

**UNITED STATES AIR FORCE
ARMSTRONG LABORATORY**

**PRO-OXIDANT BIOLOGICAL EFFECTS
OF INORGANIC COMPONENT
OF PETROLEUM: VANADIUM AND
OXIDATIVE STRESS**

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

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FOR THE DIRECTOR



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13. ABSTRACT (Maximum 200 words) Crude oil contains significant amounts of inorganic compounds of vanadium. Air Force, Army, and Navy personnel are exposed to vanadium compounds in the air, on the land, and on the sea, wherever petroleum fuel is used. Inorganic residue of fly ash resulting from combustion of some fuels may contain almost exclusively vanadium oxides. Unlike organic pollutants, vanadium is not biodegradable and it may build up in certain ecosystems to the level which may be toxic to living organisms. It has been estimated that as much as 66,000 tons of vanadium is released into the atmosphere each year. Particularly dramatic effects on the environment may result from massive incidental and/or intentional spilling of vanadium-containing crude oil into relatively confined ecosystems, as well as from massive oil burning. In addition to the vanadium exposure at the work place, the general population is also exposed increasingly to this metal, mostly as a result of increased utilization of vanadium-containing petroleum fuels. Vanadium-bearing particles may persist in the lungs for many years, raising the risk of chronic health effects. This report contains a review of the extensive literature on biochemical mechanisms of action of vanadium compounds, as well as the authors' own perspective, resulting from about two decades of laboratory research. This report provides information about adverse biological effects of vanadate, vanadium pentoxide, vanadyl, and other vanadium derivatives present in petroleum or formed during the fossil fuel combustion, on oxidative stress, cellular signal transduction, subcellular organelle functions, and on a possible interaction with polycyclic aromatic hydrocarbons and chemical co-carcinogenesis.				
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PREFACE

This report contains a review of the extensive literature on biochemical mechanisms of action of vanadium compounds, as well as the authors' own perspective, resulting from about two decades of laboratory research. This is one of a series of technical reports and publications describing results of a collaborative effort conducted by ManTech Environmental Technology, Inc., Toxic Hazards Research Unit, Located at Wright-Patterson Air Force Base, and is aimed at petroleum risk assessment.

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ABBREVIATIONS

AP-1 - activation protein-1;
B(a)P - benzo(a)pyrene;
BBPD - biologically based pharmacodynamics;
BBPK - biologically based pharmacokinetics;
DMNQ - 2,3-dimethoxy-1,4-naphthoquinone;
FMN - flavin mononucleotide;
GSH - reduced glutathione;
NAD(P)H - reduced nicotinamide adenine dinucleotide (and its phosphate);
NF κ B - nuclear factor κ B;
NDGA - nor-dihydroguaiaretic acid;
O₂⁻ - superoxide anion radical;
PBPK - physiologically based pharmacokinetics;
PTPase - phosphotyrosyl protein phosphatase;
SH2 - src homology 2 domain, a domain binding to phosphotyrosine-containing proteins;
SOD - superoxide dismutase;
TMPD - N,N,N',N'-tetramethyl-p-phenylenediamine;
V_(V) - pentavalent vanadium;
V_(IV) - tetravalent vanadium;

SECTION 1

INTRODUCTION

1.1. Vanadium as a Main Inorganic Pollutant of Petroleum

Fossil fuels, especially petroleum (crude mineral oil), contain significant amounts of vanadium compounds (Eckardt, 1971; Sokolov, 1986). Vanadium and other trace metals, unlike organic pollutants, are not biodegradable in the environment. Therefore, inorganic vanadium compounds redistributed by human activity tend to build up in the ecosystem to levels which may be toxic to living organisms. It was estimated that as much as 66,000 tons of vanadium are released and redistributed into the atmosphere each year (Nriagu and Pacyna, 1988). Devastating effects on the environment may result from massive incidental and/or intentional burning of vanadium-containing crude oil and its spilling into the sea (Sadiq and Zaidi, 1984; Kalogeropoulos et al., 1989; Vasquez et al., 1991; Madany and Raveendran, 1992; Moeller et al., 1994). Crude oil from certain locations may be especially rich in vanadium. For example, fly ash resulting from combustion of Venezuelan oil may contain up to 80% of vanadium compounds (Hudson, 1964).

In addition to fossil fuels, some ores may contain significant amounts of this metal, and thus, occupational exposure to vanadium is quite common in modern petrochemical, mining, and steel industries (Goldsmith et al., 1976; Lees, 1980; Fisher et al., 1983; Schiff and Graham, 1984; White et al., 1987; Karimov et al., 1988, 1989, 1991; Sarsebekov et al., 1994). Vanadium is often associated with uranium ore and may contribute to the increased occupational risk to those employed in uranium mines (Paschoa et al., 1987). Huge amounts of vanadium are usually deposited in the smoke-stacks and exhaust systems of engines, boilers, and generators powered by heavy fuel oils (e.g., mazut). Vanadium may be found also in diesel fuel exhaust particles (Kleinman et al., 1977; Levy et al., 1984; Rossi et al., 1986; Pisteli et al., 1991; Todaro et al., 1991; Hauser et al., 1995). Vanadium pentoxide (V_2O_5) is the most ubiquitous vanadium compound (Troppens, 1969; Nechay, 1984), although the natural oil-fired fly ash may also contain as many as a dozen other vanadium compounds (Bowden et al., 1953).

Vanadium toxicity is a true concern for industrial workers and military personnel exposed to its compounds on land and sea. In addition to vanadium exposure at the work place (Zychlinski, 1980), the general population is also increasingly exposed to this metal (Flyger et al., 1976), mostly as a result of increased utilization of vanadium-containing natural oil (Schiff and Graham, 1984). In humans, vanadium-bearing particles may persist in the lungs for many years (Paschoa et al., 1987). Thus, among the inhabitants of U.S. cities, vanadium deposits in lungs are markedly increasing with age as a result of its accumulation (Tipton and Shafer, 1964).

Acute inhalation of dust containing high concentrations of vanadium can cause harmful health effects in humans, mostly in the respiratory tract, including lung irritation, coughing, wheezing, chest pain, runny nose, and a sore throat. No comprehensive human studies are available on the health effects of chronic exposure or the carcinogenicity of vanadium; thus, the U.S. Department of Health and Human Services and the U.S. Environmental Protection Agency have not classified vanadium as to its human carcinogenicity (Agency for Toxic Substances and Disease Registry, 1992). A focussed issue, containing invited reviews and original research papers on mechanisms and biochemical effects of vanadium, edited by Srivastava and Chiasson (1995), was published in *Molecular and Cellular Biochemistry*.

1.2. Oxidative Stress

Animal studies have shown that vanadium compounds induce oxidative stress and lipid peroxidation *in vivo* (Stohs and Bagchi, 1995). An exposure of murine C3H/10T1/2 cells in culture to as low as 5 - 20 μM $\text{V}_{(V)}$ was found to induce up to 100-fold expression of the proliferin gene family, indicative of a cellular state of oxidative stress (Parfett and Pilon, 1995). Even concentrations ten times lower (0.5 - 2 μM $\text{V}_{(V)}$) stimulated growth of MCF-7 cells *in vitro*. The proliferative effect of $\text{V}_{(V)}$ on these cells reached a plateau at 1 μM , declined at 3 μM , and disappeared at 5 μM (Auricchio et al., 1996).

The significance and biological implications of chemically induced oxidative stress have been reviewed extensively by Byczkowski and Channel (1996). In summary, oxidative stress is a pathophysiological process in which intracellular balance between endogenous as well as exogenous pro-oxidants and antioxidants is shifted towards pro-oxidants, leaving cells unprotected from free radical attack. Ultimate cellular pro-oxidants are mostly free radicals (Fig. 1), defined as molecules or groups of atoms with one or more unpaired electrons and are capable of independent existence.

