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Coordination Chemistry Reviews 237 (2003) 13–22

COORDINATION CHEMISTRY REVIEWS

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(4-Hydroxypyridine-2,6-dicarboxylato)oxovanadate(V)—a new insulin-like compound: chemistry, effects on myoblast and yeast cell growth and effects on hyperglycemia in rats with STZ-induced diabetes

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Received 25 January 2002; received in revised form 24 September 2002; accepted 11 October 2002

Contents

Abstract

A new insulin mimetic vanadium(V) complex is introduced: (4-hydroxypyridine-2,6-dicarboxylato)oxovanadate(V). The compound was designed based on the desire to make a compound with more favorable chemical and insulin-enhancing properties than the parent (pyridine-2,6-dicarboxylato)oxovanadate(V). The solution chemistry was characterized and the complex was found to be more stable at neutral pH and to have a different lability pattern than the parent complex. The effect of the compound and its parent was investigated in various biological systems including cell culture, yeast and streptozotocin (STZ)-induced diabetic rats. The growth of myoblast cells (L6) was inhibited by both the parent and the modified vanadium(V) complex. Since the complexes have limited stability at neutral pH, yeast growth (pH range from 3.0 to 7.0) was employed as an adjunct cell model. The effect of the parent compound on inhibition of yeast cell growth was found to be pH dependent. These studies support the hypothesis that the

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0010-8545/02/$ - see front matter
PII: S0010-8545(02)00292-8

modified complex would be more active as an insulin-enhancing agent because of its greater stability at neutral pH. The effect of (4-hydroxypyridine-2,6-dicarboxylato)oxovanadate(V) on hyperglycemia in rats with STZ-induced diabetes was determined. This complex was found to lower the diabetic hyperglycemia and joined the ranks of the few vanadium(V) complexes that have been shown to have insulin-enhancing properties in a diabetic animal model system.

Keywords: Vanadium(V); Dipicolinate; Insulin; Diabetes; Myoblast; Yeast; Rat; Vanadate; Muscle cell; *S. cerevisiae*

1. Introduction

The most promising vanadium complexes with insulin-like effects contain desirable organic ligands. The maltol ligand (3-hydroxy-2-methyl-4-pyrone) [1] is of interest because it is an approved food additive. This vanadium(IV) complex is now commonly used as a reference compound and the related vanadium(IV) complex of the ethyl-substituted ligand is currently in clinical trials [2,3]. Picolinic acid (2-pyridinecarboxylic acid) is a desirable ligand because it is formed in the body as an intermediate in the tryptophan degradation pathway and it is also an approved food supplement. The vanadium(IV) picolinato complex is currently being investigated as an important reference compound in the Sakurai group [4–6]. In addition, Chromax®, the trade name for chromium picolinate (tris(picolinato)chromium(III)) is currently being used as a food additive and has been shown to assist diabetic patients in maintaining glycemic control [7,8]. One of our recent target ligands is pyridine-2,6-dicarboxylic acid (also referred to as dipicolinic acid) [9]. This ligand is desirable because of its low toxicity, and its amphophilic nature. Closely related derivatives, 3-pyridinecarboxylic acid (commonly known as niacin or vitamin B3) and 2,3-pyridinedicarboxylic acid (quinolinic acid) are precursors for the coenzyme NAD^+ and required in the human diet.

The vanadium(V) complex of dipicolinate, $[VO_2dipic]^-$, was originally prepared by Wieghardt [10,11] and its solution chemistry recently characterized by us [12]. This compound was found to have insulin-like properties [9]. With this compound serving as one reference compound, we are modifying its chemical properties to determine if the insulin-like properties of the compound can be both retained and enhanced. Specifically, we were interested in identifying compounds with greater stability and/or lability at the pH of the stomach. This proceeding will describe the chemistry and biological studies that we are conducting in compound design to investigate the mode of action of these compounds.

We characterized the chemical and biological characteristics of complexes of modified H_2dipic to explore if subtle changes in chemical properties influence the complexes' biological properties. A vanadium(V) complex formed with 4-hydroxypyridine-2,6-dicarboxylic acid (also referred to as 4-hydroxydipicolinic acid and abbreviated as H_2dipic–OH), $[VO_2dipic–OH]^-$, will be compared with the complex formed with H_2dipic. The Sakurai group has prepared and characterized methyl and iodo derivatives of bis(picolinato)oxovanadium(IV), and both derivatives also had promising insulin-like properties [4,5]. We are expecting our designed compounds to also have insulin-like properties. However, since we are characterizing the chemistry of these complexes in detail we are using chemical and biological studies to both deduce mechanistic information on mode of action of the complexes and to design new compounds with desirable properties.

The exact biological mechanism of the insulin-like activity of oxometalate complexes remains unknown [3]. The origin of the insulin-like action of vanadium compounds has been attributed to effects on signal transduction pathways involving inhibition of protein tyrosine phosphatases including activation of the insulin receptor-protein kinase [13]. Complicating the problem of understanding the insulin-like activities of oxometalates is the fact that the details of the interactions of insulin with cell signal transduction pathways have yet to be fully elucidated. Researchers are still looking for the missing steps in insulin signaling pathways [14].

In this report we describe the effects of the title compound and its parent in both cell growth experiments and animal studies. The cell growth studies investigating the effects of these compounds in various different biological systems were carried out with the objective of examining the true effects of the compounds when they are intact. This point is particularly critical, since the stability of the parent compound was compromised at pH 7.2–7.4, the pH range in which mammalian systems are studied. We have, therefore, used the yeast (*S. cerevisiae*) as an adjunct model system since this organism can grow well from pH 3 to 7. The comparison of cellular response at neutral pH and a pH where the compound is stable can be carried out with the yeast system. Obtaining growth data at two pH values allows one to investigate if the lack of inhibition at neutral pH may be due to the instability of the complex at that pH. In this work, we are describing such cell growth studies with $[VO_2dipic]^-$ and $[VO_2dipic–OH]^-$ Fig. 1 and showing the effect of $[VO_2dipic–OH]^-$ on diabetic hyperglycemia in animal studies.

