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Chapter 18

Biological Effects of Decavanadate: Muscle Contraction, In Vivo Oxidative Stress, and Mitochondrial Toxicity

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Decameric vanadate species (V10) can be formed at physiological pH values in vanadate solutions presumably containing only monomeric vanadate species (V1). Sarcoplasmic reticulum Ca^{2+}-ATPase and myosin are known to interact with decameric vanadate species. V10 interaction with myosin is favored by conformational changes that take place in myosin during the catalytic cycle. Apparently, V10 operates at a different protein state in comparison with monomeric vanadate (V1) that mimics the protein at the hydrolysis transition state. V10 also clearly differs from V1, by inhibiting sarcoplasmic reticulum calcium accumulation in non-damage native vesicles, besides affecting calcium efflux associated with ATP synthesis and proton ejection associated with ATP hydrolysis. Recently reported studies referred that V10 is stabilized by actin during the process of the protein polymerization since the decomposition half-life time

increases from 5 to 27 hours, suggesting that the interaction is also supported by a protein conformation induced during ATP hydrolysis followed by the formation of protein filaments. Besides affecting muscle contraction and its regulation, V10, as low as 100 nM, inhibits 50% of oxygen consumption in mitochondria, pointing that this organelle is a potential cellular target for V10, while a 100-fold higher concentration of V1 (10 μM) is needed to induce the same effect. Furthermore, *in vivo* studies have shown that following an acute exposure, decavanadate induced different changes, when compared with vanadate, on oxidative stress markers, vanadium intracellular accumulation as well as in lipid peroxidation. Putting it all together, it is suggested that the biological effects of decameric vanadate species contribute, at least in part, to the understanding of the versatility of vanadium biochemistry.

Vanadium and skeletal muscle are strongly connected to each other since almost thirty years ago when vanadium was found in commercial ATP obtained from horse skeletal muscle. Vanadium is currently used as inhibitor of E1-E2 ion transport ATPases, e.g. the sarcoplasmic reticulum (SR) Ca^{2+}- ATPase, besides being considered as a tool for the comprehension of several biochemical processes. However, the complex chemistry of vanadium difficult the interpretation of the effects promoted in biological systems and concomitantly the understanding of the role of this element in life sciences (*1*). This is not surprising if we consider that among the chemical properties of vanadium, besides the multiple oxidation states, is the capacity of vanadium(V) species to condense, forming vanadate oligomers, and with many compounds with biological interest. Certainly , serendipity combined with increasing interest from young researchers worldwide allowed to recognize motivating aims for this versatile element. This review focuses the biological effects of decavanadate. We apologize to include mainly references from our research group of "BioVanadium". In this sense, we now transmit some studies, data, ideas and future perspectives performed by our group regarding the biological effects of a vanadate oligomer, decavanadate, on: (i) contractile system and SR calcium pump, (ii) mitochondria and iii) vanadium accumulation, oxidative stress markers and lipid peroxidation following *in vivo* administration.

Formation of Decavanadate in Vanadate Solutions

Different vanadate species can occur simultaneously in vanadium(V) solutions, e.g. monomeric (V1), dimeric (V2), tetrameric (V4) and pentameric

(V5) in some cases, with different states of protonation and structures, depending on several factors such as vanadate concentration, pH and ionic strength (*2*). Besides these vanadate species, decameric vanadate can also occur in solution, in particular at acidic pH values. To our knowledge, it is not possible to prevent the formation of decameric vanadate upon acidification of vanadate solutions. In fact, many researchers working in different areas ranging from life sciences to chemical engineering, using vanadate in their studies, frequently observed the orange/yellow color in their solutions. This is due to the occurrence of decameric vanadate, for instance in a cell culture, after an acidification procedure during protein purification, after the adjustment of the pH value of the reaction medium or even in methods for vanadate quantification. Thus, if after the preparation of a vanadate solution (10 mM) an acidification occurs, it is possible to observe instantaneously the appearance of a yellow colour due to the occurrence of decameric vanadate species, even if the global pH value of the solution does not changes significantly. The formation of decameric species can be easily confirmed by NMR analysis. After a small pH acidification, from 6.8 to 6.6, of a metavanadate solution containing V1, V2, V4 and V5 vanadate species (Figure 1A), as evaluated by NMR spectroscopy, it is possible to observe NMR signals ascribed to decavanadate species (Figure 1B). Further acidification with HCl up to pH near 4, it is obtained the nominated decavanadate solution containing mainly decameric vanadate species (Figure 1C). Therefore, even at physiological pH values an eventual local acidification of a vanadate solution will induce the formation of V10 species. Once formed, decameric vanadate disintegration is in general slow enough to allow the study of its effects even in the micromolar range. Besides, it may become inaccessible to decomposition due to their stabilization upon binding to target proteins (*3*). In fact, we recently verified that in the presence of sarcoplasmic reticulum vesicles or actin, the half-life time of decameric vanadate as low as 10 μM decameric species (100 μM total vanadate), increases from 5 hours to 17 or 27 hours, respectively, at room temperature and pH 7.0, as appraised by UV/vis at 400 nm. The disintegration of decameric vanadate, follows first order kinetics, independent of concentration, and is prevented by some proteins known to interact with V10 (*3*).

