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Vanadium induced hemolysis of vitamin E deficient erythrocytes in Hepes buffer

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Abstract. Several vanadium compounds were tested for their ability to induce in vitro hemolysis of vitamin E-deficient hamster erythrocytes. Free vanadyl caused hemolysis in Hepes buffer but not in Tris or phosphate buffer, while hemolysis was inhibited by catalase, chelators such as deferoxamine mesylate and EDTA, and hydroxyl radical scavengers such as ethanol and D-mannitol. Although metavanadate itself could not induce hemolysis, metavanadate with NAD(P)H caused hemolysis in Hepes buffer only, and superoxide dismutase prevented it. Hydrogen peroxide, hydroxyl radical and Hepes radical were involved in vanadyl-induced hemolysis; superoxide anion was further involved in metavanadate plus NAD(P)H-induced hemolysis. Vitamin E prevented hemolysis under both conditions.
Key words. Vanadyl; metavanadate; vanadium; hemolysis; Hepes; vitamin E; deferoxamine mesylate.

Vanadium is a trace mineral probably essential for nutrition[1], as well as being an anthropogenic toxic metal produced by the burning of fossil fuels[2]. Vanadium shows various physiological and pharmacological properties such as an insulinomimetic effect[3–5]. It has three oxidation states in natural environments: as V(III) in sediments and rocks, and as V(IV) and V(V) in anoxic and oxic water, respectively[6]. In blood plasma it is present as V(V) anion, such as metavanadate, and intracellularly as V(IV) such as vanadyl cation. Absorbed vanadate or metavanadate is reduced to vanadyl, and intracellular vanadyl is associated with organic ligands such as iron-binding proteins and phosphate compounds[7].

Retention of orally-fed vanadium by higher animals is highly variable, but it is particularly accumulated by the kidneys and bones[8–10]. Vanadate is an inhibitor of (Na, K)-ATPase and the accumulation of vanadium in the kidneys causes renal disorders[2, 11, 12]. Vanadyl is the most effective form for inducing in vivo and in vitro hemolysis in mice[13]. Hydroxyl radicals are generated in vanadyl-catalyzed breakdown of fatty acid hydroperoxides[14]. Vanadate markedly stimulates the oxidation of NADH by the plasma membrane and this effect is eliminated by superoxide dismutase[5, 15].

Using Tween 20, we have established two hemolytic methods to detect vitamin E-deficiency in various animals[16–18]. The specific hemolysis of vitamin E-deficient erythrocytes is caused by radical species. If the toxicity of vanadium is related to the generation of harmful radical species, the hemolytic test can be used for the identification of such radicals. The object of the present work was to determine which form of vanadium might be involved in hemolyzing vitamin E-deficient hamster erythrocytes in vitro and to find protective agents against such vanadium-induced hemolysis in order to understand the underlying mechanism.

Materials and methods

The following chemicals used in experiments were obtained from the companies indicated: NAD, dithiothreitol, Hepes, superoxide dismutase (bovine serum) from Boehringer Mannheim Co.; NADH, NADP from Oriental Yeast Co.; EDTA-2Na, VO-EDTA, Tiron, DTPA, PDTS, acetylacetone from Dojindo Lab.; NADPH, deferoxamine mesylate, catalase (bovine liver), superoxide dismutase (human and dog erythrocytes) from Sigma Chem. Co.; D-penicillamine from Aldrich Chem. Co.; catechol from Tokyo Kasei Co.; bovine serum albumin from Nakarai Chem. Co., all other chemicals from Wako Pure Chem. Ind.

Mature male golden hamsters were fed a vitamin E-deficient or a vitamin E-sufficient diet for several months. The diets contained casein 19.0%, corn starch 66.7%, soybean oil 2.5%, linseed oil 2.5%, cellulose powder 5.5% and a vitamin and mineral mixture 3.8%. Vitamins (except vitamin E) and minerals were supplemented according to the NRC feeding standard for rats as shown previously[19]. The vitamin E-sufficient diet contained 100 mg of DL-alpha-tocopheryl acetate per kg. The diets and distilled water were fed freely in an air-conditioned test room.