Since the H_2dipic–OH ligand is more electron-rich than the hydrogen substituted parent, the vanadium(V)

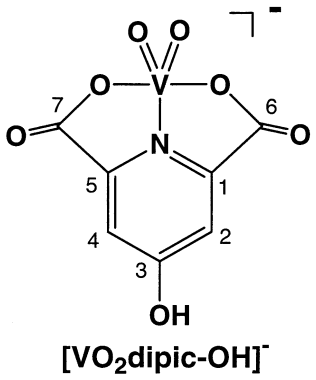

Fig. 1. The solution structure of $[VO_2dipic–OH]^-$ [12,16].

complexes may have greater stability. A compound with increased stability at pH 7 is desirable and may result in a more efficacious insulin-enhancing compound. A compound with a different lability pattern would also be desirable. We have previously proposed that compound stability at the acidic pH of the stomach may be important for compound efficacy [12]. If the ligand is to help in absorption from the intestine, it needs to remain coordinated to the metal ion as it is passed through the stomach. The three acidic protons in $H_2dipic–OH$ compared with the two acidic protons in the parent change the solution properties and the solid state properties of resulting complexes [15]. Given the emphasis on developing the means to understand the mode of action of the vanadium compounds, we will only describe here the solution properties that are directly relevant to the effects of these compounds in cellular systems. Additional details of both the solid state and solution properties of this complex are discussed elsewhere [16].

This report describes both chemical studies and biological studies in cell culture and in diabetic rats with a new vanadium(V) complex. The yeast cell growth studies were carried out to investigate the effect of pH on compound effectiveness. These studies are particularly timely since other cellular studies with V(V)-complexes have been reported recently [17]. With respect to animal experiments, the first vanadium(V) complex to be studied failed to show insulin mimetic properties in animal model studies [18], leading people to believe that this oxidation state of vanadium would not be effective. However, since then two vanadium(V) compounds in addition to vanadate have been found to have insulin-like properties. The Shechter group has reported on one V(V) compound whose structure has not yet been determined [19] and we have previously reported on the V(V)-dipic complex ($[VO_2dipic]^-$) [9], showing that some vanadium(V) compounds can have insulin-like activity. To elucidate the nature of vanadium(V) complexes that enable them to show this insulin-like activity, more examples of vanadium(V) complexes demonstrating insulin-like properties in well known animal model systems are needed.

2. Experimental

2.1. Solution studies

The samples for spectral analysis were prepared by using NH_4VO_3 and ligand or preformed complex [10–12]. The 1D ^1H and ^{51}V spectra were recorded on a Varian INOVA-300 spectrometer (7.0 T) at 300 MHz for ^1H and 78.9 MHz for ^{51}V. Routine parameters were used for the 1D ^1H-NMR experiments. 3-(Trimethylsilyl)propane sulfonic acid sodium salt (DSS) was used as an external reference for ^1H chemical shifts. ^{51}V-NMR spectra were acquired with a spectral window of 83 600 Hz, a pulse angle of 60°, and an acquisition time of 0.096 s with no relaxation delay. ^{51}V-NMR chemical shifts were referenced against an external sample of $VOCl_3$.

The 2D ^1H EXSY experiments were run on a Varian INOVA-300 spectrometer (7.0 T) at 300 MHz at 23 °C. The 2D ^1H EXSY spectra shown were recorded with a sweep width of 459 Hz, an accumulation time of 0.139 s, a delay time of 3.0 s, a mixing time of 0.5 s, and 128 increments of four scans each. The 2D ^{13}C EXSY spectra were recorded with a sweep width of 8870 Hz, an accumulation time of 0.231 s, a delay time of 2.0 s, a mixing time of 0.5 s, and 128 increments of eight scans each.

2.2. Tissue culture

L6 myoblasts, obtained from American Type Culture Collection (ATCC), were routinely maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) at pH 7.4. The complete DMEM medium is DMEM supplemented with 10% fetal bovine serum, 15 mM Hepes, 1.5 g l^{-1} sodium bicarbonate, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. The L6 cells were grown in a humidified atmosphere of 5% CO_2–95% air at 37 °C. Cultures received fresh medium 1 day prior to the day of experiment. Cells were removed from culture flasks by trypsin treatment and about 1.0×10^4 cells per well were inoculated into six-well culture plates. Cultures were grown in the presence of vanadium compounds ($[VO_2dipic]^-$ and $[VO_2dipic–OH]^-$) at doses of 0, 1, 10, 100, and 1000 μM in complete DMEM. Cells were harvested at about 35–40% confluence (day 3 or 4) and 75–80% confluence (day 5 or 7). Cell viability and viable cell counts were determined in triplicate using a hemocytometer and trypan blue exclusion procedure.

2.3. Yeast cell growth

S. cerevisiae strain LL20 (*MATα his3-11,15 leu-2,112*) was used. Yeast were grown and maintained at 30 °C with shaking and aeration as previously described on extract–peptone–dextrose (YPD) plates and experiments were done in minimal media [20]. Yeast nitrogen base medium without amino acids (Difco), 2% dextrose (Fisher), and buffer 100 mM Tris–succinate (pH 4.5 or 6.5) was the defined minimal salts media used for all experiments. Amino acids (5 mg ml^{-1}) were added as needed. $[VO_2dipic]^-$ was added as a powder directly to 10 ml of medium, dissolved by vortexing and the medium was then filter sterilized. Cells in logarithmic phase from a fresh overnight culture were added to the medium to an OD_{600} of 0.10. The pH of the culture was adjusted with filter sterilized HCl or NaOH and divided into three cultures of a volume of 3.3 ml. Growth was monitored using a spectrophotometer at OD_{600} at 3, 8, and 24 h. If visible color was observed, the OD_{600} of the sample obtained after cells were removed by low speed centrifugation in a microfuge was subtracted from the original reading. Growth was followed as OD_{600} versus time and values were plotted in a semi log plot.