Muscle Contraction

In our laboratory there is a long tradition in the study of the effects of decavanadate on molecular mechanisms involved in muscle contraction, since original findings describing that decameric species affect the myosin and actomyosin ATPase activity were reported since 1987 (*4-8*). Nevertheless, and almost twenty years after, the oligomeric vanadate species that can be present in vanadate solutions are often not accounted for consideration in most biological

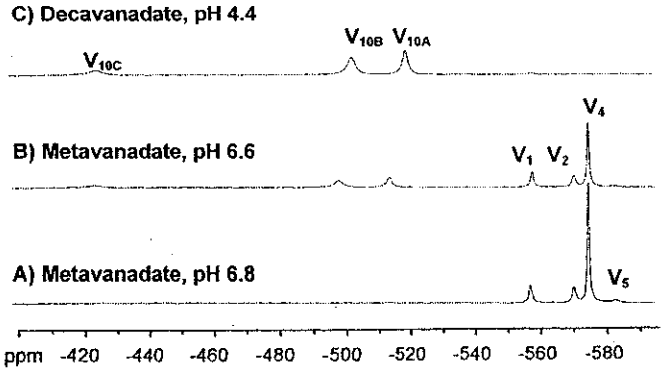

Figure 1. 105.2 MHz ^{51}V NMR spectra, at room temperature, of 10 mM metavanadate solution, pH 6.8, before (A) and after addition of HCl up to pH 6.6 (B) and to pH 4.4 (C). Decavanadate solutions correspond to spectra C. V1, monomeric; V2, dimeric; V4, tetrameric; V5, tetrameric and V10, decameric vanadate.

studies, although it is recognized that the individual species may influence enzyme activities differentially (*9*). More recently, it was suggested that the presence of vanadate oligomers, such as tetrameric ($V_4O_{12}^{4-}$) or decameric ($V_{10}O_{28}^{6-}$) species, prevent the stimulation of myosin ATPase activity by actin suggesting a different reactivity due, eventually, to different inhibition mechanisms in comparison to monomeric vanadate (*10-12*). In fact, it was shown that the ATPase activity of the actomyosin complex is inhibited (Ki = 0.27 ± 0.05 μM) by decameric but not by the monomeric form of vanadate (*13*). The results were consistent with binding of decavanadate to the conserved regions of the myosin phosphate binding-loop, adjacent to the ATP binding site, as it was subsequently showed by means of protein photocleavage studies in the presence of decavanadate (*14*). This V10 high-affinity binding site produces non-competitive inhibition of the actin-stimulated myosin ATPase activity, without causing dissociation of the ATP-free rigor actomyosin complex. Moreover, the affinity of myosin for V10 is modulated by the conformational changes that take place in myosin during the catalytic cycle, as indicated by the two to three-fold increase of the dissociation constant produced in the presence of ATP analogues such as ADP·Vi and ADP·AlF_4, which induce a conformational state close to the metastable myosin.ADP·P_i intermediate state generated during the contractile cycle (*13*). Apparently, in the mechanism of myosin ATP hydrolysis inhibition, V10 blocks the protein at a state different from the one described for monomeric vanadate. Using an uncomplicated model for the myosin ATP hydrolysis cycle, it is suggested that V10 interaction is favour by a myosin step prior to energy

transduction input, probably in the pre-hydrolysis state or upon a "back-stroke" of the myosin head, but at several myosin conformational states before ATP hydrolysis (Figure 2). Putting it all together, it is suggested that decameric vanadate species would populate myosin states with different properties in comparison to vanadate; therefore it can be used as a tool for the understanding of muscle contraction processes.