Pro-Oxidant Chemicals and Free Radicals Involved in Oxidative Stress

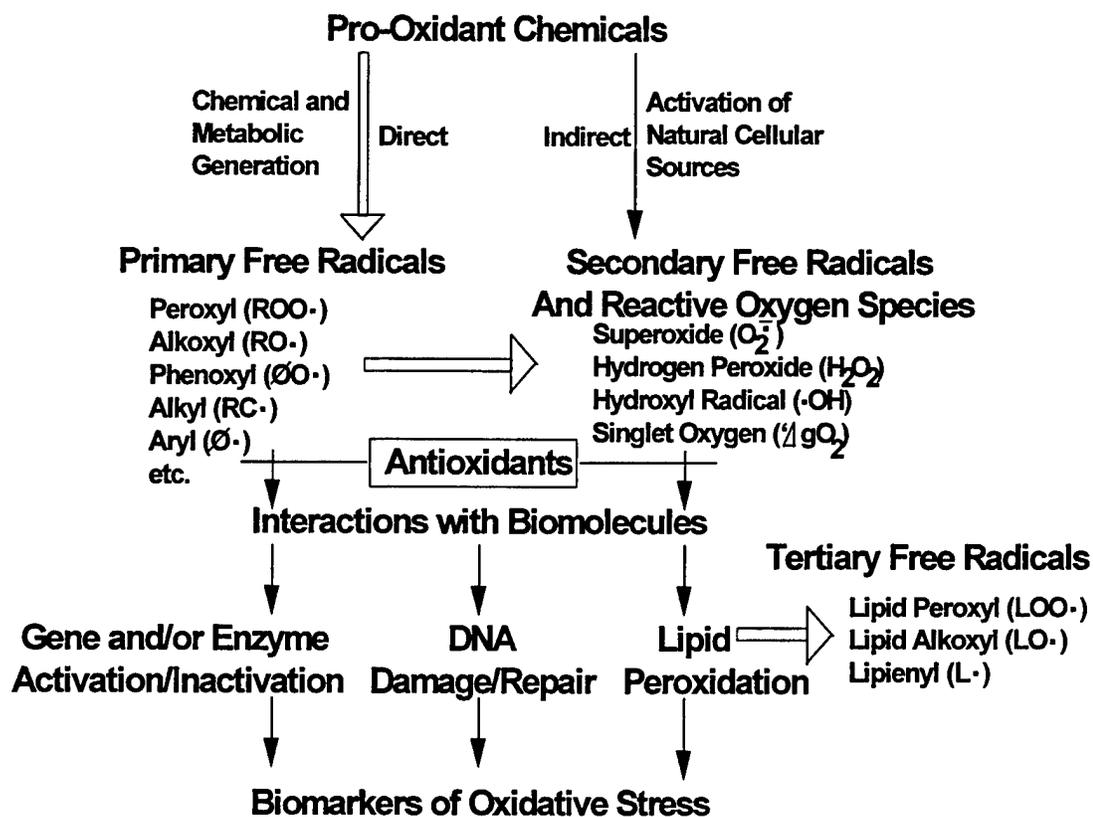


Figure 1. Generation of primary, secondary, and tertiary free radicals and reactive oxygen species involved in the oxidative stress induced by pro-oxidant chemicals (modified from Byczkowski and Channel, 1996).

These free radicals derive directly from pro-oxidant chemicals (Kehrer, 1993) or may arise indirectly from stimulated natural functions in aerobic cells (Byczkowski and Gessner, 1988); for instance, free

radicals may be produced by stimulated lipid peroxidation (Gower, 1988; Finley and Otterburn, 1993; Haegele et al., 1994). Thus, from the pro-oxidant xenobiotics, further chemical and/or metabolic reactions may generate primary free radicals (Fig. 1). Then, in an avalanche-type process, secondary free radicals and reactive oxygen species may be released (for review see Roberfroid and Calderon, 1994). Both primary and secondary free radicals may initiate lipid peroxidation, leading to the generation of tertiary free radicals (Fig. 1). Additional factors, such as aging (Stadtman et al., 1993), dietary deficiencies, or the presence of transition metals (Kulkarni and Byczkowski, 1994a, b), may augment the oxidative stress status. A depletion of cellular antioxidants such as vitamin E, C, or reduced glutathione (GSH), as well as a defective enzymatic scavenging by catalase, selenium containing GSH peroxidase, and superoxide dismutase further increase oxidative stress and may enhance damage to cellular components. Oxidative stress can be reversed by natural and synthetic antioxidants (Williams, 1993; Papas, 1993; Pratt, 1993). Oxidative stress triggers biological reactions counteracting cellular damage. It may induce enzymes with free radical scavenging and repair activities and/or activate an oxidative-stress responsive nuclear transcription factor κ B (NF κ B; Meyer et al., 1993). The importance of oxidative stress and the published literature detailing methodology employed for its measurement have been reviewed in three recent publications by Pryor (1993), Sies (1994) and Jaeschke (1995), and in a multiple author book (Spatz and Bloom, 1992).

Chemically induced oxidative stress causes derangement of antioxidant mechanisms in tissues (Videla et al., 1990), may lead to lipid peroxidation (Comporti, 1985), and may cause stimulation of cellular proliferation and/or apoptosis (Corcoran et al., 1994) that may finally result in cell injury (de Groot and Littauer, 1989). A study by Biasi et al. (1995) demonstrated that lipid peroxidation is a cause rather than an effect of necrotic tissue damage. Figure 2 summarizes dose-dependency observed in the oxidative stress induced by pro-oxidant chemicals.

Chen and Chan (1993) using 3T3-L1 cells, cultured in a serum-free medium, demonstrated that $V_{(v)}$ increases [3 H]thymidine incorporation to DNA and enhances expression of the c-fos gene in a manner analogous to a redox cycling naphthoquinone (DMNQ). The authors suggested that both pro-oxidant compounds, orthovanadate and DMNQ, increased tyrosine protein phosphorylation early in the signal transduction cascade of growth factor receptors, leading to augmentation of cell proliferation. Apparently, the common factor in the mode of action of these two completely dissimilar chemicals was an oxidative stress caused as a result of intracellular redox cycling (Chen and Chan, 1993).

Dose-Dependent Effects of Pro-Oxidants on Cellular Activities

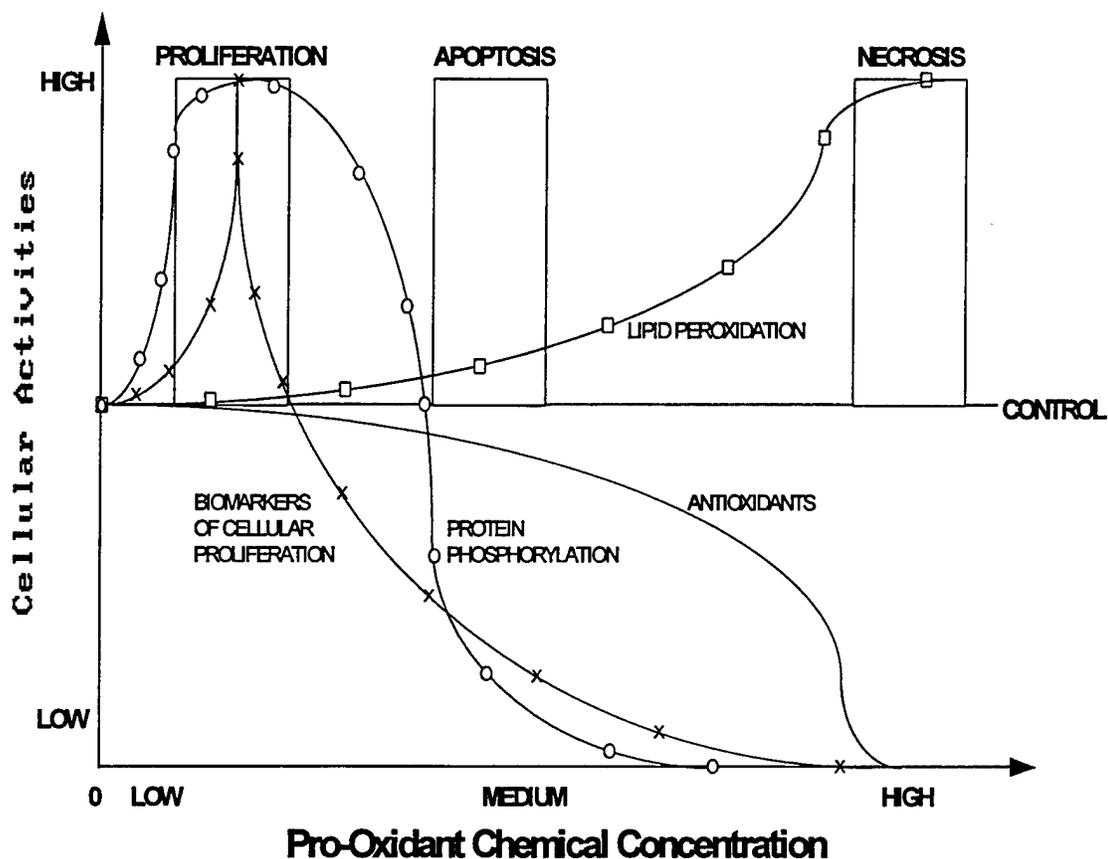


Figure 2. Effects of different concentrations of pro-oxidant chemicals on cellular function and activities:
 □—□ lipid peroxidation; — vitamin E-type antioxidant level; ○—○ protein tyrosine phosphorylation;
 X—X ornithine decarboxylase and S-adenosylmethionine decarboxylase activities. The curves are computer-generated results of simulation of: lipid peroxidation and antioxidant depletion (BBPD computer program developed by Byczkowski et al., 1995, based on data by Tappel et al., 1989), protein tyrosine phosphorylation (BBPD computer program developed by Byczkowski and Flemming, 1996, based on data by Vroegop et al., 1995 and experimental results of Heffetz et al., 1990), ornithine and S-adenosylmethionine decarboxylation (BBPD computer program developed by Byczkowski and Flemming, 1996, based on Corcoran et al., 1994).

