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One Electron Reduction of Vanadate(V) to Oxovanadium(IV) by Low-Molecular-Weight Biocomponents Like Saccharides and Ascorbic Acid: Effect of Oxovanadium(IV) Complexes on pUC18 DNA and on Lipid Peroxidation in Isolated Rat Hepatocytes

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One electron reduction of vanadate(V) to vanadyl(IV) occurs in the presence of biocomponents, such as, saccharides and L-ascorbic acid under physiological conditions. The vanadyl(IV) complexes thus generated have been demonstrated to introduce nicks in pUC18 DNA in the absence or in the presence of hydrogen peroxide and also exhibit lipid peroxidation in isolated rat hepatocytes. Thus the reducing and complexing abilities of these molecules may help in understanding the in vivo reduction and the resultant toxicity to cells incubated with vanadate(V). © 1996 Academic Press, Inc.

Vanadate(V) and vanadate-containing compounds exert potent toxic effects on a wide variety of biological systems [1]. In vitro studies have shown mutations and DNA-protein crosslinks induced by metavanadate in cultured mammalian cells [2]. Exogenously added vanadate, after permeating into cell interior, is effectively reduced to vanadyl (VO^{2+}) in the presence of a number of reducing agents common to cells like L-ascorbic acid, glutathione, cysteine etc [3]. Effect of such vanadyl species on activities of various cellular components have not been enough documentated. However, saccharides, on the other hand are widespread in nature and have been shown to effectively reduce and complex various biologically important transition metal ions [4,5]. Therefore in this communication we report the results of reduction of vanadate(V) by multihydroxy molecules such as saccharides and ascorbic acid. The oxovanadium(IV) complexes thus formed are studied for their interaction with pUC18 DNA and their effects on lipid peroxidation in isolated rat hepatocytes.

EXPERIMENTAL

$VO(L\text{-asc})_2$, **1;** $Na_2[VO(L\text{-asc})_2]$, **2;** $VO(L\text{-asc})(H_2O)_2$, **3;** $Na_2[VO(D\text{-Glc})_2]$, **4;** $Na_2[VO(D\text{-Fru})_2]$, **5;** $Na_3[VO(D\text{-Man})_2]$, **6;** $Na_4[VO(L\text{-Sor})_2]$, **7;** $Na_2[VO(D\text{-Xyl})_2]$, **8** and $Na_4[VO(D\text{-Rib})_2]$, **9** were synthesised and characterized as described elsewhere [5].

Methods and Materials

pUC18 DNA (Cesium chloride purified) was obtained from Bangalore Genei, India. Agarose, HEPES buffer and ethidium bromide were purchased from Sigma Chemical Co (USA). L-Ascorbic acid and saccharides were from Aldrich (USA) and all other chemicals were from local sources and used as received.

DNA interaction studies. The interaction of oxovanadium complexes **1-5, 8** and **9** in 1mM final concentration with pUC18 DNA and these in the presence of 4mM H_2O_2 was carried out. All the experimental details are as given earlier [4d, e].

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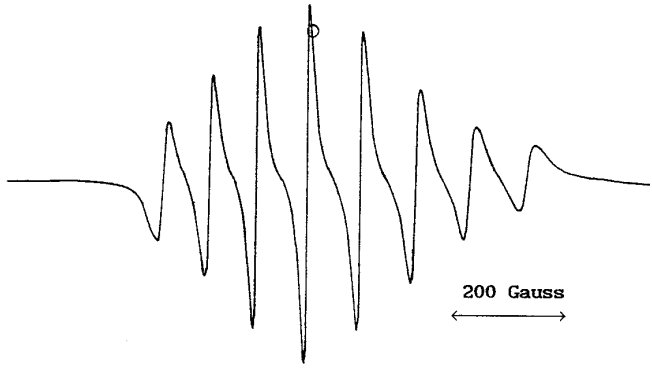

FIG. 1. X-band EPR spectrum of complex **9** in water at room temperature. Circle indicates field marker (TCNE = 3380 Gauss, g=2.00277). Instrument settings: Time constant = 0.250sec; modulation frequency = 100KHz; modulation amplitude = 2G; microwave power = 10mW; microwave frequency = 9.51GHz.

Preparation of isolated rat hepatocytes. The hepatocytes from male Wistar strain rats (200-300g body weight) were isolated and cultured according to the procedure described by Williams et al [6] with some modifications [7].