Actin Cytoskeleton

Actin is one of the two major proteins in muscle. Monomeric actin, G-actin, by itself does not stimulate myosin ATPase activity. G-actin polymerizes to F-actin, the only form known to have biological function. In muscle cells, the polymerization process is very important to maintain the thin filaments required for contraction. F-actin is the major component of muscle thin filaments and also of the microfilaments of the multifunctional cytoskeletal systems of nonmuscle cells. Besides being responsible for muscle contraction, along with myosin, F-actin filaments play very important roles in all eukaryotic cells such as locomotion, cytokinesis, structural functions and phagocytosis. In non-muscle cells, polymerization/depolymerization equilibrium regulates the process of actin filaments formation needed for specific functions such as the acrossomal process of sperm, besides others structural functions. For all eukaryotic cells if this property of actin is affected then several physiological processes would be blocked.

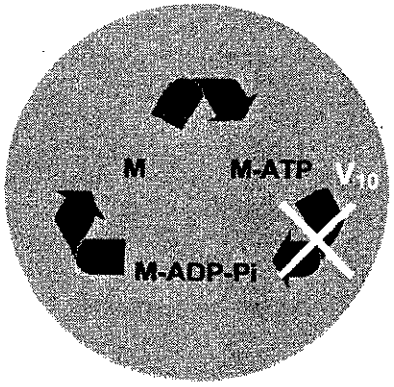

Figure 2. Scheme of the proposed decavanadate interaction with myosin during the mechanism of ATP hydrolysis. M, myosin; V10, decameric vanadate species.

It has been described that vanadate stabilizes F-actin filaments through the formation of F-actin-ADP·V1 complexes and it induces actin polymerization by inhibiting specific tyrosine phosphatases. However, the putative effects of other vanadate oligomers in actin structure and function were inexistent. Using actin from rabbit skeletal muscle we report the effects of a decavanadate solution on the capacity of actin to polymerize. Our results suggest that decameric vanadate interactions with actin inhibit G-actin polymerization and stabilize decameric vanadate species. In fact, decameric vanadate species inhibit the rate and the extent of G-actin polymerization with an IC_{50} of 68 ± 22 μM and 17 ± 2 μM, respectively, whilst they induce F-actin depolymerization at a lower extent (*3*). On contrary, no effect on actin polymerization and depolymerization was detected for 2 mM concentration of metavanadate solution that contains monomeric and metavanadate species, as observed by combining kinetic with ^{51}V-NMR spectroscopy studies. In those studies, it was also described that, at 25°C, decameric vanadate (10 μM) half-life time increases 5-fold (from 5 to 27 h) in the presence of G-actin only at experimental conditions favoring protein polymerization (Figure 3), whereas no effects were observed in the presence of phosphatidylcholine liposomes, myosin or G-actin alone. It was proposed that the decavanadate interaction with G-actin, favored by the G-actin polymerization processes, stabilizes decameric vanadate species and induces inhibition of G-actin polymerization (*3*).

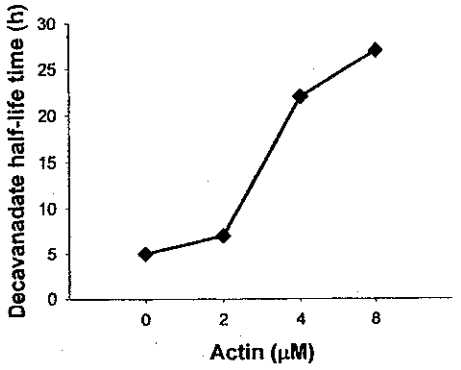

Figure 3. Effect of actin on the decameric vanadate half-life time. Decavanadate concentration of 0.1 mM (10 μM decameric species) was used in the studies (n=3).

