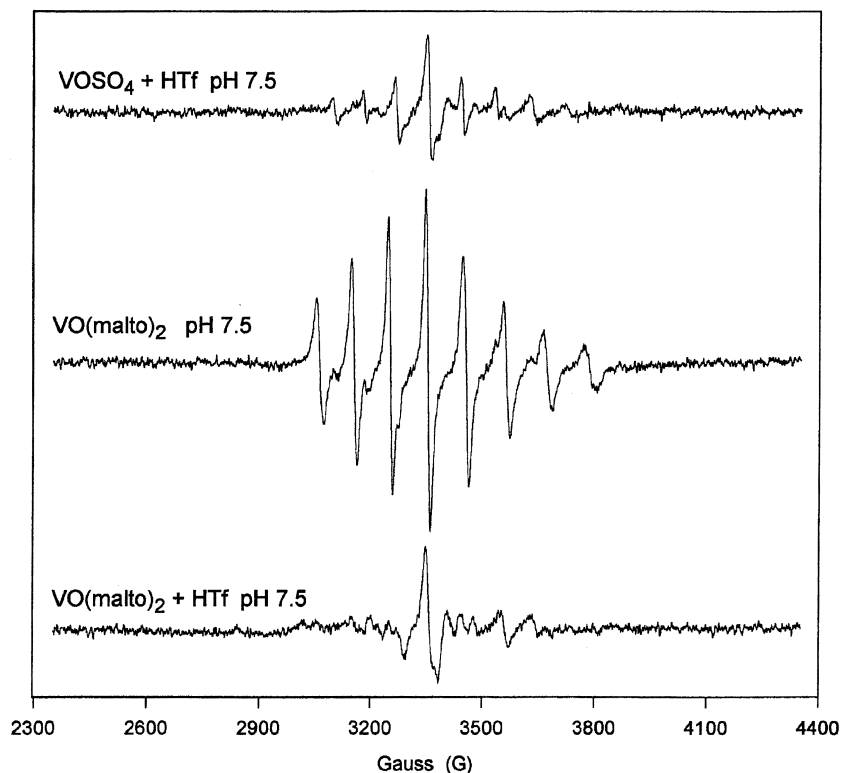


Fig. 6. EPR spectra of transferrin with VOSO_4 or $\text{VO}(\text{malto})_2$ at room temperature. Spectra listed from the top were obtained of solutions containing the following: 0.46 mM VOSO_4 and 0.31 mM apoHTf at pH 7.5; 0.45 mM $\text{VO}(\text{malto})_2$ at pH 7.5; and 0.45 mM $\text{VO}(\text{malto})_2$ and 0.41 mM apoHTf at pH 7.5.

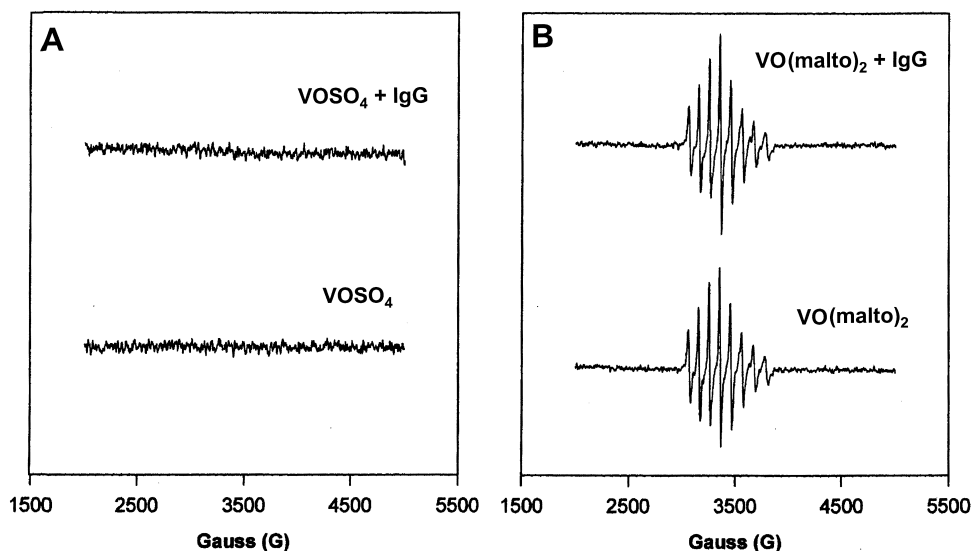


Fig. 7. EPR of VOSO_4 and VO(malto)_2 with pig IgG. Spectra were recorded at ambient temperature of solutions containing: (A) 0.44 mM VOSO_4 and 0.21 mM IgG (top); 0.44 mM VOSO_4 (bottom). (B) 0.44 mM VO(malto)_2 and 0.23 mM IgG (top); 0.44 mM VO(malto)_2 (bottom).

