THESIS

COMPARATIVE ANALYSIS OF THE ROLE OF REDOX ACTIVE MOLECULES ON BIOENERGETICALLY ACTIVE MEMBRANES

Submitted by

Connor Cathal Dolan

Department of Chemistry

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Advisor: Debbie Crans

Alan Kennan
Adam Chicco

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ABSTRACT

COMPARATIVE ANALYSIS OF THE ROLE OF REDOX ACTIVE MOLECULES ON BIOENERGETICALLY ACTIVE MEMBRANES

Transition metals play crucial roles in various biological processes, with vanadium and manganese being prominent examples due to their redox activity and impact on oxidative stress, mitochondrial function, and disease progression. This manuscript focuses on the role of transition metals, particularly vanadium, in biological functioning, with an emphasis on oxidative stress and mitochondria.

Chapter 1 of this thesis discusses the respective role that vanadium plays on oxidative stress and how that influences biological systems. Due to its variety of speciation states and its ability to redox cycle as well as its structural and electronic properties, vanadium can affect biological systems in a variety of ways. These include the generation of reactive oxygen species, lipid peroxidation, protein inhibition, changes in membrane fluidity and potential. DNA damage and cell death. The effects that vanadium has are highly dependent on the speciation and state that they exist in. This can impact the system that is being affected and the outcome. Species such as decavanadate have a unique and profound biological effect. Changing of the species, oxidation state and complexation can alter the biological consequences associated with vanadium.

Chapter 2 of this thesis explores the differences and similarities between vanadium and manganese on cardiac mitochondrial dysfunction and oxidative stress. Using varying vanadium and manganese compounds, we investigated the effects they had on isolated cardiac

mitochondria using high resolution respirometry and UV-Vis spectroscopy. We found similarities between metal salts on inhibition of respiration as well as significant differences on the metals effect on mitochondrial swelling. We further investigated the role of transport proteins on vanadium induced swelling and found that the mitochondrial calcium uniporter played an important role in vanadium induced mitochondrial swelling. We further investigated the differences in species and oxidation state on function. We tested the difference between V^{V} and V^{IV} on mitochondrial swelling and found that V^{IV} led to significantly greater swelling. We also found that there the $VO(OH)_3^-$ monomer and dimer were present in both V^{IV} compounds and the Mn^{2+} ion was present in both manganese compounds. This speciation similarity between compounds may account for some of the similar effects seen within the same metal compounds as well as the differences seen when comparing manganese and vanadium together.

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THESIS INTRODUCTION

The contents of this thesis will explore the topic of transition metals and their effects on biological systems. The contents of this thesis will explore the topic of transition metals and their effects on biological systems. Chapter 1 is a review I wrote focusing on lipid peroxidation, ROS species and vanadium speciation. In chapter 2 I describe experimental work done to determine the effects of vanadium species in mitochondria." This article was published in the International Journal of Molecular Sciences. I am the co-second author and my writing focused primarily on the sections for lipid peroxidation, biomarkers, DNA damage and apoptosis, and the effect of salts and complexes on neurodegenerative diseases. With the work from our collaborators, we published this review paper in March 2023. This work has been deemed a highly cited paper within its first year of publication and is important because it sets the groundwork for the work expounded on in Chapter 2 of this thesis. It also highlights the multiple effects that vanadium has on biological systems and the importance of understanding these effects when designing vanadium-based therapeutics. For the thesis it was taken directly from the journal, with minor editing to fit the thesis standards.

Chapter 2 discusses work from an ongoing collaborative project within the Crans group and collaborators to explore the biological properties of transition metals, particularly vanadium and manganese, on mitochondria. This project explores the differential impact that species, state and metal may have on mitochondrial dysfunction. The speciation calculations and interpretation were completed by Dr. Edgar Del Carpio and Dr. Debbie Crans. Experimental work was performed by Luke Whitcomb, Lilli Rose and me. Data analysis was performed by me and Luke Whitcomb. Data interpretation was performed by Dr. Adam J Chicco, Dr. Debbie Crans, Luke

Whitcomb and me. This project highlights the importance of speciation and metal differences on biological systems. This chapter is currently being written up for publication in the Journal of Inorganic Biochemistry.

CHAPTER 1

BIOLOGICAL CONSEQUENCES OF VANADIUM EFFECTS ON FORMATION OF REACTIVE OXYGEN SPECIES AND LIPID PEROXIDATION[1]

1.1 Introduction

Lipid peroxidation (LPO) is the autocatalyzed chain oxidation of bis-allylic bonds in the acrylic chains of fatty acids. The mechanism is well known from 30 years ago [1–3]. The important question is which radical starts the chain first. On the one hand, a radical is needed (oxidative stress environment) while on the other hand, LPO amplifies and contributes to changing the redox state towards oxidation (what was called oxidative stress). Reactive oxygen species (ROS) are well known to target major biomolecules, affecting several biological processes and inducing several pathologies [4]. LPO relates to aspects of oxidative stress in disease [2,5]. Oxidation of lipids (i.e., LPO), is a process occurring in cells and tissues. There appears to be a correlation between LPO products such as malondialdehyde, F2-isoprostanes, lipid hydroperoxides, conjugated dienes, glutathione and protein carbonyl and an imbalance between the production and accumulation of ROS leading to oxidative stress [6]. LPO effects are particularly important in organelles such as the plasma membrane and mitochondria that are surrounded by lipid-rich membranes (**Figure 1.1**) [7–9]. Major effects of LPO include both direct and indirect damage by ROS to key cellular functions as well as to the membranes and the endomembrane system [10]. The biological effects of LPO can include changes in membrane permeability, fluidity, permeability and integrity, as well as affecting the activity of proteins

[1] Aureliano M, De Sousa-Coelho AL, Dolan CC, Roess DA, Crans DC. Biological Consequences of Vanadium Effects on Formation of Reactive Oxygen Species and Lipid Peroxidation. Int J Mol Sci. 2023 Mar 11;24(6):5382. doi: 10.3390/ijms24065382. PMID: 36982458; PMCID: PMC10049017.

embedded in lipid environments (**Figure 1.1**) [11]. In addition, LPO has effects on cell signaling pathways that use membrane proteins dependent on specific lipid environments. As a result, LPO can be classified as a metabolic disease caused by the oxidative deterioration of lipids catalyzed by ROS [12]. The presence of metal ions such as vanadium impacts such processes in multiple ways as described in this review.

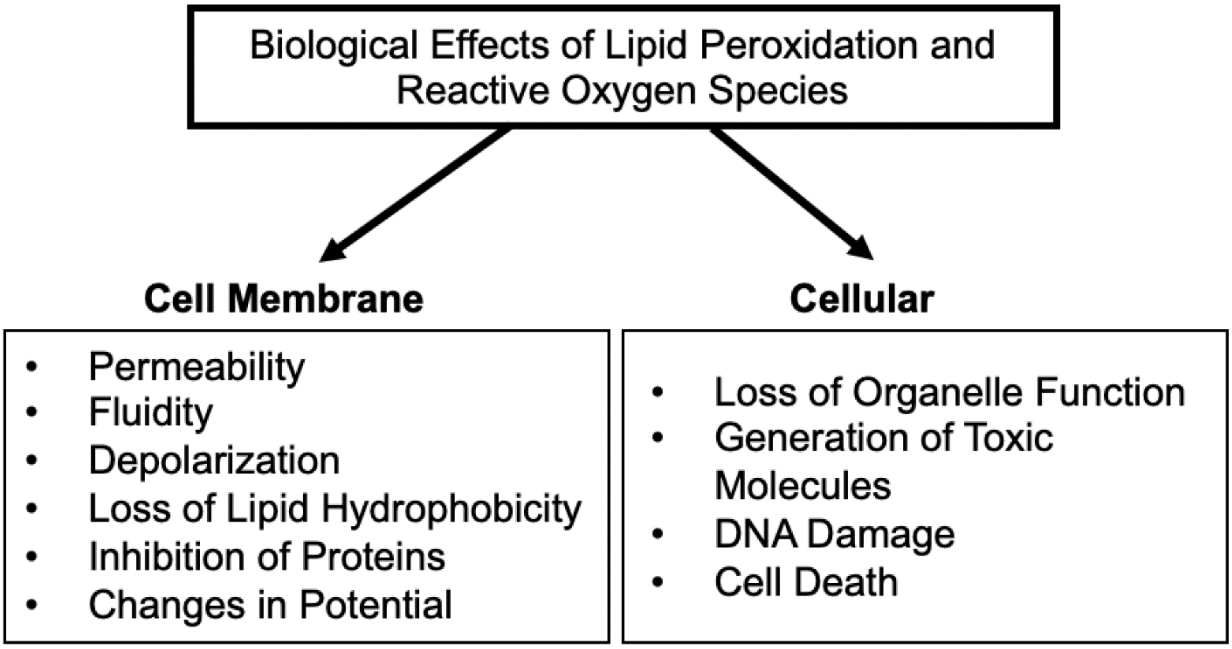

Figure 1.1. Overview of biological effects of lipid peroxidation (LPO) and reactive oxygen species (ROS). Adapted from [11] with copyright permission from Bentham.

LPO has direct consequences for human health and diseases such as cancer [6]. LPO is defined as a chemical process where alkene bonds, generally in a polyunsaturated fatty acid (PUFA), are oxidized in a chain reaction to form ROS leading to a number of products [7,8]. This is similar to the toxicological definition of LPO as an oxidative chain reaction where one lipid molecule after another is oxidized to form a lipid peroxide [9]. Products of LPO can have multiple effects on cells, not only on cell components largely composed of lipids but also on

other biomolecules (**Figure 1.1**). Oxidative stress is generally agreed to depend on concentrations of antioxidants and cellular exposure to free radicals (**Figure 1.2**) [10]. ROS form more frequently under conditions of oxidative stress with responses dependent on the oxidative damage potential of toxic biomolecules, drugs or other additives [7]. Alternatively, biomolecules, drugs or other additives may be protective [7]. As with other metals, vanadium has the ability to generate ROS that result in LPO and changes in biomarker levels for oxidative stress including the activity of antioxidant enzymes [13]. As discussed in the following sections, vanadium may either promote oxidative damage or play a role in antioxidant defense (**Figure 1.2**).

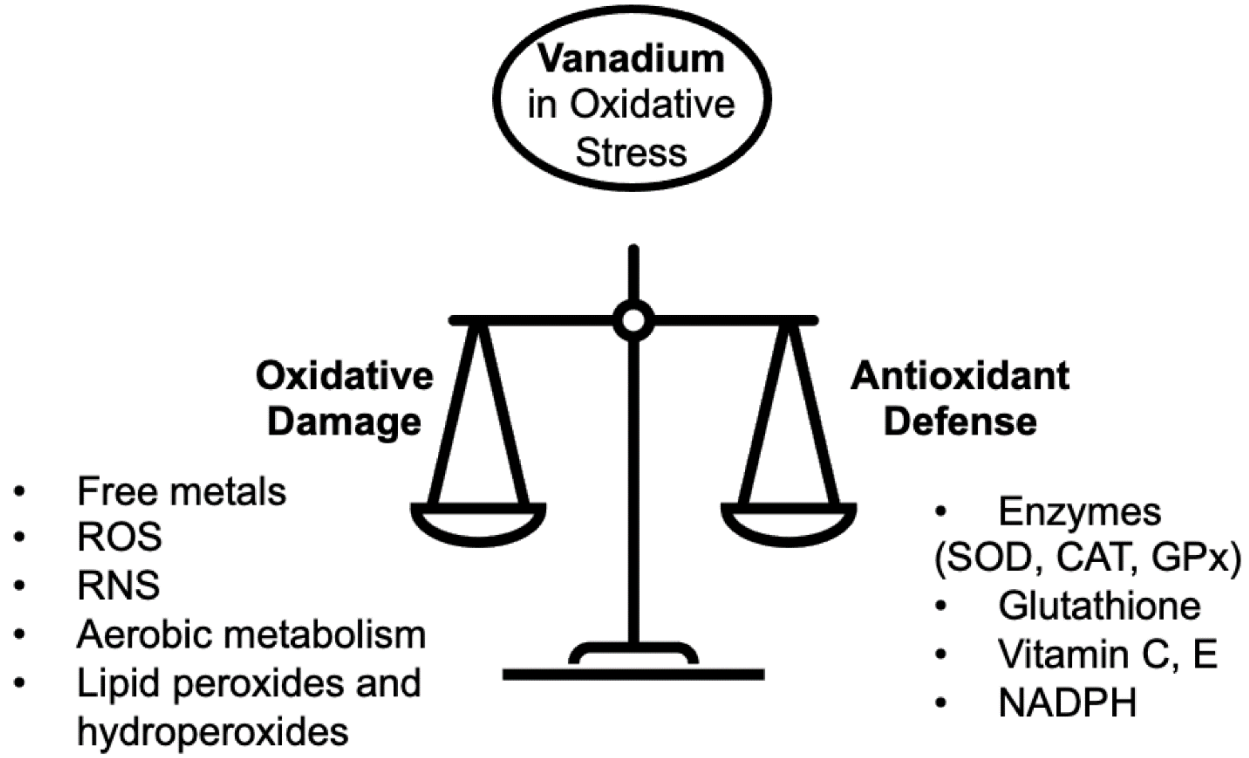

Figure 1.2. Molecules involved in oxidative stress with either unfavorable oxidative damage potential or the ability to provide antioxidant defense that is protective. Abbreviations: RNS, reactive nitrogen species; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

The primary biomarkers for LPO fall into several categories: ROS, LPO products, antioxidants and enzymes. The effects of vanadium compounds on these biomarkers have been reviewed in detail [11]. Some of these products are highly reactive, particularly the ROS derivatives, while others are more stable and can be monitored in quantitative assays reflecting the extent of LPO. It is important to note, however, that biomarkers do not always accurately describe LPO. Oxidative stress can have damaging effects on cells that are not limited to LPO, and which must be considered when analyzing data.

The extent of oxidative stress observed depends on the ratio of compounds with oxidative damage potential relative to the defense capacity of available antioxidants (**Figure 1.2**). Effects of vanadium compounds depend on whether a particular compound enhances ROS formation, in which case multiple deleterious effects on cell structure and function are observed [14,15]. Alternatively, vanadium compounds can decrease ROS formation, in which case they have protective effects on cells. For instance, it was recently described that treatment with a V complex increased the concentration of glutathione (GSH) in the visceral adipose tissue of chow-fed Wistar rats [16]. Although most studies report an increase in ROS following exposure to vanadium compounds, many have reported the protective properties of vanadium compounds [17]. This was the case for vanadyl sulfate ($VOSO_4$) where administration restored the altered levels of markers of oxidative stress in the skeletal muscle of chemical-induced diabetic male Swiss albino rats, thereby potentially protecting the animals from diabetic complications [18]. As we will discuss in this review, the observed effects of vanadium compounds on the formation of ROS and LPO depend on the specific vanadium compound being studied, the biological system or specific tissue being investigated, and details of the experiment, including the duration of treatment, pH or the solution containing the vanadium compound.

The tremendous variation in vanadium effects on cells, tissues or organisms results from vanadium's complex chemistry. Vanadium is a first-row transition metal and forms stable compounds in oxidation states IV and V under physiological conditions. Salts and coordination compounds have been investigated for effects produced under biological conditions [11]. Not only salts but also coordination complexes are found to have varying biological effects, which necessitates speciation studies together with an evaluation of biological effects. At present, several series of compounds have been studied with respect to their speciation under conditions similar to physiologic conditions and varied biological effects have been observed [19].

Most published work studying LPO effects in biological systems has been carried out using vanadium salts. However, comparisons between the effects of monomeric vanadate (V_1) and decavanadate (V_{10}) in fish highlight the marked differences in responses seen in studies using other vanadium compounds [20,21]. This review will highlight the direct and/or indirect effects of LPO induced by V_{10}, which contains 10 V atoms and 28 O atoms and forms a compact nanosized cluster that is particularly stable under slightly acidic and near physiological conditions. V_{10} has been shown to have anticancer, antiviral and antibacterial activities, among others [22,23] and, as a result, is perhaps the best-studied polyoxometalate (POM) in biology, affecting multiple biochemical and cellular processes [22,23,24]. We will also focus on the chemistry and cellular responses to vanadium compounds as they relate to LPO and oxidation stress. We and others have demonstrated different properties for various vanadium species, but an analysis that considers the formation of vanadium species over the course of an experiment has rarely been done. As an example, vanadium effects on mitochondria are of interest considering vanadium's potential to induce both LPO directly and indirectly through the formation of ROS which then can initiate LPO. Vanadium accumulation in in vivo studies has

suggested that mitochondria are a subcellular target for vanadate, especially when vanadium is administered in the form of V_{10} [25-29]. Because many vanadium salts and compounds have been reported to have both beneficial and toxic effects in biological systems and, in some cases, are being considered as potential therapeutic agents, it is particularly important to understand the effects of different vanadium species on LPO processes in both cell culture and animal model systems. Vanadium's ability to form ROS through Fenton and Haber–Weiss chemistry as well as to induce LPO products directly makes it important to highlight when each mode of action occurs.

1.2 Reactive Oxygen Species and Lipid Peroxidation

1.2.1 Definition of a Reactive Oxygen Species

Radicals are reactive species with a free electron formed from the breaking of one or more bonds in the parent molecule. When the radical resides on an oxygen atom, the resulting oxygen species is very reactive. These reactive oxygen species (ROS) are fundamental to aerobic life because oxygen oxidizes both carbon- and hydrogen-rich substrates to provide energy essential for cellular functions. Important examples of oxygen-based radicals, the hydroperoxyl radical (HOO**.**), the superoxide anion radical (O_2^{-}**.**) and the hydroxyl radical (HO**.**), are products of fundamental reactions discussed below and important intermediates in lipid peroxidation.

The reduction of oxygen in the presence of a proton can lead to a number of radicals as illustrated in reactions (1)–(5). When starting with the hydroperoxyl radical shown in reaction (1), the hydroperoxyl radical can dissociate as shown in reaction (2) to form the superoxide radical anion (O_2^{-}**.**) which, upon reaction with two protons, forms hydrogen peroxide (H_2O_2) as shown in reaction (3). In reaction (4), the reduction of H_2O_2 forms one molecule each of

OH^- and hydroxyl radical (HO**.**). HO will react with an electron and a proton to form one molecule of water as shown in reaction (5).

$$O_2 + e + H^+ \rightarrow HO_2\textbf{.} \quad (1)$$

$$HO_2^- \rightarrow H^+ + O_{2-} \quad (2)$$

$$O_2^- + 2H^+ + e \rightarrow H_2O_2 \quad (3)$$

$$H_2O_2 + e \rightarrow HO^- + HO\textbf{.} \quad (4)$$

$$HO.+ e + H^+ \rightarrow H_2O \quad (5)$$

1.2.2. ROS Formed by Metal Catalysis

Reactions can occur with superoxide or H_2O_2 and a metal ion, a process referred to as Fenton chemistry when the metal ion is iron (Fe) [30-33]. The reaction of Fe^{3+} with superoxide is shown in reaction (6), and the disproportionation reaction catalyzed by Fe^{3+} is shown in reaction (7). Vanadium participates in a similar reaction in which V^V reacts with superoxide to form a peroxyradical V^{IV}-OO. or with NADPH, a cofactor in anabolic reactions, to form V^{IV} and $NADP^+$. The disproportionation reaction (10) is catalyzed by V^{IV} and analogous to the Fe-catalyzed reaction (6). These reactions are related to the overall Haber–Weiss detoxification mechanism beginning from superoxide and shown in reaction (11) [34]. This process and the relationship between transition metals, Fenton reactions and the Haber–Weiss reaction is illustrated in **Figure 1.3**.

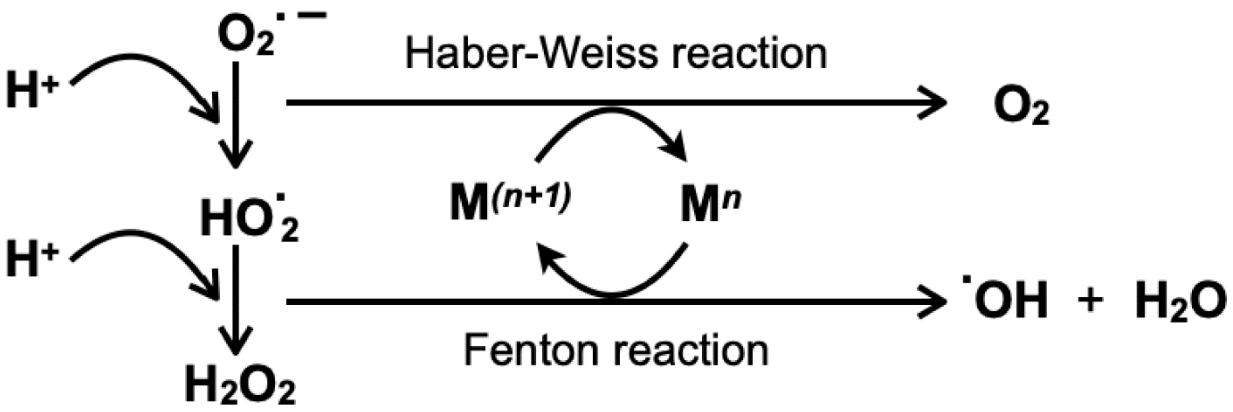

Figure 1.3. The relationship between the Fenton reaction and reactions catalyzed more generally by transition metals forming oxygen radicals. Metal-catalyzed radical formation is linked to the Haber–Weiss reaction as shown. Adapted with permission from [8]. Hindawi Copyright 2014, Antonio Ayala et al.

Fenton reactions:

$$Fe^{3+} + O_2^{-}\cdot \rightarrow Fe^{2+} + O_2 \quad (6)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{-} + HO\cdot \quad (7)$$

$$V^{V} + O_2^{-}\cdot \rightarrow [V^{IV}OO\cdot] \quad (8)$$

$$V^{V} + NADPH \rightarrow V^{IV} + NADP^{+} + H \quad (9)$$

$$V^{IV} + H_2O_2 \rightarrow V^{V} + HO^{-} + HO\cdot \quad (10)$$

Haber–Weiss metal-catalyzed reaction:

$$O_2^{-}\cdot + H_2O_2 \rightarrow O_2 + HO + HO^{-} \quad (11)$$

Some controversy exists in the vanadium literature resulting from reports showing that the superoxide is not kinetically competent to generate a free ROS from vanadate as shown in reactions (8) and (9) unless NADPH or phosphate is present [35]. However, recycling has been proposed with V^{IV} reacting with O_2 to form V^{V} and the superoxide shown in reaction (12)

[30,33]. Functional studies showing differences in various V compounds and their speciation are discussed in more detailed below [17,30,33].

$$V^{IV} + O_2 \rightarrow V^{V} + O_2^{-}\bullet \quad (12)$$

1.2.3. Lipid Peroxidation and Oxidized Lipid Species

LPO can be defined broadly as a chemical process in which alkene bonds, generally in a polyunsaturated fatty acid, are oxidized in a chain reaction of lipids to their respective products. At the molecular level, LPO occurs in a three-step reaction involving lipid double bonds and oxidation by ROS. This reaction results in direct and indirect damage to the cell and plays a role in several disease processes. Indirect effects of LPO are due to the reactive nature of the intermediate and final products of the peroxidative process, although, in practical terms, it is difficult to distinguish indirect effects from direct effects of LPO [36]. The effects of LPO on membranes are clearer and recognized as a common process in various pathological conditions [37]. More importantly, the continued propagation of ROS contributes to various diseases. This confirms the notion that an organism's innate defense system is not always sufficient to protect it from damage or cell death [38,39,40].

LPO often occurs in response to oxidative stress in a reaction with three phases: initiation, propagation and termination (**Figure 1.4A**). A basic chemical description of the three-step mechanism of the LPO process is shown below. During each phase, specific lipid oxide intermediates are formed and reactions take place, as has been extensively reviewed [36,41-44].

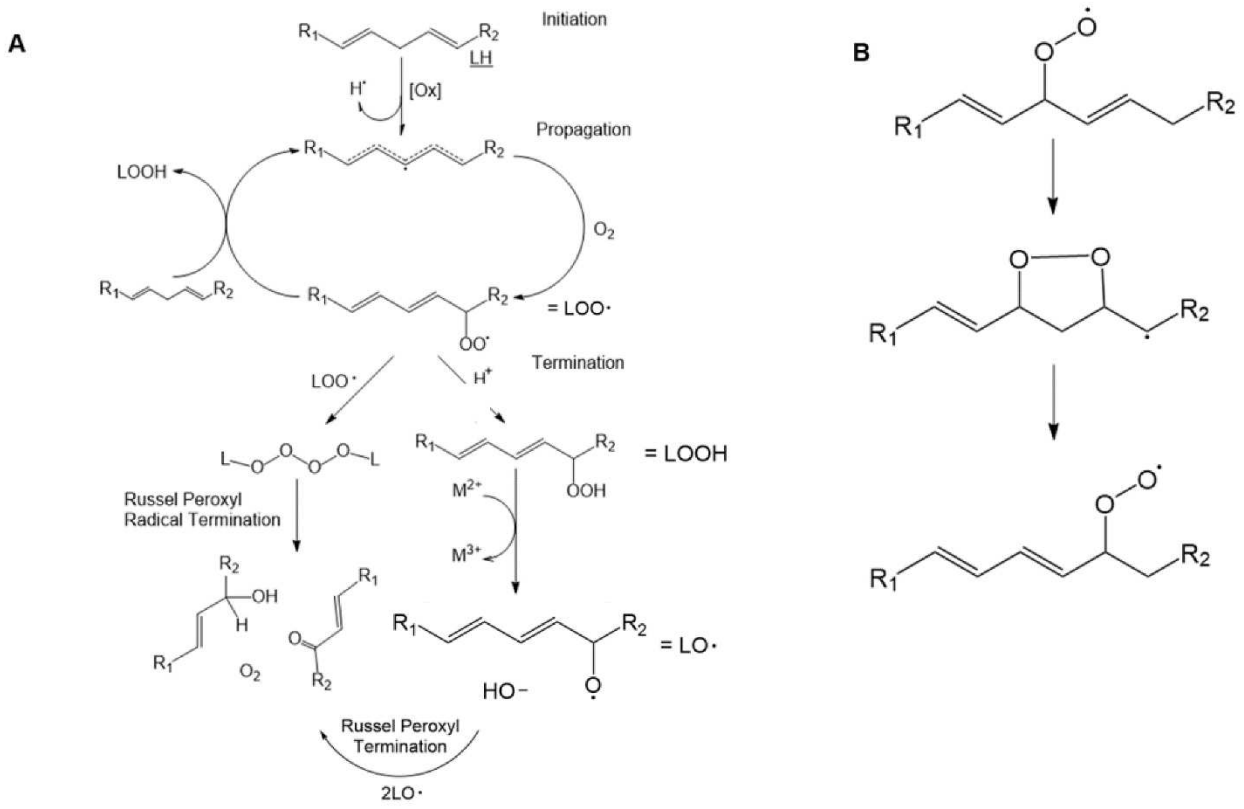

Figure 1.4. The overall mechanism of LPO. (**A**) Three phases of lipid peroxidation: initiation, propagation and termination, where LH = polyunsaturated fatty acid; LOOH = lipid hydroperoxide shown in scheme; LOO· = lipid peroxyl radical shown in scheme; LO· = lipid alkoxyl radical shown in scheme; [Ox] = an oxidant and could be L·, HO·, UV Light, HOO·; M = metal (Fe, V, Cu). (**B**) Peroxyl radical cyclization rearrangement, where $R_1 = C_5H_{11}$; $R_2 = (CH_2)_7COOH$.