Sarcoplasmic Reticulum Calcium ATPase

Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase is a transmembrane transport system, which accumulates Ca^{2+} at expense of ATP splitting during the process

of muscle relaxation. Therefore, there is an important physiological relationship between the contractile system constituted also by the proteins myosin and actin described above and the SR in the process of muscle contraction/relaxation. In our country there is a tradition in the study of the molecular mechanisms of the Ca^{2+} translocation by the SR calcium pump. More recently, significant additional data were obtained on the interaction of species present in vanadate solutions with ionic pumps (*15-17*). In these studies, it was shown that in non damage native vesicles, for instance, without the presence of calcium ionophores, decameric vanadate clearly differs from other oligomeric species in inhibiting Ca^{2+} uptake by SR coupled to ATP hydrolysis, Ca^{2+} efflux coupled with ATPase reversed activity (ATP synthesis) and H^+ ejection promoted by the SR ATPase (*9, 15-16*).

In native vesicles, the measurements of Ca^{2+} accumulation by the SR calcium pump reflect simultaneously the uptake of Ca^{2+} through the pump and the Ca^{2+} efflux (Figure 4). At this condition, where a gradient of calcium modulated the calcium pump activity, only decameric vanadate (V10) inhibits the calcium pump (Figure 4A, coupled uptake). The Ca^{2+} efflux could be passive when not associated with the pump activity or active when directly associated with intrinsic reactions to the SR Ca^{2+} pump mechanisms. In a passive efflux of calcium the ATPase works as a Ca^{2+} channel and vanadate might behave as natural ligands of the enzyme. For an active efflux of calcium, coupled to ATP synthesis, it was demonstrated that in SR vesicles loaded with radioactive Ca^{2+}, sediment by centrifugation and diluted into media containing EGTA, ADP and phosphate, Ca^{2+} active efflux from the vesicles was strongly depressed (85%) by 40 μM decameric species while milimolar concentrations of V1 has no effect (Figure 4B, right side, active efflux). In another different experimental condition, it was observed that when the gradient of calcium is destroy, meaning using phosphate or oxalate to reduce the calcium concentration inside the vesicles to almost zero, and see only accumulation, the calcium ATPase is inhibited by both V10 and V1 solutions (Figure 4C, left side).

When the vesicles are damaged, for example by the presence of a calcium ionophore, the ATP hydrolysis is not associated with calcium translocation, the calcium leaks, and in this condition the ATPase activity is also inhibited by both vanadate solutions (*9,15,18*). Therefore, "decavanadate" exert noticeable effects, on comparison to metavanadate, on "physiological" calcium accumulation, e.g. coupled with ATP hydrolysis besides on the efflux of Ca^{2+} in particular when coupled with ATP synthesis (Figure 4).

Vanadium ions and complexes are known to affect the activity of various enzymes. Sarcoplasmic reticulum E1-E2 Ca^{2+}-ATPase (SR calcium pump) is one of the proteins known to interact with vanadate (Figure 5). As a transmembranar transport system, it accumulates Ca^{2+} at expense of ATP during the process of muscle relaxation. The catalytic mechanism includes a covalent phosphorylated enzyme intermediate, which is formed by transfer of the ATP terminal

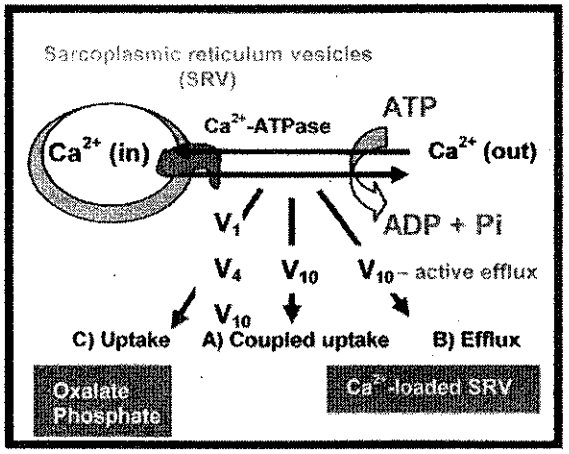

Figure 4. Schematic representation of calcium translocation by sarcoplasmic reticulum at 3 different experimental conditions: (A) coupled uptake (centre), (B) active efflux (right side) and (C) uncoupled accumulation (left side) as affected by different vanadate oligomers.