brought to market as viable treatment alternatives for diabetic patients. A better understanding of the pharmacokinetics and bio-availability would assist in product development. The $t_{1/2}$ for disappearance of V from serum was obtained using a one-compartment exponential model. At steady state (four to five half-lives) the amount of V excreted in the urine (urinary clearance) is an estimate of the amount absorbed into the serum. For the three doses of V used in this study absorption was less than 1% (data not shown). Data was fitted to a one-compartment model due to the reasonable fit of the data and a lack of a terminal long-term component to the decay curves. This, of course, does not preclude a large number of compartments into which the V is actually distributed.

Diabetic subjects taking 100 mg V/day had significantly reduced $t_{1/2}$ compared to those taking 50 mg V/day. This could be indicative of dose-dependent changes in V biotransformation or excretion as the body adjusts to the ingestion of higher levels of V. Alterations in the distribution of V in serum and erythrocytes at the higher level of dosing was also observed (data not shown). There was no individual correlation between the concentration of V in serum and any of the three clinical responses evaluated, reduction in mean fasting glucose, or increased glucose sensitivity when measured by euglycemic clamp at both low and high insulin levels. A striking example of this can be seen in the data of patient 100V-6, who responded well for all three clinical variables, yet accumulated little V above his baseline levels (Fig. 2). However more patients did show improvement in the clinical variables assessed as the dose of V was increased. These data strongly imply that neither total serum V levels nor another compartment in equilibration with total serum V is the effective compartment in gauging the clinical response to V.

So what might be the relevant V pool? Experiments in

yeast [8,9] have supported the idea that there is no 'free' metal within cells. The variable response to the metal salt VOSO_4 may be due to free metal quickly binding to various ligands in the serum (such as proteins). If V coordinates to the ligands present in serum, both the relative abundance of the protein and V affinity to that protein would affect bio-availability. This could contribute to the variable effectiveness of V demonstrated when administered as a salt. Given the controlled chemistry of coordinated V complexes, total serum V might better correlate to clinical outcome if V were administered as a coordinated derivative.

To pursue this point, we investigated the correlation of blood V and plasma glucose in STZ-induced diabetic rats treated with both the simple salt VOSO_4 and the coordinated VO(malto)_2 when administered by i.p. injection. The effect of V compounds in alleviating the symptoms of STZ-induced diabetic rats has been widely studied [30]. The concentration of V in the blood is frequently used in rodent studies and allows one to take less fluid from the animal compared to using an equivalent volume of serum. For the type 2 diabetic patients dosed with 50 and 100 mg V/day discussed in this paper the concentration of V in serum correlated well ($R^2=0.86$) with the concentration of V in blood (Willsky, unpublished). Although there appeared to be no relationship between plasma glucose levels and blood V in rats treated with VOSO_4 , there was a trend observed in animals treated with VO(malto)_2 in which lower plasma glucose V levels were associated with higher blood V levels. These data are consistent with our hypothesis that providing the appropriate ligand for the administered V might decrease the binding of the V in circulation to other ligands, increasing the bio-availability of serum V and reducing the variability in patient response. It is also possible that a ligand could bind the V so tightly that it

would be unavailable to the biological target. The goal of future work would be to identify ligands that increase rather than decrease bio-availability.

Proteins, some of which are known to bind V compounds tightly, could bind V under physiological conditions. We have examined the interactions of VOSO_4 and VO(malto)_2 with three serum proteins at ambient and low temperature and demonstrate that both VOSO_4 and VO(malto)_2 form complexes with both HSA and apoHTf. Solutions of VOSO_4 at pH 7.4 yield EPR silent species, however, a signal is observed in the presence of protein. Given the differences observed between the solutions of VO(malto)_2 alone, VO(malto)_2 in the presence of HSA, and VOSO_4 in the presence of HSA, we conclude that a different complex forms between HSA and VOSO_4 compared to that formed with VO(malto)_2 . The complexes that VOSO_4 and VO(malto)_2 form with HSA have very similar spectroscopic signatures, and low temperature EPR studies at variable pH are necessary to demonstrate that the complexes formed are actually different. One interpretation of this observation is that VO(malto)_2 may not lose both maltol ligands, but rather the HSA binds the V while at least one of the maltol ligands remain coordinated.