The initiation phase is characterized by the removal of the bis-allylic hydrogen and formation of a conjugated diene system and either a pentadienyl radical (L·) or a lipid peroxyl radical (LOO·) (reactions (13) and (14) and **Figure 1.4A**). This can occur through several different mechanisms characterized as existing oxidative reactions. Oxidation can occur through reactions containing metals, pre-existing lipid hydroperoxides (see LOOH in **Figure 1.4A**), enzymes such as lipoxidase, ultraviolet light irradiation or ROS reactions. An exception is the superoxide radical which cannot initiate LPO by itself [45,46].

Lipid peroxidation mechanism reactions

Initiation

$$LH + HO\cdot \rightarrow L\cdot + H_2O \quad (13)$$

$$LOOH + HOO\cdot \rightarrow LOO\cdot + H_2O_2 \quad (14)$$

Propagation

$$L + O_2 \rightarrow LOO\cdot \quad (15)$$

$$LOO + LH \rightarrow LOOH + L\cdot \quad (16)$$

$$LOOH + M^{2+} \rightarrow LO\cdot + HO\text{-} \quad (17)$$

Termination

$$LOO\cdot + L \rightarrow LOOL \quad (18)$$

$$LOO\cdot + LOO\cdot \rightarrow LOOOOL \quad (19)$$

$$LOOOOL \rightarrow LOH + L = O + O_2 \quad (20)$$

$$L\cdot + L \rightarrow L\text{-}L \quad (21)$$

$$LOO\cdot + AOX \rightarrow LOOH + AOX\cdot \quad (22)$$

Initiation through the formation of a free radical allows for the propagation of LPO. The propagation phase consists of a radical abstraction step that can continue indefinitely in a cyclic reaction (reactions (15)–(17)). Regeneration of the lipid radical is accompanied by the formation of a lipid peroxyl radical in reaction (15) in which the reaction of L· with molecular oxygen (O_2)

forms LOO· [42]. LOO· then reacts with a lipid through the removal of the bis-allylic hydrogen to create LOOH and regeneration of L· in reaction (16). The lipid hydroperoxide can react with a metal to form a lipid alkoxyl radical (LO·) and a hydroxide ion. This cycle is repeated until the overall reaction is terminated.

Termination of LPO propagation occurs through multiple steps and the formation of several products including, but not limited to, peroxides, aldehydes and alcohols [47]. The presence of the radical is the driving force behind the continued oxidation of lipids. Removal of the radical will cause the chain reaction to cease. There are several ways for termination to occur (**Table 1.1**, reactions (18)–(22)) and a variety of products that can result. These include the Russell peroxyl radical termination [48] to form a ketone and alcohol along with molecular oxygen, quenching of the radical via antioxidants to form lipid hydroperoxides, reactions with other lipids and lipid peroxides and the reaction of lipid hydroperoxides with metals. Termination steps and products formed are summarized in **Table 1.1**. Yin, Xu and Porter (2011) and de Groot and Noll (1987) describe the termination reactions in depth [49,50].

Table 1.1. Potential steps and products formed in the termination reaction of lipid peroxidation.

Mechanism	Products
Russell peroxyl radical termination (4 oxygen)	Aldehyde/ketone, alcohol, molecular oxygen
Russell peroxyl radical termination (2 oxygen)	Aldehyde/ketone, alcohol, pentane
Antioxidant quenching	Lipid hydroperoxide, lipid alcohol
Self-termination	Crosslinked lipids
Cross-termination	LOOL
Metal-mediated	Hydroxyl radical, aldehyde/ketone, pentane

Lipid oxide products of LPO vary, with major diene hydroperoxide products dependent on the PUFA being oxidized. The reactions of the side products can be seen in reactions (18)–(22). LPO oxidation results in LPO products that are sterically either trans or cis and which can also vary due to the continued reaction of the peroxyl radical converting to L· [47]. Another reaction that increases variety in LPO products occurs in the oxygen cycle cyclization step in which the peroxyl radical is transferred to an adjacent carbon atom [51] (**Figure 1.4B**). This oxygen scrambling step increases product variations and can be monitored via HPLC and UV-Vis spectroscopy [47,52]. The steric variation of these hydroperoxides determines which end products are formed and whether they are subsequently oxidized to aldehydes, ketones or remained alcohols (**Figure 1.5**).

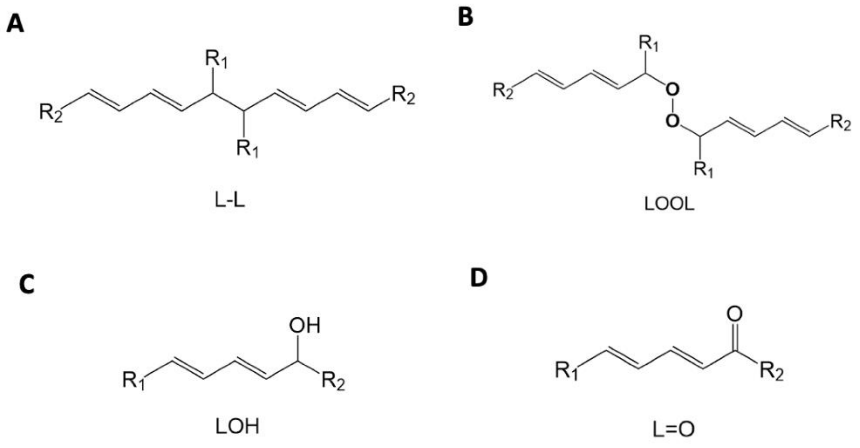

Figure 1.5. Core termination products shown in reactions (17), (18), (20) and (21) with R_1 = C_5H_{11}; R_2 = $(CH_2)_7COOH$. The stereochemistry of products through the following reaction steps are (**A**) self-termination, (**B**) cross-termination, (**C**) lipid hydroxyl and (**D**) lipid ketone.

1.2.4. Effects of Vanadium Compounds and Speciation on Lipid Peroxidation

As with other toxic metals, vanadium has the ability to produce ROS resulting in LPO and changes in biomarker levels including the activity of antioxidant enzymes discussed

in Section 1.2.6 below [13]. LPO is initiated by metal ions or metal compounds through Fenton chemistry and affects LPO reactions. The Fenton reaction produces HO· through a reaction of H_2O_2 with a transition metal. Vanadium, in the form of V^{IV} (vanadyl), reacts similarly with H_2O_2, as do other transition metals such as iron and copper, to form the HO·. This reaction initiates LPO which is followed by propagation and termination for PUFAs and lipid hydroperoxides. Due to its reactivity, the vanadium ion is often bound to ligands in complexes, with studies demonstrating anticancer or antidiabetic properties of these vanadium complexes [17,24,53-55].

Because vanadium's role in LPO depends on its oxidation state and whether the vanadium is complexed with ligands, it is necessary to consider the speciation of vanadium, particularly under cellular conditions. Vanadium exists in multiple oxidation states (II-V), with oxidation states IV and V being most prevalent under physiological conditions [56-58], although oxidation state III may also be present but difficult to observe [59]. The properties of these different oxidation states affect the reactivity of the observed ROS. Vanadium has been reported to redox cycle between V^{IV} and V^{V}, leading to the generation of ROS [17,56,58,60-62]. Keller and others have shown that vanadyl (V^{IV}) as VO^{2+} is better at generating ROS in the form of HO· than vanadate (V^{V}) [17,56,58,60-62]. Vanadium in oxidation states IV and V forms oxo-ions which exist as several oligomeric ions. In oxidation state IV, VO^{2+} ($VO(H_2O)_5^{2+}$ and the deprotonated form $VOOH^{+}$ ($VO(H_2O)_5OH^{+}$) are the most common species considered, although it is known that these forms do not exist at neutral pH but are instead bound to proteins, DNA, lipids or other available metabolites and referred to generally as VO^{2+} or V^{IV}. In oxidation V, oxovanadates are well defined, although several interconvert. The most common ions have nuclearities of 1, 2, 4, 5 and 10, each of which is likely to have different effects on lipid peroxidation. Vanadate, when its nuclearity is one (V_1), resembles phosphate and exists in three

different protonation forms, $H_2VO_4^-$, HVO_4^{2-} and VO_4^{3-}. These forms are readily generated when dissolving metavanadate and orthovanadate into solutions at neutral pH. Ammonium metavanadate and sodium metavanadate form V_1 solutions and increase LPO products along with V^{IV} (as $VOSO_4$) [11]. This may be due to the recycling between V^{IV} and V^V systems reported under physiologic conditions.

The effects of decavanadate (V_{10}) on lipid peroxidation have been well studied in comparison with monomeric vanadate (V_1) [19,25-28]. Lipid peroxidation by V_{10}, unlike V_1, requires longer times, perhaps due to the need for decomposition of V_{10} to vanadate before redox cycling and lipid peroxidation can occur. The effects of V_{10} on lipid peroxidation are also longer lasting than those of vanadate, although the mechanism is not clear. This may be due to the length of time needed for the conversion of V_{10} to V_1, this is not consistent with other differences in the effects of these two oxovanadates. These results suggest that different LPO mechanisms are utilized by vanadate and V_{10} [19,25-28].

Similar trends in reactivity are not always observed for complexed forms of vanadium. Although metavanadate salts increase LPO products along with $VOSO_4$, complexes containing both V^V and V^{IV} do not increase LPO products [11]. A study examining vanadium complexes with anticancer activity containing V^{III}, V^{IV} or V^V showed that V^{IV} complexes induced higher levels of ROS necessary for LPO than did V^V complexes [63]. Another study investigating V^{III-V} complexes and the production of reactive oxygen and nitrogen species showed that V^{IV} produced the lowest levels of reactive oxygen and nitrogen species in colon cancer Caco-2 cells, a result attributed to V^{IV} being more inert under cellular conditions [63]. This result was also consistent with observations that V^{IV} when polymerized is slow to react as in solutions of $VOSO_4$ at pH 7 [56,58,64]. Thus, if a solution of V^{IV} is polymerized before treatment of the

Caco-2 cells, V^{IV} would not be available for initiation of LPO. Together, these results suggest that product formation is sensitive to the nature of the V^{IV} solutions and whether V^{IV} is polymerized [19]. They are supported by the observation that the addition of a reducing agent such as glutathione or NADPH contributes to ROS and LPO production, presumably by allowing V^{V} to cycle to V^{IV} where it initiates LPO.

In summary, vanadium can induce ROS formation in biological systems through (1) Fenton-type reactions [36], (2) bioreduction of vanadate by the action of glutathione (GSH), flavoenzymes or NAD(P)H oxidases with subsequent formation of ROS [65,66], and (3) the indirect promotion of ROS, probably by interactions with mitochondria [67]. Several hypotheses have been formulated to address the relationships between vanadate and other vanadium compounds and ROS production, including how the oxidation state and presence of ligands may impact ROS production. There remains much work to be done to investigate which species are present in vivo, the redox chemistry of the relevant vanadium compounds and their mechanisms and pathways involved in physiologic function that are affected by various vanadium species.

1.2.5. Biomarkers Associated with Lipid Peroxidation

Lipid peroxidation creates multiple products, some of which serve as biomarkers indicative of oxidative stress. The primary biomarkers for LPO fall into several categories: ROS, LPO products, antioxidants and enzymes. Some of these products, for example, ROS derivatives, are highly reactive, while others are more stable and can be monitored in quantitative assays that accurately reflect the extent of LPO. As a caveat, biomarkers may not always accurately describe LPO simply because oxidative stress can have damaging effects on cells that are not limited to LPO and should be considered when analyzing data. Regardless, biomarkers can provide important details on cell status and serve as useful reporter groups.

Because of the integral role of ROS in LPO, the production of ROS is linearly correlated with LPO formation. Flow cytometry with suitable probes has been used to measure the presence of ROS. Specific LPO products exist that reflect LPO formation and can be monitored, some of which are shown in **Figure 1.6**. As an example, malondialdehyde (MDA) is commonly used in the thiobarbituric acid (TBA) assay. Additional products that can be monitored are 4-hydroxy-2-nonenal (4-HNE), acrolein, isoprostanes and neuroprostanes [68,69]. Each of these compounds is reactive in vivo both chemically and after processing. However in vivo processing can be problematic, as is the case for MDA, which is further metabolized in vivo, making cellular metabolism an important consideration when interpreting in vivo results with this agent [**70**].

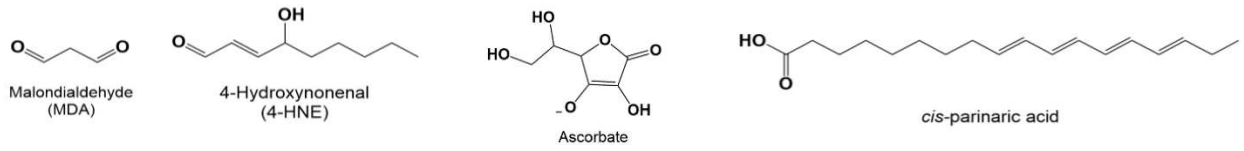

Figure 1.6. Structures, names and abbreviations of biomarkers for LPO products induced by vanadium salts or vanadium compounds including MDA, 4-HNE, ascorbate and *cis*-parinaric acid. MDA and 4-HNE are LPO products and excellent biomarkers. Ascorbate is an antioxidant, and *cis*-parinaric acid is a fluorescent probe sensitive to the presence of peroxyl radicals in cell media.

Biomarkers used for studying vanadium effects on LPO include MDA, ROS production, ascorbate [11] and quantification of fluorescence from *cis*-parinaric acid [27,71]. Since vanadium is known to generate ROS, monitoring ROS production offers insight into which vanadium species plays a role in ROS generation and subsequent LPO. Fluorescence decay in the presence of cis-parinaric acid (**Figure 1.6**) can be used to determine the rate of LPO in cells. The addition of peroxyl radicals to cell media containing cis-parinaric acid will cause a decrease in fluorescence emission [72]. Both V^{IV} and vanadate solutions generate ROS, although V^{IV} generates ROS more quickly and in greater amounts.

The biomarker ascorbic acid was used in two animal studies described in a review by Scibior and Kurus [11]. Mice were treated with $NaVO_3$ and NH_4VO_3 to determine whether there was a difference between treatments using these two salts. Since these salts differ only in having a Na^+ versus NH_4^+ counter ion, no major difference in LPO was expected. However, as Scibior and Kurus reported, there were large variations in LPO responses in liver tissues, as shown in **Figure 1.7**. While lower ascorbic acid (L-AA) levels were observed in the liver with NH_4VO_3 treatment, the L-AA content doubled with $NaVO_3$ treatment. This was an unexpected result, particularly given similar responses to these salts in erythrocytes and plasma samples (**Figure 1.7**). However, these differences could be attributed to a different length of administration, the concentration of vanadate salts used, and animal age and weight, all factors that may affect L-AA content [11]. Nonetheless, it is reasonable to conclude that there are similarities between the $NaVO_3$ and NH_4VO_3 effects on erythrocytes and in plasma but differences may be observed in the liver (**Figure 7**).

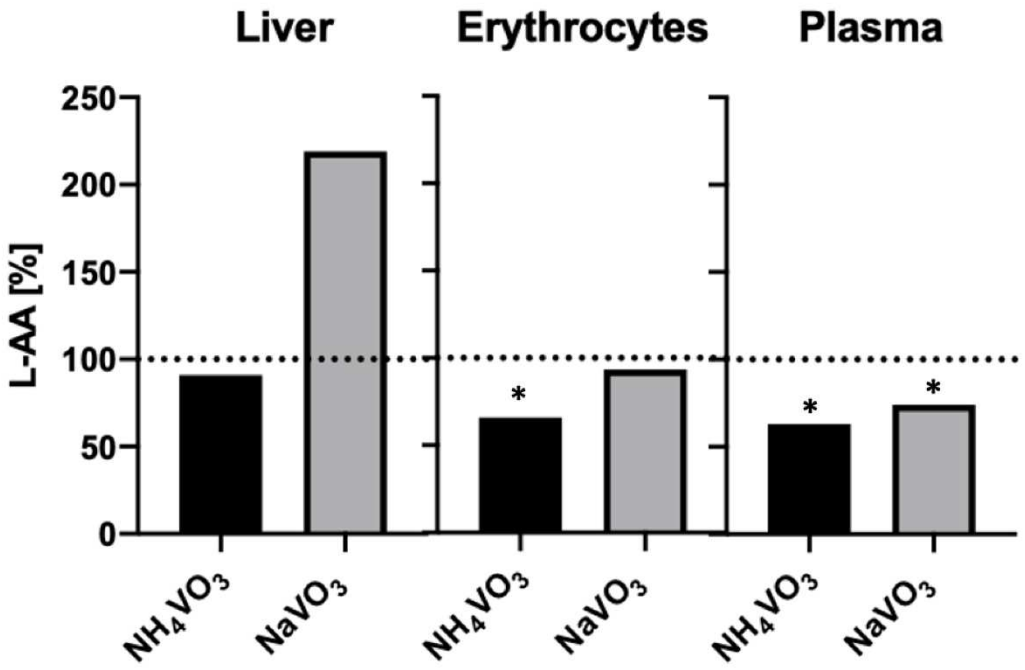

Figure 1.7. Levels of L-ascorbic acid (L-AA) in the liver, erythrocytes and plasma of rats receiving selected vanadate compounds expressed as the percent of changes compared to the control. The NH_4VO_3 dose (mg/body weight/24 h) was 10.7 mg for 4 weeks (wks) for all samples evaluated (liver, erythrocytes and plasma). The $NaVO_3$ dose was 12–13 mg for 12 wks for liver and 10.7 mg for 6 wks for erythrocytes and plasma. L-AA = L-ascorbic acid; NH_4VO_3, ammonium metavanadate; $NaVO_3$, sodium metavanadate. * designates statistical significance relative to control ($p < 0.05$). Adapted from [11] with copyright permission from Bentham.

Enzyme activity can also be used as an LPO biomarker, as has been done using superoxide dismutase (SOD) and catalase (CAT) in studies of fish treated with vanadium [26-28,71]. SOD protects against the superoxide anion while CAT degrades H_2O_2. Both are involved in ROS metabolism and are affected by vanadium species. One study evaluating the effect of vanadate and V_{10} on the activity of SOD and CAT in cardiac tissue showed a 115% increase in SOD activity and no change in CAT activity with exposure to metavanadate and a 20% increase in SOD activity and a 55% decrease in CAT activity with exposure to V_{10} [28]. A study looking at vanadium pentoxide exposure in rabbits found a decrease in SOD (14.3%) and CAT (30.0%) activity with a 42.9–60.0% increase in LPO [73]. No study to date has compared the effects of vanadium on LPO using different biomarkers. Thus, while providing valuable details on the metabolic state of the system, there is a possibility that different biomarkers may be responding differently to various vanadium compounds.

1.2.6. The Effects of Vanadium on LPO of Proteins and Enzymes

When discussing the effects of vanadium on the LPO of proteins, it is important to consider whether an isolated protein is being studied [24] or whether the protein is studied in cells, tissues or intact animals [74]. Furthermore, the form of vanadium involved and its speciation affect the interpretation of the results [21,23-28,32,71,75]. Several common forms of V^V include sodium metavanadate, ammonium metavanadate, sodium orthovanadate (abbreviated

SOV with a formula Na_3VO_4) and V_{10} which, when prepared in aqueous solutions, will form the appropriate oxovanadates as determined by solution pH [23]. In studies with isolated enzymes, one would expect similar responses regardless of the initial form of vanadium. Results from work with V^{IV} are more varied, which has been attributed to the fact that V^{IV} can polymerize to generate a slowly accessible form of V^{IV}. Early studies by the Chasteen and Crans group demonstrated that buffer used experimentally could interact with V^{IV} and form a complex that prevented polymerization of V^{IV} and resulted in effects of V^{IV} in enzyme studies [64,76,77].

Vanadate is competent to reduce enzymes with, for example, thiol groups, as has been reported for protein tyrosine phosphatases [78,79]. Accordingly, one would expect these proteins to be particularly sensitive to LPO in in vivo systems and in cells. Vanadium coordination compounds may be less likely to show similar responses because the redox properties of the complex have been changed by the ligand [80,81]. Some work has been done with various vanadium compounds and isolated enzymes together with accompanying speciation studies [82]. Of particular interest when considering speciation effects is a study showing that V_{10} binding to G-actin prevents V_{10} decomposition [83]. Incorporating vanadium in a polyoxidovanadate may protect the vanadium from hydrolysis while still allowing redox chemistry. A number of studies with this class of compounds have appeared recently showing that some of these compounds are very active in vivo and have properties that suggest their effects may involve LPO [22,23,25-28].

Independent animal studies and studies in fish have allowed for a direct comparison of the effects of vanadate and V_{10}, with results showing different effects on myosin, actin and P-type ATPases [22-28]. Only V_{10} induced the oxidation of the so-called “fast” cysteines, or exposed cysteine Cys-374, when actin was in the polymerized and active form [84]. For P-type ATPases, quercetin prevented protein cysteine oxidation induced by V_{10} [85]. For myosin,

V_{10} strongly inhibited actomyosin ATPase hydrolysis and the mechanism involved in muscle contraction due to a specific interaction at the myosin backdoor [86,87]. In addition, V_{10} was found to have both direct and indirect effects on oxidative stress markers and LPO [22-28,71], leading to the proposed mechanisms shown in **Figure 1.8**. The redox cycling of V^V and direct interactions with H_2O_2 are likely to be both important and consistent with expected vanadium chemistry.

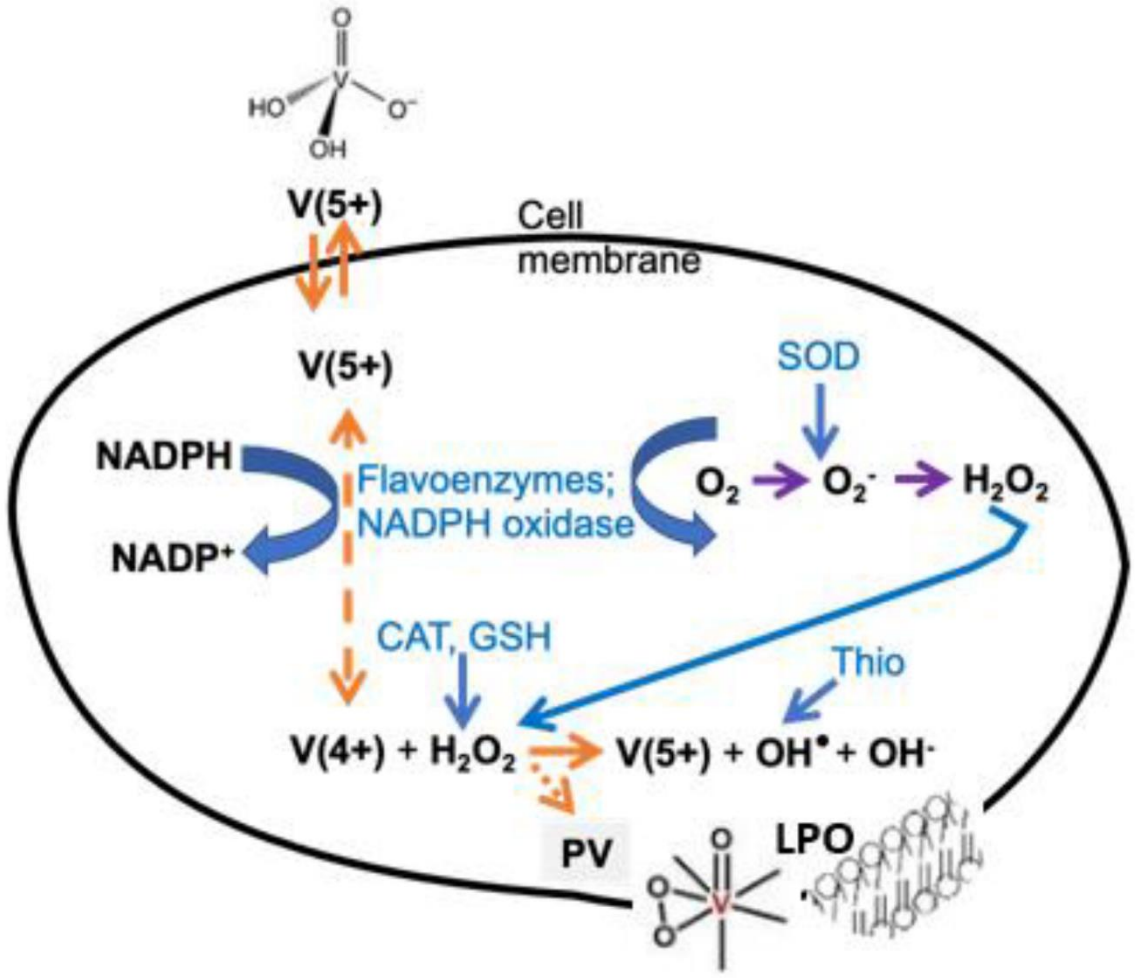

Figure 1.8. Vanadate (V(5+)), vanadyl (V(4+)) and peroxovanadate (PV) effects on intracellular oxidative stress. Representation of the mechanisms described in text for redox cycling under oxidation of NADPH, reaction with H_2O_2 undergoing oxidation and formation of vanadium peroxides that may interact directly with lipids. CAT, catalase; SOD, superoxide dismutase; PV,

peroxovanadate; LPO, membrane lipid peroxidation. Adapted from [66] with copyright permission from Elsevier.