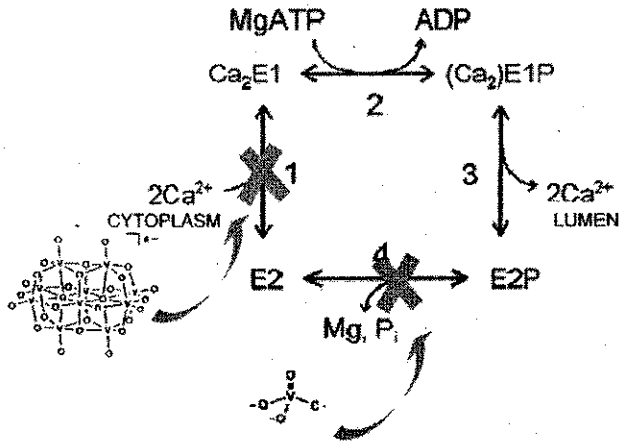

Figure 5. Effects of decavanadate on the mechanism of calcium translocation by the SR calcium ATPase. V10 block calcium efflux and calcium accumulation associate with ATP hydrolysis whereas monomeric vanadate interaction is favoured by E2 conformation.

phosphate to an aspartyl residue at the catalytic site. Several kinetic studies have suggested that, in the absence of ATP, orthovanadate can bind to the SR-ATPase and form a transition state analogue of the phosphorylated intermediate blocking the E2 conformation of the protein (Figure 5). In addition to monovanadate (V1), it has been reported that other vanadate oligomers, such as decameric vanadate, interact with the SR calcium pump (*9*) at distinct sites from the phosphorylation site. Some of these interactions, e.g. decavanadate (V10), as described above strongly inhibit calcium accumulation. Besides, decameric vanadate also stabilized the protein conformation at conformation E2, although others conformation may be also favourable, as described by ^{51}V- NMR spectroscopy (*9*). Being clearly different in inhibiting the SR calcium pump it is proposed a different mode of action for decavanadate in the mechanism of calcium accumulation associated with ATP hydrolysis (Figure 5).

In vivo Studies Following Decavanadate Administration

Several biological studies associate vanadium with the ability to produce reactive oxygen species (ROS), resulting in antioxidant enzymes alterations and leading to lipid peroxidation. In order to explore the hypothesis that the vanadate effects on antioxidante stress markers and on cellular responses are dependent on the oligomeric species, several kinetic studies following decavanadate administration were performed combining NMR and UV/vis spectroscopy analysis. To our knowledge, only our group has performed *in vivo* administration of decavanadate in order to understand the contribution of V10 to the toxic effects of vanadate (*19-25*). In these studies, a metavanadate solution not containing V10 was also administered as a comparison group, besides a placebo group. Until now, following *in vivo* administration of V10, several parameters were analysed such us: subcellular vanadium distribution; lipid peroxidation; antioxidants enzymes activities besides several oxidative stress markers. Among the different experimental conditions described, it was included different: mode of V10 administration (intraperitoneal, i.p. versus intravenous, i.v.); animal species (*Halobatrachus didactylus* and *Sparus aurata*); vanadate concentration (1 and 5 mM); tissues (cardiac, hepatic, renal, blood); subcellular fractions (cytosol, mitochondria, red blood cells, blood plasma); exposure time (1, 6, 12, 24 hours, 2 and 7 days). Although decameric vanadate is unstable in the assay medium, it decomposes with a half-life time from 5 to 16 hours (*19-25*), depending on the experimental conditions, allowing studying its effects not only *in vitro* but also *in vivo*. Moreover, besides the interest of piscine models to oxidative stress studies, being more sensitive to heavy metals toxicity than mammals, it has been shown to be very useful to study decavanadate toxicity, since at the fish physiological temperature decameric vanadate species is stable enough to induce different effects than vanadate itself.

By analysing the vanadium subcellular distribution following *in vivo* administration, it was described that the amount of vanadium in *Sparus aurata* cardiac tissue (46 ± 11 ng/g dry tissue) and blood (231 ± 45 ng/g dry tissue) depends on total vanadium concentration administration (25). After 1 and 6 hours of 1 mM vanadate i.v. administration, individuals intoxicated with metavanadate exhibits a higher amount of vanadium in heart (114 ± 28 ppb and 94 ± 16 ppb) relatively to those injected with decavanadate (31 ± 7 ppb and 41 ± 7 ppb), respectively, whereas after 12 hours a similar value was obtained for both solutions, approximately 80 ppb (Figure 6A). However, upon administration of a higher vanadate concentration (5 mM), the amount of vanadium detected in cardiac tissue does not depend on the type of vanadate solution administrate (Figure 6B). Therefore, decavanadate does only affect vanadium distribution at the earlier times and at lower vanadate concentrations in comparison to metavanadate (*19-25*).