Determination of lipid peroxidation. Twenty four hours after plating, the cells were rinsed twice with salts-glucose medium (SGM; 50mM HEPES, pH 7.2 with 100mM NaCl; 5mM KCl; 2mM $CaCl_2$; and 5mM glucose), and the cells were then treated with vanadium compounds **1-5, 7** in the concentration range 125-1000μM at 37 °C in this maintainence medium. Following treatement with vanadium, cells were rinsed twice with SGM and then dislodged from the dishes by scraping. The scrapped cells were resuspended in ice cold PBS and sonicated. They were pulsed for 1min and the lipid peroxidation was assayed according to the thiobarbituric acid (TBA) method by Buege and Aust [8]. As an indicator of lipid peroxidation, amounts of TBA reactive substances (TBARS) were expressed in terms of nmol malondialdehyde (MDA)/mg protein. Proteins were measured by the method of Lowry et al [9], using bovine serum albumin as the standard. All the results are expressed as means and standard errors of 3 or 4 experiments.

RESULTS

Vanadyl(IV) generation from the reaction of sodium orthovanadate with hexoses, pentoses and L-ascorbic acid in aqueous medium. The reduction of vanadate as sodium orthovanadate was carried out with the hexoses: D-glucose (D-Glc), D-fructose (D-Fru), D-mannose (D-Man), L-sorbose (L-Sor) and the pentoses: D-xylose (D-Xyl), D-ribose (D-Rib); as well as L-ascorbic acid (L-asc) in water or in phosphate buffer at near neutral pH (7.4-8.0). Three ratios of metal:ligand, 1:4, 1:8 and 1:16 reactions were carried out and the time taken for reduction with each ligand was monitored by UV-visible studies through following the increase in the intensities of two d-d bands at 676$\pm$10 nm and 510$\pm$10 nm [5]. The rates of the relative reducing ability of these molecules towards vanadate was calculated as per the procedure reported earlier [4a,b]. Based on these values the relative reducing abilities are found to be: L-Asc $\gg$ D-Fru $>$ D-Rib $\geqslant$ L-Sor $>$ D-Xyl $>$ D-Man $\gg$ D-Glc. The EPR of the final solutions exhibited an isotropic eigth line spectrum characteristic of oxovanadium(IV) species [5,10] as shown for complex **9** in Fig. 1.

Synthesis and isolation of oxovanadium(IV) complexes of saccharides and ascorbic acid. The vanadyl(IV) complexes have been synthesised, isolated in the solid state from methanol and characterized [5]. These were found to be identical to the ones generated in aqueous medium possessing formulae given in experimental section and were examined for their effect on pUC18 DNA and on lipid peroxidation.

DNA nicking in pUC18 mediated by oxovanadium(IV) complexes 1-5, 8 and 9. Incubation of ascorbate complexes **1-3** with pUC18 DNA (90% form I) showed different extents of cleavage for each complex as shown in Fig 2. Typical agarose gel photograph showing the DNA nicking by complex **1** under different conditions is shown as an inset in Fig 2. Complexes