Vanadium compounds have also been reported to be effective initiators of signal transduction by a G protein-coupled receptor (GPCR), specifically the luteinizing hormone receptor (LHR) [88,89], and a protein tyrosine kinase, the Type I Fcε receptor [89,90]. The LHR signals in the presence of some vanadium-containing compounds as a result of vanadium compound interactions with the membrane lipid interface. Three different types of interactions that initiate cell signaling have been identified. The first class of coordination complexes was hydrophobic complexes that initiated signaling by interacting with the outside surface of the membranes, causing changes in lipid organization that indirectly drove receptor dimerization and production of a second messenger, cyclic AMP (cAMP). Evidence for this type of signaling included measuring lipid packing in the bilayer and intracellular levels of cAMP. Compounds that interact with membranes through this mechanism included $VOSO_4$ [88]. The second class of compounds was vanadium coordination complexes that interacted with the cellular interface and caused changes in lipid packing. These compounds showed evidence for uptake and internalization by the cell. Vanadium compounds that were found to interact with the membrane interface and potentially be internalized included vanadate, $VO(acac)_2$, and BMOV, a compound that was in clinical trials for the treatment of type 2 diabetes. In separate studies, the addition of DIDS [91] stopped the transport of these compounds across the erythrocyte membrane [67,92]. This group of compounds may also include vanadate monomer V_1, V_{10}, a polyoxovanadate with 14 V atoms (V_{14}) and a polyoxovanadate with 15 V atoms (V_{15}) which, because they cannot be readily removed from cells, may be internalized [93]. Finally, the third class of compounds was hydrophilic compounds that interacted only transiently with the membrane, changed lipid

packing and initiated receptor signaling without penetrating the membrane. These compounds, specifically two monosubstituted V_{10}s, V_9Mo and V_9Pt [94], could be quickly separated from cells. Although these studies provided no direct information on the impact of these compounds on LPO, membrane interactions are a feature of both vanadate and $VOSO_4$ which do impact LPO.

Recently new classes of vanadium coordination complexes have been reported that are mild radical initiators and should be able to initiate LPO and induce oxidation [14]. Interestingly, they are not cytotoxic to embryonic mouse fibroblasts (NIH/3T3) [14], Cal33 cells or HeLa cells. One class of compounds is vitamin E chelate siderophores with a lipid portion and a metal ion, amphiphilic properties and hydrolytic stability [15]. The other class of compounds are vanadium complexes with a sterically hindered catechol that have low toxicity while remaining reactive, suggesting that such vanadium compounds may be active as LPO inducers and, in the case of this latter complex, demonstrate some efficacy against cancer cells [95,96].

1.3 Effects of Vanadium and LPO on Plasma Membranes, Organelles, Mitochondria and DNA

Vanadium salts and compounds can affect LPO in intact cells by enhancing ROS formation. Multiple deleterious effects of LPO include effects on enzymes involved in redox cycling of vanadium, inhibition of enzymes and proteolysis as described above in **Figure 1.8**, and damage to cellular membranes including the plasma membrane, mitochondrial membranes and membranes making up the endomembrane systems as summarized in **Figure 1.9**.

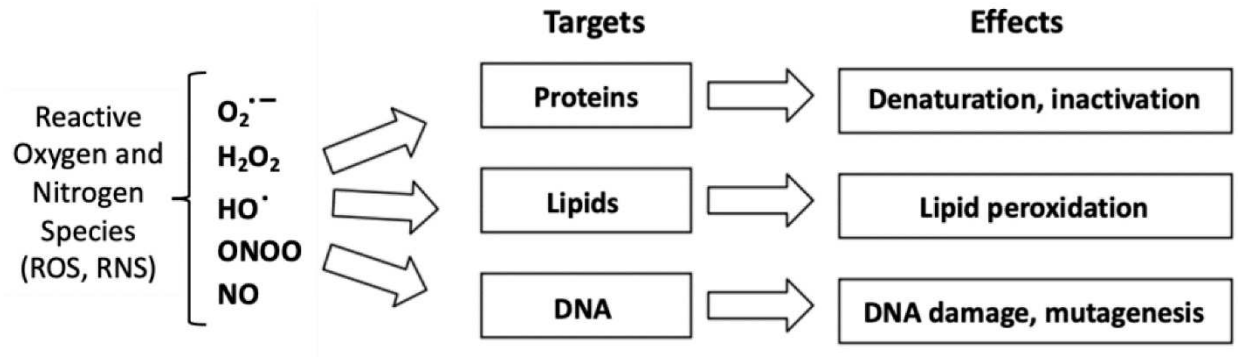

Figure 1.9. The consequences of reactions of reactive oxygen species (ROS) and reactive nitrogen species (RNS) with major biomolecules such as proteins, lipids and nucleic acids, promoting global structural modifications leading to denaturation and/or inactivation of proteins, lipid peroxidation, DNA damage and mutagenesis.

ROS and/or reactive nitrogen species (RNS)-mediated oxidative modifications in proteins [69] that produce partial inactivation or affect structure and function have been described for myosin and actin [97-99]. More recently, the effects of V_{10} and peroxynitrite on myosin have been compared. Peroxynitrite strongly inhibits actomyosin-ATPase activity with an IC_{50} of inhibition of 47 μM. This inhibition is due to the oxidation of the highly reactive Cys on myosin. In contrast, exposure to V_{10} induces the oxidation of a core Cys on myosin and not the highly reactive Cys together with inhibition of actomyosin-ATPase activity (IC_{50} = 2.7 μM) which is 17 times lower. These results suggest that myosin is more sensitive to oxidative modifications mediated by V_{10} than by peroxynitrite [100].

DNA effects involve changes in DNA structure which are, as a result, categorized as mutations. The general effects on the cell membrane, endomembranes and organelles including mitochondria and DNA are summarized below. When discussing the effect of vanadium on plasma membranes and cells, it is important to discuss the speciation and form of vanadium. Several commonly studied forms associated with LPO in cells are salts and include sodium

metavanadate, ammonium metavanadate, vanadyl sulfate, SOV and V_{10}, but as described above under Section 2.4, comparisons between studies are difficult. Much less is reported about LPO in response to vanadium coordination complexes.

1.3.1. Effects of LPO and Vanadium on the Plasma Membrane

The plasma membrane contains up to 75% phospholipids. Polyunsaturated fatty acids (PUFAs) are major lipid components in the sn2-FA position of membrane phospholipids [101,102]. LPO can break down the FAs to ketone-, alcohol- or aldehyde-containing products which affect membrane fluidity, increase membrane permeability and decrease lateral mobility of membrane proteins [103,104]. An increase in the membrane permeability changes the distribution of salts across the membrane which in turn affects the membrane potential. Increased permeability also allows undesired species to penetrate the cell membrane such as additional ROS and metals such as vanadium. Studies have shown that some LPO products change the structural conformation within the bilayer to reduce bilayer thickness and increase toxic molecule penetration of the membrane [103].

LPO in healthy cells reduces defense mechanisms protecting the cell from oxidative stress and increases the damage done by ROS. Several studies have shown that vanadate and vanadyl lower L-ascorbic acid and glutathione levels in cells, reducing overall oxidative stress in the body, decreasing organ weight and increasing LPO products [105]. Additionally, an increase in $HO\cdot$ concentration is observed with the addition of vanadium salts to cells, perhaps as a result of vanadium converting molecular oxygen first to H_2O_2 and then to $HO\cdot$. Such continued imbalances in the oxidative defense mechanisms and the amount of ROS in the body will perpetuate LPO in healthy cells and cause continued damage.

Studies using vanadium in several systems including cell plasma membranes have been reported. For example, an early study investigated the interaction of vanadate with membrane preparations of the Na^+/K^+ ATPase using ^{51}V NMR spectroscopy [106]. Interactions between vanadium and membranes showed selective interactions with V_{10}, although no direct effect on LPO was reported [106]. Numerous studies with vanadate and V_{10} in membrane model systems have been carried out [85,107,108], without any evidence for reduction, and these results are consistent with the requirement for NADPH/phosphate or another reducing agent for the formation of V^{IV} leading to LPO [109].

1.3.2. The Effect of LPO and Vanadium on the Endomembrane System

Damage caused by LPO extends to organelles within the cell as well as fragmented pieces of the endoplasmic reticulum called microsomes [110]. Microsomes can be oxidized by metal ions, including vanadium, as a result of high concentrations of PUFAs in their lipid membrane [110-112]. The endoplasmic reticulum, part of the endomembrane system, is essential for protein synthesis and metabolism of molecules and elements such as lipids and calcium [113]. An important role of the endoplasmic reticulum is to encapsulate molecules to be transported to the Golgi apparatus [113]. For suitable vesicle formation, a mobile membrane is essential, and LPO will limit the mobility of the endoplasmic reticulum (ER) membrane components. LPO has been measured in microsomes from placenta, brain and liver cells [111,112,114]. LPO in microsomes requires vanadyl or vanadate, the latter in the presence of NADPH which is needed to reduce vanadate (V^V) to V^{IV} so that it can initiate the LPO. The proposed mechanism is shown in **Figure 1.10** [112].

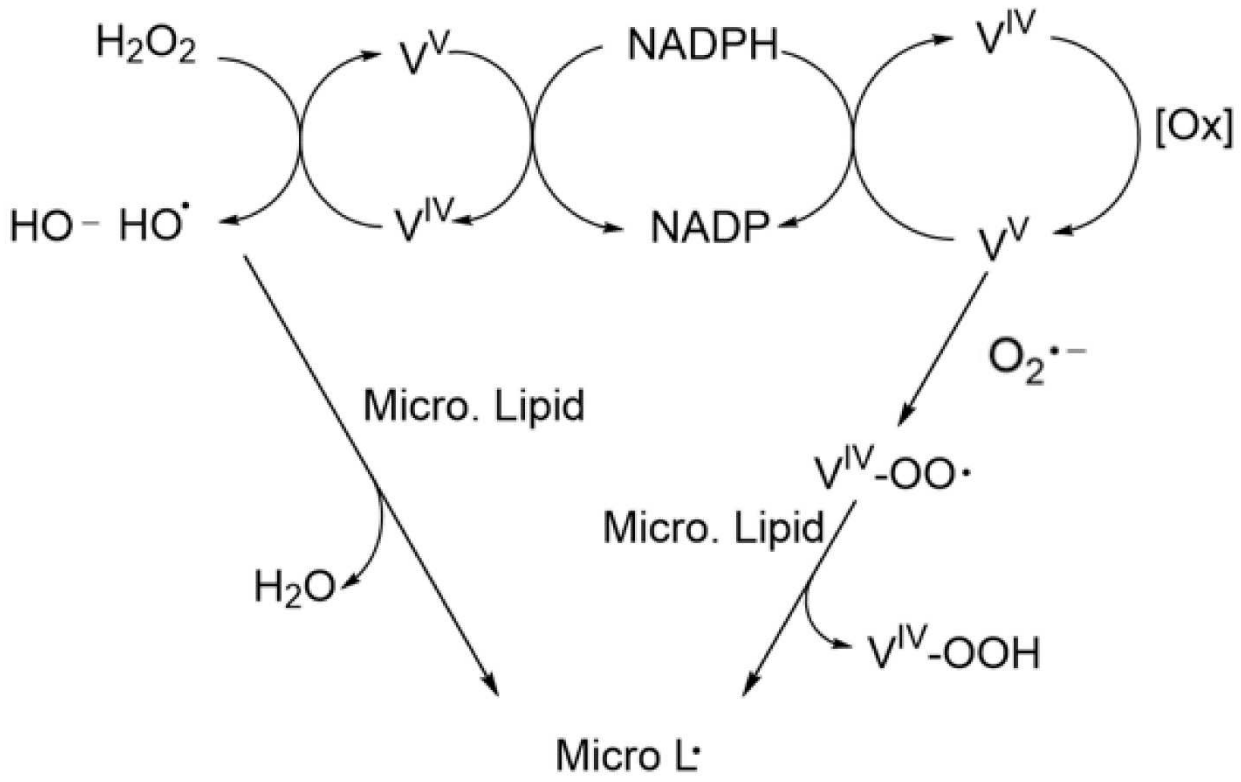

Figure 1.10. The recycling of V^{V} to V^{IV} catalyzed by H_2O_2 or NADPH is shown, as is the similar conversion of V^{IV} to V^{V} catalyzed by an oxidant or NADP. The proposed mechanism of vanadium redox cycling and conversion to LPO products (Micro L.) occurs in microsomes. Inspired by Ref. [112].

1.3.3. The Effects of Vanadium and LPO in the Mitochondria

1.3.3.1. LPO in the Mitochondria

Oxidative phosphorylation is a biochemical process used to meet the energy requirements of cells. Mitochondria, sites of oxidative phosphorylation, are typically found in high numbers in tissues with high energy requirements such as the heart and skeletal tissues. Any compound inhibiting oxidative phosphorylation causes dramatic, negative effects on metabolism in organs such as the heart, kidney, liver and brain. LPO increases mitochondrial membrane permeability and the permeability of the transition pore in the mitochondrial membrane as a result of the

oxidation of the lipids in the membrane and an accompanying decrease in membrane thickness. This affects mitochondrial energy production which is dependent on the membrane potential and the synthesis of ATP driven by the proton gradient powering the ATP synthase. Additionally, LPO of the mitochondrial membrane can cause the release of cytochrome C, a complex necessary for electron transport [115,116]. Complex II, also known as succinate dehydrogenase, is also critical for electron transport [117] and for metabolism in the Krebs cycle [117]. The vanadium-induced decrease in complex II activity is toxic to the cells and results in cell death. Assessing various mitochondrial activities, such as oxygen consumption, ATPase activity and NADH oxidase activity, is important in monitoring the degree of mitochondrial dysfunction induced by an external, toxic agent.

1.3.3.2. The Effect of Vanadium Salts and Complexes on the Mitochondria

Vanadium is reported to affect mitochondria and subsequent energy production, contributing to cell death [118,119]. For vanadate to generate ROS, it needs to be reduced to V^{IV} which occurs in the presence of NADPH and phosphate. The effects of different vanadium species on mitochondria have been investigated, and different responses to vanadium salts and vanadium complexes provide insight into the function of these species. LPO and both V^{IV} and V^{V} treatments [115,120] cause an increase in ROS production, with V^{IV} producing a greater increase in ROS than V^{V}. LPO was measured for both types of vanadium, and although vanadyl caused more LPO than vanadate, vanadate-induced LPO could be increased with the addition of NADPH (and phosphate) [109,121], consistent with the reduction of vanadate to vanadyl to generate ROS and initiate LPO.

The total pro-oxidant activity after in vivo exposure to vanadate was evaluated through the quantitative analysis of ROS production in a study comparing the effect of two types of

V^V vanadates, namely stable, compact V_{10} and simple vanadate, V_1. In fish cardiac mitochondria, only the V_1 solution caused a significant and delayed increase in the ROS production of about 198% 12 h after administration of V_1 solution [28]. There was no effect on oxidative activity levels in V_{10}-treated animals (**Figure 1.11**). In hepatic tissue [27], vanadate oligomers similarly affected the pro-oxidant activity differently than V_{10} (**Figure 1.11**). The V_{10} solution increased ROS production by about 80% during the entire exposure period (12 h), while the V_1 solution induced a 150% increase after the first hour of exposure, registering a blockage in its pro-oxidant capacity over the 12 h of exposure with values of 40% and ~0% 6 and 12 h after exposure to vanadium, respectively [27,28] (**Figure 1.11**). The most likely interpretation for the latter result is that putative decomposition of V_{10} causes gradual, delayed exposure of the liver to a V_1 species whose toxicity is prevented by glutathione (GSH). In fact, a similar suppression in GSH levels has been reported in the liver using the same experimental conditions for V_1 administered in the form of metavanadate [27,28].

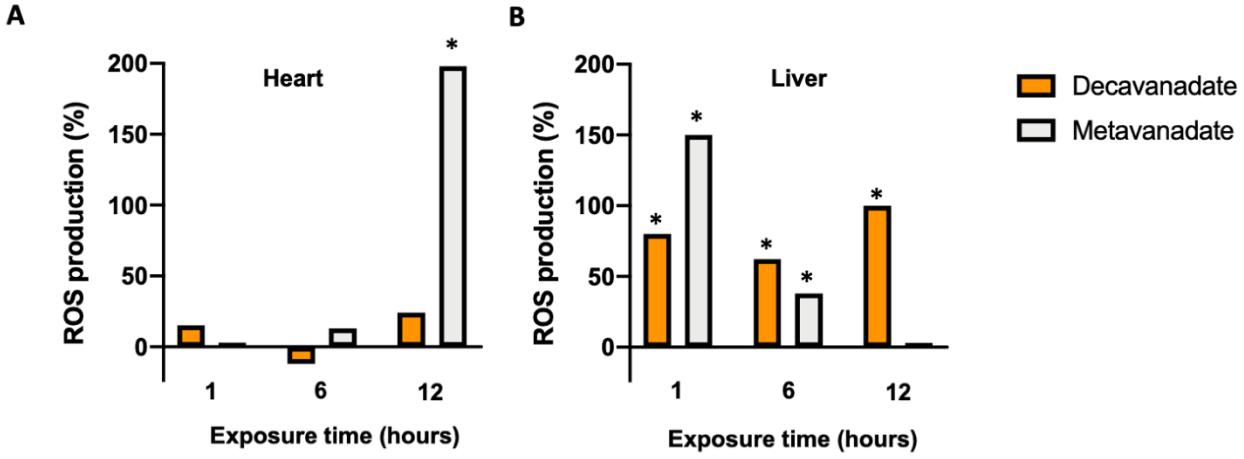

Figure 1.11. Percent ROS production is shown as a function of time in (**A**) cardiac and (**B**) hepatic mitochondria of *Sparus aurata*. Animals (n = 6) intravenously received solutions of V_{10} or metavanadate (1 mM total vanadium). * designates significance compared to control ($p < 0.05$). Adapted from [21,28] with Research Signpost and Elsevier copyright permission.

These effects on mitochondria have been studied using vanadium species in different oxidation states and with different ligands bound [115]. Varying responses were observed depending on the vanadium compound used. The coordination complexes $VO(acac)_2$ and VOcitrate (VOcit) reacted differently than the coordination complex VOdipic with respect to the release of cytochrome C. No mitochondrial swelling was seen in the VOdipic-treated system, indicating that cytochrome C was released via a channel rather than through an increase in membrane permeability. In contrast, the two other V^{IV} complexes $VO(acac)_2$ and VOcit increased membrane permeability. VOdipic had little effect on mitochondrial membrane swelling or changes in membrane potential. $VOSO_4$ and $VO(acac)_2$ caused swelling, eliminated the membrane potential and generated ROS. These experiments showed that the V^{IV} complexes exerted different effects depending on the ligand bound to vanadium, with the greatest effects observed for $VO(acac)_2$ and the V^{IV} salt ($VOSO_4$). A smaller effect was observed using $NaVO_3$ and VOcit. $NaVO_3$ eventually had similar effects to those of $VOSO_4$, suggesting that vanadate had to be reduced to V^{IV} before becoming toxic. Effects with V^{IV} complex VOcit took longer, which was attributed to the high stability of this complex; the slow dissociation of the ligand was necessary before more pronounced effects of the complex were observed. Together, these results demonstrate that vanadium coordination with a ligand can produce subtle differences in biological activity.

1.3.3.3. The Effect of V_{10} on the Mitochondria

As described above, vanadium can affect biological systems including mitochondria directly or through the formation of ROS, both of which can take place simultaneously [21,27,28]. In practical terms, both the direct and indirect effects of vanadium must be considered. To experimentally test the possibility that V_{10} inhibits the electron transport chain

through a complex IV inhibition mechanism, the effect of V_{10} on the oxidation-reduced state of purified ferrocytochrome C was examined [122]. Neither V_{10} nor V_1 induced changes in the oxidation-reduced state of cytochrome C. Furthermore, none of the species caused changes in cytochrome C oxidase activity in rat liver or fish heart [122,123], in agreement with previous studies [124]. These results suggest that the oxidation of reduced cytochrome C during vanadate-stimulated NADH oxidation requires the presence of vanadate and H_2O_2, since it occurs by the action of hydroxyl radicals formed in the vanadate/H_2O_2 mixture.

After excluding the notion that mitochondrial depolarization and inhibition of oxygen consumption promoted by V_{10} resulted from the inhibition of cytochrome oxidase, the effects of V_{10} on the oxidation-reduced state of the mitochondrial cytochrome b were considered [122]. Changes recorded between 500 and 550 nm induced by V_{10} in mammalian liver mitochondria indicated that V_{10}, but not V_1, altered the oxidation-reduced state of cytochrome b, suggesting that mitochondrial complex III was a target of V_{10} and that V_{10} inhibited the respiratory chain of hepatic mitochondria in a manner similar to antimycin-A, a complex III specific inhibitor. Thus, the reduction of cytochrome b is a consequence of the blockade induced by V_{10} in the respiratory chain [122].

Vanadate affects mitochondrial respiration by altering electron transfer between complexes III and IV [125]. Several studies have associated vanadium toxicity with its ability to induce ROS formation, probably through interactions with mitochondrial oxidative–reductive centers [31,67,125]. Vanadate blocked the transfer of electrons in the respiratory chain between cytochromes b1 and C, causing inhibition of succinate oxidation and oxidation of substrates associated with NADH [126]. Vanadate ions have also been implicated in the inhibition of mitochondrial succinate dehydrogenase as well as ATP-dependent succinyl-CoA synthetase from

rat brain mitochondria [127]. Studies involving V_{10} and other oxovanadate species have demonstrated that V_{10}, but not another oxovanadate, stimulates NADH oxidation in the erythrocyte plasma membrane and in rat liver microsomes [124,128,129], induces cytochrome c reduction [130], exhibits α-adrenergic agonist activity in aortic rings in rats [131] and is reduced by $NADP^+$-specific isocitrate dehydrogenase (IDH) [130].

The effects of V_{10} and V_1 on mitochondrial function in preparations of rat liver and fish heart were compared with respect to depolarization of the mitochondrial membrane [26,28,29]. V_{10} strongly depolarized the mitochondrial membrane potential in rat liver mitochondria [122] and fish heart mitochondria [123], compared to V_1 species. An attempt was made to determine the site and nature of V_{10} effects on the electron transport chain by examining oxidation–reduction changes in the cytochromes of the mitochondrial respiratory chain. The effects promoted by the V_{10} and V_1 on the potential of the mitochondrial membrane in the presence of a physiological concentration of GSH (5 mM) were further analyzed. A 10 min exposure to increasing concentrations of vanadate increased mitochondrial depolarization. V_{10} induced mitochondrial depolarization in rat liver mitochondria at very low concentrations, with an IC_{50} value of 38.7 ± 10.2 nM, while 5.4 ± 2.5 μM monomeric vanadate was required to induce 50% depolarization in rat liver mitochondria [122]. Likewise, V_{10} induced depolarization of fish cardiac mitochondria at very low concentrations, with an IC_{50} of 196 nM, while 55 μM V_1 induced a 50% depolarization [123]. Because the V_{10} species was found to affect mitochondrial membrane repolarization (IC_{50} ~1 μM V_{10}, i.e., 10 μM total vanadium) and since mitochondrial membrane hyperpolarization has been described as an early mitochondrial response during apoptotic events [132,133], these results suggest that V_{10} should be explored as an anti-apoptotic agent. Interestingly, the antibiotic cyclosporine A (CsA) did not protect

mitochondria from depolarization induced by the V_{10}, suggesting that, at least in isolated mitochondria, V_{10} depolarization of the mitochondrial membrane is not due to the opening of membrane transition pore inhibitor (MMTP) [134,135].

The effects of V_{10} and V_1 on the mitochondrial function in preparations of rat liver and fish heart were compared with respect to oxygen consumption by the mitochondrial membrane [26,28,29]. V_{10} appeared to be about 100-fold more effective than V_1 in inhibiting oxygen consumption in hepatic mitochondria, as indicated by the IC_{50} values, 98.5 ± 5.1 nM for V_{10} and 9.7 ± 1.4 μM for V_1 [122]. Likewise, V_{10} inhibited oxygen consumption in fish cardiac mitochondria more strongly than V_1, with an IC_{50} of approximately 400 nM for V_{10}, while a 60 times higher value was determined for V_1 (23 μM) [123]. Both vanadate solutions inhibited mitochondrial respiration without uncoupling mitochondria; there was no effect on the respiratory control ratio (RCR), which in coupled mitochondria from rat liver, breathing in pyruvate and malate, was 5.1 ± 0.1 and 5.0 ± 0.1, respectively, in the absence or presence of both V_{10} and V_1. These results are in agreement with previous studies [29], where there was no uncoupling of oxidative phosphorylation but a slight increase in the ADP/O ratio in rat liver mitochondria, due to an inhibition of adenylate kinase activity by V_{10} (1 mM).

1.3.3.4. Effects of V_{10} on LPO in Mitochondria

Although the indirect effects of vanadium on LPO resulting from the reactive nature of the intermediate and final products in the peroxidative process are difficult to distinguish from the direct effects of vanadium [36], indirect effects of V_{10} and V_1 that arise from ROS formation can be evaluated. V_{10}, when administered in vivo, produces a subcellular distribution of

vanadium in mitochondria that differs from that observed after exposure to vanadate [29]. Furthermore, a distinct pattern of LPO and markers of oxidative stress was observed in response to V_{10} different than that induced by V_1 (see below) [28]. Neither V_{10} nor V_1 affected the production of ROS, specifically superoxide anion ($O_2.^-$), in rat liver mitochondria except when NADH was used. V_{10} decreased $O_2.^-$ production in hepatic mitochondria by 40% (5 μM), whereas a 10-fold higher concentration of V_1 was required to promote similar inhibition [27]. In cardiac mitochondria from fish, V_{10} had more potent antioxidant activity than V_1, inhibiting the production of $O_2.^-$ in the absence (IC_{50} = 610 nM) and in the presence of NADH (IC_{50} = 15 nM) [28]. In the presence of NADH, V_1 caused inhibition of $O_2.^-$ formation on the order of 50% (237 nM) [122,123].

This decrease in $O_2.^-$ formation was due, at least in part, to the inhibition of mitochondrial respiration. $O_2.^-$ arising from mitochondrial respiration is a stoichiometric precursor of mitochondrial H_2O_2. Even a small decrease in the membrane potential of rat brain mitochondria, which in turn decreases mitochondrial respiration, strongly inhibits ROS formation [136], which depends on the mitochondrial membrane potential. Since the V_{10} vanadate strongly depolarizes mitochondria [122,123], it should also decrease $O_2.^-$ production, which, in fact, has been observed in V_{10}-treated isolated mitochondria.

LPO occurs after the induction of ROS production in the cell medium. There were several studies that, without specifying the species of vanadate present, have reported that vanadate can attenuate lipid peroxidation in hepatic tissue affected by hepatocarcinogenesis [137] and mammary carcinogenesis [138] without altering lipid peroxidation in control animals. Aureliano's research group has demonstrated that a high concentration of vanadate (5 mM total vanadium) significantly increased LPO propagation in cardiac tissue 1 and 7 days after

intravenous (i.v.) exposure to V_{10} (**Figure 1.12B**) [21]. In hepatic tissue, V_{10} had almost no effect on LPO 12 h after administration but by 24 h increased LPO to levels similar to metavanadate (**Figure 1.12C**) [27].