Vanadate toxicity is known to induce oxidative stress that leads to lipid peroxidation. Recent unpublished results demonstrated that for higher vanadate concentration (5 mM), different longer exposure times, fish species (*H. didactylus*) but for the same tissue (heart), a significant increase ($P < 0.05$) in cardiac tissue lipid peroxidation propagation was observed after 1 (+123%) and 7 (+64%) days after decavanadate exposure, whereas no effects where observed for metavanadate (Figure 7). In liver tissue from a same fish species, the same vanadate concentration (5 mM) and mode of administration (intravenous, i.v.) an

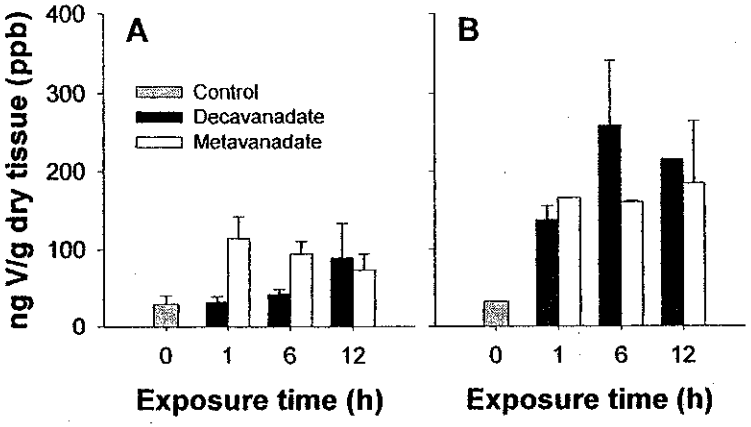

*Figure 6. Total vanadium amount in cardiac tissue of Sparus aurata individuals (n=4) 1, 6 and 12 hours after 1 mM (A) and 5 mM (B) total vanadate concentration of decavanadate and metavanadate exposure. *Significantly different from Control (P <0.05).*

80% increase ($P < 0.05$) in lipid peroxidation was observed 24 hours after i.v. administration of both vanadate solutions (*23*).

Therefore, the reactivities of decavanadate and metavanadate leading to lipid peroxidation are different according to the different organs. Putting it all together, at specific experimental conditions the administration of decameric vanadate clearly induces different biological responses such as vanadium accumulation and lipid peroxidation than other labile oxovanadates pointing out the importance of taking in account the V10 species in the evaluation of vanadate effects.

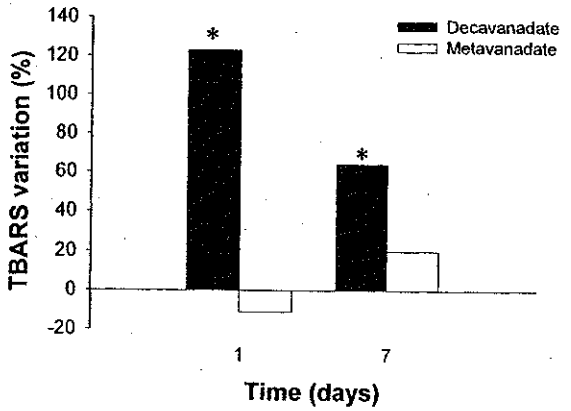

*Figure 7. Lipid peroxidation propagation variation in H. didactylus heart, 1 and 7 days following decavanadate and metavanadate (5 mM total vanadate) in vivo administration. Variation is calculated based on basal values. Values are present as means ± SD (n=6). * Significantly different from Control (P <0.05).*

Decavanadate *in vivo* administration also differs from metavanadate in not inducing cardiac mitochondrial ROS production and superoxide dismutase (SOD) activity besides decreasing catalase (CAT) activity (*25*). Apparently, more pronounced prooxidant effects occur in cardiac mitochondria following i.v. metavanadate exposure whereas decavanadate administration seems to prevent this effect. Decavanadate *in vivo* exposure also induce a decrease in cardiac mitochondrial CAT activity (-60%), 7 days following 5 mM decavanadate i.p. administration in *H. didactylus* (*18*). In liver, it was described that decavanadate and metavanadate administration clearly induce different changes in oxidative stress markers (*23*). Therefore, the antioxidants responses induced by vanadate may depend on the total vanadium concentration administered, on the way of exposure and/or vary between fish species, besides the vanadate species composition of vanadate solutions.