2.4. Animal protocol

Modifications of the previously published procedure for the induction of diabetes and animal care were used [21]. The protocol, approved by the SUNY at Buffalo IACUC Committee, is in compliance with State and Federal regulations. Male rats from three different Wistar rat strains were examined in this study. The commonly used Wistar strain is an outbred strain and will be referred to as Wistar (Outbred). In addition, two inbred rat strains: Wistar (Kyoto) and Wistar (Furth) were also used. Wistar (Outbred) rats weighed 186–252 g, whereas age-matched Wistar (Kyoto) weighed 154–192 g and Wistar (Furth) 166–190 g. Diabetes was induced by administration of 60 mg ml^{-1} freshly prepared streptozotocin (STZ) injected either intravenously in 0.9% saline or intra-peritoneally in 100 mM sodium citrate, pH 4.5 at a dose of 60 mg kg^{-1} body weight. Blood glucose levels were determined 4 days after streptozotocin injection with an Accu-Chek® blood glucose monitor as previously described [21]. The diabetic animals were randomly assigned to treated or untreated groups. Normal untreated animals were matched in age and weight. Treatment protocol consisted of adding compounds to the drinking water in concentrations from 1.6 to 6.2 mM. Daily intake of water was monitored and for the first 6 days the concentration of metals in the water was adjusted to try to achieve similar dosing as measured by mmol kg^{-1} per day. From day 6 to 14 the concentrations of metal were as follows: $VOSO_4$ ($n=4$) 6.2 mM; $[VO_2dipic–OH]-$ ($n=8$) 3.3 mM, or $dipic–OH^{2}-$ ($n=4$) 5.0 mM. The diabetic untreated control group consisted of five animals ($n=5$). The experiment with the Wistar outbred male rats is part of an ongoing study using a protocol in which normal, diabetic and treated animals are carefully handled in the same manner in different sets of animals. Each set includes ca. 50 animals. This allows us to have only two normal rats per set of animals and include data from normal animals in another set of animals. For comparison of different strains of rats 6.2 mM $VOSO_4$ was added to the drinking water daily for 14 days.

Blood glucose levels were determined twice a week and urine ketones and glucose determined once a week using Ketodiastix®. Any animal appearing to be dehydrated as monitored by loss of weight or physical examination, was re-hydrated subcutaneously behind the neck with 10–30 ml per day of lactated Ringer's bicarbonate solution (0.84%).

2.5. Statistics

Data for blood glucose levels was analyzed using a one way ANOVA with Dunnet's multiple means test. To achieve a more balanced ANOVA with $n=4$–6, if more than six animals were included in a group, samples were deleted randomly from the analysis to achieve an n of 6.

3. Results and discussion

3.1. Compound design: rationale

Compound design is based on knowledge of compound efficiency and chemistry. A series of organic vanadium(V) complexes [12,22,23] were found to be labile [24–28]. These complexes include vanadium(V) compounds with polydentate O,N-donor containing functionalities [24,26–28]. The $[VO_2dipic]^-$ complex, recently shown to lower blood glucose in cats [9] and in STZ-induced diabetic rats [29], is the first well characterized vanadium(V) compound [12] with insulin-enhancing activity. Variable temperature and EXSY spectroscopic studies described above show, perhaps not surprisingly, that the $[VO_2dipic]^-$ complex also is labile [12]. Given the insulin-like activity of this complex, and the fact the compound had very low stability and high lability at neutral pH, the insulin-like properties of this compound in animals were not anticipated to even reach the level of simple salts [9]. Since it was found to have pH dependent lability but that the pH region of lowest lability was at the pH of the stomach, we set out to identify compounds with different lability patterns. If compounds with different lability patterns also have insulin-like properties, this information will be important in evaluation of the role of the ligand.

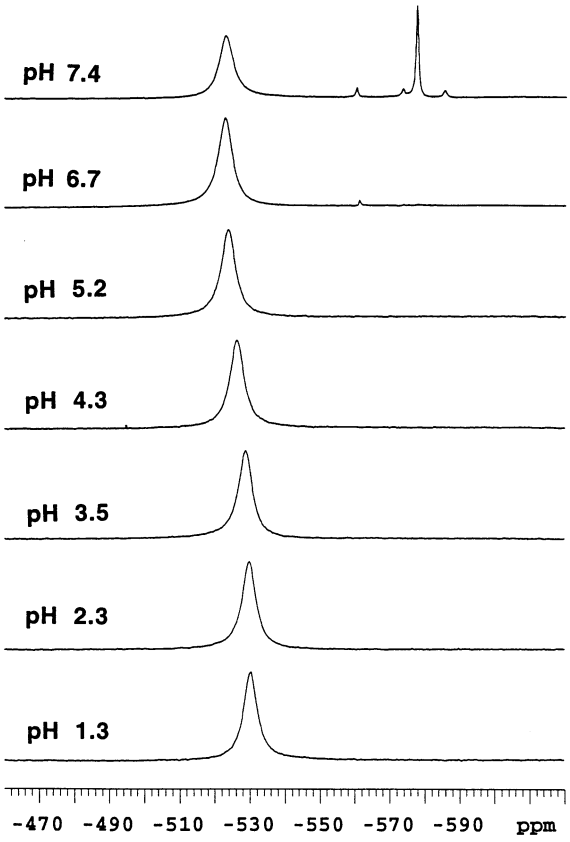

Fig. 2. ^{51}V-NMR spectra for solutions of dissolved 40 mM NH_4VO_3 and 69 mM H_2dipic–OH from pH 1.3–7.