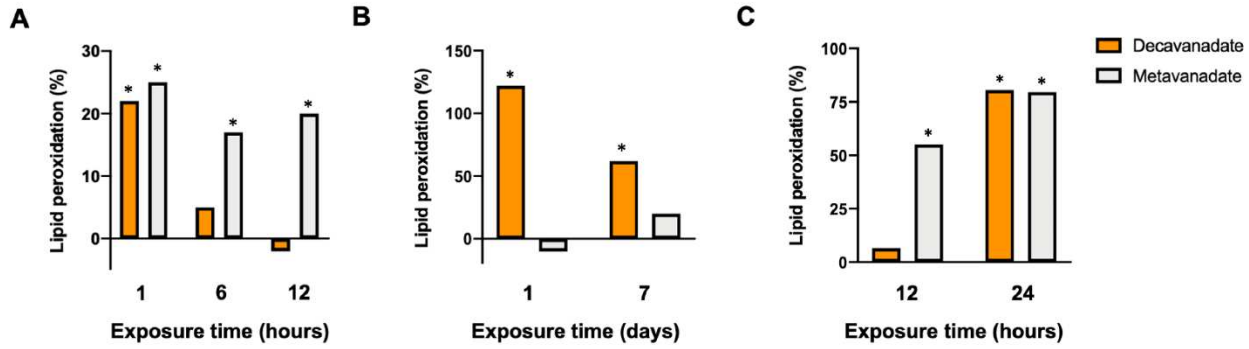

Figure 1.12. Lipid peroxidation products following in vivo administration of V_{10} and metavanadate (V_1) as a function of time depended on the animal model used, the mode of administration, tissues being studied and vanadium concentrations. (**A**) Cardiac tissue (1 mM total vanadium), intravenous administration, *Sparus aurata*; (**B**) cardiac tissue (5 mM total vanadium), intraperitoneal administration, *Halobactracus didactylus*; (**C**) hepatic tissue (5 mM total vanadium), intravenous administration, *Halobactracus didactylus*. * designates significance compared to control ($p < 0.05$). Adapted from [21,27,28] with Research Signpost and Elsevier copyright permission.

Whole heart tissues were similarly studied following in vivo intravenous (i.v.) administration using shorter exposure times (1, 6 and 12 h) and lower vanadium concentrations (1 mM) (**Figure 1.12A**). After 1 h, both V_{10} and V_1 increased ($p < 0.05$) the baseline value, 3.02 ± 0.51 μmol TBARS/g tissue, by about 20% [28]. Only V_1 sustained this increase in LPO at 6 and 12 h. V_{10} treatment had no prolonged effect. In a different species (*H. didactylus*), a higher vanadate concentration (5 mM total vanadium) with intraperitoneal administration and longer exposure times caused an increase in the propagation of LPO in cardiac tissue 7 days after administration (about 80% and 60% after treatment with V_{10} and V_1, respectively) [26]. Together, these results suggest that V_{10} induces peroxidation through a different mechanism or perhaps

even prevents V_1 effects since it never achieves levels as high as those recorded 6 and 12 h after exposure to the V_1 solution. Even with time, the total decomposition of V_{10} into V_1 does not cause the same effects as those seen following the administration of V_1. This suggests that interactions promoted by labile oligomeric oxovanadates are different from those induced by V_{10}. Furthermore, when the V_{10} is completely decomposed into other oligomers of vanadate, targets may no longer be available that produce the effects noted previously. Still, at higher concentrations, V_{10} is unequivocally a promoter of LPO since V_1 has less pronounced effects. Peroxidative damage could be related to a decrease in the activity of antioxidant enzymes as described in complementary studies following changes in antioxidant enzymes [21,27,29,71,122,123]. Although in most vanadium toxicity studies, the contribution of V_{10} is often not considered, it appears that, due to its longer stability at physiological pH, V_{10} does not completely decompose to V_1 before causing marked changes in oxidative stress markers in vitro and in vivo. In fact, the V_{10} solution exhibits different patterns of LPO response and markers of oxidative stress than those resulting from V_1 exposure [19,25-28].

In vivo studies underscore the fact that V_{10} induces consequential responses by antioxidant enzymes and LPO which appear to be related to observed increases in intracellular vanadium [19,25-28]. After administration of the V_{10} solution, metabolism was affected, with mitochondria subsequently identified as the main subcellular target. Thus, based on the in vivo results described here, it appears that V_{10} exhibits pro-oxidant activity by promoting the formation of superoxide anion ($O_2^{\bullet -}$). The increase in radical production leads to a concomitant increase in superoxide dismutase (SOD) activity and an increase in H_2O_2. Increased H_2O_2 causes increased activity of glutathione peroxidases (GPx) which leads to an increase in glutathione

(GSH) content. $O_2^{\cdot -}$ also promotes the propagation of LPO through Haber–Weiss chemistry (**Figure 1.13**).

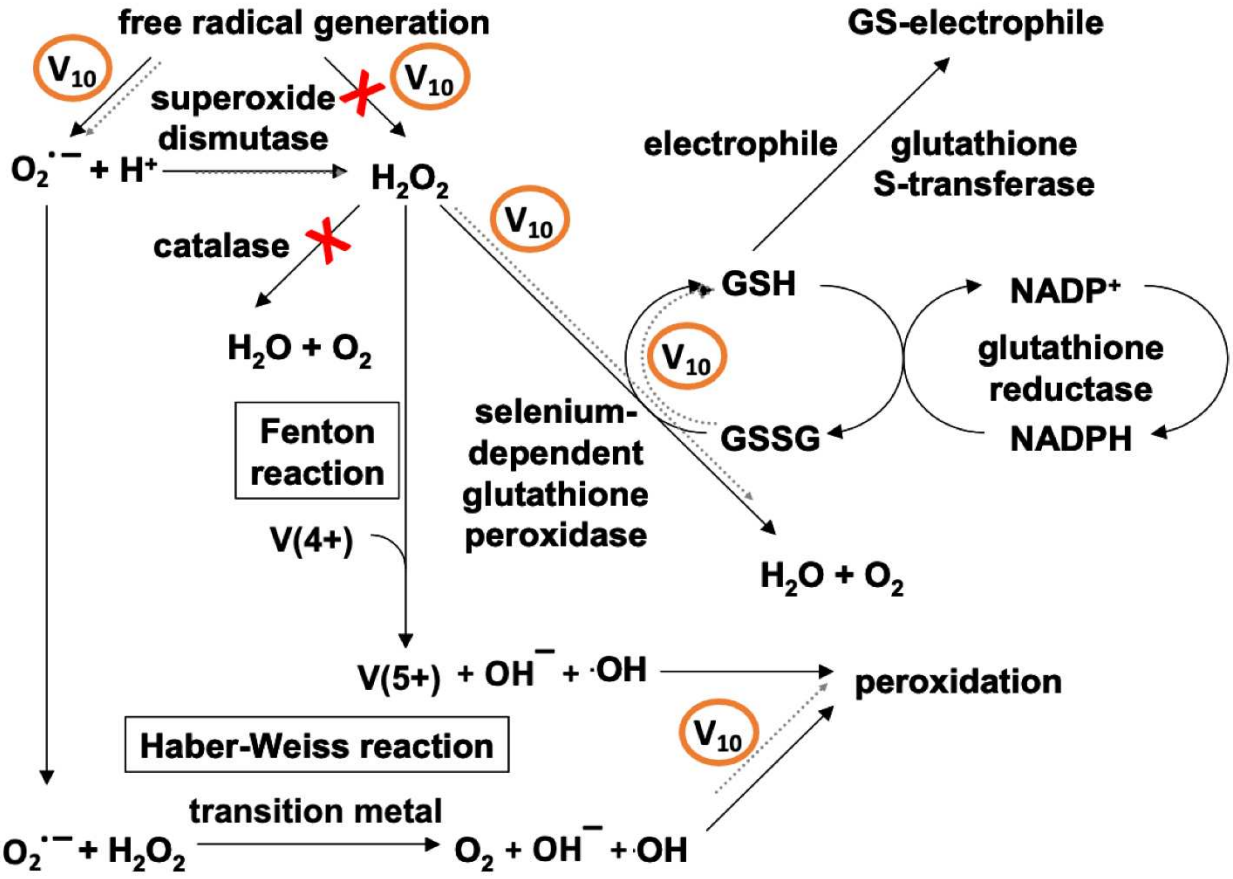

Figure 1.13. Putative V_{10} pathways for the generation of reactive oxygen species and of the actions in some of the enzymes involved in antioxidant defense mechanisms in cells. In vivo V_{10} studies point to the formation of the superoxide anion ($O_2^{\cdot -}$), leading to increases in superoxide dismutase (SOD) activity, H_2O_2, the activity of glutathione peroxidases (GPx) and a subsequent increase in glutathione (GSH) content. Adapted from [21] with Research Signpost copyright permission.

Importantly, the oxidative activity of V_{10} does not result directly from the induction of H_2O_2 production since the antioxidant activity of CAT appears to be unrelated to responses to oxidative stress induced by the toxic effects of V_{10}. This suggests that the peroxidation of membrane lipids recorded after in vivo administration of V_{10} does not result from a stimulation

of Fenton reactions. Results obtained after in vivo exposure to V_{10} differ from the effects of V_1 and underscore the importance of taking into account the speciation of vanadium when evaluating its toxicity.

1.3.4. The Effect of LPO on DNA and Apoptosis

Vanadium affects DNA through multiple mechanisms. Vanadium can have direct interactions with DNA [139-142] or with proteins associated with DNA (histones), as well as indirect consequences through the generation of ROS. The direct interaction of vanadium complexes with DNA often reflects the ability of vanadium compounds to be intercalated into the 3D structure of the DNA double helix [143-147]. Vanadium salts and compounds have been shown to interact directly with proteins as discussed in Section 2.6. ROS are known to react with DNA as well [4,101,148]. Since vanadium enhances ROS formation, vanadium salts and complexes are also able to interact indirectly with DNA by first forming ROS that react in turn with DNA [4,118,149].

The main site of ROS interaction with DNA is the guanine base, where the product of the oxidation reaction is 8-hydroxyguanine. However, other sites of DNA interaction have been identified, including, for example, 2-deoxyribose. These reactions all cause changes in the basic DNA structure corresponding to mutations and potentially leading to programmed cell death and apoptosis. Both experimental studies and theories have emerged to explain the effect that ROS have on DNA and, as a consequence, on programmed cell death [4,118,148,150]. Since ROS are linked to LPO, the direct interaction of ROS with DNA must be considered as well.

Because ROS are increased by vanadium, they should be considered when evaluating vanadium effects on LPO while also recognizing that the direct effects of vanadium may be toxic

as well. Some studies have found that vanadium, in the form of vanadate, can cause damage to DNA through DNA scission, phosphorylation inhibition and oxidation of DNA bases [151-153]. Individuals exposed to vanadium pentoxide through inhalation had greater amounts of DNA base oxidation [151]. In addition, DNA tail strands were lengthened due to increased purine oxidation (7%) and pyrimidine oxidation (30%). Desaulniers and coworkers found that vanadium in the form of sodium metavanadate decreased DNA phosphorylation and changed DNA unwinding due to breaks in the DNA strands [152]. Rodriguez-Mercado and coworkers found that V^{IV} in vanadium tetraoxide (V_2O_4) caused double-strand DNA breaks in human leukocytes, although V^{III}, V^{IV} and V^{V} caused some DNA damage as well [153].

Damage to the DNA can lead to cell damage and death [154,155]. Similarly, ROS can play a role in apoptosis [156]. There are a number of pathways that lead to apoptosis that are affected by ROS and, in some cases, by LPO. Two pathways known to be affected by vanadium-generated ROS are the ERK/MAPK and the NF-kB pathways [33,157]. Vanadium inhibits the NF-kB pathway which causes cell proliferation in healthy cells and apoptosis in cancer cells. Molinuevo et al. found that two vanadyl compounds were related to the activation of the ERK pathway and enhanced apoptosis through a mechanism that was unclear, although treatment with antioxidants inhibited the pathway [157]. The effect on proteins (p53 and $Cdc25B_2$) and on mitochondrial membrane permeability by vanadium also contributed to cell death [33,119,158]. These toxicological effects on otherwise healthy intact cells may also lead to cell death through effects on cell growth checkpoints [159].

1.3.5. The Oxidative Damage by V_{10} in Biological Systems

Several biological studies have shown that vanadium has the ability to produce ROS resulting in LPO and changes in antioxidant enzymes indicative of oxidative stress [40,65,66].

Despite accumulated knowledge to date, the oxidative effects of vanadium observed after acute exposure in vivo in cardiac muscle have not been thoroughly investigated, and the contribution of different oligomeric species to the toxic effects promoted by V^V is not known. In fact, most studies on the toxicological effects of vanadate in biological systems do not account for the contributions of different vanadium species even when the stable V_{10} is being used. Moreover, V_{10} can form intracellularly [62] and affect the activity of several enzymes [22-24]. The decomposition of V_{10} is sufficiently slow to allow the study of its effects on biochemical systems not only in vitro but also in vivo [20,25-29,71,122,123,160]. Furthermore, V_{10} interacts with certain cytoskeletal proteins such as actin [83,84,161] and with membrane proteins such as the Ca^{2+}-ATPases [85,119,120] which increase its stability [84]. Interactions with other proteins such as myosin and with membrane vesicles do not affect the half-life time of V_{10} decomposition [83,162]. Therefore, it is hypothesized that V_{10} exists under physiological conditions for a period of time sufficient to induce several biological effects that differ from those promoted by V_1. Careful evaluation of this possibility would require experimental conditions where V_{10} is stable such as a lower pH [160] or where V_{10} is associated with a protein [24]. Open questions are whether V_{10} exists under physiological conditions and, if so, whether this species induces specific in vivo effects such as LPO. In fact, a limited number of in vivo studies have been carried out with V_{10}. Since 1999, Aureliano's research group and its collaborators have carried out in vivo studies administering V_{10} solutions in an attempt to assess the contribution of the V_{10} to the toxic effects of vanadate [20,25-29,71,122,123,160,163], and more recently, Trevino and collaborators have investigated the therapeutic potential of V_{10} [80,164].

1.4. Vanadium Effects on Lipid Peroxidation and Disease Processes

The number of metal-based therapeutics is steadily increasing as the chemical space continues to be expanded. Vanadium is one of the less utilized elements to date with fewer ongoing clinical trials [54]. However, as dogma continues to be challenged and new discoveries are reported, this element is becoming more attractive to the pharmaceutical industry. Vanadium is, in general, known to induce oxidative stress. Although vanadium compounds can impact LPO, few investigations have been reported using compounds other than salts. Since ligands allow for the fine-tuning of the properties of vanadium, it is surprising that LPO studies with more compounds have not been undertaken.

1.4.1. Role of Vanadium in Lipid Peroxidation Related to Cancer

Despite somewhat controversial reports suggesting that vanadium may be an essential trace element for humans [165,166], pharmacological amounts of vanadium needed to observe efficacy may be 10 to 100 times the normal intake [167]. Under certain levels, some vanadium complexes/compounds have shown anticancer and/or antidiabetic activity in mammals [23,53,54,74,168], while higher levels can cause toxicity. Under some conditions, vanadium can act as a pro-oxidant molecule, which interacts with other oxidants and synergistically enhances oxidative stress and potentially lipid peroxidation (LPO) [11].

Reports from the early 1990s showed that V complexes/compounds induced LPO, which was associated with tissue toxicity and carcinogenicity [169,170,171]. Tissue-specific responses were shown for vanadate, which produced a cytotoxic response in the murine osteoblast-like MC3T3E1 nontransformed cell line [172]. This level of cytotoxicity was higher than that in vanadate-treated osteosarcoma cancer UMR106 cells with respect to both time- and

concentration-dependent responses [172]. Osteoblastic cells were more sensitive to the vanadate-induced free radical and biomarker thiobarbituric acid (TBARS) formation, particularly at low concentrations. Nevertheless, higher basal TBARS was observed in untreated osteosarcoma cells [172]. Other vanadium compounds ($VOSO_4$) and a complex of vanadyl with aspirin (VO/Aspi)) were found to be more potent than vanadate in inducing TBARS and inhibiting cellular growth, in both cell lines tested [172] (**Table 1.2**). However, when an equivalent low concentration of VO/Aspi was released from a controlled delivery system (poly(β-propiolactone) (PβPL) film), less TBARS formation was observed [173] (**Table 1.2**), which reflects lower cytotoxicity compared to that previously reported for the metallodrug in solution [172].

The development and testing of vanadium derivatives with different ligands and with improved bioavailability and toxicity profiles continues. Both naproxen- and glucose-complexed vanadium compounds (NapVO and GluVO) had antiproliferative effects that were more pronounced in osteosarcoma UMR106 cells than in the normal MC3T3E1 osteoblasts [157]. This supported the observation that a low level of GluVO and NapVO increased TBARS production in tumoral cells but not in the nontransformed cells [157] (**Table 1.2**), suggesting LPO was involved in the antineoplastic action observed. Interestingly, neither the free vanadyl cation nor ligands induced an antimitogenic effect in cells at the concentrations tested [157]. At low concentrations, a large number of different complexes/compounds of vanadium were found to be therapeutically active [64,166]. Possible mechanisms for the anticancer activity of vanadium complexes/compounds included an increase in ROS generation, hyperactivation of the Ras-Raf-MEK-ERK pathway and cell cycle arrest [174,175,176]. It is also possible that vanadium may confer protection against chemical-induced carcinogenesis or toxicity in normal tissues by normalization of increased pathogenic LPO and oxidative stress. While an increase in hepatic

LPO was observed in a group of carcinogen-treated female Sprague Dawley rats, this increase was lowered towards normal values by vanadium co-administration [138] (**Table 1.2**) and was associated with a significantly lower percentage of rats with tumors after vanadium treatment. In these experimental groups, SOD activity in the liver paralleled LPO. By contrast, hepatic glutathione (GSH) and cytochrome P450 (CYP) enzyme content and glutathione S-transferase (GST) activity decreased with carcinogenic treatment compared to control rats and recovered with vanadium treatment [138]. Similarly, in a model of hepatocarcinogenesis induced in rats by chronic feeding of 2-acetylaminofluorene (2-AAF), continuous vanadium administration inhibited LPO and suppressed cell proliferation [177] (**Table 1.2**), suggesting vanadium was chemopreventive.

The chemoprotective role of vanadium against cancer chemotherapy-induced toxicity is also relevant. Many chemotherapeutic agents such as cyclophosphamide (CP) and cisplatin (CDDP) are toxic due to multifactorial mechanisms that include increased oxidative stress in normal tissues and organs, namely the liver and kidney. The co-administration of compounds with antioxidant potential may be beneficial to patients. For example, the simultaneous treatment of female Swiss albino mice with CP and either vanadium(III)-L-cysteine complex (VC-III) [178] or oxovanadium(IV)-L-cysteine methyl ester (VC-IV) [179] reduced ROS levels when compared to the increase in ROS observed in CP-treated group vs. control [178,179]. With respect to LPO, partial normalization of TBARS in CP/VC-III- or CP/VC-IV-treated mice was observed (**Table 1.2**) [178,179]. After treatment with CP, there was a decrease in GSH levels and in GST, glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) activities, while a recovery was observed with vanadium treatment [178,179]. Similar protective effects were observed with concomitant treatment with cisplatin (CDDP) and VC-III (**Table 1.2**)

[180]. These results suggest that vanadium may be beneficial as an adjunct therapy to protect against the toxicity of anticancer drugs.

Table 1.2. Effects of vanadium in lipid peroxidation related to cancer. Main outcomes of studies using different V compounds in various organs/tissues of animal models or cancer cells.

Vanadium Compound	Combined/Complexed	Carcinogenic/Toxic Agent or Cell Lines	Tissue/Model	Main Results/Outcome	Ref.
V^{IV}; VO	Aspirin; polymeric film	Osteosarcoma UMR106 cells in culture	Bone	Cytotoxic effects	[172,173]
V^{IV} derivatives	Naproxen (Nap-VO); Glucose (GluVO)			Apoptosis mediated by lipid peroxidation	[157]
Ammonium monovanadate (NH_4VO_3, +V oxidation state) (vanadium supplemented in drinking water)		7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary carcinogenesis in rats	Mammary gland	Prevention of mammary cancer	[138]
Vanadium (in the form of ammonium vanadate)		2-acetylaminofluorene (2-AAF)-induced hepatocarcinogenesis in rats	Liver	Vanadium was chemopreventive; inhibition of lipid peroxidation	[177]
Oxovanadium(IV)-L-cysteine methyl ester (VC-IV)		Cyclophosphamide (CP)-induced hepatotoxicity in mice	Liver	Protective role of VC-IV against CP-induced toxicity	[179]
Vanadium(III)-L-cysteine complex (VC-III)				Protective role of VC-III against CP- and CDDP-induced toxicity	[178]
		Cisplatin (CDDP)-induced nephrotoxicity in mice	Kidney		[180]

Even though vanadium participates in Fenton-type reactions [40] and the mechanisms proposed for vanadate action involve redox cycling and the production of ROS [65,66], some results show a depression in ROS and the rate of ROS formation [21]. Previous results show that in certain experimental conditions, for example in rats with induced hepatocarcinogenesis [137] and diabetes [138], vanadate may decrease oxidative stress. Strong evidence supports the observation that V_{10} alters the production of mitochondrial $O_2.^-$ differently from V_1 and suggests the possibility that different pathways are involved in the biological activity of different vanadium species. Of the proposed intracellular pathways for vanadate, several involve the production of $O_2.^-$ mediated by oxidoreductases of NADPH in the respiratory chain [65,66]. Considering the proposed mechanisms of action and detoxification of vanadate, which include reducing vanadate to vanadyl with $O_2.^-$ production, the available data support the interpretation that V_{10} may participate in bioprocessing and metabolism differently than V_1.

1.4.2. Effect of Vanadium in Diabetes-Induced Lipid Peroxidation

Diabetes mellitus is a complex metabolic disease characterized by a chronic state of hyperglycemia [181]. Although the impaired function of the pancreatic islets might be relevant in its etiology, other tissues are affected and may present complications in uncontrolled disease [182]. Diabetes can be generally classified into different categories with distinct clinical features. Type 1 diabetes is an autoimmune disease in which beta cells in the pancreas are unable to produce the hormone insulin while in type 2 diabetes, the most common form of diabetes, the body is either resistant to insulin or incapable of producing sufficient amounts of insulin [181].

An imbalance between the production and removal of ROS and RNS may contribute to insulin resistance and pancreatic beta cell dysfunction, which ultimately leads to the development of type 2 diabetes [183,184]. Increased levels of TBARS, a biomarker of LPO, were more highly elevated in type 2 diabetes patients than in healthy control subjects [185]. In patients with type 2 diabetes, hyperglycemia was associated with increased oxidative stress and free radical-mediated LPO [186-188] both of which may affect the development of micro- and macrovascular complications related to the intensification of systemic inflammation in these patients [184,189,190]. Compounds that modulate LPO and oxidative stress and have an antioxidant potential may contribute to improving the metabolic health in patients with diabetes and be a valuable therapeutic approach.

In 1979, Tolman et al. showed that vanadium salts exhibited insulin-mimetic effects which led to an interest in vanadium chemistry for the treatment of diabetes [191]. Since then, a series of reports have been published describing the insulin-like effects of various vanadium compounds, mainly V^{IV} and V^{V} salt and coordination complexes. One coordination complex, an organic vanadium compound, bis(ethylmaltolato)oxovanadium(IV) (BEOV), exhibited excellent

efficacy in streptozotocin (STZ)-diabetic rats [192] and entered Phase I and II clinical trials [193,194].

Using different animal models of diabetes and analyzing diverse tissues, many reports showed effects of vanadium compounds on the activity of antioxidant enzymes and the levels of LPO (**Figure 1.14**). Early studies from the 1990s showed that treatment of STZ-induced diabetic Sprague Dawley rats with sodium metavanadate ($NaVO_3$), did not lead to changes in the antioxidant defense system [195]. However, the tissue level of vanadium positively correlated with the TBARS level [195]. By contrast, sodium orthovanadate (SOV) treatment of STZ-induced diabetic male Wistar rats led to the STZ-induced decrease in the hepatic activities of SOD, CAT and GPx being restored to normal levels, while the elevated plasma lipid peroxides (as measured by MDA) were decreased almost to basal values [196] (**Figure 1.14**). This same pattern was observed in the liver enzymes of alloxan-induced diabetes female Wistar rats (**Figure 1.14**), but not in all the tissues evaluated [137]. SOV treatment also almost normalized the chemical-induced increase in the levels of TBARS in the brain, along with the normalization of the activity of the brain GST, which was decreased in the diabetic rats [197] (**Figure 1.14**). Interestingly, subsequent studies have used SOV combined with *Trigonella graecum* seed powder (TSP) which makes it possible to use lower concentrations of vanadate. Most authors have shown a reversal of non-physiologic antioxidant levels and peroxidative stress in different tissues from diabetic animals [198-202] (**Figure 1.14**).

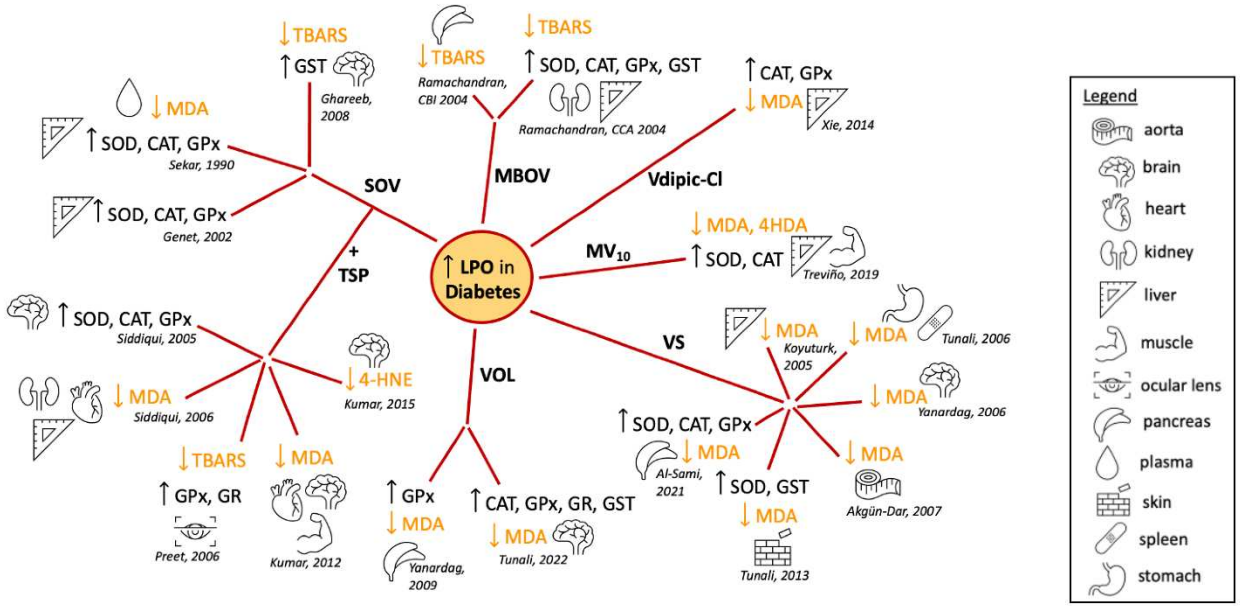

Figure 1.14. Summary of the reported effects of different vanadium compounds in lipid peroxidation and antioxidant enzyme activity, evaluated in different tissues of diabetes-induced animal models [137,196-214]. Abbreviations: superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx; glutathione reductase, GR; glutathione S-transferase, GST; malondialdehyde, MDA; 4-hydroxy-2-nonenal, 4-HNE; thiobarbituric acid reactivity, TBARS; 4-hydroxyalkenals, 4HDA; sodium orthovanadate, SOV; Trigonella graecum seed powder, TSP; macrocyclic binuclear oxovanadium complex, MBOV; N(1)-2,4-dihydroxybenzylidene-N(4)-2-hydroxybenzylidene-S-methyl-thiosemicarbazidato-oxovanadium (IV), VOL; vanadyl sulphate, VSO_4; metformin-decavanadate, MV_{10} [137,196-214].