Blood was collected in heparinized tubes. One volume of blood was diluted with 25 volumes of saline, and the erythrocyte portion obtained by centrifugation was resuspended in the same volume of Hepes buffer (pH 7.4). By measuring the hematocrit, the final erythrocyte concentration was adjusted to 0.04% of the total incubation

medium. Protocols for standard hemolysis[17], H_2O_2-induced hemolysis and vanadium-induced hemolysis were adopted. In the standard procedure the final concentration of Tween 20, L-ascorbic acid and sodium azide were 0.001%, 1.16 mM and 5.26 mM, respectively, and the erythrocyte suspension was incubated for 30 min at 37.5 °C. In H_2O_2-induced hemolysis the final concentration of H_2O_2 added was 5 mM and the incubation time at 37.5 °C was 1 h. For vanadium-induced hemolytic procedures, the final concentrations of chemicals used and the incubation time at 37.5 °C are shown in each result section. In some experiments the incubation time was prolonged as the severity of the vitamin E deficiency state became diminished.

Absorbances of the hemoglobin-containing supernatants released from the lysed erythrocytes were read at 540 nm using a Gilford spectrophotometer (Model 240). Hepes buffer (pH 7.4) contained 25 mM Hepes (Good's buffer) and 125 mM NaCl. Other buffers used were Tris buffer (pH 7.4) containing 25 mM Tris and 125 mM NaCl, and phosphate buffer (pH 7.4) containing 0.1 M phosphate and saline at a ratio of 1:1. Plasma vitamin E was determined by measuring the fluorescence of plasma hexane extract at 295 nm excitation and 340 nm emission as shown previously[18].

Data were analyzed by one-way analysis of variance using a statistical computer program (StatView's ANOVA, Abacus Concepts, Inc., Berkeley, CA, USA 1987), and Fisher's PLSD method was used to assess the significant differences between treatment groups. The level of significant difference was set at $p < 0.05$ throughout the experiments.

Results

The average plasma vitamin E concentrations in vitamin E-deficient and vitamin E-sufficient animals were 0.87 and 4.52 μg/ml, respectively ($p < 0.01$). The average percentages hemolysis of vitamin E-deficient and vitamin E-sufficient erythrocytes by the standard hemolytic procedure were 75.0 and 3.5%, respectively ($p < 0.01$). The average percentages of H_2O_2-induced hemolysis of vitamin E-deficient and vitamin E-sufficient erythrocytes were 78.3 and 3.1%, respectively ($p < 0.01$).

Table 1 shows the effects of various vanadium compounds on the hemolysis of vitamin E-deficient erythrocytes in Hepes buffer. Each vanadium compound was added to a final concentration of 1 mM and incubated at 37.5 °C for 1 h. Only free vanadyl ($VOSO_4$, +4 oxidation state) caused hemolysis in vitamin E-deficient erythrocytes, which was similar to both the standard and H_2O_2-induced hemolysis, whereas VCl_3 (+3 oxidation state), metavanadate ($NaVO_3$, +5 oxidation state) and vanadyl-EDTA complex (VO-EDTA, +4 oxidation state) did not. Vitamin E prevented vanadyl-induced hemolysis.

Table 1. Effects of vanadium compounds on hemolysis of hamster erythrocytes in Hepes-saline buffer.

	Mean	SD
E-deficient RBC		
VCl_3	5.3[a]	0.5
$VOSO_4$	82.0[b]	2.6
VO-EDTA	6.5[a]	0.6
$NaVO_3$	6.3[a]	0.3
E-sufficient RBC		
$VOSO_4$	11.7[c]	0.5

Final concentration of each compound was 1 mM and incubation time was 1 h. Each determination was in triplicate. There were significant differences ($p < 0.05$) between mean values with different superscripts.