SECTION 2

PRO-OXIDANT PROPERTIES OF VANADIUM

A pro-oxidant effect of vanadium in tissue preparations was first described by Bernheim and Bernheim (1938). Since then, the biologically relevant redox properties of vanadium stirred both extensive research activity and controversy. Especially vanadium-stimulated NAD(P)H oxidation, reported for the first time by Byczkowski and Zychlinski (1978) in mitochondrial preparations, attracted much attention and became a subject of passionate discussions (Erdmann et al., 1981; Liochev and Fridovich, 1990, 1996). Because the progress in this field has been rapid, it was not possible, in the format of only one chapter, to review all of the work and cite each relevant publication. Instead, only selected experimental and review papers which we thought were key to understand and substantiate the phenomenon of oxidative stress caused by vanadium are discussed. We apologize to those whose work is not cited here.

2.1. Compartmentalization of $V_{(V)}$ and $V_{(IV)}$ in the Organism

In oxygenated blood, the absorbed vanadium circulates as poly-vanadate ($V_{(V)}$, isopolyanions containing vanadium in the +5 oxidation state). Different tissues retain vanadium mainly as vanadyl ($V_{(IV)}$, cationic form of vanadium in the +4 oxidation state) (Erdmann et al., 1984) in the presence of endogenous reducing compounds (such as glutathione-SH; Bruech et al., 1984). The highest concentration of vanadium in rats fed either $V_{(V)}$ or $V_{(IV)}$ was found in the kidneys while the liver and spleen contained about three times lower concentrations. The lowest accumulations of vanadium were found in the lung, blood plasma and blood cells. The half-life for vanadium elimination from the body was about 12 days in rats (Ramanadham et al., 1991). To date, apparently, there is no systematic pharmacokinetic study conducted on vanadium absorption, distribution, metabolism, and disposition, and no pharmacokinetic model is available describing comparative kinetics and toxicity of vanadium administered by different routes.

A successful attempt to isolate an inhibitor of (Na^+ , K^+)-ATPase, inherent to the Sigma Grade commercial ATP preparations isolated from muscle, lead Cantley et al. (1977) to the discovery that sodium ortho-vanadate (Na_3VO_4 , a salt containing $V_{(V)}$) inhibits this activity at a very low concentration. In rat cardiac subcellular preparations, $V_{(V)}$ was an even more potent inhibitor of the (Na^+ , K^+)-ATPase than cardiac glycoside (ouabain) which is used as a "specific inhibitor" of the sodium pump (Erdmann et al., 1984). However, a high degree of inhibition of (Na^+ , K^+)-ATPase activity was observed only in the purified enzyme systems or when damaged subcellular preparations were employed. Since no evidence of inhibition was noted in intact cells or *in vivo*, it has been proposed that some kind of compartmentalization and/or "detoxification" of vanadium exists in the undamaged tissue. It was suggested that the inhibitory $V_{(V)}$ may be intracellularly reduced to vanadyl ($V_{(IV)}$) which was believed to be non-inhibitory (Erdmann et al., 1984). Inside the cell, $V_{(IV)}$ may further bind to several endogenous ligands, and be protected against reoxidation (Nechay et al., 1986). Although, as shown in the hog kidney (North and Post, 1984) and rat brain (Svoboda et al., 1984) microsomal preparations, vanadyl may also act as an inhibitor of (Na^+ , K^+)-ATPase.

Using the inhibition of substrate oxidation in liver mitochondria (Byczkowski et al., 1979) as an indicator of $V_{(V)}$ accumulation, it was possible to demonstrate a significant amount of vanadate accumulation in the liver during experimental administration of V_2O_5 to rats (Zychlinski and Byczkowski,

1990). Although under physiological conditions, the partial oxygen pressure in liver is low (pO_2 from about 30 mm Hg in the perivenous zone to about 56 mm Hg in the periportal zone), the actual O_2 concentration in different compartments across the hepatic acinus may vary more than two-fold (Kulkarni and Byczkowski, 1994b). Due to preferential partitioning, it may be even three times higher in a lipid phase of biomembranes than in the water phase (Antunes et al., 1994). This suggests that the process of "detoxification" by reduction of $V_{(V)}$ to $V_{(IV)}$ in the rapidly perfused liver tissue may be insufficient to prevent its deleterious effects on biomembranes and bioenergetic functions, especially under conditions of chronic exposure to V_2O_5 .

These apparent discrepancies may be explained using computer simulations of vanadyl oxidation with a biologically based pharmacokinetic (BBPK) model, considering the oxygen availability (Fig. 3). The model was calibrated with the experimental data from North and Post (1984) as shown in Fig. 3C. Under conditions of chronic oxidative stress tissue reserves of GSH may be depleted. Under these circumstances, for example, 1 mM vanadyl may be completely reoxidized to $V_{(V)}$ by the available oxygen within 10 hours (Fig. 3A), provided that the tissue, stripped of antioxidants, is receiving a constant supply of oxygen (assumed to be initially no more than $124 \mu M$ around arterial vessels in a rapidly perfused tissue).

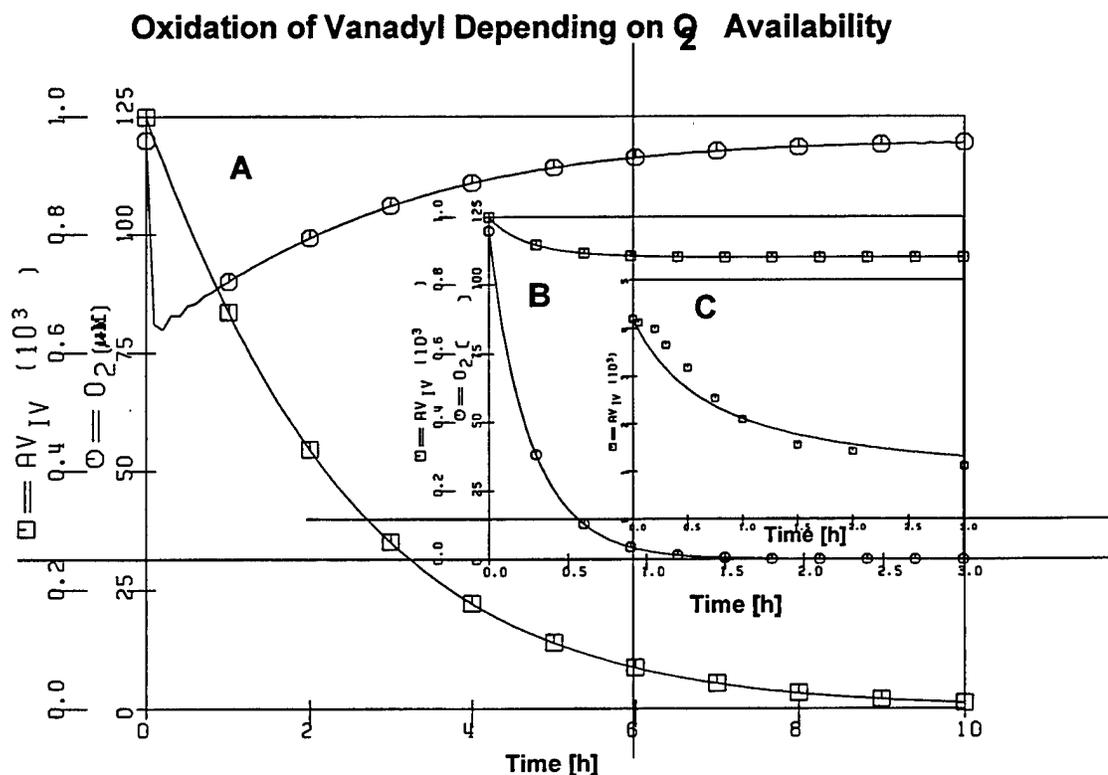
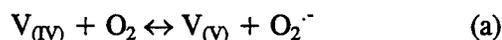


Figure 3. Oxidation of dissolved vanadyl, at near neutral pH, depending on oxygen availability. The curves are computer-generated results of simulation with biologically based pharmacokinetic model (BBPK computer program based on North and Post, 1984; and Byczkowski et al., 1988), assuming replenishment of oxygen by diffusion from the air (C), from perfusing blood (A) or with oxygen supply turned off (B). The curves marked with small squares show vanadyl concentration ($AV_{IV} [\mu M]$), and curves with small circles show oxygen concentration ($O_2 [\mu M]$). The small squares in Fig. 3 C depict experimental data, from North and Post (1984), used for model calibration.