The vanadium salt in oxidation state IV, vanadyl sulfate ($VOSO_4$), has also been studied extensively. An early study showed that TBARS levels were elevated in vanadyl-treated animals, although cataract development was suppressed in STZ-diabetic Wistar rats [215]. By contrast, many reports showed in different tissues that the treatment with $VOSO_4$ reversed the increased levels of LPO in response to diabetes induction [205-208,211,212] (**Figure 1.14**). These results were recently expanded to show similar normalization of the oxidative state in cardiac, lung, skeletal muscle and eye lens tissue [216]. Similar antioxidant effects were observed in the pancreas, liver and kidneys of diabetic rats treated with a macrocyclic binuclear oxovanadium

complex (MBOV) [213,214] and in the pancreas and brain of diabetic rats treated with the N(1)-2,4-dihydroxybenzylidene-N(4)-2-hydroxybenzylidene-S-methyl-thiosemicarbazidato-oxovanadium (IV) (VOL) compound [209,210] (**Figure 1.14**).

Oxidative stress in the liver and muscle tissues of alloxan-induced diabetic rats was addressed after treatment with $(H_2Metf)_3[V_{10}O_{28}]$ (metformin-decavanadate, MV_{10}) [204]. After 60 days, decreased activity levels of SOD and CAT induced by alloxan were restored to normal levels (**Figure 1.14**). Furthermore, the increased levels of LPO markers in the diabetic animals were normalized after Metf-V_{10} treatment. This was observed for both MDA and 4-hydroxyalkenal (4HDA) levels in a similar fashion to treatment with insulin, while metformin alone had very limited effects [204] (**Figure 1.14**). Decavanadate was previously reported to increase the glucose uptake in rat adipocytes, in the presence or in the absence of insulin [217]. Together, these findings suggest that vanadium compounds are not only insulin mimetics but may also enhance the activity of insulin [23,164,218].

Changing the oxidation state of the vanadium compound changes the redox properties of the complex alters the formation of LPO products as described above for vanadate (V^V) and $VOSO_4$ (V^{IV}). One study compared the effects of vanadium in oxidation states III, IV and V in a series of coordination complexes with the same ligand, chloro-substituted dipicolinic acid [203]. V^{IV}dipic-Cl and V^Vdipic-Cl complexes in liver tissues produced improved blood glucose levels, while there were lesser effects of V^{III}dipic-Cl [203] (**Figure 1.14**). This demonstrated that even high-oxidation-state vanadium compounds are beneficial in changing MDA levels toward normal and reducing ROS levels presumably through redox cycling. For complexes with the dipic-Cl ligand, it was surprising that the V^V complex showed a trend towards being slightly better at

normalizing the redox state of diabetic cells, though V^V complexes would need to undergo Fenton chemistry first [203].

It is important to note that the animal models of diabetes using diabetogenic chemicals cause the destruction of β-cells resulting in type 1 diabetes, so it is unclear whether such effects would be observed in type 2 diabetes animal models or patients. Moreover, in some cases, the diabetic animals did not show a reduction in the activity of enzymes involved in antioxidant defense in all tissues analyzed. As an example, an increase in enzyme activity was observed in diabetic heart tissues when compared to normal animals [137,201], although even in such cases, treatment with vanadium compounds restored levels close to normal (non-diabetic) values [137,198,201,206].

Taken together, these data suggest treatment with vanadium compounds may contribute to alleviating oxidative stress in patients with diabetes and contribute to an overall improvement in metabolic function. However, more evidence of vanadium antioxidant beneficial effects and safety is still required.

1.4.3. Vanadium Lipid Peroxidation and Neurodegenerative Diseases

Vanadium is known to have neurotoxic effects and contribute to a number of neurodegenerative diseases presumably through the introduction of oxidative stress and LPO production. The brain contains high amounts of PUFAs, making it a prime target for LPO, which can cause the destruction of the myelin sheath, loss of neurons via cell death, disruption of the cell membrane potential, depletion of dopamine, and inactivation of phosphatase enzymes. Neurons are surrounded by a myelin sheath which is important for the development of the electric potential and the ability to transmit electrical impulses in the form of action potentials

quickly. Vanadium exposure has been reported to cause damage to the myelin sheath [219] and, as a result of LPO, neuronal death. LPO in the mitochondria also leads to cell death through effects on mitochondrial membranes. Vanadium accumulates in the brain after exposure [220], indicating that the toxic effects of vanadium relating to membrane destruction may play a role in the reported neurodegenerative diseases such as Parkinson's and Alzheimer's. The metal content and transporters in the rat brain have been reported to be sensitive to the presence of other metals, including Mn, chromium, zinc, cobalt, aluminum, molybdenum and vanadium [221].

1.4.3.1. Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disease that has been associated with several failures in brain function. A decrease in the neurotransmitter dopamine has been correlated with the onset of Parkinson's which, with disease progression, leads to a failure in the dopaminergic system. Some basis of knowledge around metals, specifically manganese (Mn), and the onset of Parkinson's or the onset of similar symptoms called Parkinsonism exists [222]. The latter is a condition that results in loss of motor and neurological function similar to that of Parkinson's but does not exhibit the symptoms of Parkinson's disease. Symptoms produced by Mn are called manganism [223]. Mn, like vanadium, undergoes redox cycling and is known to have many neurotoxic effects. The ability of Mn to produce ROS has been well characterized [224] and shown to cause effects on mitochondrial function similar to those observed with vanadium treatment, including the loss of the mitochondrial membrane potential and the release of Cyt C [225]. Additionally, LPO products have been observed in mitochondria and the endoplasmic reticulum system and are similar to the oxidative stress in response to manganese exposure. With high doses of Mn, symptoms of Parkinson's disease are seen and correlated with the onset of Parkinson's [226]. Given the similarities between vanadium and manganese, the

effects of vanadium on the onset of Parkinson's are likely to be similar. Ngwa and coworkers have reported a link between vanadium neurotoxicity and its effect on the dopaminergic system due to its effect on protein kinase C-delta and its function in cell signaling mechanisms [227]. Ohiomokhare and coworkers (2020) found that vanadium increased ROS and decreased motor function in Melanogaster drosophila, both wild-type and PD models, and that these effects were alleviated with chelators or the administration of L-DOPA [228].

1.4.3.2. Alzheimer's Disease

Alzheimer's disease (AD's) is a neurodegenerative disease characterized by loss of memory. Although no single cause of AD's has been discovered, there is evidence that metals, lipid peroxidation and oxidative stress can play a role in disease progression. The disease is associated with the accumulation of β-amyloid plaques in the brain that have the capability of interacting with redox-active metals, such as copper, zinc and iron [229]. These metal ions induce the disease because of their ability to generate ROS and damage the brain through DNA damage and oxidation of lipids and proteins. Studies have shown that 4-HNE, a product of LPO, is present in the brains of AD's patients [230]. Mitochondrial ROS production and mitochondrial dysfunction have been associated with AD [231]. All of these are known products of vanadium-based oxidative stress and offer a basis for vanadium having a role in AD.

As is the case for Parkinson's, there are only a limited number of studies characterizing vanadium effects on the development of AD's disease or its progression. However, due to vanadium's redox properties and ability to generate ROS, it has the potential to induce at least some similar effects. Montiel-Flores and coworkers found that the inhalation of vanadium pentoxide caused AD-like neuronal cell death in rats [232].

There is also a growing body of work investigating the use of vanadium in treating AD's disease. Although vanadium has toxic effects, studies reported some potential of vanadium-based therapeutics for AD's disease [233]. Vanadyl acetylacetonate was found to promote glucose and energy metabolism in neuronal cells but did not reduce β-amyloid plaque production [234]. Two peroxovanadium complexes were reported to inhibit β-amyloid fibril formation. He et al. (2015) showed that two complexes were able to inhibit the aggregation of amyloids using PrP106–126 and $A\beta_{1–42}$, where PrP is from the prion disease and refers to protein-prion protein. Inhibition was more effective in PrP than in Aβ, but there was not much difference in its effects on cell toxicity. Peroxovanadium complexes increased cell viability perhaps due to the ability of peroxovanadium complexes to reduce methionine residues [235]. This group also found that BEOV was able to ameliorate AD symptoms through a number of mechanisms including inhibition of Aβ aggregate formation [236]. These results should encourage studies on the use of vanadium in the treatment of neurodegenerative diseases.

1.4.4. The Potential for LPO as a Future Target for Therapeutic Treatments

The ability of vanadium compounds to impact oxidative stress and the formation of LPO products is well documented [237]. Since vanadium remains a comparatively underexplored metal [238], new compounds are being assayed to determine their potential for alleviating oxidative stress [239,240]. Novel compounds are being designed which affect LPO but lack cellular toxicity. New pathways are discovered by investigating organisms not traditionally investigated [241]. New approaches are being developed based on combatting oxidative stress in disease processes. For example, a 2D vanadium carbide synthetic enzyme referred to as V2C MXenzyme has been reported to alleviate ROS-mediated inflammation [224]. Specifically, the 2D V2C MXenzyme can replace SOD, CAT, POD, TPx, GPx and HPO, thus mimicking the

intracellular antioxidant defense system against ROS-mediated oxidative damage including protein carbonylation, lipid peroxidation and DNA damage. In vitro and in vivo experiments demonstrated that V2C MXenzyme was biocompatible and exhibited ROS-scavenging capability, protecting cellular components against oxidative stress. Future investigations are likely to involve the characterization of novel biological systems, new compounds and agents such as the V2C MXenzyme and related systems [224] designed to combat the effects on oxidative stress and LPO.

1.5. Conclusions

Lipid peroxidation (LPO) is a process that affects human health and can be modulated by vanadium compounds. Some forms of vanadium salt and complexes exacerbate LPO, while other compounds have protective effects. Vanadium salts and compounds can sometimes generate radicals and ROS directly but can also act indirectly through effects on LPO. LPO typically affects the structure and function of cellular membranes, as do vanadium compounds. The formation of ROS also impacts other cellular functions in ways that are modulated by vanadium compounds. The protective effects of vanadium compounds reported for cancer and neurodegenerative diseases are likely to involve LPO products and ROS.

The effects of vanadium compounds on several biological processes have been tested, and a number of biomarkers have been identified. The most common ones are malondialdehyde (MDA) and hydroxynonenal (4-HNE), although cis-parinaric acid and ascorbate have been used, as have enzymes such as superoxide dismutase (SOD) and catalase (CAT). The effects of LPO on enzymes, DNA and membrane uptake or signaling in the presence of vanadium compounds have been characterized. Enzymes with redox-active amino acids such as cysteine can be reduced in the presence of vanadium compounds, and hence the enzymes are irreversibly

affected. Similarly, LPO can affect these enzymes. In the case of DNA, guanine is generally converted to 8-hydroxyguanine, and similarly, the direct interactions of vanadium impact the guanine base in DNA, although vanadium compounds can also be intercalated in the 3D-organized DNA strand.

Vanadium accumulation in cells suggests that mitochondria are frequently subcellular targets for vanadium, particularly when it is administered as V_{10}. This conclusion is based on distribution studies of vanadium in the mitochondria of cardiac, hepatic and renal tissue. The extent of vanadium accumulation depends on the system investigated, the total concentration of vanadium administered and the mode of administration. Decavanadate (V_{10}) is consistently 10 to 100 times more potent than monomeric vanadate (V_1) both as a mitochondrial membrane-depolarizing agent and as an inhibitor of oxygen consumption by hepatic and cardiac mitochondria and may contribute to the antioxidant effect through the partial inhibition of ROS production. Although mitochondrial effects have generally been the target of vanadium studies, other cellular components and organelles should not be ignored.

Because vanadium salts and complexes can induce ROS formation both directly and indirectly, studies of mechanisms leading to LPO should include investigations of both direct and indirect processes. This is, of course, complicated by the fact that different vanadium species exist under physiological conditions and can have different effects. Thus, studies evaluating the mechanism of action of vanadium compounds should involve speciation studies of vanadium to evaluate the direct and indirect effects of the species that exist during the course of study. In addition, studies relating to how these molecules interact in other biological systems are relevant to these considerations. Undoubtedly, understanding LPO is important for better understanding how vanadium exerts its effects in biological systems and gives rise to some of the beneficial

effects reported in cells and tissues in cancer, diabetes and neurodegenerative diseases, important therapeutic targets that will hopefully encourage future work with this important metal.

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FOOTNOTES

1. Aureliano M, De Sousa-Coelho AL, Dolan CC, Roess DA, Crans DC. Biological Consequences of Vanadium Effects on Formation of Reactive Oxygen Species and Lipid Peroxidation. Int J Mol Sci. 2023 Mar 11;24(6):5382. doi: 10.3390/ijms24065382. PMID: 36982458; PMCID: PMC10049017.

CHAPTER 2
VANADIUM AND MANGANESE'S IMPACT ON CARDIAC MITOCHONDRIAL DYSFUNCTION

2.1 Introduction

Mitochondrial function is imperative for cell survival and is associated with several diseases. Due to the electron transfer system (ETS), mitochondria provide most cellular ATP and cellular reactive oxygen species (ROS). Dysfunction in the mitochondria have been associated with several diseases such as cancer, diabetes, and neurodegenerative diseases such as Parkinson's and Alzheimer's [1,2]. The presence of redox active components in the cell can contribute to mitochondrial dysfunction and the growth of these diseases. One class of redox components is transition metals in the environment, of interest in this work are vanadium and manganese.

Several transition metals are essential elements and have been associated with biological effects, both beneficial and toxic effects [3]. Transition metals can be structural or as functional as in the case of cofactors in several enzymes necessary for proper biological functioning. Transition metals are also used in diagnostics or therapeutics, such as for cancer treatment [4–8]. The primary example of a successful therapeutic is cis-platin which uses platinum to treat several cancers. A significant problem with transition metals is that they can show toxic effects and are associated with several diseases [3,9]. Having insight into the effects of the transition metals is important due transition metals in the environment and their increased use in therapeutics and diagnostic applications. Specifically, vanadium and manganese are associated with environmental exposure and significant biological effects associated with cancer, Parkinson's, diabetes, and Alzheimer's and have been explored for potential therapeutic use [10–14].

Transition metals show different activity based on the speciation and potential for finetuning beneficial or toxic effects. It is well known that different forms, oxidation, and protonation states [15], nuclearity [16] and coordination to potential metabolites and ligands can dramatically affect their activity [17,18]. For example, organic ligands coordinating to the transition metals forming coordination complexes can alter their biological activity from their salts. Dramatic differences have also been reported with simple salts [19]. It has been reported that vanadate tetramer specifically inhibits 6-phosphogluconate dehydrogenase [20], both vanadate dimer and tetramer inhibit glycerol-3-phosphate dehydrogenase [21] and glucose-6-phosphate [22], decavanadate inhibits phosphofructokinase [23] and ribonuclease [24], and although most phosphatases are inhibited by vanadate monomer [25], the prostatic acid phosphatase is inhibited by both vanadate monomer and dimer [22]. An x-ray with a vanadate dimer in the active site has been reported [26]. Recent vanadium and manganese complexes have shown activity against cancer cell lines; [10–12,27] however, little is known about the species involved in a particular mode of action, but prevailing data indicates trends [11]. Significant amounts of literature indicate mitochondria as potential targets for transition metal mode of action and due to the redox properties of these cells, ROS release is important [28]. Studies have shown that different vanadium complexes have varying effects on mitochondria [29] and some literature shows that Mn^{2+} is more likely to have a biological effect than Mn^{3+} [30] but little has been done to characterize the effects of Mn salts and complexes to understand the role that manganese speciation and state may play on biological activity. Furthermore, these two metals have shown to have similar biological effects including the loss of mitochondrial membrane potential, effects on mitochondrial protein, promotion of neurodegenerative diseases and cell death [31–36]. However, some forms of vanadium have been reported to be protective against

neurodegenerative disease [37,38] and cardiovascular disease [39]. With such similar responses they are two interesting metals ions to study to identify the potential similarities and differences in their biological effects

In this study we investigate the effects that vanadium(IV) and (V) and manganese(II) compounds has on mitochondrial function. Using $VOSO_4$, $VO(acac)_2$, $NaVO_3$, $MnCl_2$, and $Mn(acac)_2$ we monitored real-time mitochondrial respiration and ROS release. Specifically, we determined the effects of different mitochondrial pores and channels on the uptake of vanadium and manganese species. We supported these mitochondrial studies with speciation calculations to better understand what the species are that are affecting mitochondrial function.

2.2.1 Materials and Methods

2.21 Materials

Amplex^{TM} UltraRed Reagent, manganese(II) chloride ($MnCl_2$), and Vanadyl sulfate ($VOSO_4$) was purchased from Thermo Fisher Scientific (Waltham, MA). Manganese(II) acetylacetonate ($Mn(acac)_2$) was purchased from Aldrich Chemical Company (now Sigma Aldrich (Milwaukee, WI)). Acetylacetone was purchased from Oakwood Chemical (Estill, SC). Bis(acetylacetonato)oxovanadium ($VO(acac)_2$), sodium metavanadate ($NaVO_3$), L-(-)-Malic Acid sodium salt, sodium pyruvate, L-Glutamic Acid, sodium succinate dibasic hexahydrate, adenosine 5'-diphosphate monopotassium salt dihydrate (ADP), and horseradish peroxidase (HRP) were purchased from Sigma Aldrich (St. Louis, MO). (3-[3-[[[3-(2,6-dichlorophenyl)-5-methyl-4-isoxazolyl]methyl]amino]benzoyl]-1H-indole-1-butanoic acid (DS16570511) and N-[[1-(1-naphthalenylmethyl)-4-(phenylamino)-4-piperidinyl]carbonyl]-glycine (VBIT-12) were purchased from Cayman Chemicals (Ann Arbor, Michigan) and prepared in DMSO.

2.2.2 Isolation of cardiac mitochondria

Adult (>8-month-old) C57Bl/6 mice were obtained from Jackson Labs for use in these studies. Animals were housed in a temperature and humidity-controlled facility on a 12:12 hour light:dark cycle and provided water and chow (Purina 2918) ad libitum and were euthanized for tissue collection by CO_2 inhalation followed by midline thoracotomy and removal of the heart following confirmation of deep unconsciousness (absence of respiration or tail pinch reflex). All procedures were approved by the Colorado State University Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cardiac mitochondria were isolated from fresh heart tissue by differential centrifugation protocols as previously described [32]. All samples and reagents were kept on ice or controlled at 4°C for the duration of the protocol. Briefly, hearts were harvested immediately after sacrifice and rinsed with ice-cold Chappell-Perry (CP1) buffer consisting of (in mM) 1 ATP, 100 KCl, 50 MOPS, 1 EDTA, 5 EGTA, and 5 $MgSO_4 \cdot 7H_2O$ at pH 7.4 with KOH. Minced tissue was homogenized for 15 seconds using a polytron, incubated for 7 minutes in CP1 containing trypsin (~5 mg/g tissue), quenched with CP2 (CP1+2mg/mL Bovine Serum Albumin), then subjected to 12 passes with a glass Teflon Potter Elvehjem homogenizer before centrifugation at 600 g. The mitochondria-rich supernatant was collected and centrifuged at 7,000 g. Supernatant was discarded and the pellet resuspended in CP2, before being followed by three 7,000-g clarifying spins in CP2 and then one spin in KME buffer (100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA). Final mitochondrial pellets were suspended in 350 uL of KME. A bicinchoninic acid protein assay (BCA; Thermo Fisher Scientific, San Jose, CA) was utilized to determined specific

protein concentration of mitochondrial isolates before 30 ug protein was added to each respiratory chamber (15 ug/mL).

2.2.3 Monitoring mitochondrial dysfunction and ROS release via high resolution respirometry

Mitochondrial respiration and ROS production was investigated using two Oxygraph O2k-FluoRespirometer (Oroboros Instruments, Innsbruck, AT). The FluoRespirometer consists of two temperature-controlled chambers, sealed to maintain oxygen concentration, and is fitted with an oxygen sensor and a fluorometric probe (O2k-Fluo LED2 module) which allow for measurement of total O_2 and H_2O_2 in the chamber. Samples were run in MiR05 respiration medium containing (in mM) 0.5 EGTA, 3 MgCl2 hexahydrate, 60 lactobionic acid, 20 taurine, 10 KH2PO4, 20 HEPES, 110 sucrose, and 0.1% BSA, pH 7.1 with KOH. The instrument was calibrated using the Datlab software (Oroboros Instruments, Innsbruck, Austria) to accurately measure O_2 concentration in the chamber and the rate of oxygen consumption (JO_2) which is calibrated prior to sample addition. Each chamber was allowed to equilibrate to room O2 concentration and kept at a temperature of 37 degrees C. Following stabilization 5ul of HRP and 2.5ul Amplex Red were added to the chamber. Following O2 stabilization, 30ug of isolated mitochondria were added to the chamber and were treated with substrates (1mM Malate/5mM Pyuvate/10mM Glutamate/10mM Succinate/10mM ADP). Each sample was treated separately with titrations of $VOSO_4$, $MnCl_2$, $VO(acac)_2$ and $Mn(acac)_2$ from 1uM to 275uM. Hydrogen peroxide formation was measured by accumulation of resorufin in the chamber (Ex/Em 571/585 nm).

2.2.4 Evaluation of mitochondrial swelling induced by $VOSO_4$, $VO(acac)_2$, $NaVO_3$, $MnCl_2$, $Mn(acac)_2$, and AcAc

Changes in the volume of mitochondria induced by shifts in osmotic pressure from metal uptake were measured by ultraviolet absorbance at 540nm (A_{540}). Mitochondria isolated from the heart of 10–12-month-old mice were diluted in 2RX respiration buffer (500 mM Sucrose, 20 mM Tris-MOPS, 0.02 mM EGTA, 10 mM pyruvate, 2 mM malate, adjusted to 7.4pH with KOH) to a concentration of 60ug/100ul 2RX buffer. In a clear 96 well plate, 100ul of the mitochondria/RX buffer sample were added. $VOSO_4$, $VO(acac)_2$, $NaVO_3$, $MnCl_2$, and $Mn(acac)_2$, at concentrations of 3.2uM, 20uM, 200uM, and 400uM were prepared fresh in distilled water for each experiment. Acetylacetonate was tested as a control with concentrations of 6.4uM, 40uM, 400uM, and 800uM. Each well was brought to a final volume of 200ul with the addition of 100ul of each metal sample so that the final concentrations of metals were 1.6uM, 10uM, 100uM, 200uM. Absorbance at 540nm was measured every 2 mins for 60mins with a microplate spectrophotometer (VersaMax, Molecular Devices).

2.2.5 Effect of inhibiting mitochondrial pores and channels on vanadium and manganese induced swelling

In a 96 well plate 100ul of master mix of 60ug/100ul mitochondria and 2RX buffer (500 mM Sucrose, 20 mM Tris-MOPS, 0.02 mM EGTA, 10 mM pyruvate, 2 mM malate, adjusted to 7.4pH with KOH). Three inhibitors were tested (cyclosporin A, VBIT-12, DS16570511) were tested. A 2mM stock of Cyclosporin A (CsA) was prepared in DMSO and 4mM stocks of VBIT-12 and DS16570511 were created in DMSO. At the bottom of separate wells 1ul of the 2mM CsA stock, 5ul of a 4mM VBIT-12 stock, and 5ul of a 4mM DS16570511 were added. followed

the addition of 100ul of the master mix. The assay was started by the addition of metal to bring the well to a final volume of 200ul and a concentration of 200uM metal. Each inhibitor was run with an N of 3-6. Each well was brought to a final volume of 200ul and a final concentration of 200uM metal ($VOSO_4$, $NaVO_3$, $VO(acac)_2$, $MnCl_2$, $Mn(acac)_2$,). As a control 400uM acetylacetonate was tested. Absorbance at 450nm was measured every 2mins for 60mins using a microplate spectrophotometer (VersaMax, Molecular Devices).

2.2.6 Speciation calculations of $VOSO_4$, $VO(acac)_2$, $NaVO_3$, $MnCl_2$, and $Mn(acac)_2$

Transition metal ions undergo hydrolytic, protonation and redox reactions, and as have been shown in details differences have been reported regarding which species interaction with a protein, with interphases and with intact cells. In this work we sought to investigate the effects of these coordination complexes and their respective salts in the various systems we were investigating. However, since most of these studies were carried out at uM or nM concentrations, it is not possible to measure the different species observed because they are present below detection limits by most methods. Thus, we were resorting to the estimation of the speciation based on the known reported speciation constants. In such case the speciation will not be exact, but since the comparisons we have done with speciation constants in different biological systems yielded concentrations within a factor of two, the evaluations determined by use of known speciation constants will be representative and provide some information on the systems we have been investigating. In the following we will briefly describe how we obtained the speciation profiles for each of the two salts and the two coordination complexes. Some of the considerations, such as the speciation observed in the stock solutions (10uM) used as well as under the conditions of the study (uM) are provided in the supplemental material.