3.2. Stability and structure of $[VO_2$dipic–OH$]^-$

The ammonium, potassium and methyl amine salts of the vanadium(V) complex were synthesized and investigated. The solution properties are identical whether the complex is formed in situ or by dissolution of crystalline compound. Spectroscopic studies were conducted and ^{51}V-NMR spectra showed only one pH dependent signal for solutions of NH_4VO_3 and H_2dipic–OH [16,29] in the pH range 1.3–7 (Fig. 2). Above pH 6.7, signals for oligovanadates show that the complex hydrolyzes to form vanadate and dianionic hydroxydipicolinate (dipic–OH^{2-}). However, more $[VO_2$dipic–OH$]^-$ exists at neutral pH than previously reported for $[VO_2$dipic$]^-$ [12].

The stoichiometry of the compound was found to be 1:1. Since the chemical shift changes when describing the dianionic form at −522 ppm and the monoanionic form at −530 ppm (< pH 3), the complex contains an acidic proton. The concentration of $[VO_2$dipic–OH$]^-$ changes gradually following a bell curve with a maximum around 3. The complex formed in the pH range 6.6–7.9 can be described in reaction (1) and the formation constant based on equation (1) was found to be $K = 1.6 \pm 0.1 \times 10^{10}$ M^{-2} (data not shown). Given the change in the ^{1}H chemical shift, a pK_a value for the complex can be calculated to be 4.1 (±0.1) [30].

$$H_2VO_4^- + \text{dipic–}OH^{2-} + 2H^+ \rightleftarrows [VO_2(\text{dipic–OH})]^- + 2H_2O \quad (1)$$

^{1}H-, ^{13}C- and ^{17}O-NMR spectra were recorded to obtain information on the solution structure, and the data show that $[VO_2$dipic–OH$]^-$ (Fig. 1) has the same solution structure as the parent complex $[VO_2$dipic$]^-$ [12].

3.3. Lability of the VO_2(Hdipic–OH)$^-$ anion

Variable temperature ^{1}H-, ^{51}V- and the ^{13}C-NMR spectra were recorded of solutions added crystalline $[VO_2$dipic–OH$]^-$, solutions with both crystalline $[VO_2$(dipic–OH)$]^-$ and excess free ligand, and solutions with both NH_4VO_3 and free ligand, respectively. The variable temperature ^{13}C-NMR spectra (from 298 to 328 K) of a solution containing 515 mM NH_4VO_3 and 836 mM ligand H_2dipic–OH at pH 3.6 show broadening of all ^{13}C signals as the temperature was increased (data not shown). In contrast, in the corresponding experiment at pH 6.7 the exchange broadening was decreased beyond detection (data not shown).

To obtain additional information regarding the nature of this process, homonuclear ^{1}H and ^{13}C EXSY spectroscopy were obtained for a solution of 40 mM NH_4VO_3 and 69.3 mM free complex at pH 3.6 (±0.1), 3.7 (±0.1), 3.9 (±0.1) and 5.0 (±0.1) (Fig. 3). The ^{1}H-NMR EXSY spectra were recorded at pH 3.1, 3.5, 3.7, 3.9, 4.3, 4.6, 4.9, 5.0, 5.2 and 6.7. Although the chemical shifts for the free ligand and complex varied significantly with pH, some of these changes are attributed to chelated ligand exchanging with free ligand. In Fig. 4 the ratio of total volume integrals of exchange cross-signals between $[VO_2$dipic–OH$]^-$ and free ligand and the total diagonal-signals obtained from ^{1}H EXSY-NMR spectra are plotted as a function of pH.

The unique insulin-like properties of the $[VO_2$dipic$]^-$ complex are of interest for three reasons: (1) $[VO_2$dipic$]^-$ is charged, which suggests absorption may utilize different pathways; (2) $[VO_2$dipic$]^-$ is unstable at the physiological pH of intercellular fluids and (3) $[VO_2$dipic$]^-$ is labile. As a result it has been proposed, that the $[VO_2$dipic$]^-$ complex is reduced to a form that has greater stability at the pH of the cellular target [12]. Such a hypothesis would imply that the role of the dipic-ligand is to transport the vanadium through the stomach and across the plasma membrane of cells in the intestinal tract. In several of the vanadium(IV) complexes in use, the role of the ligand is believed to be related to metal ion delivery to the cellular targets [3]. These hypotheses show the need for the design of compounds with different stability and lability patterns, that will allow testing of such hypotheses. [VO_2(dipic–