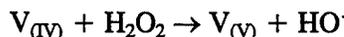
However, the same initial concentration of oxygen may be completely depleted within 1 hour in the presence of the same 1 mM vanadyl when the diffusion of fresh oxygen is restricted (Fig. 3B). This process may be drastically accelerated by the presence of traces of catalytically active transition metals, e.g., copper (Byczkowski et al., 1988). Moreover, in the perfused rat liver, $V_{(V)}$ caused significant vasoconstriction which resulted in a progressive decrease in perfusion rate (Younes and Strubelt, 1991) that was irreversible by antioxidants (Younes et al., 1991). Thus, under the conditions of reduced rate of perfusion or hypoxia, at best, up to 12% of vanadyl may be oxidized, assuming that $V_{(IV)}$ oxidation is the only process using oxygen. In reality, the tissues become anaerobic much faster (for instance, an average estimated O_2 concentration in the liver was found to be only about 35 μ M; Antunes et al., 1994), which may inhibit further reoxidation of intracellular vanadyl in poorly perfused or hypoxic tissues, even without the contribution of GSH and other cellular reductants.

2.2. Generation of Free Radicals

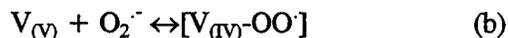
Autooxidation of $V_{(IV)}$ with O_2 from the air is a potentially deleterious, but rather a slow process, producing superoxide anion radical ($O_2^{\cdot -}$) and $V_{(V)}$:



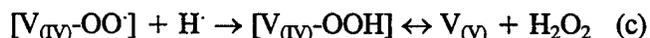
This reaction may either be drastically accelerated by the presence of catalytic amounts of transition metal cations (e.g., Cu^{2+}) or slowed down by chelators (e.g., EDTA; Byczkowski et al., 1988). Superoxide anion radicals may dismutate spontaneously or enzymatically (by superoxide dismutase, SOD), producing hydrogen peroxide (H_2O_2). It may, in turn, react with remaining $V_{(IV)}$ producing hydroxyl free radical (HO^{\cdot}) in a Fenton-type reaction:



Theoretically, HO^{\cdot} may react with H_2O_2 to generate $O_2^{\cdot -}$ in a Haber-Weiss reaction. While the Fenton-type oxidation of $V_{(IV)}$ can easily be demonstrated under anaerobic conditions *in vitro* (Stankiewicz et al., 1991), its biological significance seems to be rather low (Byczkowski and Kulkarni, 1992b). On the other hand, in a chelating environment (Crans et al., 1989), superoxide may remain complexed within the vanadium moiety, forming a peroxy-vanadyl-type intermediate:



It seems that amino acids, peptides, and other natural ligands (analogous to Tris buffer) may stabilize the product of one-electron reduction of $V_{(V)}$ (Byczkowski and Kulkarni, 1992b). In contrast to superoxide, this hypothetical peroxy-vanadyl intermediate probably disappears very quickly under the biologically relevant conditions (Liochev and Fridovich, 1990) via hydrogen abstraction from biomolecules:



This reaction, through the pervanadate (vanadyl hydroperoxide) intermediate, may regenerate $V_{(V)}$ necessary for continuous operation of the redox cycle. The vanadium redox cycling, initiated by one-electron oxidation of vanadyl or one-electron reduction of vanadate with superoxide anion

radical, may operate as a chain reaction providing primary, secondary, and tertiary free radicals and reactive oxygen species as long as $O_2^{\cdot-}$ and/or biomolecules capable of reducing $V_{(V)}$ back to $V_{(IV)}$ are available. A summary of the reactions involved in the vanadium redox cycling is shown schematically in Fig. 4.

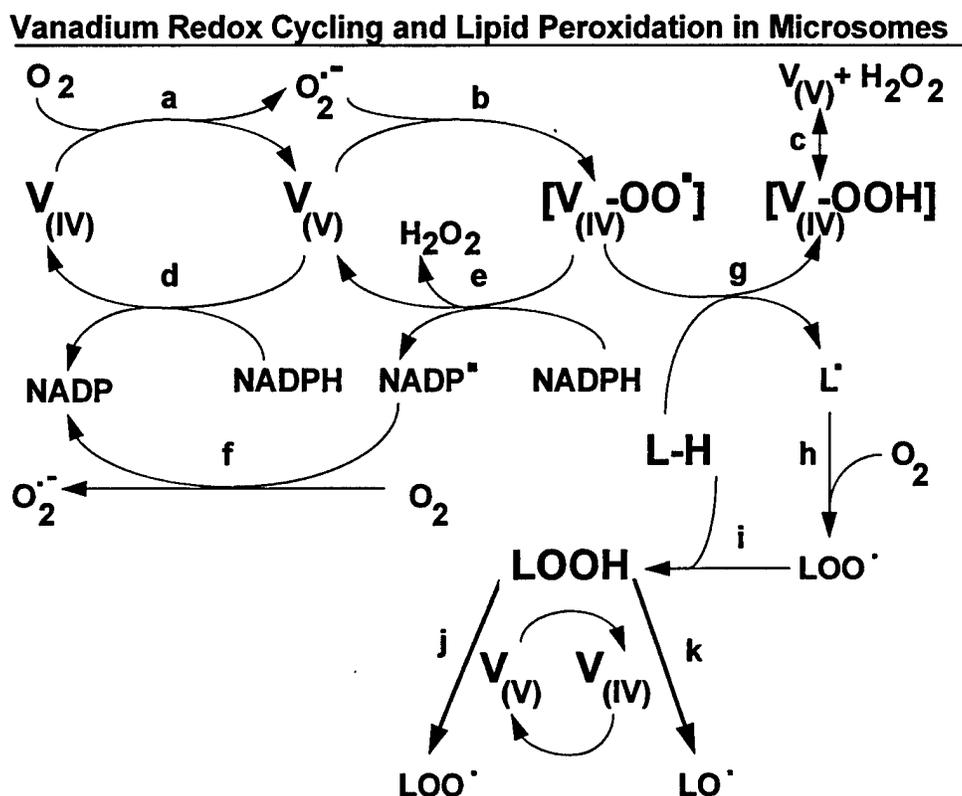
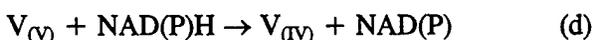


Figure 4. A summary scheme of the postulated reactions participating in vanadium redox cycling, NAD(P)H oxidation and lipid peroxidation (according to Byczkowski et al., 1988; Byczkowski and Kulkarni 1992b).

2.3. NAD(P)H Oxidation

The stimulatory effect of vanadate on oxidation of NADH was originally described by Byczkowski and Zychlinski (1978) in the sonicated rat liver mitochondrial preparations (Zychlinski and Byczkowski, 1978; Byczkowski et al., 1979). Subsequently, a similar $V_{(V)}$ stimulated NADH oxidation was reported in cardiac cell membranes (Erdmann et al., 1979) followed by many claims for NADH and NADPH oxidation in several other subcellular preparations (Ramasarma et al., 1981) and enzymatic systems (Darr and Fridovich, 1984).