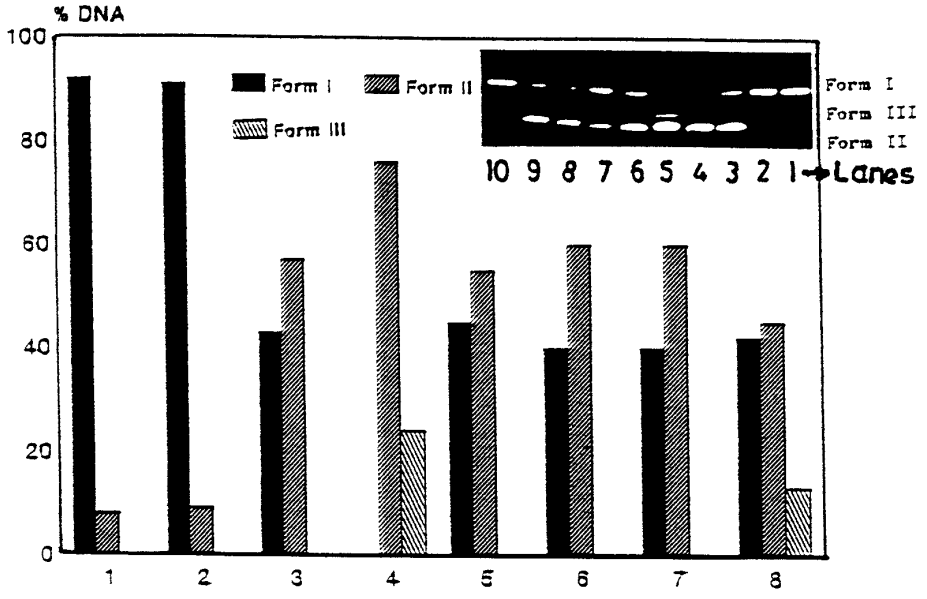

FIG. 2. Histogram depicting the nicking of pUC18 DNA in the presence of oxovandium(IV)-ascorbate complexes **1–3.** Lanes (1), DNA; (2), DNA + H_2O_2; (3), lane 1 + complex **1;** (4), lane 3 + H_2O_2; (5) lane 1 + complex **2;** (6) lane 5 + H_2O_2; (7) lane 1 + complex **3;** (8), lane 7 + H_2O_2. (Inset) Typical 0.8% agarose gel photograph showing the effects of vanadyl complexes **1** and **9** on pUC18 DNA. Lanes: (1), DNA; (2), DNA + H_2O_2; (3), lane 1 + complex **1;** (4), lane 3 + H_2O_2; (5) lane 4 + ME; (6) lane 4 + NaN_3; (7), lane 1 + complex 9; (8), lane 7 + H_2O_2; (9) lane 8 + ME; (10) lane 8 + NaN_3.

1-3 generated form II by a single nick in the plasmid DNA. Addition of H_2O_2 greatly increased the nicking ability of these complexes. While complex **1** showed no form I, 76% form II and 24% form III, complex **2** showed 40% form I, 60% form II and **3** showed 42% form I, 45% form II and 13% form III respectively, in presence of 4mM H_2O_2. However, addition of mercaptoethanol did not have any effect on DNA nicking. In case of complex **3** at 2mM concentration and in presence H_2O_2, a smear was seen on the gel indicating extensive DNA nicking. Addition of 0.1M NaN_3 inhibited the formation of form III, though form I and form II were generated to the same extent. Addition of 0.2M Mannitol did not effect the formation of form III, in case of complex **1**. This indicates that the generation of singlet oxygen occurs in the presence of vanadyl diascorbate complex **1,** and peroxide, probably by a psuedo-Fenton reaction. The DNA cleaving ability of these complexes in the absence of added peroxide is due to the generation of ascorbate radicals, which are formed in the presence of transition metal ions [3b]. As expected, the neutral complexes **1** and **3** showed greater interaction with DNA as compared to the anionic complex **2**.

Saccharide complexes **4, 5, 8** and **9** did not show appreciable cleavage of plasmid DNA in the absence of H_2O_2. The different amounts of forms I and II in the presence of complex and H_2O_2 (wherever indicated) are shown in Fig 3. Inset of Fig 2 also shows the DNA nicking activity of complex **9** under different conditions. However no form III was seen even in the presence of peroxide. Addition of 0.1M NaN_3 to an aliquot containing DNA, complex and hydrogen peroxide completely inhibited the nicking effects of these complexes, thus indicating the involvement of active oxygen species ($^1O_2$) in the DNA nicking ability of these complexes in presence of peroxide. Mannitol (0.2M) was found to inhibit the nicking to some extent indicating the involvement of hydroxyl radicals also.

Effect of vanadyl complexes 1-5 and 7 on lipid peroxidation. Lipid peroxidation *in vivo* is

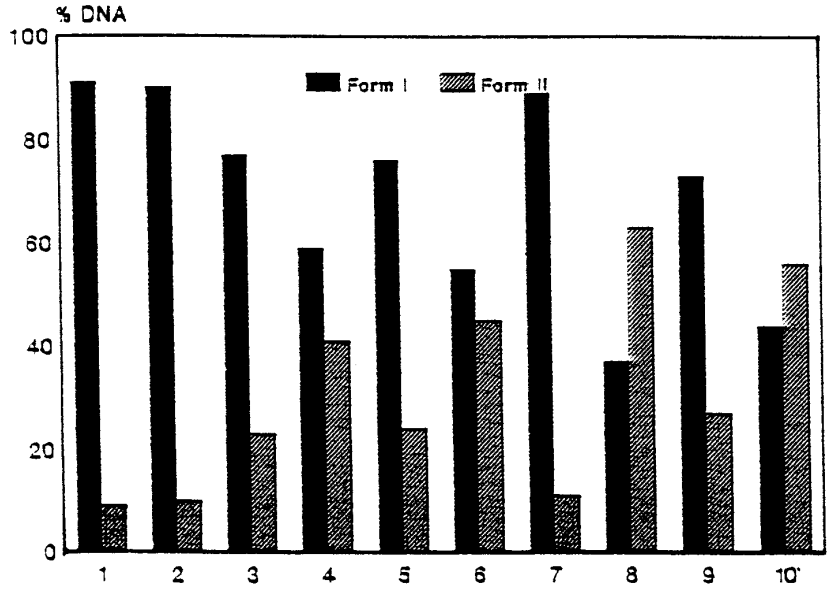

FIG. 3. Histogram depicting the nicking of pUC18 DNA in the presence of oxovandium(IV)-saccharide complexes **4, 5, 8** and **9.** Lanes: (1), DNA; (2), DNA + H_2O_2; (3), lane 1 + complex **4;** (4), lane 3 + H_2O_2; (5) lane 1 + complex **5;** (6) lane 5 + H_2O_2; (7) lane 1 + complex **8;** (8), lane 7 + H_2O_2. (9) lane 1 + complex **8;** (10), lane 9 + H_2O_2.