$VOSO_4$ speciation. The speciation diagrams were calculated using the HYSS program and the IUPAC Stability Constants Database Software (Version 5.81) [Data version 4.62] (2000). Speciation profiles were constructed using the following formation constants: $[VO(OH)]^+$ ($\log\beta_{-1,1} = -5.94$) [41], $[(VO)_2(OH)_2]^{2+}$ ($\log\beta_{-2,2} = -5.94$) [41] , $[VO(OH)_3]^-$ ($\log\beta_{-3,1} = -18.0$) [41], $[(VO)_2(OH)_5]^-$ ($\log\beta_{-5,2} = -22.5$) [41], $\{VO(OH)_2\}_{n\,(s)}$ solubility product value of $Ksp = 6.6 \times 10^{-23}\ M^3$, $[VO(HSO_4)]^+$ ($\log\beta_{1,1,1} = 1.74$) [42], $VOSO_4$ ($\log\beta_{0,1,1} = -2.51$). [41]

$VO(acac)_2$ speciation. The speciation diagrams were calculated using the HYSS program and the IUPAC Stability Constants Database Software (Version 5.81) [Data version 4.62] (2000). The speciation profiles illustrates the concentration of $VO(acac)_2$ and hydrolytic species using the following formation constants: $[VO(OH)]^+$ ($\log\beta_{-1,1} = -5.94$) [41], $[(VO)_2(OH)_2]^{2+}$ ($\log\beta_{-2,2} = -5.94$) [41], $[VO(OH)_3]^-$ ($\log\beta_{-3,1} = -18.0$) [41], $[(VO)_2(OH)_5]^-$ ($\log\beta_{-5,2} = -22.5$)[41], $\{VO(OH)_2\}_{n\,(s)}$ solubility product value of $Ksp = 6.6 \times 10^{-23}\ M^3$, $[VO(acac)]^+$ ($\log\beta_{0,1,1} = 17.67$) [43], $VO(acac)_2$ ($\log\beta_{0,1,2} = 33.62$) [43].

$MnCl_2$ speciation. The speciation diagrams were calculated using the HYSS program. The diagrams illustrate the concentration of $MnCl_2$ and hydrolytic specias. Diagrams have been constructed based on the most abundant species at physiological pH (pH = 7.4) using the following formation constants: $[Mn(OH)]^+$ ($\log\beta_{-1,1} = -10.58$) [44], $Mn(OH)_2$ ($\log\beta_{-2,1} = -22.18$) [37], $[Mn(OH)_3]^-$ ($\log\beta_{-1,1} = -34.34$) [44], $[Mn(OH)_4]^{2-}$ ($\log\beta_{-4,1} = -48.28$) [44], $Mn(OH)_{2(s)}$ and $MnO_{(s)}$ solubility product value of $\log\beta = 15.19$ and $\log\beta = 17.94$ respectively [44], $[MnCl]^+$ ($\log\beta_{0,1,1} = 3.69$) [45], $MnCl_2$ ($\log\beta_{0,1,2} = 6.09$) [45], $[MnCl_3]^-$ ($\log\beta_{0,1,3} = 10.02$) [45], $[MnCl_4]^{2-}$ ($\log\beta_{0,1,4} = 12.63$) [45].

$Mn(acac)_2$ speciation. The speciation diagrams were calculated using the HYSS program. The diagrams illustrate the concentration of $Mn(acac)_2$ and hydrolytic species using the following

formation constants: $[Mn(OH)]^+$ ($log\beta_{-1,1}$ = -10.58) [44] , $Mn(OH)_2$ ($log\beta_{-2,1}$ = -22.18) [44], $[Mn(OH)_3]^-$ ($log\beta_{-1,1}$ = -34.34) [44], $[Mn(OH)_4]^{2-}$ ($log\beta_{-4,1}$ = -48.28) [44], $Mn(OH)_{2(s)}$ and $MnO_{(s)}$ solubility product value of logβ = 15.19 and logβ = 17.94 respectively [44], $[Mn(acac)]^+$ ($log\beta_{0,1,1}$ = 13.20) [42], $Mn(acac)_2$ ($log\beta_{0,1,2}$ = 25.28) [42] .

2.3 Results and Discussion

2.3.1 $MnCl_2$ and $VOSO_4$ inhibit mitochondrial respiration while acac-complexes show mitigated inhibition of respiration, but show little effect on ROS release

The effect on mitochondrial respiration showed a varied response depending on metal and complexation state. To account for individual variations and limitations caused by depleting oxygen concentrations in the chamber, the data was expressed as a % respiration from the max respiration followed by the addition of ADP. Titrations were stopped after 275uM addition due to volume displacement effecting mitochondrial concentration in the chamber. $VOSO_4$ and $MnCl_2$ inhibited mitochondrial respiration by nearly 28% at 15uM concentration but had no significant differences between vanadium or manganese (**Fig. 2.1C**). Both $VO(acac)_2$ and $Mn(acac)_2$ inhibit respiration only 15-20% and begin to differ significantly above 175uM ($p < 0.05$) with $Mn(acac)_2$ showing significantly less inhibition. There was a metal dependent effect on acac-complex respiration inhibition ($p < 0.0001$) (**Fig. 2.1D**). Both manganese compounds inhibited respiration, however, there was a state dependent affect ($p < 0.0001$) with $Mn(acac)_2$ having greater inhibition of respiration than $MnCl_2$ above 1uM concentration ($p < 0.05$) (**Fig. 2.1B**). Vanadium compounds showed no significant difference in inhibition of respiration at any concentration but there was a state dependent difference on respiration inhibition between $VOSO_4$ and $VO(acac)_2$ ($p < 0.05$) (**Fig. 2.1A**). The metal and type of compound play asignificant role in controlling the inhibition of mitochondrial respiration.

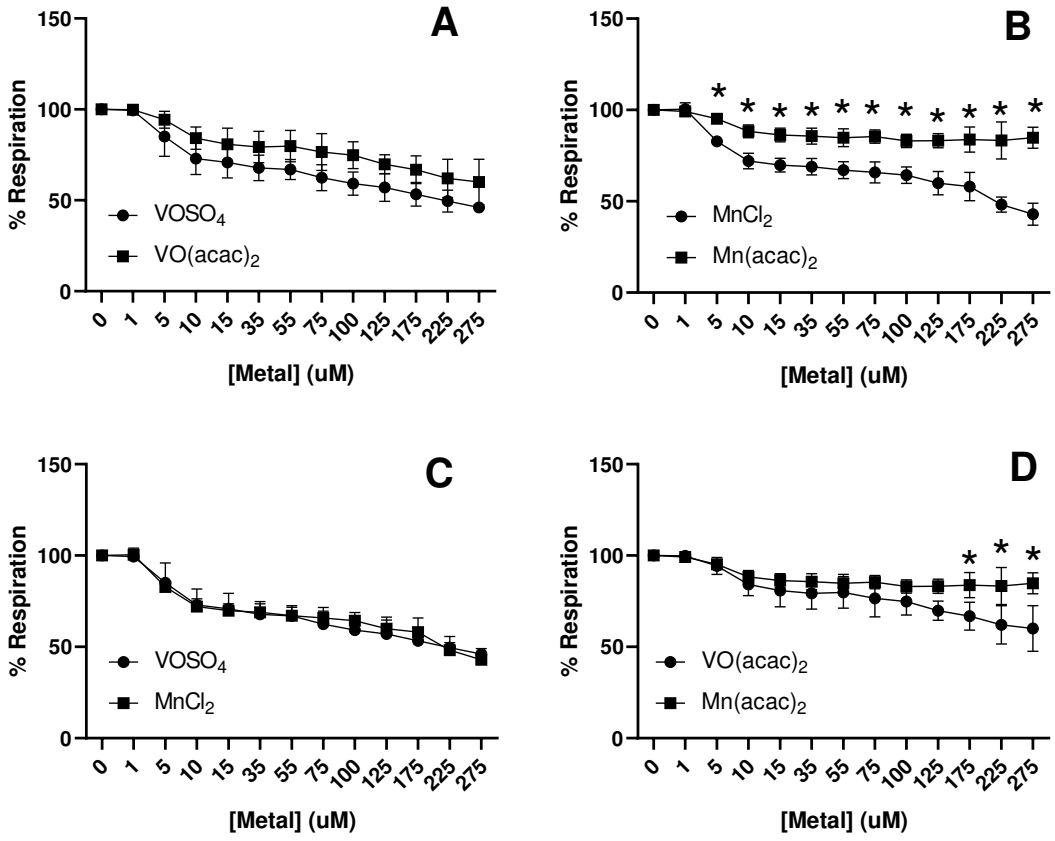

Figure 2.1. Concentration dependent effects of $VOSO_4$, $MnCl_2$, $VO(acac)_2$, and $Mn(acac)_2$ on mitochondrial respiration. (A) $VOSO_4$ and $VO(acac)_2$ (B) $MnCl_2$ and $Mn(acac)_2$ (C) $MnCl_2$ and $VOSO_4$ (D) $VO(acac)_2$ and $Mn(acac)_2$. Respiration was measured using two Oxygraphs using a Clark type electrode. Statistical analysis was performed using a 2-Way ANOVA using GraphPad Prism8 2Way ANOVA with Sidak's multiple comparisons test. Each experiment was completed with an N = 3. (* $p < 0.05$)

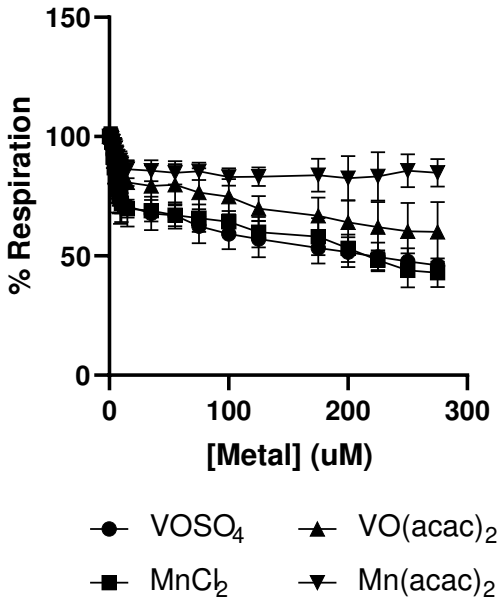

Figure 2.2. Comparison of vanadium and manganese titrations on mitochondrial respiration.

The release of ROS varied depending on metal and state. ROS release was measured through the fluorescence of resorufin, the byproduct of the reaction between hydrogen peroxide and Amplex Red catalyzed by horseradish peroxidase (HRP). Measurement of ROS release by manganese was stopped after 150uM because max ROS signal had been reached. $VOSO_4$ and $VO(acac)_2$ showed significant differences in ROS release at concentrations under 200uM (**Fig. 2.3A**). There was a state dependent effect on vanadium ROS release ($p < 0.0001$). $MnCl_2$ and $Mn(acac)_2$ showed no significant difference in ROS release at any concentration (**Fig. 2.3B**). $MnCl_2$ showed significantly higher ROS release than $VOSO_4$ above 150uM with the type of metal having a significant effect ($p < 0.0001$) (**Fig. 2.3C**). Metal acac complexes had a significant metal dependent differences on ROS release ($p < 0.0001$). $Mn(acac)_2$ had a significantly higher ROS release rate than $VO(acac)_2$ above 10uM (**Fig. 2.3D**). The data shows

that both the metal and state (salt vs complex) matter in mitochondrial ROS release. To determine if this release of ROS was due to interaction with the mitochondria, a control was run without any mitochondria and the same ROS release was seen, indicating a chemical reaction instead of an interaction with mitochondria (**Fig. 2.4**).

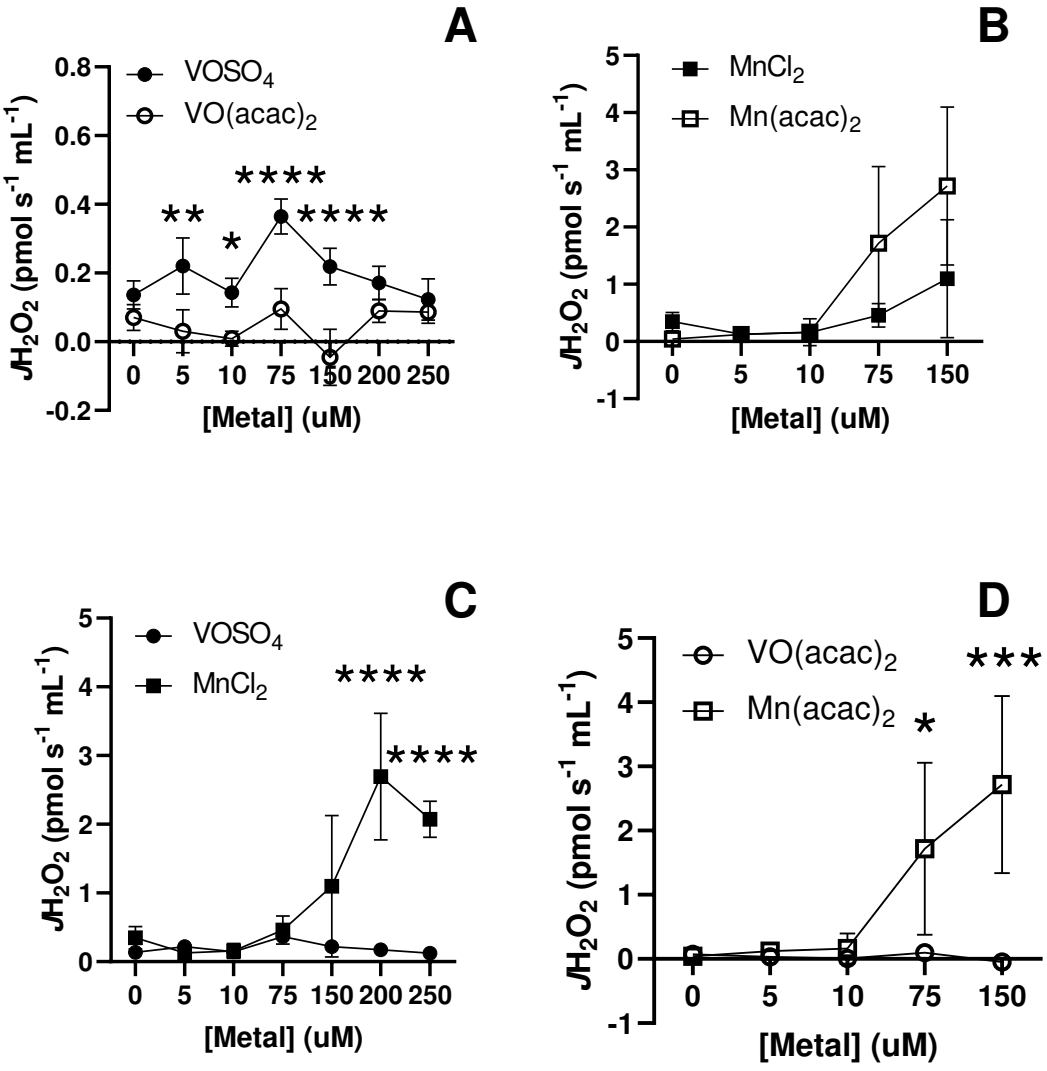

Figure 2.3. Concentration dependent effects of vanadium and manganese on the rate of mitochondrial ROS production. (A) $VOSO_4$ and $VO(acac)_2$ (B) $MnCl_2$ and $Mn(acac)_2$ (C) $MnCl_2$ and $VOSO_4$ (D) $Mn(acac)_2$ and VO(acac) The rate of ROS release was measured using fluorometric probe (O2k-Fluo LED2 module) to monitor the production of resorufin from the

reaction of with HRP and H_2O_2 (Ex/Em 571/585 nm). Statistical analysis was performed using a 2Way ANOVA using GraphPad Prism8 with Sidak's multiple comparisons test. Each experiment was completed with an n=3 per group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$)

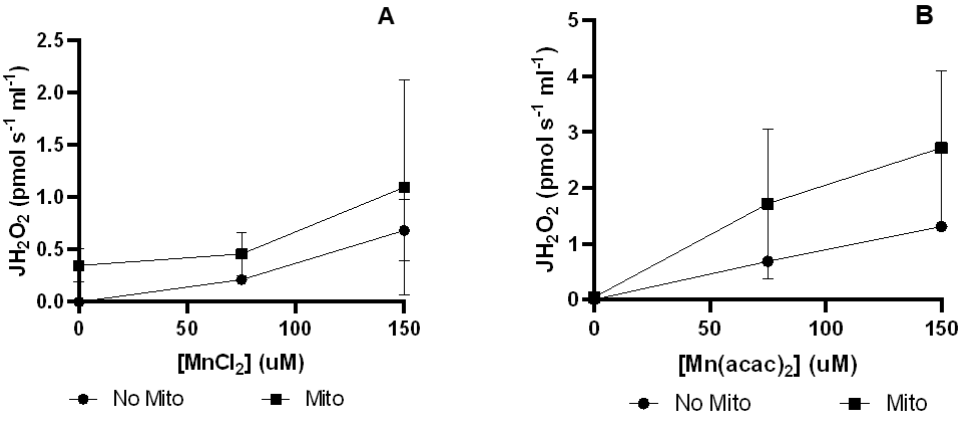

Figure 2.4. Manganese induced ROS release background check. (A) $MnCl_2$ (B) $Mn(acac)_2$ The rate of ROS release was measured using fluorometric probe (O2k-Fluo LED2 module) to monitor the production of resorufin from the reaction of Amplex Red with HRP and H_2O_2 (Ex/Em 571/585 nm). A sample with mitochondria and without mitochondria were tested. No statistical difference between in ROS release was seen. Statistical analysis performed on GraphPad Prism8 using a using a 2Way ANOVA with Sidak's multiple comparisons test.

2.3.2 $VOSO_4$, $VO(acac)_2$ induce mitochondrial swelling while $NaVO_3$, $MnCl_2$ and $Mn(acac)_2$ do not

Swelling induced by metal species is indicative of the opening of ion channels and pores in the mitochondria membrane allowing water to enter changing osmotic pressure. This increase in osmotic pressure can cause mitochondria to rupture, ultimately leading to a reduction in cellular respiration. The ability of vanadium salts and complexes to induce mitochondrial swelling has been previously reported; however, the species present is solutions causing the swelling induced by the vanadium has not been determined. Though a manganese(III) compound was shown to induce cellular swelling [46] and manganese(II) acetate was shown to induce mitochondrial swelling in animal cells [47], whether the complexes remained intact

during the studies were not determined. Further, the current literature lacks investigations to determine the comparison of the action of manganese salts compared to coordination complexes in cellular swelling as well as comparison with a similar metal ion.

We tested the effect of three vanadium compounds ($VOSO_4$, $NaVO_3$, $VO(acac)_2$ and two manganese compounds ($MnCl_2$, $Mn(acac)_2$) on mitochondrial swelling with the objective of identifying similar effects as well as differences. It is our hypothesis, that similarities will be observed if speciation allows for similar ionic charges and structures. However, the comparison of the salt with the metal ion may allow for a study of free metal ion and compare it to a chelated metal ion which may result in different responses as observed previously for other systems.

The V^{IV} salt and coordination compound showed rapid and severe mitochondrial swelling starting at 100uM when compared to the control mitochondrial sample (**Fig. 2.5A, 2.5B**). The testing of both $VOSO_4$ and $VO(acac)_2$ allowed for direct comparison of V^{IV} in salt and a coordination complex, and the speciation studies below demonstrate whether the $VO(acac)_2$ convert to the same species as found with $VOSO_4$. To study the effects of speciation differences of the vanadium in different oxidation states, we tested both V^{IV} and V^{V} salts effects on swelling. While the V^{V} salt, $NaVO_3$, had negligible effects as shown in **Figure S1** and **Figure 2.6**, the V^{IV} salt showed a significant effect documenting the importance of oxidation state.

The manganese(II) compounds show insignificant effects on mitochondrial swelling at 200uM concentration at 20-30 min timepoints (**Fig. 2.5C, 2.5D, 2.6**) whereas some effect on mitochondrial swelling was observed at the 200uM concentration at the 60 min timepoint. The results with $MnCl_2$ and $Mn(acac)_2$ provide the comparison between a salt and complex and at 200 uM show similar results at 60 min compared to the control. When comparing the Mn^{II} with V^{IV} (in the form of VO^{2+}) both as a salt and complex, the V^{IV} forms are more efficacious than the

Mn^{II} forms. The results show the V^{IV} salt and the coordination complex have similar effects, while there are not great differences between Mn^{II} salt and complex.

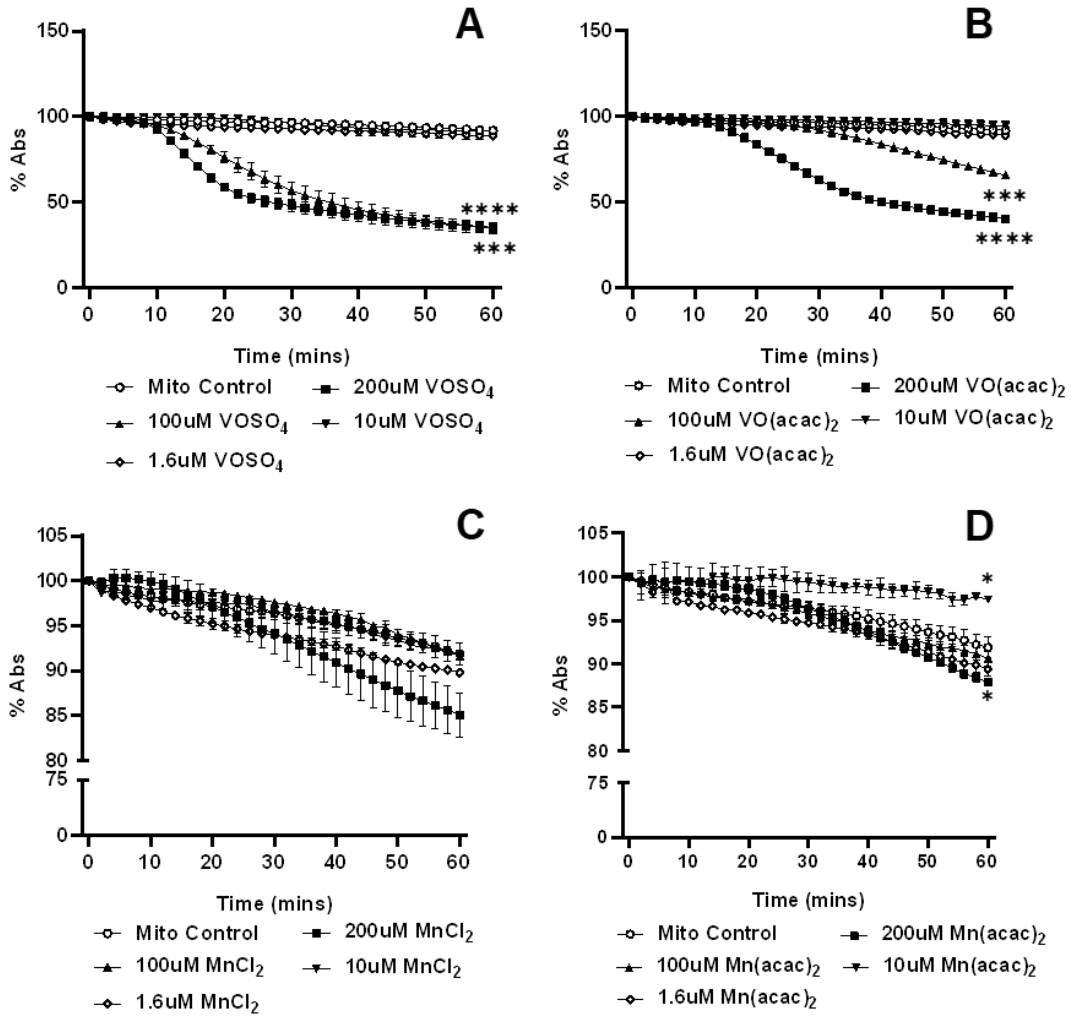

Figure 2.5. Concentration dependent effect of vanadium and manganese complexes on mitochondrial swelling. Mitochondrial swelling was measured through absorbance at 540nm while incubated at 25ºC with vanadium compounds ($VOSO_4$, $VO(acac)_2$) and manganese compounds ($MnCl_2$, $Mn(acac)_2$). Mito control had no metal added. Each experiment was run for 60 minutes with readings every 2 mins and completed with an n=3 per group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$)

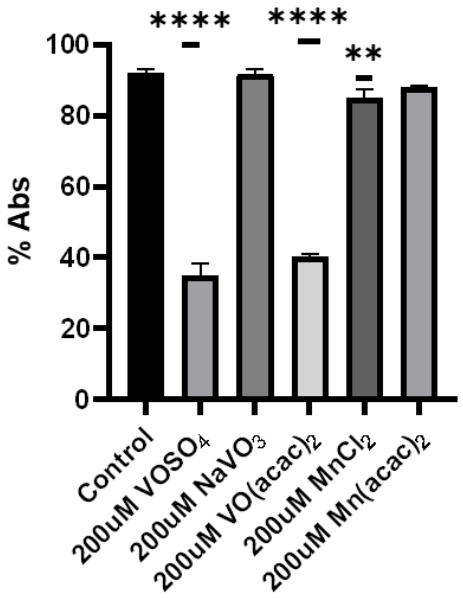

Figure 2.6. Mitochondrial swelling caused by metals after 60 mins. Statistical analysis performed was a 1way ANOVA with Dunnett's Multiple Comparison Test using GraphPad Prism8. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$)

2.3.3 Differential role of mitochondrial pores and channels on metal induced mitochondrial swelling

Entry of metal ions into the mitochondria is primarily accomplished through pores and channels. For calcium ions (Ca^{2+}) several pores and channels exist to facilitate uptake including the mitochondrial calcium uniporter (MCU) and the voltage dependent anionic channel (VDAC). The mitochondrial permeability transition pore (MPTP) is a major way for water to enter the mitochondria and is activated when exposed to considerable amounts of calcium ions [48]. Previous studies suggest that the MPTP plays a significant role in vanadium(IV) induced mitochondrial swelling and influences manganese(II) swelling while the MCU also appears to play a minimal role with manganese [49]. We hypothesized that the differences in metals,

oxidation states, and complexation states may use different pathways to influence mitochondrial swelling. We investigated this through inhibition of several pores and channels. Inhibition of the MCU by DS16570511 completely inhibited mitochondrial swelling seen at 200uM addition of $VOSO_4$ and $VO(acac)_2$ (**Fig. 2.7A, 2.7B**). In contrast both CsA and VBIT-12, used to inhibit the MPTP and VDAC, showed a small, delayed effect on $VOSO_4$ and $VO(acac)_2$ induced mitochondrial swelling (**Fig. 2.7A, 2.7B**). In contrast there was no effect on $NaVO_3$ by DS16570511, VBIT-12 or CsA documenting the different response by vanadium in a different oxidation state (**Fig. S3**). $MnCl_2$ and $Mn(acac)_2$ induced swelling was not affected significantly by the inhibitors (**Fig. 2.7C, 2.7D**).