Vanadate can be directly reduced by NAD(P)H at a biologically relevant pH, but this reaction is very slow (Byczkowski et al., 1979; Vyskocil et al., 1980):



In the presence of oxygen and an appropriate biological preparation, this reaction can be increased by

several hundred-fold. This reaction exhibits specific pH optima (Vyskocil et al., 1980) and is sensitive to specific inhibitors and boiling (Ramasarma et al., 1981). No wonder that many researchers thought the reaction (d) to be catalyzed by either "NAD(P)H : vanadate reductase" or "vanadium-dependent NAD(P)H oxidase". In fact, this reaction, which is insensitive to inhibition by SOD or catalase, is a "general property of endomembranes" (Ramasarma et al., 1981) containing flavin coenzymes capable of accepting electrons from NAD(P)H. Vanadate can accept electrons in a way similar to ferricyanide (Ramasarma et al., 1981). The oxidation-reduction potential for one-electron reduction of ferricyanide under standard conditions (E'_0) is +0.26 V; whereas the E'_0 for one-electron reduction of $V_{(V)}$ is even more positive (about +1.0 V). The biological importance of reaction (d) lies in its ability to provide reduced vanadium $V_{(IV)}$.

Under aerobic conditions, autooxidation of $V_{(IV)}$ may generate $O_2^{\cdot -}$ (reaction a) which, reacting with $V_{(IV)}$, may turn on a fast and destructive one-electron oxidation of NAD(P)H:



As demonstrated by Liochev and Fridovich (1988), $O_2^{\cdot -}$ generated in the system containing $V_{(IV)}$ and NADH initiates a free radical reaction and causes a rapid oxidation of NADH with the estimated chain length of 15 NADH oxidized per $O_2^{\cdot -}$. As expected, the overall process was inhibited by SOD (because of the reaction b), but not by catalase. Under aerobic conditions, this process may be self-sustaining, as the next step can re-generate $O_2^{\cdot -}$. Thus, in the presence of oxygen, the free radical intermediate NAD(P) $^{\cdot}$ may pass its unpaired electron on dioxygen, generating more $O_2^{\cdot -}$:



Obviously, in a well-perfused tissue, any endogenous compound (e.g., GSH) capable of reducing the initial amount of vanadate (generated endogenously or introduced) to $V_{(IV)}$ may also initiate the vanadium redox cycling and depletion of NAD(P)H. This deleterious redox cycling may be broken by anoxia or by chain-breaking antioxidants. A summary of the main reactions involved in the vanadium-mediated NAD(P)H oxidation is shown in Fig. 4.

2.4. Depletion of Cellular Antioxidants

Both water and lipid soluble antioxidants, along with enzymatic scavengers, represent the cellular defense mechanisms which allow, for instance, the liver tissue to detoxify as much as 60 - 72 μ mole $O_2^{\cdot -}$ per gram liver without significant injury (Jaeschke, 1995). However, significant differences exist in the tissue levels of antioxidant protection and the ratio of water soluble vs. lipid soluble antioxidants in different species. For instance, human liver contains only 1 - 2 μ moles GSH per gram as compared to 7 - 8 μ moles GSH per gram in rat liver (Purucker and Wernze, 1990). On the other hand, physiological levels of lipid soluble antioxidants and natural free radical scavengers in a human liver are within an order of magnitude higher than in a mouse liver (Cutler, 1991; Sohal, 1993).

It was shown by Bruech et al. (1984) that exposure to $V_{(V)}$ results in the depletion of cellular thiol pool. GSH is the main intracellular thiol compound which also plays an important role as a main water-soluble cellular antioxidant. GSH is involved in the homeostasis of the intracellular redox state and is coupled to the oxidation state of cysteine residues in proteins (Ziegler, 1985). Tissue GSH was shown to participate directly in vanadate inactivation (Kretzschmar and Braunlich, 1990). As was observed *in vitro*, $V_{(V)}$ caused the oxidation of thiols, including GSH and cysteine, and the formation of thiol radicals in

this reaction was suggested (Shi et al., 1990). Depletion of GSH not only decreases the antioxidant defense in the cytosol, but also prevents regeneration of a vital lipid-soluble antioxidant, α -tocopherol (vitamin E), increasing the vulnerability of phospholipid-rich biomembranes to oxidative stress and lipid peroxidation.

Experimental treatment of rats with $V(V)$ in drinking water (0.15 mg of ammonium metavanadate/mL) for 14 days resulted in a decrease in the activity of two essential "antioxidant enzymes," catalase and glutathione peroxidase, in the liver and kidneys. This decrease was linked with the increased spontaneous lipid peroxidation measured in liver and kidney homogenates. However, there was no change in the activity of cytosolic and mitochondrial superoxide dismutase (Russanov et al., 1994).

SECTION 3

EFFECTS ON MITOCHONDRIA

Experiments performed on rat liver and wheat seedling mitochondria showed that, depending on the localization of vanadate, in the intermembrane space or at the inner side of inner mitochondrial membrane, either the inhibition of the respiration with NAD-linked substrates and succinate (but not ascorbate) or short-circuiting of the respiratory chain can be observed (Fig. 5; Byczkowski et al., 1979).

Mode of Action of Vanadium in Mitochondria

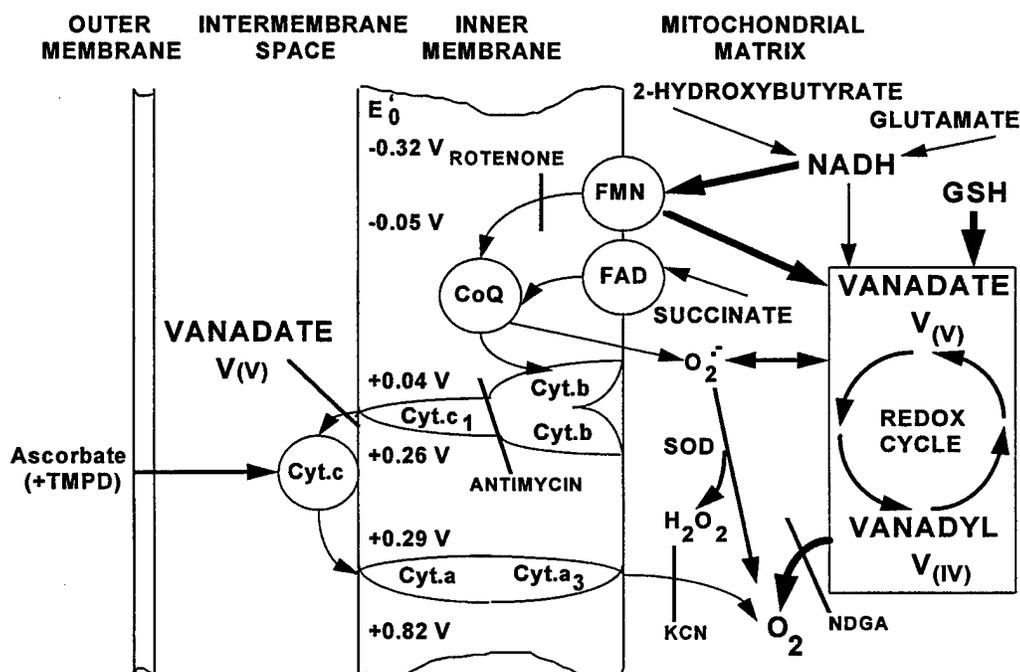


Figure 5. A summary scheme of the mode of action of vanadium in mitochondria (according to Byczkowski and Sorenson, 1984).

3.1. Inhibition of the Respiratory Chain by $V_{(V)}$

In intact mitochondria, vanadate accumulates in the intermembrane space, blocks the electron transfer through the respiratory chain between cytochrome c_1 and cytochrome c which causes inhibition of NADH-linked substrates and succinate (Byczkowski and Zychlinski, 1978; Zychlinski and Byczkowski, 1978; Byczkowski et al., 1979). In the isolated undamaged rat liver mitochondria, the respiration with glutamate and succinate at state 3 was more sensitive to inhibition by $V_{(V)}$ than at state 4, whereas the efficiency of oxidative phosphorylation (measured as ratio ADP to O) was not affected significantly up to the 0.1 mM concentration of $V_{(V)}$. Similarly, mitochondrial respiration with ascorbate (+ N,N,N',N'-tetramethyl-p-phenylenediamine, TMPD), which supplies electrons to cytochrome c , was not inhibited (Zychlinski and Byczkowski, 1990). The oxygen uptake by mitochondria isolated from the livers of rats treated intratracheally with a massive single dose of 5 mg V_2O_5 /kg was significantly inhibited for up to

48 hours with glutamate and succinate, but not with ascorbate. Even more pronounced inhibition was observed after chronic treatment of rats with 0.56 mg V_2O_5 /kg monthly for 12 months (Zychlinski and Byczkowski, 1990). Other significant changes in the rats from the chronic treatment group included the decreased blood glucose and cholesterol concentrations, and increased (almost two-fold) average hydroxyproline content in the lungs (Zychlinski et al., 1991).