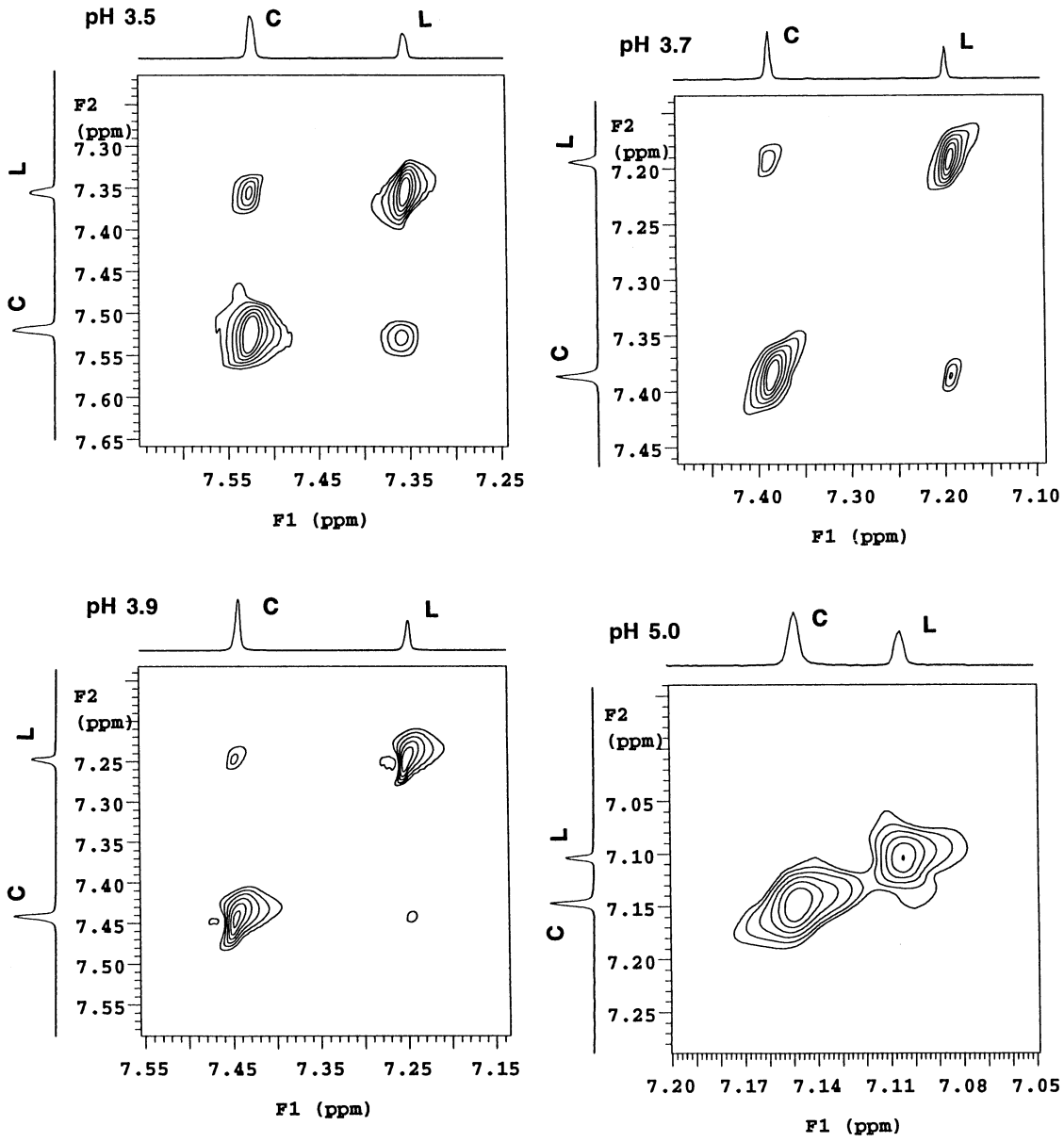

Fig. 3. ^{1}H EXSY spectra of the solution containing 40 mM NH_4VO_3 and 69 mM ligand H_2dipic–OH at pH 3.5 (±0.1), 3.7 (±0.1), 3.9 (±0.1) and 5.0 (±0.1).

OH)]$^-$ is an example of a complex that has a different pH dependent profile, and evaluation of it's insulin-enhancing activity will address the proposed mode of action of the parent complex.

3.4. Effects of [VO_2dipic]− and [VO_2(dipic–OH)]$^-$ on the growth of mammalian cells

The addition of [VO_2dipic]− to a rat myoblast cell line (L6) at 0.001 mM results in growth stimulation, while inhibition is observed at 0.010 mM (Fig. 5). No growth is observed in the presence of 0.10 mM (Fig. 5) or 1.0 mM [VO_2dipic]− (data not shown). The effect of [VO_2dipic–OH]− on myoblast cell growth is also shown in Fig. 5. The [VO_2dipic–OH]− complex shows a greater inhibition at 0.010 mM than [VO_2dipic]− and does not show growth stimulation at 0.001 mM. No growth was observed at 0.10 and 1.0 mM (data not shown for 1.0 mM). Ligands alone ([dipic–OH]$^{2-}$ and [dipic]$^{2-}$) had no effect on the growth of L6 cells at all concentrations tested (data not shown).

As described above [VO_2dipic–OH]− is more stable than [VO_2dipic]− at neutral pH and more labile from pH 2 to 4. The enhanced effect of [VO_2dipic–OH]− on inhibition of tissue culture cell growth compared with [VO_2dipic]− could be due to the increased stability of [VO_2dipic–OH]− at pH 7.4 values. Tissue culture studies must take place at pH 7.4. Both [VO_2dipic]−

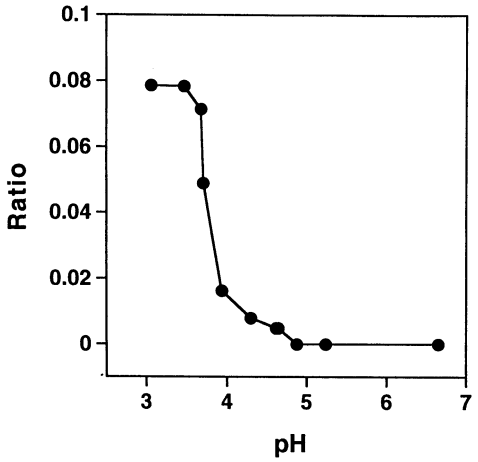

Fig. 4. The ratio of total volume integrals of exchange cross-signals between $[VO_2dipic–OH]^-$ and free ligand and the total diagonal-signals obtained from ^1H EXSY-NMR spectra are plotted as a function of pH. The results depicted by solid circles represent data obtained from solutions 40 mM NH_4VO_3 and 69 mM ligand $H_2dipic–OH$.