The spectra in Fig. 5 show that VOSO_4 and VO(malto)_2 form similar amounts of protein complexes with HSA. The concentration of VO^{2+} is very small in the neutral pH range, and the concentration of VO(malto)_2 is significant. Given the dimerization and polymerization of VO^{2+} there is only very little VO^{2+} in solution, and the concentration of VO(malto)_2 is all the compound that is not complexed to the protein. Therefore, the protein has a significantly greater affinity for VO^{2+} than for VO(malto)_2 under physiological conditions. Thus, when discussing the strength of the formation constant the VOSO_4 forms a much stronger complex.

The spectra in Fig. 6 show that VOSO_4 and VO(malto)_2 also form nearly equal amounts of protein complexes with apoHTf. Since the protein concentrations are similar, it may appear that the apoHTf has a similar affinity for the two compounds. However, given the concentration of free VOSO_4 and VO(malto)_2 in solution, the apoHTf- VO^{2+} is the stronger complex.

The HSA-V complex is very sensitive to specific solution conditions. The V(IV) complex at neutral pH is readily oxidized and forms other EPR invisible species. Thus, although the preparation of the solutions by adding stock solutions of VOSO_4 (or VO(malto)_2) to a solution containing HSA followed by the addition of Hepes buffer reproducibly leads to observation of two different complexes as presented in this paper, the order of component addition did seem to seriously affect complex formation under these conditions (which did not stringently attempt to exclude oxygen). We therefore attribute the differences in our observations with those observed by Orvig and Thompson (personal communication) to the conditions of solution preparation and the difficulty of recording spectra

of frozen aqueous solutions which do not form perfect gels.

EPR spectral parameters of a protein complex reflect the protein residues that coordinate to the V. If the V in VOSO_4 and VO(malto)_2 binds similarly to apoHTf, the complexes would have identical parameters. The complexes formed between apoHTf and VOSO_4 and VO(malto)_2 have different EPR spectra as documented in both ambient and low temperature studies. The difference in the coordination environment of these complexes, presumably reflect that at least one maltol ligand remained coordinated to the V in the protein complex, allowing an EPR spectrum to be recorded. Since carbonate coordinates to the vanadyl bound to the apoHTf, lack of observation of spectral enhancement with the complex derived from HTf is consistent with a maltol ligand coordinating to the V and thus preventing the carbonate from binding. An alternative explanation is the possibility that maltol acts as a synergistic anion as reported for carbonate [31,32]. There are several reports detailing such an interaction for Fe(III) binding to HTf. We favor the former explanation since we know from the speciation profiles that a VO(malto)_2 complex forms [24]. Since the pK_a value for formation of the maltol anion is 8.46 [24], the anion is present in significant concentrations.

Finally, we did not observe any interaction between VOSO_4 and VO(malto)_2 and IgG, ruling out the possibility that these immune proteins form weak complexes with vanadyl cation as the albumins do. These results suggest that should a complex form with IgG in vivo, it would likely be significantly weaker than those formed with HSA. The implications of all these EPR studies are that should the VO(malto)_2 and other organic complexes survive the acidity of the stomach and transport into the blood stream, the VO(malto)_2 would be expected to distribute differently in the blood than VOSO_4 .

These results demonstrate that further work needs to be done to both to identify which patients would best respond to V therapy, and to determine whether coordinated forms of V would be more effective therapeutically than the simple salt, as suggested in the rodent model. The human and animal studies described here combined with the in vitro studies with isolated serum proteins suggest that significant differences exist in the biotransformation of the V salts and coordinated V compounds. The observation that the plasma glucose levels of STZ-induced diabetic rats treated i.p. with VOSO_4 do not correlate with the blood V levels, while there is a strong correlation in similar animals treated with VO(malto)_2 , is promising for future studies with coordinated V compounds.

5. Notation

apoHTf, human apotransferrin; EPR, electron paramagnetic resonance; GFAAS, graphite furnace atomic absorption spectrometry; Hepes, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-

ethanesulfonic acid]; HSA, human serum albumin; IgG, immunoglobulin (purified IgG fraction); i.p., intraperitoneal; NMR, nuclear magnetic resonance; STZ, streptozotocin; V, vanadium; VO(malto)₂ frequently seen as BMOV, bis(maltolato)oxovanadium(IV); VO₄, vanadyl sulfate

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