3.2. Short-circuiting of the Respiratory Chain

In the rat liver mitochondria damaged by sonication, $V_{(V)}$ caused a dramatic stimulation of oxygen uptake with NADH-dependent substrates (Byczkowski and Zychlinski, 1978; Zychlinski and Byczkowski, 1978; Byczkowski et al., 1979). It seems that when the inner mitochondrial membrane permeability barrier to vanadate polyanions was broken, $V_{(V)}$ undergoes a redox cycling. Most likely, $V_{(V)}$ at the inner side of inner mitochondrial membrane is reduced by NADH at the level of flavoprotein center of the dehydrogenase (reaction "d"). Although reduction of $V_{(V)}$ by NADH may proceed non-enzymatically, it is much slower than the reduction with dihydroflavin in a FMN-containing flavoprotein (Byczkowski et al., 1979). The oxidation-reduction potential of flavoprotein under standard conditions (E_0) is less negative than that of NADH (-0.05 V versus -0.32 V, respectively; Lehninger, 1965); by comparison, the E_0 for one-electron reduction of $V_{(V)}$ is about +1.0 V, but may be further affected by chelation with amino acids, Tris, and other buffers.

It should be emphasized that any reaction capable of reducing $V_{(V)}$ to $V_{(IV)}$ (with NADH, GSH, sugars, etc.) under aerobic conditions would initiate vanadium redox cycling and eventually lead to the destructive reaction (e) and $O_2^{\cdot-}$ generation (f) (Fig. 4). Similarly, even trace amounts of $O_2^{\cdot-}$ may initiate vanadium redox cycling and produce even more $O_2^{\cdot-}$ in a chain reaction (Liochev and Fridovich, 1988). Accordingly, it was proposed that NAD(P)H oxidation in the presence of $V_{(V)}$ amplifies the initial generation of $O_2^{\cdot-}$ and thus may be used as a sensitive assay method for $V_{(V)}$ (Liochev and Fridovich, 1990). While the idea of $O_2^{\cdot-}$ "amplification" appears attractive, its application to the results observed with mitochondrial preparations is very difficult. First of all, mitochondria contain a highly active SOD and even in isolated submitochondrial sonic particles, washed and recentrifuged, a residual SOD activity enriched the reaction mixture with O_2 (from the dismutated $O_2^{\cdot-}$). The additional amount of O_2 initially caused a substantial delay in the recorded oxygen uptake when compared to the NADH oxidation measured spectrophotometrically (Byczkowski, et al., 1979). Eventually, the traces of SOD became overwhelmed and, finally, destroyed by free radicals, and only then the oxygen uptake achieved its fastest rate. Moreover, when NADH oxidation was initiated by high concentrations of $V_{(IV)}$, the reaction was supported by boiled mitochondria, purified cytochrome c oxidase, or even inorganic Cu^{2+} (Crane, 1975; Byczkowski and Zychlinski, 1978; Zychlinski and Byczkowski, 1978; E_0 potential for cytochrome a is +0.29 V, and for Cu^{2+} is +0.15 V).

The proposed mode of action of vanadium on mitochondria is schematically shown in Fig. 5. This scheme is based on the results of our experiments and the evidence presented by other investigators (Byczkowski and Sorenson, 1984). The overall process may be triggered either with $V_{(V)}$ by one-electron reduction or with $V_{(IV)}$ by one-electron oxidation. However, Liochev and Fridovich (1996) have recently proposed that the role of a biological membrane in this process is to produce $O_2^{\cdot-}$, implying that the vanadium redox cycling may be initiated only by $O_2^{\cdot-}$. Their argument does not explain why, in intact plant mitochondria and sonicated rat liver mitochondria, the $V_{(V)}$ -stimulated oxygen uptake is insensitive to rotenone with malate and/or glutamate (+ NAD). The insensitivity of this process to rotenone (which in mitochondria blocks the respiratory chain at the site before ubiquinone, and keeps flavin-dependent dehydrogenases in the reduced state but prevents the leakage of $O_2^{\cdot-}$) rules out the involvement of $O_2^{\cdot-}$ in

the $V_{(v)}$ stimulated mitochondrial oxygen uptake at least in liver and plant mitochondria (Byczkowski et al., 1979).

SECTION 4

EFFECTS ON MICROSOMES

Analogous to the process described above for mitochondria, the addition of $V_{(V)}$ to the isolated rat hepatic or human placental microsomes, in the presence of NADPH, also evoked a significant increase in the oxygen uptake and NADPH oxidation rate (Byczkowski et al., 1988; Zychlinski et al., 1991). The experiments performed with the microsomes isolated from the human placenta showed that $V_{(V)}$ and $V_{(TV)}$ trigger lipid peroxidation due to redox cycling, increase oxygen uptake, and cause the depletion of NADPH (Byczkowski et al., 1988). Although the mechanism offered for this phenomenon was criticized by Liochev and Fridovich (1990), to date, it still remains the best possible explanation for the results obtained (Byczkowski and Kulkarni, 1992b).

4.1. Microsomal Lipid Peroxidation

In microsomes from human tissue, vanadyl and vanadate triggered lipid peroxidation due to redox cycling and formation of the reactive peroxy-vanadyl complex with superoxide (Byczkowski et al., 1988). On the basis of our experiments and the evidence presented by other workers, the following mechanism was postulated (Byczkowski et al., 1988): 1. Vanadyl_(TV) oxidizes nonenzymatically, generating $V_{(V)}$ and superoxide ($O_2^{\cdot-}$)(a); 2. $V_{(V)}$ with superoxide forms instantaneously a peroxy-vanadyl complex ($V_{(V)}-OO^{\cdot}$) (b); 3. The peroxy-vanadyl complex attacks microsomal polyunsaturated lipid (L-H), abstracting hydrogen atom and initiating lipid peroxidation (g). The overall process (Fig. 4) leads to the vanadium redox cycling, consumption of O_2 , depletion of NAD(P)H, and destruction of microsomal lipids and represents a source of reactive oxygen species.

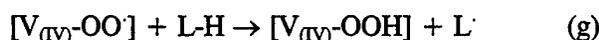
SECTION 5

CARCINOGEN CO-OXYGENATION

In several target tissues, vanadium is very likely to undergo one-electron redox cycling and initiate lipid peroxidation (Byczkowski et al., 1988). Under these prooxidant conditions, aromatic hydrocarbon co-pollutants such as benzo(a)pyrene (B(a)P), may be subjected to co-oxygenation and activation to their reactive intermediates that bind to the vital macromolecules (Dix and Marnett, 1983). This process of co-oxygenation was observed earlier by Byczkowski and Gessner (1987 a-d) during the interaction of B(a)P with asbestos and/or catalytically reactive iron in mouse liver microsomes. The results of the experiments with isolated lipoxygenase supplemented with linoleic acid suggested an involvement of lipid peroxyl and other free radical products of lipid peroxidation in activation of B(a)P-7,8-dihydrodiol to the ultimate muta- and carcinogenic epoxide (Byczkowski and Kulkarni, 1989, 1990a, 1992a). Therefore, co-occurrence of vanadium and benzo(a)pyrene as environmental pollutants raises a major concern for the possibility of synergistic interaction between them (Byczkowski and Kulkarni, 1990b).