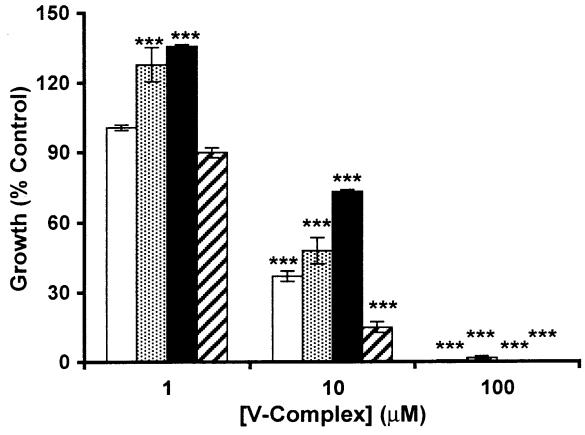

Fig. 5. The effect of $[VO_2dipic]-$ and $[VO_2dipic–OH]^-$ on growth of L6 myoblast cells in culture. Cells were grown in complete DMEM in the presence of 0, 1, 10 or 100 μM complex as described in the Section 2. An initial inoculation of 1.2×10^4 cells per well was plated in triplicate at day 0. Viable cell counts for each dose group were done in triplicate and cells harvested at ca. 30–40 and 70–85% confluence. Results are shown as percent growth of controls with ca. $1.5–6.6 \times 10^5$ viable cells for 30–40% confluence harvested at day 3 or 4 and $1.5–2.5 \times 10^6$ viable cells for 70–85% confluence harvested at day 5 or 7. Bars represent: at 30–40% confluence (white) treated with $[VO_2dipic]-$ or (grey) treated with $[VO_2dipic–OH]^-$, while at 80% confluence (black) treated with $[VO_2dipic]-$ and (striped) treated with $[VO_2dipic–OH]^-$. Data is presented as the mean ± S.E.M. *, $P \leq 0.001$ vs. percent viable cell growth for cells treated with 1 μM $[VO_2dipic]-$ at 30–40% confluence.

and $[VO_2dipic–OH]-$ have limited stability at this pH and probably hydrolyzed to vanadate. The effect of complex persists even though only little complex may exist intact in solution.

The interpretation of these results is complicated by the fact that $[VO_2dipic]^-$ and $[VO_2dipic–OH]^-$ are not

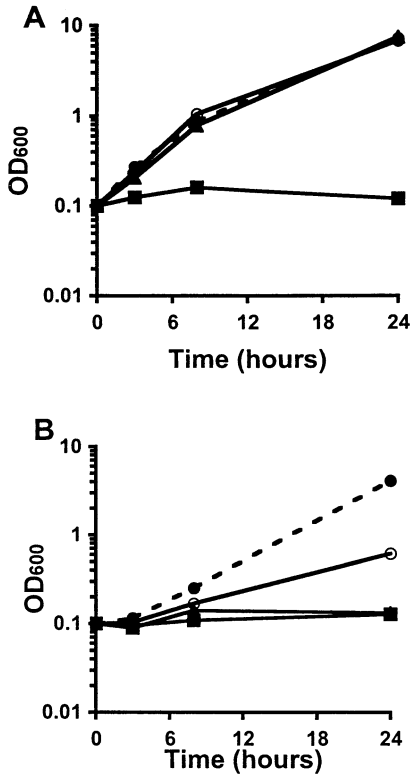

Fig. 6. The effect of $[VO_2dipic]^-$ on growth of *S. cerevisiae* at pH 6.5 and 4.5 is shown. Cells were grown in minimal salts medium as described in the Section 2. The symbols represent: (●) control; (■) addition of 5.0 mM $[VO_2dipic]-$; (▲) addition of 1.0 mM $[VO_2dipic]-$, and (○) addition of 0.50 mM $[VO_2dipic]-$ at pH 6.5 (A) and at pH 4.5 (B). Data was presented as mean ± S.D. of three samples except for the 24 h time point in panel A, which was the average of two samples. When error bars are not seen they are smaller than the symbols.

particularly stable at physiological pH and mammalian cells can only thrive in a narrow pH window (i.e. at around pH 7.4). Use of an adjunct model system is important to investigate whether a compound truly lacks activity or whether it falls apart at pH 7.4.

3.5. Effects of $[VO_2dipic]-$ on yeast cell growth

The eukaryotic microbe, *S. cerevisiae*, grows very well in a minimal salts buffered medium at pH values from 3.0 to 7.0. Therefore, the biological effect of vanadium complexes can be evaluated at both a pH where the compound is intact, and at neutral pH. The inhibitory effect of $[VO_2dipic]-$ on the growth of *S. cerevisiae* at pH 6.5 is shown in Fig. 6A. Inhibition was only observed at 5.0 mM $[VO_2dipic]-$. Most of the $[VO_2dipic]-$ should be hydrolyzed at pH 6.5 and we suggest that the inhibition observed at 5 mM complex is due to the combined effects of complex and vanadate formed when the complex hydrolyzes. The inhibitory effect of $[VO_2dipic]-$ on the growth of *S. cerevisiae* at pH 4.5 is shown in Fig. 6B: inhibition was observed at

all concentrations tested. Yeast cell growth is much more inhibited at pH 4.5, a pH where the complex is stable, than at pH 6.5, a pH where the complex has hydrolyzed. The possibility cannot be ruled out that the lower inhibition observed at neutral pH could be attributed to differential compound inhibitory effect at varying pH. However, these results are consistent with the interpretation that intact complex is inhibiting the growth of yeast and that less inhibition is seen at neutral pH due to compound instability. These studies suggest that growth of *S. cerevisiae* is a useful adjunct model system to investigate whether observed effects on cell growth are due to compound decomposition or inherently low activity.