Mitochondria: a Target for Decavanadate Toxicity

Mitochondria tend to be concentrated in tissues in which the energy demand is high. Thus, a compound that inhibits mitochondrial oxidative phosphorylation can therefore have a profound effect on the metabolism of important organs like the heart, kidney, liver and brain. As described above, decavanadate *in vivo* administration point out to specific effects in mitochondrial activity besides affecting mitochondrial anti-oxidants enzyme activities (*25*). Apparently, the mitochondria seem to be a target for decavanadate. This hypothesis was further explored and recent results are now described. Mitochondria isolated from rat liver have the advantage to be a well-characterized model for toxicological studies, very easily obtainable and the preparation produces large quantities of functionally intact mitochondria. Thus, it was show that V10 inhibits mitochondrial respiration and induces mitochondrial membrane depolarization in a larger extent that metavanadate, in both hepatic and cardiac mitochondria (Sandra *et al.*, unpublished results). For instance, decavanadate concentration as low as 100 nM, inhibits 50% of oxygen consumption in mitochondria, while a 100-fold higher concentration of V1 (10 μM) is needed to induce the same effect pointing out mitochondria as a potential cellular target for V10 toxicity (Figure 8).

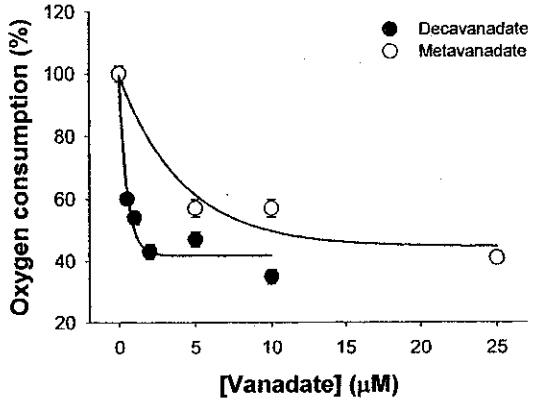

Figure 8. Effect of decavanadate and metavanadate solutions on rat liver mitochondrial oxygen consumption (n=4).

Concluding Remarks and Future Perspectives

This review focuses biological effects of decameric vanadate species on phosphohydrolases and ATPases such as the Ca^{2+}-pump of sarcoplasmic

reticulum (SR) and myosin from skeletal muscle. Current studies are in progress in order to evaluate decavanadate toxic effects on neonatal rat cardiac myocytes and in the modulation of muscle contraction since it was proposed that decavanadate inhibits actomyosin ATPase and SR ATPase activity by a different mechanism than the one described for monomeric vanadate, besides affecting actin polymerization. Recently results suggesting that mitochondria are a potential target for decameric toxicity also associate the effects of decavanadate with bioenergetics processes. Once decavanadate solutions contain decameric vanadate species, whose dissociation is slow enough to study its *in vivo* effects in piscine models, it was observed that antioxidant stress markers, lipid peroxidation and vanadium subcellular distribution is dependent on the administration of solutions containing or not decameric vanadate species. In fact, decavanadate *in vivo* intoxication clearly induces different changes on oxidative stress markers and lipid peroxidation than others oligomers. Upon intoxication with decavanadate the metabolism of vanadium is affected, being mitochondria a potential toxicity target.

These studies point to the importance of taking into account decavanadate, which once formed may not completely fall apart, in the evaluation of vanadium biological effects. Questions that remain to be addressed include for instance: i) How can it be prevented the formation of decavanadate? ii) Once formed, can it be prevented the disintegration of decavanadate? iii) How can it be formed in cells an *in vivo*? iv) Does decavanadate interaction with myosin induce different *in vivo* populated states from the ones induce by monomeric vanadate? v) Can be those myosin conformation states induced by V10 relevant for the understanding of muscular contraction processes? vi) Is mitochondria a preferential target for decavanadate toxicity? vii) How can decavanadate be stabilized by actin polymerization? viii) Does V10 enter into SR vesicles or does it operate through the outside? ix) Are the *in vivo* effects induced by V10 due to interactions with membrane proteins? x) Does V10 induce the *in vivo* effects due to specific citoplasmic protein targets?

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