5.1. Polyunsaturated Fatty Acid Peroxidation

In the air saturated incubation medium containing polyunsaturated fatty acid, the autooxidation of $V_{(IV)}$ initiates lipid peroxidation, analogous to that described above for microsomes (Zychlinski et al., 1991). The reaction can be partially inhibited by chain-breaking antioxidant (nor-dihydroguaiaretic acid, NDGA) and accelerated by pre-formed hydroperoxides (Byczkowski and Kulkarni, 1990b). Under identical conditions, $V_{(V)}$ was without effect. Apparently, the reactive intermediate responsible for initiation of lipid peroxidation was not a pentavalent vanadium itself, but rather its complex with O_2^- (peroxy-vanadyl):



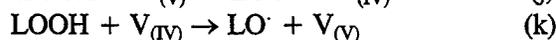
The generation of lipid-derived free radicals was sufficient to propagate lipid peroxidation in the linoleic acid system *in vitro*:



The next step in the process is expected to yield lipid hydroperoxide:



However, the endproducts of $V_{(IV)}$ -initiated peroxidation of linoleic acid did not contain linoleate hydroperoxide (Byczkowski and Kulkarni, 1992b). Therefore, it was postulated that vanadium depletes lipid hydroperoxides as shown in the following reactions:



Both reactions (j) and (k) are not dependent on superoxide ion radicals; they generate reactive lipid

alkoxyl and peroxy radicals and may further propagate lipid peroxidation (Fig. 6). It is believed that lipid-derived epoxy-peroxy radicals serve as the ultimate oxidants in the co-oxygenation of B(a)P-7,8-dihydrodiol (Hughes et al., 1989). Understanding of these reactions is crucial for explanation of the limited sensitivity of the vanadium-initiated B(a)P and its 7,8-dihydrodiol co-oxygenation to the inhibition by SOD (Byczkowski and Kulkarni, 1992b).

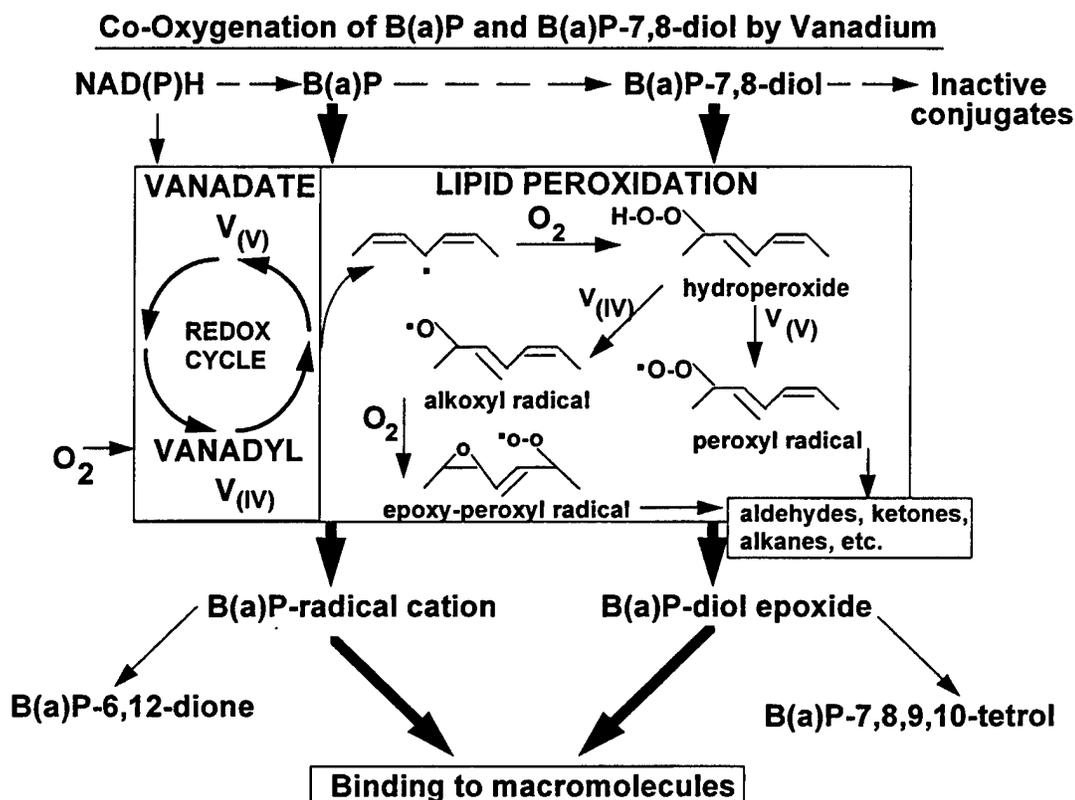


Figure 6. A summary scheme of the coupling between vanadium-initiated lipid peroxidation and benzo(a)pyrene (B(a)P) metabolism. Broken lines depict normal microsomal B(a)P metabolism, which may be inhibited by lipid peroxidation and vanadium redox cycling. Thick arrows depict activation of B(a)P and its 7,8-dihydrodiol epoxide by co-oxygenation (according to Byczkowski and Kulkarni, 1992b; 1994).

5.2. Benzo(a)pyrene Co-oxygenation

Several transition metal cations affect oxygen activation and influence the B(a)P metabolism in mammalian tissues (Byczkowski and Gessner, 1987 a,b,c,d). For instance, there is a growing body of evidence that iron-initiated lipid peroxidation may enhance the formation of the ultimate carcinogenic B(a)P metabolite (Byczkowski and Kulkarni, 1994). Byczkowski and Gessner (1987 a,c) have shown that reactive oxygen species involved in the peroxidation of hepatic microsomal lipids (reviewed by Byczkowski and Gessner, 1988) change the balance between bioactivation and conjugation of B(a)P metabolites, causing an accumulation of the B(a)P-7,8-diol epoxide and other intermediates capable of binding to macromolecules (Fig. 6). Further studies conducted on mouse liver microsomes (Byczkowski and Gessner, 1987 c,d) suggested that even the trace amount of iron present in the asbestos fibers can activate B(a)P under conditions of oxidative stress. Experimental data also showed that vanadium redox cycling effected a dose-dependent co-oxygenation of B(a)P and its 7,8-dihydrodiol thereby increasing

the generation of metabolites capable of interaction with macromolecules (Byczkowski and Kulkarni, 1992b).

SECTION 6

EFFECTS ON SIGNAL TRANSDUCTION

At the tissue level the extracellular signals conveyed by hormones, growth factors, cytokines, etc., are transmitted to the cellular nucleus by a network of protein intermediates which are sequentially phosphorylated in response to the extracellular signaling molecule. The cellular system involved in signal transduction consists of transmembrane receptors, protein kinases, phosphoprotein phosphatases, transcription factors, and the regulatory or promoter regions of genes. Several oxidants can cause deregulation of signal transduction and simultaneous activation of protein kinase C and some transcription factors (e.g., activation protein-1 or AP-1, and NF κ B; Klaassen, 1996). In this regard, it is interesting to note that the oxidative stress induced by redox cycling of naphthoquinones or orthovanadate was found to result in the activation of a membrane-associated phosphatidylinositol kinase through the tyrosine-protein phosphorylation (Chen et al., 1990).

The regulation of protein phosphorylation represents a balance between protein kinase and protein phosphatase activities (Fischer et al., 1991). The SH2 domain (src homology 2 domain) binds to phosphotyrosine residues. The SH2 domain, containing phosphotyrosyl protein phosphatases (PTPases), causes down-regulation of growth-factor receptor-initiated tyrosine phosphorylation and down-stream signal transduction pathways. The PTPases have dual specificity, hydrolyzing not only phosphotyrosyl esters, but also phosphoseryl and phosphothreonyl esters. These PTPases are involved in the regulation of cell proliferation and modulation of cell cycle (Byczkowski and Channel, 1996). Inhibition of receptor dephosphorylation and stimulation of MAP kinase activity by pro-oxidant chemicals may lead to activation of p21^{ras} and the transmission of the signal to the nucleus. This mechanism was enhanced significantly by the depletion of intracellular GSH (Lander et al., 1995).

6.1. Inhibitor of Protein Tyrosine Phosphatase

Vanadium has been shown to inhibit, *in vitro*, several enzyme systems: ATPases, adenylate kinase, phosphofructokinase, glucose-6-phosphate dehydrogenase, ribonuclease, squalene synthetase, and PTPases (see review by Nechay, 1984; and references therein). Among these, the inhibition of (Na⁺, K⁺)-ATPase by vanadate (Rifkin, 1965) has drawn the most attention. Vanadate can replace phosphate as a substrate for glyceraldehyde 3-phosphate dehydrogenase, which leads to the formation of the unstable analogue of 1,3-diphosphoglycerate (Simons, 1979). As a phosphate analog, vanadate at (sub)micromolar concentrations inhibits several other phosphatases and ATPases such as (Ca²⁺, Mg²⁺)-ATPase, (H⁺, K⁺)-ATPase, dynein ATPase, etc. (Nechay, 1984; Jandhyala and Hom, 1983; Shimizu, 1995), and thus interferes with the phosphate transfer or release reactions. On the other hand, vanadyl V(IV) seems not to affect the (Na⁺, K⁺)-ATPase, but it inhibits alkaline phosphatase activity (Simons, 1979).