3.6. Effect of [VO_2dipic–OH]− on alleviating the hyperglycemia of STZ-induced diabetes in Wistar rat strains

As anticipated, [VO_2dipic–OH]− similarly to [VO_2dipic]− has an insulin-enhancing effect on the hyperglycemia of rats with STZ-induced diabetes (Fig. 7). In the experiment shown in Fig. 7 both $VOSO_4$ (1.6 ± 0.5 mmol V kg^{-1} per day ingested) and [VO_2dipic–OH]− (1.0 ± 0.02 mmol V kg^{-1} per day ingested) reduced the elevated blood glucose level to the same extent and with the same statistical significance ($P \leq 0.001$). In a different experiment, [VO_2dipic]− was found to reduce the elevated blood glucose level to a lesser extent but the same statistical significance ($P \leq 0.001$) as $VOSO_4$ [16]. Free ligand $(dipic–OH)^{2}-$ did not have any effect on lowering blood glucose in this experiment (Fig. 7).

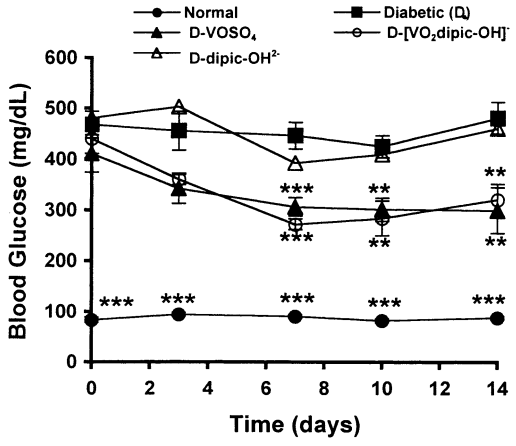

Fig. 7. The effect of $[VO_2dipic–OH]^{-}$ on hyperglycemia in Wistar outbred rats with STZ-induced diabetes is shown. Compounds were administered in the drinking water and blood glucose measured as described in the Section 2 for (●) normal, $n = 6$; (■) untreated diabetic, $n = 6$; (▲) $VOSO_4$ treated diabetic, $n = 6$; (○) [VO_2dipic–OH]− treated diabetic, $n = 6$; (□) $[dipic–OH]^{2-}$ treated diabetic rats ($n = 4$). Data is presented as the mean ± S.E.M. **, $P \leq 0.01$ vs. diabetic; ***, $P \leq 0.001$ vs. diabetic.

Evaluation of compound efficacy requires not only a diabetic control group in each experiment, but also a positive control for therapeutic response to metal treatment. In addition, the inclusion of some normal animals documents that nothing in the treatment of the animals effects the parameters monitored. Due to variability in animal experiments, comparison with a positive control is crucial for evaluating and comparing the effects of compounds. For example, if the compound tested has no effect, the positive control group rules out the possibility that that whole group of animals would not respond to therapy (resulting from some as of now unidentified parameter). The results presented here show that both $VOSO_4$ and [VO_2dipic–OH]− are effective in lowering elevated glucose levels. In addition, the fact that similar lowering of blood glucose values is achieved by a lower dose of V when administered in the organic complex compared with the salt is consistent with the previous reports [3] that organic complexes of vanadium are more effective than the simple salts.

The effect of individual rat and general strain differences on evaluating the effects of metal complexes in alleviating the symptoms of a multicomponent disease such as diabetes. To study the effect of genetic heterogeneity and individual variability on the different response of individual animals and strains in evaluating insulin-like effects of vanadium complexes, the effect of $VOSO_4$ in three rat strains was determined. One outbred strain, the Wistar (Outbred) rat, was investigated. This strain is used by most researchers using Wistar rats in this field and was used in the studies described with [VO_2dipic–OH]−. In addition, two inbred Wistar strains, Wistar (Kyoto) and Wistar (Furth), were also examined. The effect of $VOSO_4$ administration for 14 days (6.2 mM in the drinking water) in twelve rats of each strain was determined and the results are shown in Fig. 8. The average amount of metal ingested was 0.55 ± 0.023 mmol V kg^{-1} per day for Wistar (Furth), 0.52 ± 0.024 mmol V kg^{-1} per day for Wistar (Outbred) and 0.61 ± 0.017 mmol V kg^{-1} per day for Wistar (Kyoto). Over the course of the experiment no toxic effects were observed. Of the three strains Wistar (Furth) animals did not respond to treatment (Fig. 8A), whereas both Wistar (Outbred) and Wistar (Kyoto) animals showed a statistically significant response to treatment as shown in Fig. 8B.

The Wistar (Outbred) rats showed highly variable glucose levels with four of the 12 rats responding well to the treatment (Fig. 9A), while all but one of the twelve Wistar (Kyoto) rats showed a similar response to treatment with $VOSO_4$ (Fig. 9B). These results indicate the importance of using a genetically homogenous strain and a large enough number of animals to compensate for individual variability. It may be possible to use a smaller number of animals if the experiment involves a genetically inbred strain. The use of genetically hetero-