In Figure 8 the effects of inhibitors on mitochondrial swelling over time was investigated for $VOSO_4$ and $VO(acac)_2$ as well as for the $MnCl_2$ and $Mn(acac)_2$ treated mitochondria compared to the control with no inhibitors. We find that the $MnCl_2$ and $Mn(acac)_2$ showed no effects were observed as a function of time whereas, all the inhibitors showed an effect on slowing swelling seen with $VOSO_4$ and $VO(acac)_2$ after 20 min. The effect on slowing mitochondrial swelling disappeared after 40 mins with only DS16570511 completely stopping mitochondrial swelling (**Fig. 2.8**). These results show a differential role that mitochondrial pores and channels have on swelling related to vanadium treatment.

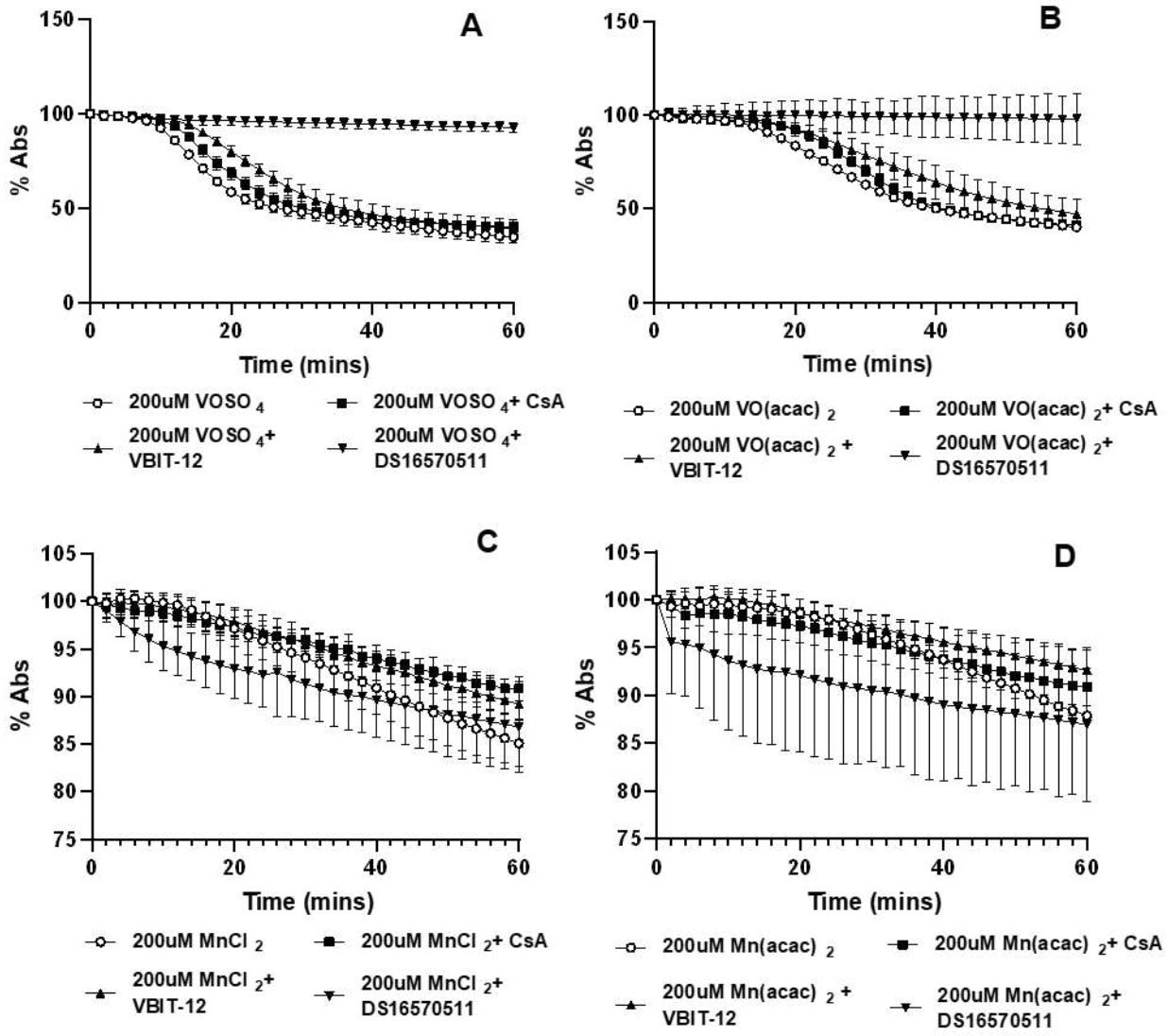

Figure 2.7. Effect of inhibition of mitochondrial pores and channels on swelling induced by treatment with vanadium(IV) and manganese(II) compounds. (A) $VOSO_4$ (B) $VO(acac)_2$ (C) $MnCl_2$ (D) $Mn(acac)_2$ Mitochondrial swelling was measured through absorbance at 540nm incubated at 25ºC. Mitochondrial channel and pore inhibitors (CsA, VBIT-12, DS16570511) were added. The samples were treated with vanadium compounds ($VOSO_4$, $VO(acac)_2$) and manganese compounds ($MnCl_2$, $Mn(acac)_2$). Each experiment was run for 60 minutes with readings every 2 mins and completed with an N = 3-6 per group.

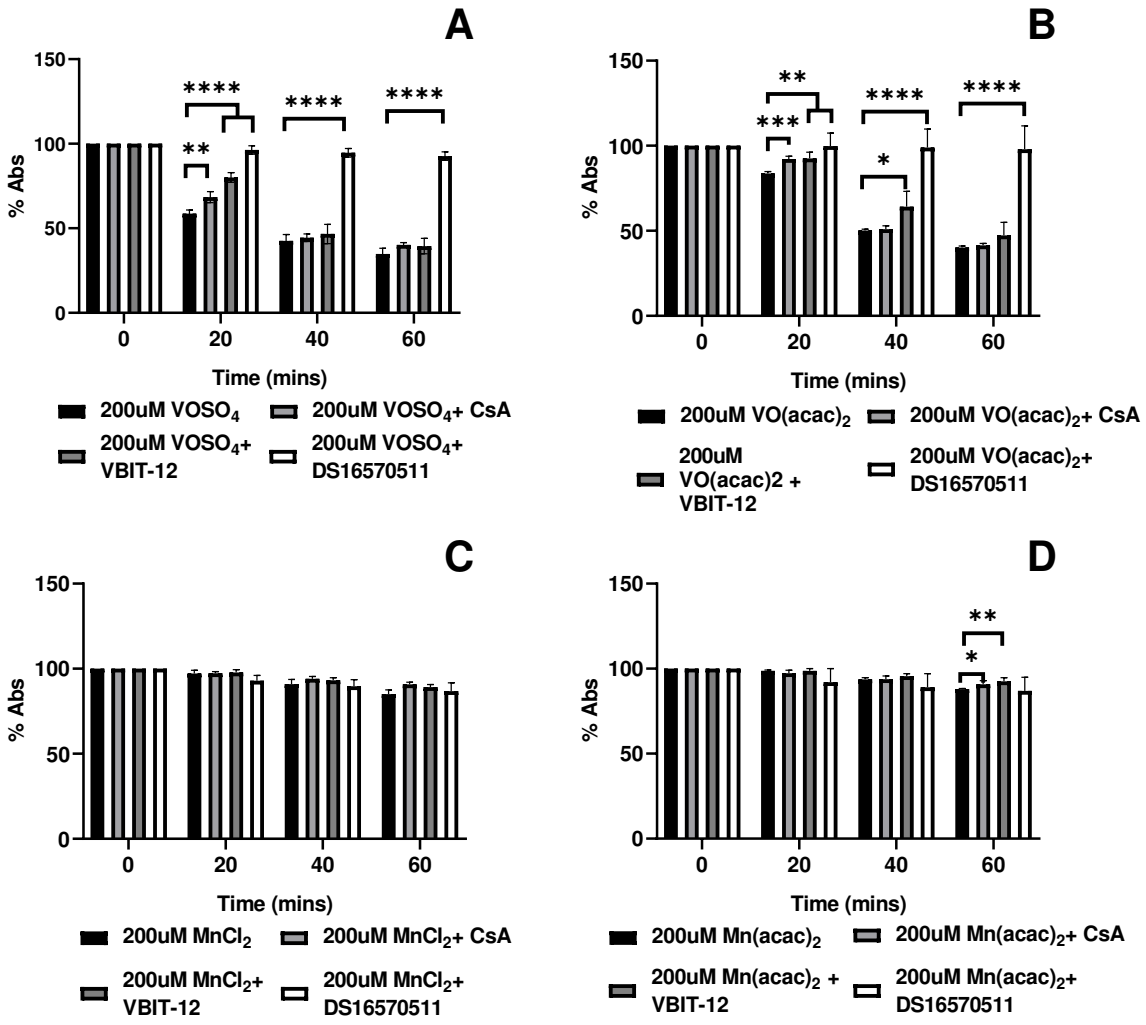

Figure 2.8. Inhibitor effect on metal induced mitochondrial swelling at different time points. Statistical analysis performed was a 2way ANOVA with Dunnet's Multiple Comparison's test using GraphPad Prism8. N = 3 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$)

2.3.4 Speciation differences in vanadium and manganese salts and complexes

In the supplemental material we provide speciation diagrams for the salts and coordination complexes used in this work. These profiles illustrate the diversity of the systems, and the fact that several different species varying in protonation states and composition. These profiles illustrate that both the $VOSO_4$ and $VO(acac)_2$ and the $MnCl_2$ and $Mn(acac)_2$ contain

several species which varies dramatically with pH. Since the studies with the mitochondria are mainly done at pH 7.4 the speciation plots provided here are focusing on the changes as a function of added metal ion concentration in the swelling experiments. The speciation diagrams added here shown in **Figures 2.9A-8D** shows the distribution of species as a function of concentration used in the swelling assays.

For $VOSO_4$ it is shown that two major species are present namely the monomer $VO(OH)_3^-$, and a dimeric species $(VO)_2(OH)_5^-$. Since the dimer contains two vanadium atoms – we show both the concentration of molecule as well as the concentration of V-atoms in the dimeric species $(VO)_2(OH)_5^-$. Based on Figure 9A the species in solution is mainly a dimeric form of V^{IV}. For $VO(acac)_2$ it is shown that three major species are present namely the $VO(acac)_2$, the hydrolyzed monomer $VO(OH)_3^-$, and a dimeric species $(VO)_2(OH)_5^-$. Since the dimer contains two vanadium atoms – we show both the concentration of molecule as well as the concentration of V-atoms in the dimeric species $(VO)_2(OH)_5^-$. Based on these speciation estimations it would be clear that most of the observed effects should be attributed to the hydrolyzed $VO(acac)_2$, and thus the monomeric and dimeric forms of V^{IV}.

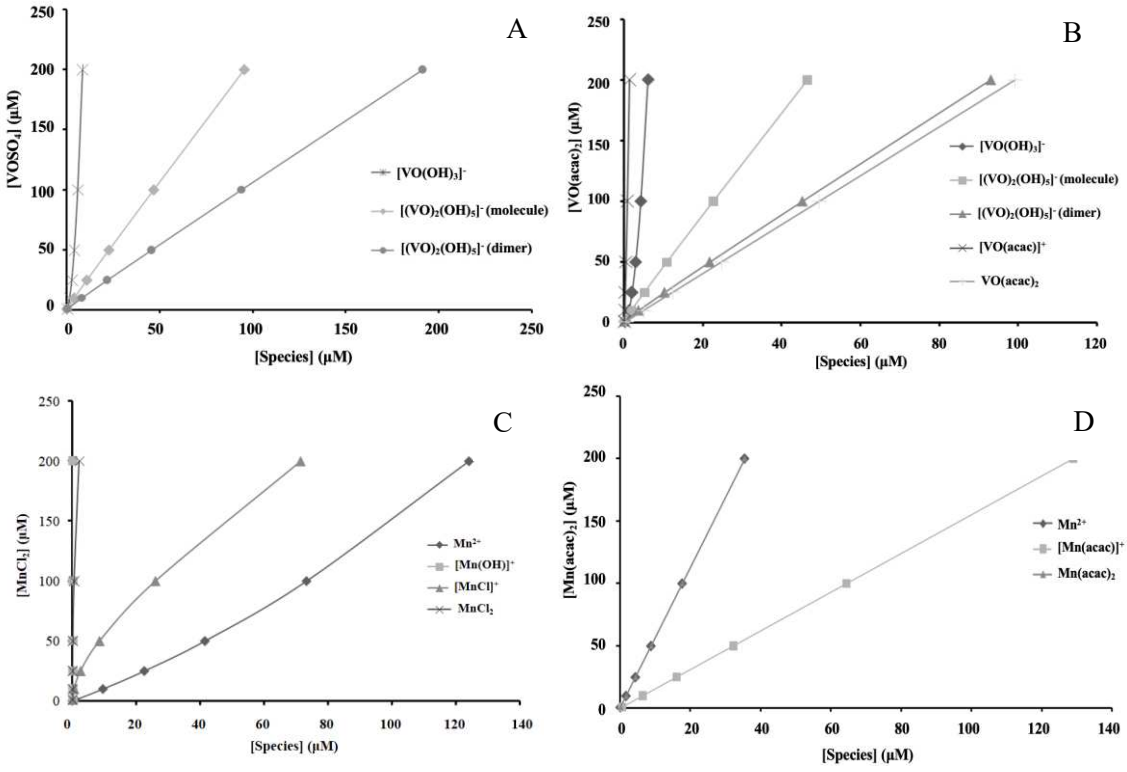

Figure 2.9. Speciation profiles illustrate the concentration of metal ion species as a function of the overall metal ion concentration. (A) $VOSO_4$ (B) $VO(acac)_2$ (C) $MnCl_2$ (D) $Mn(acac)_2$ Diagrams have been constructed based on the most abundant species at physiological pH (pH = 7.4).

For $MnCl_2$ it is shown that two major species and two minor species are present at pH 7.4 in the concentration range up to 200uM. The two major species is the intact Mn^{2+} ion and the partially hydrolyzed species $MnCl^{+}$. The two minor species present are $Mn(Cl)_3^{-}$ and intact $MnCl_2$. Based on Figure 9C the species in solution is mainly the Mn^{2+} ion and the partially hydrolyzed species $MnCl^{+}$. For $Mn(acac)_2$ shown in Figure 8D it is shown that three major species are present namely the $Mn(acac)_2$, the hydrolyzed $Mn(acac)^{+}$, and the Mn^{2+} cation. Based on these speciation estimations it would be clear that most of the species present is the

hydrolyzed $Mn(acac)^{+}$ cation, however the two other ions present are in sufficient amounts that any observed effects should be attributed to the combination of these forms of Mn^{II}.

The speciation estimates have been calculated using the formation constants reported and they were not measured under the same ionic strengths. As reported previously formation constants were reported for a series of different conditions for vanadium(V) speciation [50]. Thus, although the constants would vary, the differences in the speciation concentrations were measurable but not dramatically different. As a result, these speciation calculations provide a qualitive understanding of the speciation that exists at the pH at which the experiments were conducted in this work.

2.3.5 Implication of the speciation differences of the effects of vanadium and manganese salts and complexes

The studies shown here illustrate that there is a profound effect of speciation on the swelling of cardiac mitochondria treated with vanadium and manganese salts and vanadium and manganese complexes. We observe a difference in effects regarding oxidation state, salts, and coordination complexes. However, since they are both first row transition metals, they are likely to have some similar chemistry while also show some differences and this was observed. Although vanadium can redox cycle, different biological effects, where observed for vanadium in oxidation states IV and V [17]. Vanadium V is generally reported as the more toxic species, regarding the swelling of cardiac mitochondrial cells the vanadium IV was more effective [51,52]. Our comparison of the effects of manganese and vanadium in similar transition states was accomplished on cardiac mitochondria virtue of the fact that vanadium forms oxoions and thus oxidation state IV would form VO^{2+}-ion derivatives [41] like the Mn^{2+} ions. The comparison of both salts and complexes in the 4+/2+ transition state allowed us to further investigate if

organic ligands offer the ability to control biological effects through manipulating and altering the metal ion speciation states. This was indeed observed for the $MnCl_2$ and $Mn(acac)_2$ systems on mitochondria respiration. The fact that this was not observed for $VOSO_4$, and $VO(acac)_2$ can be rationalized by the fact that $VO(acac)_2$ converted to mainly species formed for $VOSO_4$ as was demonstrated by speciation calculations at concentration used for the swelling studies.

Both manganese and vanadium are not very prevalent metal ions in the biosphere although manganese is an essential element for human, animals, and plants [3,53,54]. Manganese functions as a cofactor in MnSOD in the conversion of superoxide into H_2O_2. Vanadium, however, is a trace element and only essential for algae, tunicates, fan worms and plants containing vanadium nitrogenase. Each of these metals has two physiologically relevant transition states that can redox cycle between oxidation states generating different biological responses [31,53,54]. A primary location for this redox cycling and biological response is the mitochondria of cells, which earlier studies have shown a multifaceted effect on mitochondrial dysfunction. A primary mechanism of toxicity by these metals reported in the literature are ROS release, changes in mitochondrial membrane potential and effect on proteins in the electron transfer system (ETS) [55–57].

Concentration and state of metals can play a significant role on biological systems, changing from essential to toxic [51]. Considering the roles of the mitochondria as the organ creating energy and the fact. To investigate this, we monitored mitochondrial function and ROS release simultaneously with increasing concentrations of $VOSO_4$, $VO(acac)_2$, $MnCl_2$, and $Mn(acac)_2$. Both salts showed similar effects on inhibiting mitochondrial respiration while $VO(acac)_2$ and $Mn(acac)_2$ showed significantly less inhibition on respiration when compared to the respective metal salts but not compared to each other. Earlier reports have shown that cell

culture with 100uM $MnCl_2$ [59] or 10-100uM Mn(II) acetate [60]will inhibit mitochondrial function over time but acute additions of metals under 15uM $MnCl_2$ have not been previously reported to inhibit mitochondrial function. A major amount of the inhibition occurred under 15uM metal, near physiologically relevant concentrations. A metal dependent difference was the change in ROS released with the addition of metals. Both $MnCl_2$ and $Mn(acac)_2$ showed significant ROS release compared to $VOSO_4$ and $VO(acac)_2$ at high concentrations. This differs than previously reported literature which indicates that vanadium causes ROS dependent damage in cells and mitochondria [28,52]. Previous reports have shown that to inhibit mitochondrial respiration excessive amounts of manganese are needed [61], in contrast to our findings. However, it has been previously reported that manganese induces high amounts of ROS [62] which is consistent with our findings and may be associated with the calcium uniporter [63]. This difference in ROS release was unexpected, especially considering the insignificant difference in inhibition of respiration by the vanadium and manganese salts.

Mitochondrial swelling allows for further investigation into mechanism of metal induced mitochondrial dysfunction. Mitochondrial swelling can be caused by rapid influx of calcium ions into the matrix. Calcium ions can enter mitochondria and cause shifts in osmotic pressure. These shifts can occur to such an extreme that mitochondria can burst, leading to a loss of function. This can also lead to the loss of the membrane potential limiting respiratory capacity of the ETS. This is mediated by the MPTP which controls water flux into and out of the cell. Previous literature reports vanadium, particularly $VOSO_4$ and $VO(acac)_2$, induce significant mitochondrial swelling which can be mediated through inhibition of the MPTP [52]. Little has been done to investigate the impact of species on mitochondria swelling. Inhibition of vanadium and manganese induced swelling by the blocking of ion channels and pores help elucidate potential

mechanisms of mitochondrial swelling by vanadium and manganese complexes. DS16570511 is a novel inhibitor of MCU which can block calcium transport into the mitochondria [64] and VBIT-12 acts as an inhibitor for VDAC [65], neither of which have been tested with vanadium or manganese. Previous literature has used a range of concentrations for these molecules and to ensure max inhibition we used 100uM. DS16570511 completely inhibited vanadium induced swelling while CsA and VBIT-12 delayed swelling without any mediation of maximal swelling. There was no significant effect of these inhibitors on swelling by manganese. These findings show that while having their similarities, the mechanism of vanadium and manganese induced mitochondrial dysfunction are radically different, and this can partially be attributed to speciation.

Speciation differences may account for different effects of vanadium and manganese causing mitochondrial effects. Both metal ions inhibit mitochondrial respiration. It is however particularly noteworthy that the major forms of V-species are anionic, and the major forms of Mn-species are cationic and may be related to the observed low ROS release of ROS and high mitochondrial swelling for the V-species whereas high ROS release and low mitochondrial swelling was observed with manganese. Interestingly, the V(IV) are impacted by the presence of the mitochondrial calcium uniporter, but not sensitive to and only slightly affected by the mitochondrial permeability transition pore. The variation seen shows a metal difference and potential species difference.

Metals, including vanadium and manganese, are known to form toxic byproducts of industrial processes such as fossil fuel extraction and refinement and thus pose a risk to human health [66,67]. In contrast, some forms of vanadium and manganese complexes have been reported to have medicinal applications, with manganese being an essential element [3]. The

present study emphasizes the importance of metal species in biological metabolism. An important aspect for the therapeutic potential of these complexes is stability and the organic ligand which can affect biological impact. If manipulation of the ligand can alter biological effects this could lead toward controllable toxicity and targeted treatments. Further investigation of different vanadium and manganese complexes into their biological effects on cells and mitochondria will be necessary to test the differences in mitochondrial swelling, ROS release, membrane potential and lipid peroxidation damage. Manganese's natural ROS release and inhibition of mitochondrial respiration may make it potentially useful for transition metal-based therapeutics for treatments of some diseases if the effects on cardiac mitochondria are consistent across tissues.

Further investigation into the differences between damage induced by these metals needs to be done using multiple speciation states and complexes. Cell cultures studies tested with these metals could allow for more insight into the mechanistic differences of vanadium and manganese, specifically about state and species, on biological effects. Measuring ROS induced damage, cellular respiration and protein expression would provide more in-depth information of the role that ROS might play and mitochondrial swelling. Earlier reports show that lipid peroxidation damage occurs in response to vanadium and manganese treatment. This would also help to elucidate if the lack of a ROS response was because of vanadium peroxyl complex formation interfering with the Amplex Red/HRP reaction and if ROS damage is occurring and causing mitochondrial dysfunction. This information could prove useful for future therapeutic design by offering a mode of action.

2.4 Conclusion

Vanadium and manganese both are first row transition metals, and both have redox properties and similar reports with biological responses attributed to these metal ions have been made. We investigated the effects of the vanadium(IV), vanadium(V) and manganese(II) salts and the respective metal complexes with acetylacetonate of Vanadium(IV) and manganese(II) and compared the responses on cardiac respiration, mitochondrial respiration, and ROS release. Both vanadium(IV) and manganese(II) showed acute inhibition of mitochondrial respiration at low concentrations that was altered through complexation with the acac anion. Mitochondrial swelling and ROS release on the other hand showed different effects depending on the metal and whether the metal was a salt or in a coordination complex. Specifically, vanadium(IV) showed low ROS release and significant mitochondrial swelling that could be mitigated through inhibition of the MCU. Manganese(II) showed less mitochondrial swelling and ROS release, though this was due to a chemical reaction not an interaction with the mitochondria. These studies do show that there is a difference depending on metal oxidation state and whether the metal ion is present as a salt or a coordination complex.

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APPENDIX I
SUPPORTING INFORMATION

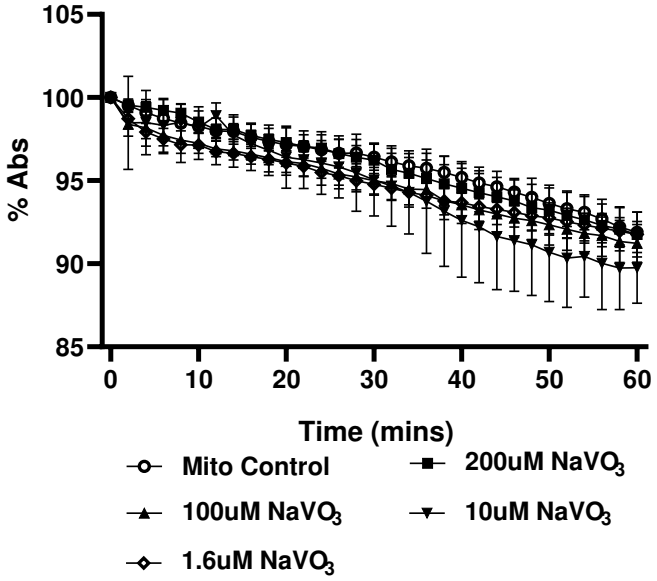

Figure S1. Measurement of mitochondrial swelling caused by $NaVO_3$. Absorbance at 540nm was measured every 2 mins for 60 mins. N of 3.

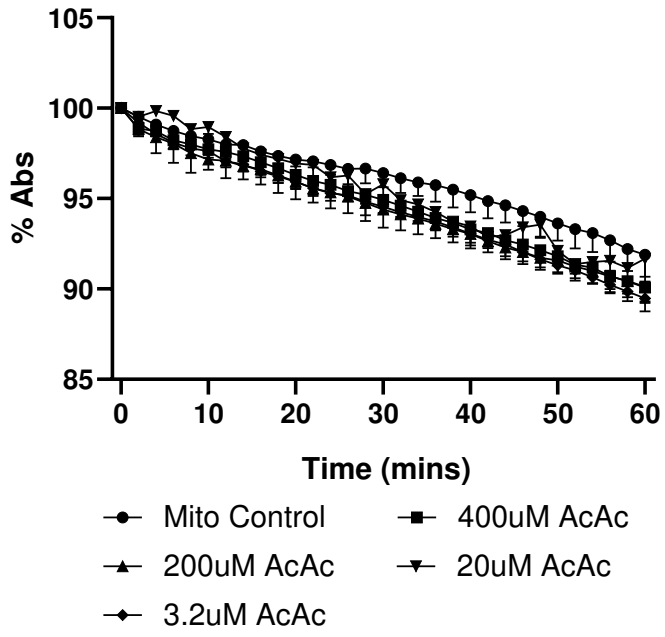

Figure S2. Measurement of mitochondrial swelling caused by AcAc. Absorbance at 540nm was measured every 2 mins for 60 mins. N of 3.