Vanadate and pervanadate (vanadyl hydroperoxide) are potent protein tyrosine phosphatase inhibitors (Fig. 7). Pervanadate may be produced, *in vitro*, in the reaction of orthovanadate with hydrogen peroxide (c) (Ianzu et al., 1990). However, in the intracellular compartments with reductive environments, it is very likely to undergo the redox cycling, yielding a family of reactive and free-radical compounds (Fig. 4). It was demonstrated that an alteration of the intracellular redox balance by decreasing the GSH levels selectively increases protein tyrosine phosphorylation, while it does not alter the serine/threonine phosphorylation (Staal et al., 1994). This effect was accounted for by selective effects on redox-sensitive

protein tyrosine phosphatase. Typically, PTPases contain nucleophilic cysteinyl residue in their catalytic center (Stone and Dixon, 1994). Their enzymatic activities are rapidly inhibited by small disulfides (Ziegler, 1985). It seems that the cysteinyl residue must be kept in the reduced -SH form, therefore, thiol-directed reagents that oxidize it cause inhibition of PTPases (Fischer et al., 1991). It is therefore possible that, at millimolar concentrations, vanadium can affect intracellular redox potential and can lead to the inhibition of PTPase activity by causing oxidation of essential cysteinyl thiols. This pro-oxidant action of $V_{(V)}$ is independent of its interference with phosphate groups and, thus, it seems that at least two independent sites of inhibition should exist within the protein tyrosine phosphatase for $V_{(V)}$ and other pro-oxidants (Hecht and Zick, 1992). Accordingly, it was suggested that $V_{(V)}$ induced the expression of two categories of genes in mouse C127 cells by two separate mechanisms (Yin et al., 1992). The stimulation of the synthesis of mRNA of c-jun was dramatically enhanced by either NADH or H_2O_2 and partly inhibited by catalase, suggesting the involvement of vanadium redox cycling, while the vanadate-stimulated synthesis of mRNA of actin and c-Ha-ras was unaffected by oxidants, reductants, or antioxidants.

Dose-Dependent Effects of Pervanadate on PTPase Activity

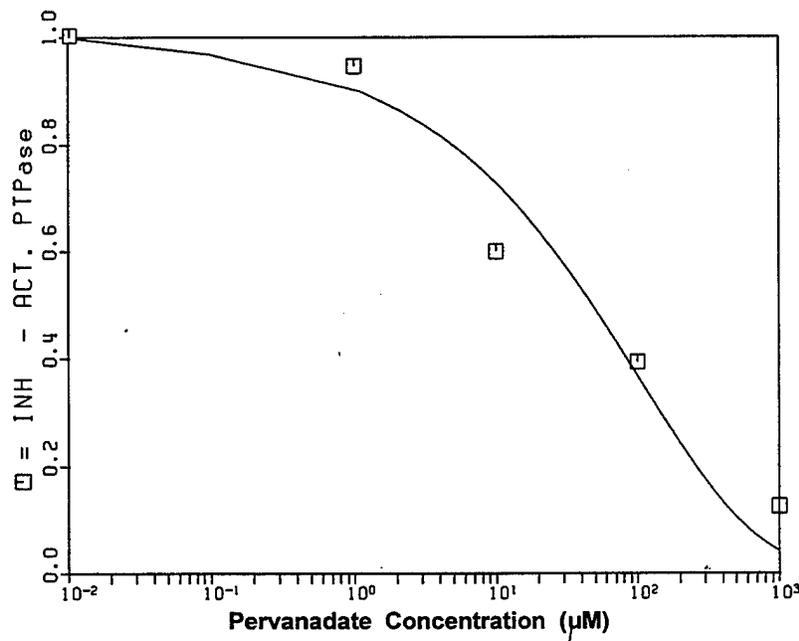


Figure 7. Dose-dependent effects of vanadate in the presence of excess H_2O_2 (pervanadate) on PTPase activity in intact rat hepatoma (Fao) cells. The curve is a computer-generated result of simulation with computer program (BBPD computer program developed by Byczkowski and Flemming, 1996, based on Heffetz et al. 1990). The small squares depict experimental data from Heffetz et al. (1990).

6.2. Inhibition of Receptor and Phosphoprotein Dephosphorylation

Increased phosphorylation of several soluble proteins and membrane receptors by vanadate and pervanadate was reported in diverse biological systems (Heffetz et al., 1990; Purushotham et al., 1995; Rokhlin and Cohen, 1995; Haque et al., 1995; Lin and Grinnell, 1995; Yamaguchi et al., 1995). Similar stimulatory effects on receptor phosphorylation status and enzymatic protein phosphorylation also were reported for other pro-oxidants (Tan et al., 1995). On the other hand, at high concentrations free radicals generated by the pro-oxidants caused nonspecific crosslinking, polymerization, and/or fragmentation of proteins, changes in membrane fluidity, and a decrease in the number of binding sites and/or binding affinity for specific ligands. In macrophages, $V_{(V)}$ reduced their ability to interact with interferon γ as well as other cytokines, while increased their basal spontaneous release of O_2^- and H_2O_2 (Cohen et al., 1996).

6.3. Nonspecific Interactions

Paradoxically, under certain conditions vanadate was shown to stimulate Ca^{2+} -ATPase from pig heart sarcoplasmic reticulum (Erdmann et al., 1984). Reportedly, toxic doses of vanadium stimulated activity of monoamine oxidase (see review by Jandhyala and Hom, 1983; and references therein) and also the secretion of pancreatic enzymes (Proffitt and Case, 1984a, b).

Vanadate, at reasonably low concentrations, enhanced adenylyl cyclase activity in several cell membrane preparations, thereby increasing the levels of cyclic AMP (Erdmann et al., 1984). This mechanism seems responsible for the secretion of Cl^- , Na^+ and water in rat jejunum (Hajjar et al., 1986). However, in some isolated multicellular model systems like toad skin or bladder, vanadate inhibited effects of cyclic AMP on osmotic water flow (Nechay, 1984). Also, both vanadate and vanadyl *in vitro*, decreased cyclic AMP production in the luteinizing hormone-treated rat corpora lutea and the involvement of vanadium-stimulated tyrosine phosphorylation in this process was proposed (Lahav et al., 1986). Involvement of tyrosine kinase stimulation was also suggested in pervanadate-stimulation of respiratory burst in neutrophils (Yamaguchi et al., 1995). Therefore, it seems that stimulation of tyrosine phosphorylation of phospholipase C, activation of phospholipase C, increased production of diacylglycerols (DAG), and possibly, protein kinase C and/or tyrosine kinase activation (Yamaguchi et al., 1995) may be all involved in nonspecific effects of high concentrations of $V_{(V)}$ on several cellular systems, analogous to more specific action exerted, for instance, by a classical tumor promoter, phorbol acetate (Byczkowski and Channel, 1996). On the other hand, drastic oxidation of purified protein kinase C damaged both regulatory and catalytic domains (Ramasarma, 1990) which may cause inactivation of the protein kinase C-dependent signal transduction *in vivo*.

SECTION 7

CONCLUSIONS

Although in a trace amount vanadium may be essential to normal metabolic functions of the organism, excessive exposure to this element is potentially deleterious, and may even be toxic. Due to its complex chemistry and ability to undergo a free radical redox cycling, vanadium may adversely affect several biochemical functions and is capable of causing oxidative stress, co-oxygenating carcinogens, and interfering with signal transduction. The results reported in the literature are sometimes based on a treatment with a single dose or *in vitro* experiments in the presence of just one concentration of vanadium. A very few systematic dose-response studies have been conducted with vanadium to date. Clearly, more experimental data are necessary to assess fully its toxicological potential and a possible health hazard from exposure to vanadium compounds.

7.1. Future Research Needs

A systematic pharmacokinetic study should be conducted on vanadium absorption, distribution, metabolism, and disposition. The development of a pharmacokinetic/dynamic model should be very helpful to understand the kinetics and toxicity of vanadium administered by different routes (analogous to the pharmacokinetic model described for chromium by O'Flaherty, 1993, 1996). Currently, there is no information available about the microcompartmentalization and the redox state of vanadium in different subcellular fractions of the target tissues. More experimental data are necessary to understand the mode of action of vanadium on the cellular signal transduction pathway and DNA transcription as well as carcinogenicity, if any.

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