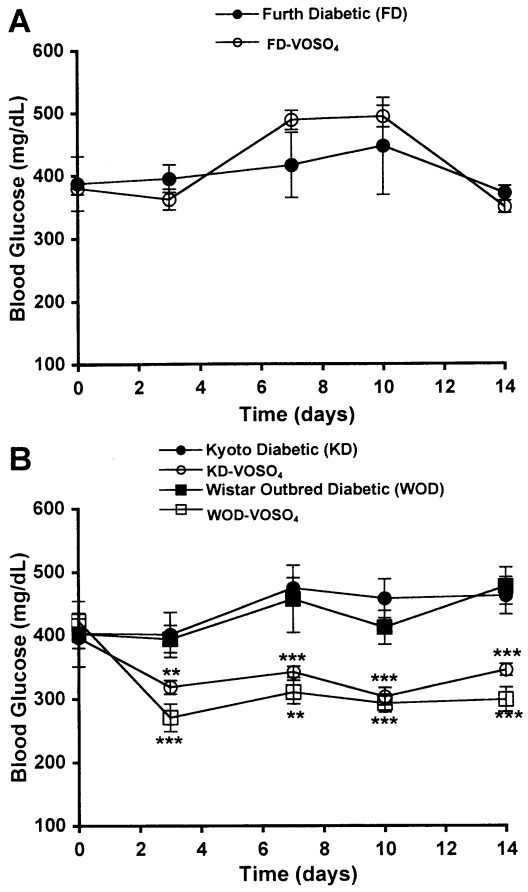

Fig. 8. The effect of administration of $VOSO_4$ treatment on hyperglycemia in three strains of Wistar rats with STZ-induced diabetes is shown. $VOSO_4$ (6.2 mM) was administered in the drinking water and blood glucose level was measured as described in the Section 2. Each treatment group contained 12 animals, while each untreated diabetic group contained five animals. Data is presented as the mean ± S.E.M. Graph A depicts (●) Wistar (Furth) untreated diabetic rats and (○) $VOSO_4$ treated Wistar (Furth) diabetic rats. Graph B represents (●) Wistar (Kyoto) untreated diabetic rats; (○)Wistar (Kyoto) $VOSO_4$ treated diabetic rats; ○Wistar (Outbred) untreated diabetic rats; and (□) Wistar (Outbred) $VOSO_4$ treated diabetic rats. Data is presented as the mean ± S.E.M. *, $P \leq 0.05$ vs. diabetic; **, $P \leq 0.01$ vs. diabetic, ***, $P \leq 0.001$ vs. diabetic. The treated animals for each strain were compared with the untreated diabetic animals of that same strain.

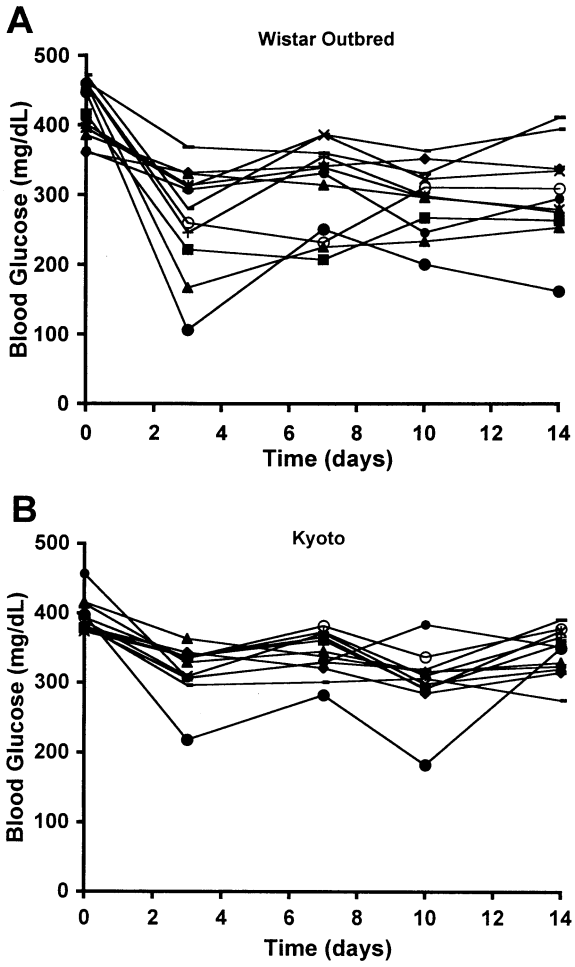

Fig. 9. The effect of $VOSO_4$ on the hyperglycemia of individual Wistar (Outbred) and Wistar (Kyoto) rats with STZ-induced diabetes is shown. $VOSO_4$ (6.2 mM) was administered in the drinking water and blood glucose measured as described in the Section 2. Each line represents an individual animal and $n = 12$ for both groups. Data for Wistar (Outbred) rats are shown in Graph A and Wistar (Kyoto) in Graph B.

zygous strains and low numbers of animals in each experimental group, may be contributing to some of the conflicting studies reported in the literature.

4. Summary

The $[VO_2(dipic–OH)]^-$ complex shows a greater stability than $[VO_2dipic]^-$ at neutral pH, since it is extending the stability range of the complex by 0.5–1.0 pH units. The $[VO_2dipic]^-$ complex exhibits the lowest lability from pH 2.5 to 4.5. This pH pattern was different than that observed for $[VO_2(dipic–OH)]^-$, which was found to be labile below pH 4. Given the hypothesis that the $dipic^{2-}$ ligand's role is to deliver the vanadium(V) to the cells, demonstration of the insulin-enhancing properties of a compound with a different lability profile than $[VO_2dipic]^-$ was desirable. Cell culture studies were used to examine the effects of the compounds in various biological systems. Regardless of the stability of $[VO_2dipic]^-$ and $[VO_2(dipic–OH)]^-$ inhibition of myoblast (L6) cell growth was observed. However, using an adjunct method, a comparison of the effects of the parent compound in yeast at pH where the complexes were intact and at neutral pH could be carried out. The effect of (4-hydroxydipicolinato)-oxo-vanadate(V) on hyperglycemia in rats with STZ-induced diabetes was demonstrated. Even though the degree of lowering of elevated glucose levels was similar for $[VO_2(dipic–OH)]^-$ and $VOSO_4$ the complex was found to be more potent than the salt with regard to dose

needed to attain the observed lower blood glucose values.

Acknowledgements

D.C.C. and G.R.W. thank the American Diabetes Association and the General Medical Institute of the National Institutes of Health for funding this project.

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