thought to cause membrane damage and to play an important role in the induction of cellular and/or tissue injuries induced by various chemicals including metal ions [12]. Since oxovanadium(IV) is one of the chemical form of vanadium in the cell, we have studied the effect of various vanadyl complexes on isolated rat hepatocytes for inducing lipid peroxidation. The data is presented in Fig. 4. In concentration range studied, 125-1000μM, the vanadyl complexes produced an increase in TBARS. The neutral ascorbate complexes **1** and **3** showed highest activity of lipid peroxidation.

DISCUSSIONS AND CONCLUSIONS

We have demonstrated that V(IV) can be generated under physiological conditions, from vanadyl salts and vanadate reductions. It has been suggested that ascorbic acid reduces V(V) to V(IV) and generates species possessing high oxidizing potential [12]. Such vanadyl complexes can potentially be strong oxidants generated in vivo having the ability to cause single strand DNA nicks by generation of active oxygen species like $^1O_2$ and also exhibit lipid peroxidation. Free radicals generated from lipid hydroperoxides have been demonstrated to cause damage to cellular systems [11]. It has been previously demonstrated that strong oxidants like peroxynitrite can cause DNA nicks [13] and apoptosis in rat thymocytes [14]. Recently an insulin mimicking oxovanadium(IV) complex has been demonstrated to induce B cell apoptosis [15]. Thus the toxicity in cells treated with vanadate might well be due to its **reduction** *in vivo* by cellular components and its concomitant effect on DNA, lipid peroxidation and apoptosis.

The saccharide and ascorbate complexes were found to be hydrolytically and oxidatively stable in solution for over a period of 48h as studied using UV-vis and EPR [5], although various other complexes as VO^{2+} are known to be easily oxidized to vanadates in solution [1]. We have recently demonstrated that these oxovanadium(IV) complexes can inhibit protein

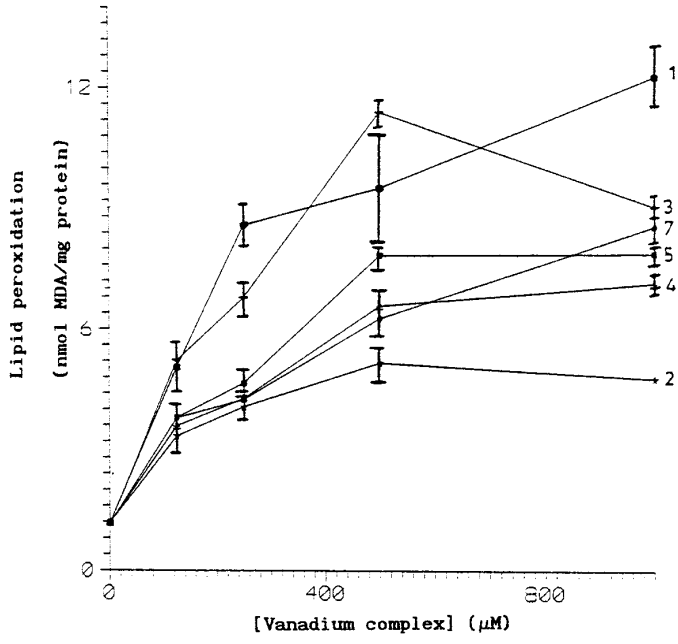

FIG. 4. Effect of oxovanadium(IV) complexes **1–5** and **7** on lipid peroxidation in isolated rat hepatocytes, as a function of concentration. Numbers indicate the compound numbers (see text).

synthesis in heme-defecient and heme supplemented rabbit reticulocyte lysates [16]. Efforts are in progress for bringing a correlation between the cytotoxicity, lipid peroxidation, *in vivo* DNA single strand breaks and apoptosis with these vanadyl complexes. While it appears that vanadium containing compounds may exhibit toxicity via initiation of lipid peroxidation, such results are scanty. Our results are the first of its kind in the literature to demonstrate that vanadyl(IV) complexes, especially those generated in presence of ascorbic acid, exhibit substantial single strand breaks in DNA and cause lipid peroxidation.

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