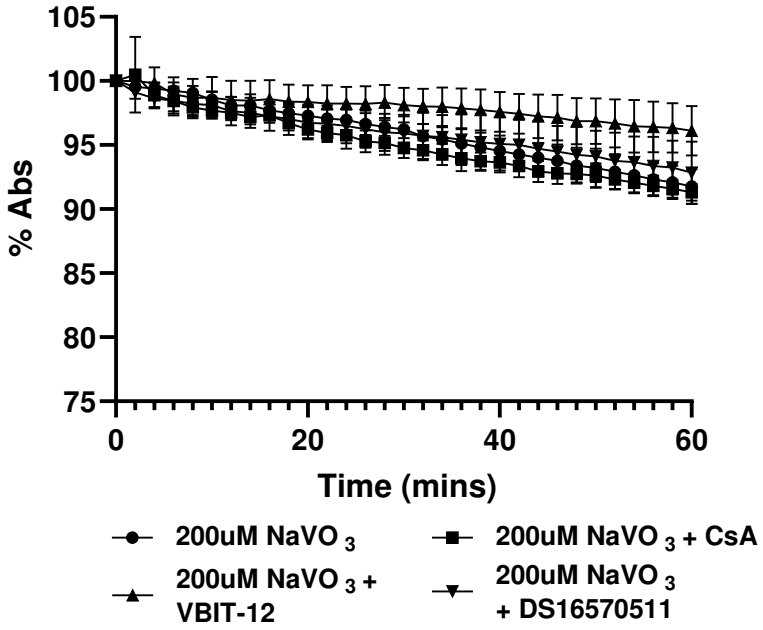

Figure S3. Measurement of mitochondrial swelling caused by $NaVO_3$ with mitochondrial pore and channel inhibitors present. Mitochondrial channel and pore inhibitors (CsA, VBIT-12, DS16570511) were used. Absorbance at 540nm was measured every 2 mins for 60 mins. N of 3-6.

Table S1. Chemical equilibria and formation constants involved in speciation distribution diagrams of $VOSO_4$

Equilibria	Logβ
$H_2O \rightleftharpoons H^+ + OH^-$	-13.77
$VO^{2+} + H_2O \rightleftharpoons [VO(OH)]^+ + H^+$	-5.94[1]
$2VO^{2+} + 2H_2O \rightleftharpoons [(VO)_2(OH)_2]^{2+} + 2H^+$	-5.94[1]
$VO^{2+} + 3H_2O \rightleftharpoons [VO(OH)_3]^- + 3H^+$	-18.0[1]
$2VO^{2+} + 5H_2O \rightleftharpoons [(VO)_2(OH)_5]^- + 5H^+$	-22.5[1]
$VO(OH)_{2(s)} \rightleftharpoons VO^{2+} + 2OH^-$	$K_{sp} = 6.6 \times 10^{-23}$ M^3
$VO^{2+} + SO_4^{2-} + H^+ \rightleftharpoons [VO(HSO_4)]^+$	1.74[2]
$VO^{2+} + SO_4^{2-} \rightleftharpoons VOSO_4$	2.51[2]

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Table S2. Chemical equilibria and formation constants involved in speciation distribution diagrams of $VO(acac)_2$

Equilibria	Logβ
$H_2O \rightleftharpoons H^+ + OH^-$	-13.77
$VO^{2+} + H_2O \rightleftharpoons [VO(OH)]^+ + H^+$	-5.94[1]
$2VO^{2+} + 2H_2O \rightleftharpoons [(VO)_2(OH)_2]^{2+} + 2H^+$	-5.94[1]
$VO^{2+} + 3H_2O \rightleftharpoons [VO(OH)_3]^- + 3H^+$	-18.0[1]
$2VO^{2+} + 5H_2O \rightleftharpoons [(VO)_2(OH)_5]^- + 5H^+$	-22.5[1]
$VO(OH)_{2(s)} \rightleftharpoons VO^{2+} + 2OH^-$	$K_{sp} = 6.6 \times 10^{-23}$ M^3
$VO^{2+} + acac^- \rightleftharpoons [VO(acac)]^+$	17.67[2]
$VO^{2+} + 2acac^- \rightleftharpoons VO(acac)_2$	33.62[2]

[1] Crans, D.C., Smee, J.J., Gaidamauskas, E. and Yang, L. (2004). The Chemistry and Biochemistry of Vanadium and the Biological Activities Exerted by Vanadium Compounds. Chemical Reviews, 104(2),pp.849–902. https://doi.org/10.1021/cr020607t
[2] Martell, A.E. and Smith, R.M. (1977). Other Organic Ligands. Boston, MA: Springer US. https://doi.org/10.1007/978-1-4757-1568-2

Table S3. Chemical equilibria and formation constants involved in speciation distribution diagrams of $Mn(acac)_2$

Equilibria	Logβ
$H_2O \rightleftharpoons H^+ + OH^-$	-13.77[1]
$Mn^{2+} + H_2O \rightleftharpoons [MnOH]^+ + H^+$	-10.58[1]
$Mn^{2+} + 2\ H_2O \rightleftharpoons Mn(OH)_2 + 2\ H^+$	-22.18[1]
$Mn^{2+} + 3\ H_2O \rightleftharpoons [Mn(OH)_3]^- + 3\ H^+$	-34.34[1]
$Mn^{2+} + 4\ H_2O \rightleftharpoons [Mn(OH)_4]^{2-} + 4\ H^+$	-48.28[1]
$Mn(OH)_{2(s)} + 2\ H^+ \rightleftharpoons Mn^{2+} + 2\ H_2O$	15.19[1]
$MnO_{(s)} + 2\ H^+ \rightleftharpoons Mn^{2+} + H_2O$	17.94[1]
$Mn^{2+} + acac^- \rightleftharpoons [Mn(acac)]^+$	13.20[2]
$Mn^{2+} + 2\ acac^- \rightleftharpoons Mn(acac)_2$	25.28[2]

[1] P.L. Brown and C. Ekberg, Hydrolysis of Metal Ions. Wiley, 2016, pp. 557−561.

[2] Martell, A.E. and Smith, R. (1982). Critical Stability Constants. Springer eBooks. Springer Nature. https://doi.org/10.1007/978-1-4615-6761-5

Table S4. Chemical equilibria and formation constants involved in speciation distribution diagrams of $MnCl_2$

Equilibria	Logβ
$H_2O \rightleftharpoons H^+ + OH^-$	-13.77[1]
$Mn^{2+} + H_2O \rightleftharpoons [MnOH]^+ + H^+$	-10.58[1]
$Mn^{2+} + 2\ H_2O \rightleftharpoons Mn(OH)_2 + 2\ H^+$	-22.18[1]
$Mn^{2+} + 3\ H_2O \rightleftharpoons [Mn(OH)_3]^- + 3\ H^+$	-34.34[1]
$Mn^{2+} + 4\ H_2O \rightleftharpoons [Mn(OH)_4]^{2-} + 4\ H^+$	-48.28[1]
$Mn(OH)_{2(s)} + 2\ H^+ \rightleftharpoons Mn^{2+} + 2\ H_2O$	15.19[1]
$MnO_{(s)} + 2\ H^+ \rightleftharpoons Mn^{2+} + H_2O$	17.94[1]
$Mn^{2+} + Cl^- \rightleftharpoons [MnCl]^+$	3.69[2]
$Mn^{2+} + 2Cl^- \rightleftharpoons MnCl_2$	6.09[2]
$Mn^{2+} + 3Cl^- \rightleftharpoons [MnCl_3]^-$	10.02[2]
$Mn^{2+} + 4Cl^- \rightleftharpoons [MnCl_4]^{2-}$	12.63[2]

[1] P.L. Brown and C. Ekberg, Hydrolysis of Metal Ions. Wiley, 2016, pp. 557−561.

[2] Hitoshi Ohtaki (1988). Calorimetric and spectrophotometric studies of chloro complexes of manganese(II) and cobalt(II) ions in N,N-dimethylformamide. Journal of the Chemical Society, 84(7), pp.2409–2409. https://doi.org/10.1039/f19888402409

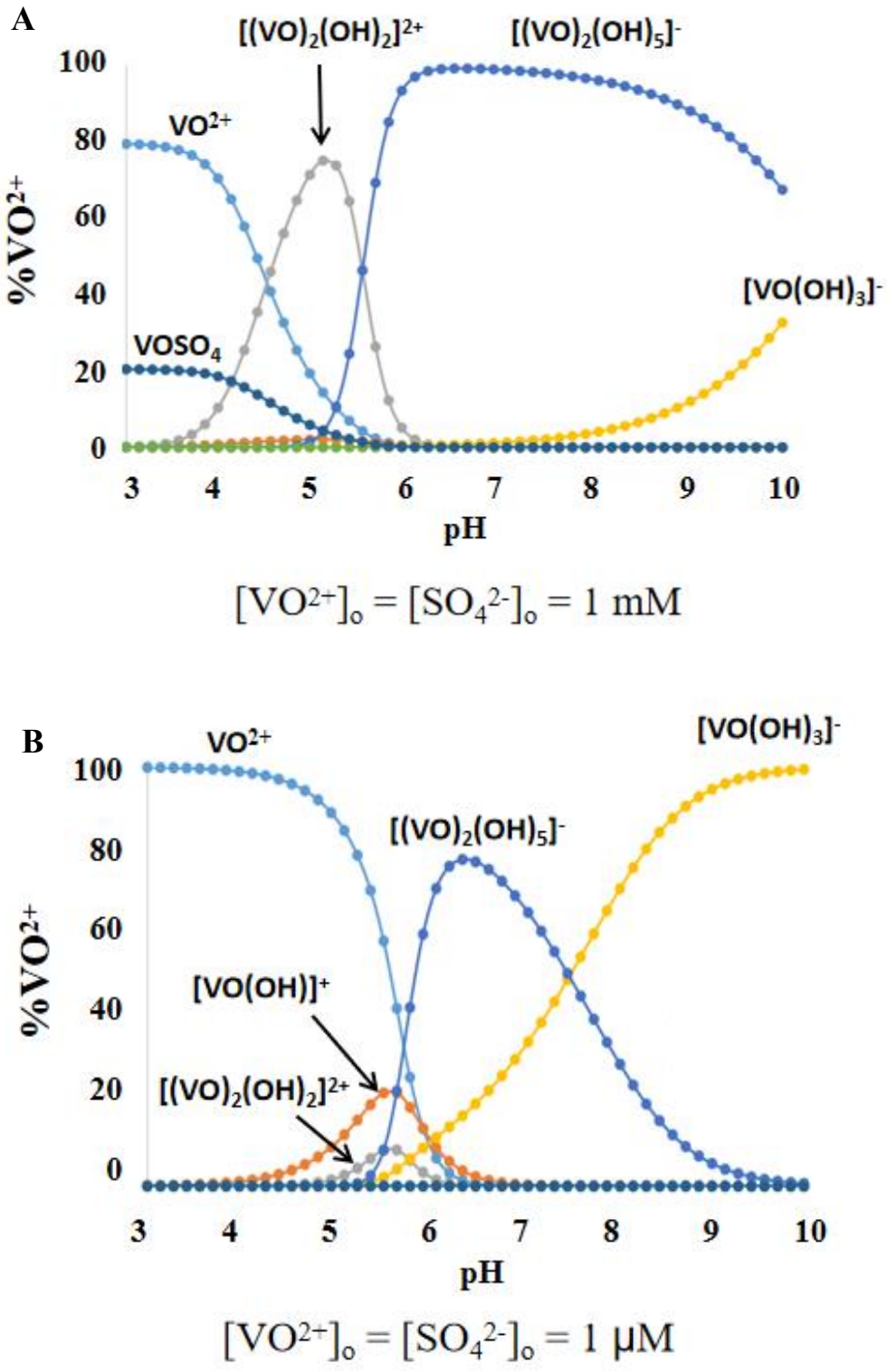

Figure S4. Speciation distribution diagrams for $VOSO_4$ system. (A) 1mM $VOSO_4$ (B) 1uM $VOSO_4$)

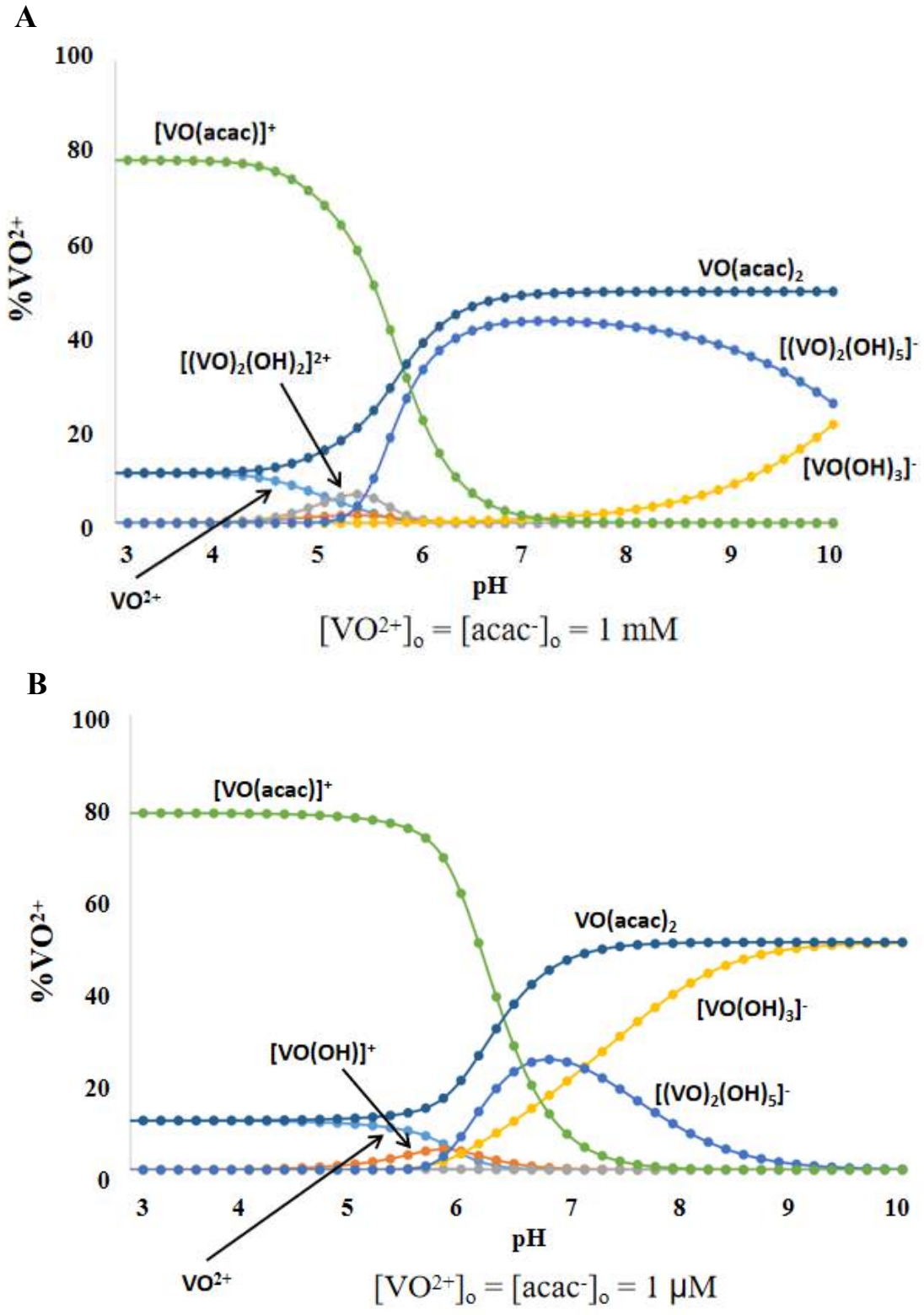

Figure S5. Speciation distribution diagrams for $VO(acac)_2$ System. (A) 1mM $VO(acac)_2$ (B) 1uM $VO(acac)_2$

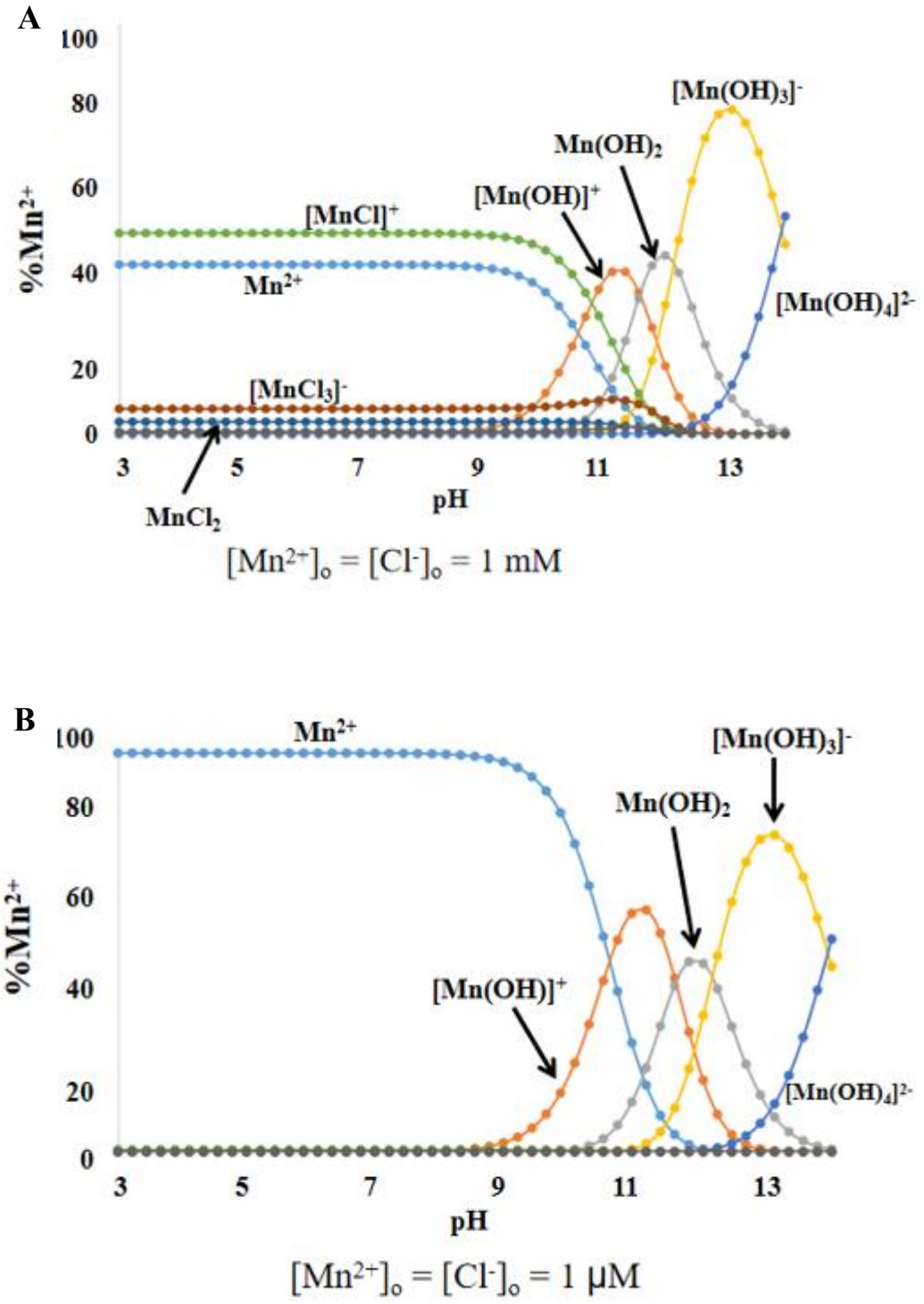

Figure S6. Speciation distribution diagrams for $MnCl_2$ System. (A) 1mM $MnCl_2$ (B) 1uM $MnCl_2$

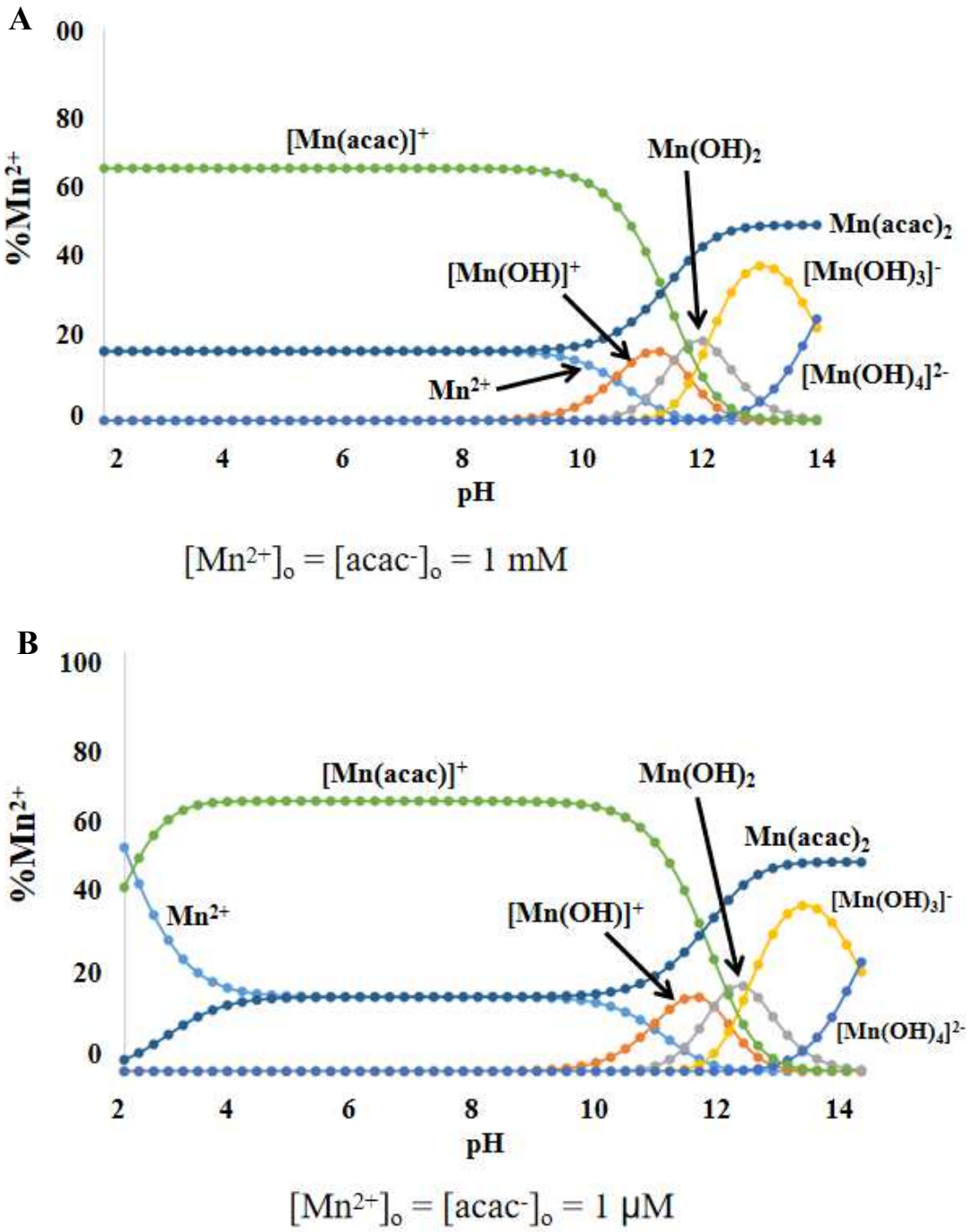

Figure S7. Speciation distribution diagrams for $Mn(acac)_2$ System. (A) 1mM $Mn(acac)_2$ (B) 1uM $Mn(acac)_2$

ABBREVIATIONS

AA	Ascorbic Acid
AcAc	Acetylacetonate
ADP	Adenosine diphosphate
AOX	Antioxidant
ATP	Adenosine triphosphate
BEOV	Bis(ethylmaltolato) oxidovanadium(IV)
Ca^{2+}	Calcium
Caco-2	Colorectal adenocarcinoma cells
CAL-33	Centre Antoine Lacassagne-33
CAT	Catalase
CDDP	Cisplatin
CP	Cyclophosphamide
CsA	Cyclosporin A
Cu	Copper
CYP	Cytochrome P450
Cyt	Cytochrome
DNA	Deoxyribonucleic acid
DS16570511	(3-[3-[[[3-(2,6-dichlorophenyl)-5-methyl-4-isoxazoloyl]methyl]amino] benzoyl]-1H-indole-1-butanoic acid
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinase
ETS	Electron transfer system
FA	Fatty acids
Fe	Iron
GluVO	Glucose vanadate
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione S-transferase
Hacac	Acetylacetic acid
HeLa	Henrietta Lacks cell line
HRP	Horseradish peroxidase
HOO·	Hydroperoxyl radical
HO·	Hydroxyl radical
H_2O_2	Hydrogen peroxide
HPLC	High-performance liquid chromatography
i.p.	Intraperitoneal
i.v.	Intravenous
K^+	Potassium
LOOH	Lipid hydroperoxide
LOO·	Lipid peroxyl radical
L·	Lipid pentadienyl radical

LPO	Lipid peroxidation
MAPK	Mitogen-activated protein kinase
MBOV	Macrocyclic binuclear oxovanadium complex
MCU	Mitochondrial calcium uniporter
MEK	Mitogen-activated protein kinase ERK kinase
MDA	Malondialdehyde
MMTP	Membrane transition pore inhibitor
Mn	Manganese
$MnCl_2$	Manganese(II) chloride
Mn(acac)2	Manganese(II) acetylacetonate
MnSOD	Manganese superoxide dismutase
MPTP	Mitochondrial permeability transition pore
MV_{10}	Metaformin-decavanadate
Na2+	Sodium cation
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NapVO	Naproxen vanadate
$NaVO_3$	Sodium metavanadate
NF-kB	Nuclear factor kappa B
NH_4VO_3	Ammonium metavanadate
NH_4^+	Ammonium
NMR	Nuclear magnetic resonance
NO·	Nitric oxide
$O_2\cdot^-$	Superoxide radical
O_2	Molecular oxygen
Ox	Oxidizers
PβPL	Poly(β-propiolactone)
POMs	Polyoxometalate
PUFA	Polyunsaturates fatty acid
RAF	Rapids accelerated fibrosarcoma
Ras	Rat sarcoma
RCR	Respiratory control ratio
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SOV	Sodium orthovanadate
STZ	Streptozotocin
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactivity
TSP	Trigonella graecum seed powder
UV	Ultraviolet
V	Vanadium
V^{IV}	Vanadyl
V^{V}	Vanadate
V_1	Monomeric vanadate
V_{10}	Decavanadate

VBIT-12	N-[[1-(1-naphthalenylmethyl)-4-(phenylamino)-4-piperidinyl]carbonyl]-glycine
VC-111	Vanadium(III)-L-cysteine complex
VC-IV	Oxovanadium(IV)-L-cysteine methyl ester
VDAC	Voltage dependent anionic channel
$VO(acac)_2$	Vanadyl acetylacetonate
VO(citrate)	Vanadyl citrate
VOdipic	Vanadyl dipicolinate
VOL	N(1)-2,4-dihydroxybenzylidene-N(4)-2-hydroxybenzylidene-S-methyl-thiosemicarbazidato-oxovanadium (IV)
$VOSO_4$	Vanadyl sulfate
2-AAF	2-Acetylaminofluorene
4-HNE	4-Hydroxy-2-nonenal
4HDA	4-Hydroxyalkenals