Table 2. Effects of added reagents on vanadyl-induced hemolysis of vitamin E-deficient erythrocytes (mean ± SD).

Treatment	Hemolysis (%)
$VOSO_4$	82.1 ± 7.7 (11)
+deferoxamine mesylate	5.1 ± 0.7 (9)*
+EDTA-2Na	7.2 ± 0.2 (3)*
+Tiron	7.0 ± 0.7 (3)*
+DTPA	22.7 ± 2.5 (3)*
+D-penicillamine	95.5 ± 3.2 (3)**
+catechol	11.3 ± 0.4 (3)*
+PDTS	96.8 ± 1.4 (6)**
+acetylacetone	8.3 ± 1.1 (3)*
+L-ascorbic acid	84.3 ± 1.1 (3)
+dithiothreitol	9.0 ± 0.3 (3)*
+catalase	17.4 ± 5.1 (3)*
+superoxide dismutase	97.5 ± 2.9 (6)**
+serum albumin	98.4 ± 1.0 (3)**

Numbers in parentheses are the number of repetitions of each determination. The values with single asterisks were significantly ($p < 0.05$) less than the value (82.1%) of $VOSO_4$-induced hemolysis; the values with double asterisks were significantly ($p < 0.05$) greater than the value of $VOSO_4$-induced hemolysis.

Table 2 shows the effect of various reagents on vanadyl-induced hemolysis of vitamin E-deficient erythrocytes in Hepes buffer. Deferoxamine mesylate, EDTA-2Na, Tiron, DTPA, catechol, acetylacetone, dithiothreitol and catalase prevented hemolysis, while D-penicillamine, PDTS, L-ascorbic acid, serum superoxide dismutase and serum albumin did not. Deferoxamine mesylate, EDTA-2Na, Tiron, DTPA, catechol and acetylacetone formed complexes with vanadyl and prevented radical formation. Dithiothreitol, a reducing agent, and catalase protected the cell membrane by eliminating radicals.

Vanadyl-induced hemolysis occurred in Hepes buffer (table 1), while in Tris and phosphate buffers no such hemolysis occurred. Figure 1 shows the effect on vanadyl-induced hemolysis of replacing Tris or phosphate buffer with Hepes buffer. The presence of more than 2.5 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) was necessary for inducing the hemolysis in Tris or phosphate buffer, and Tris buffer appeared to be more inhibitory than phosphate buffer.

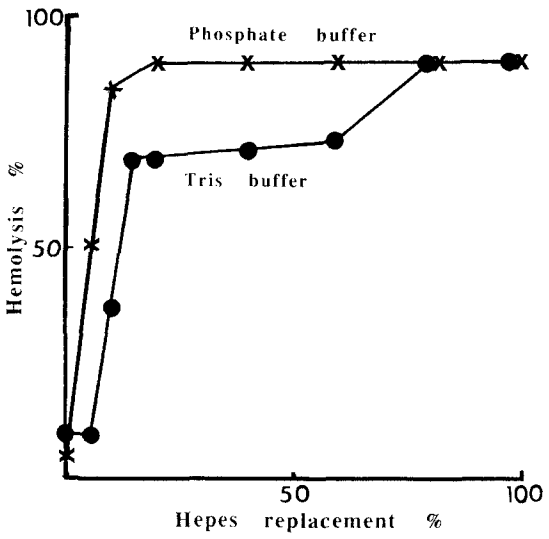

Figure 1. Effect of replacement of phosphate buffer or Tris buffer with Hepes buffer on vanadyl-induced hemolysis of vitamin E-deficient erythrocytes. Zero and 100% of Hepes replacement values indicate only phosphate or Tris buffer and only Hepes buffer, respectively. Experimental conditions were the same as thoese in table 1.

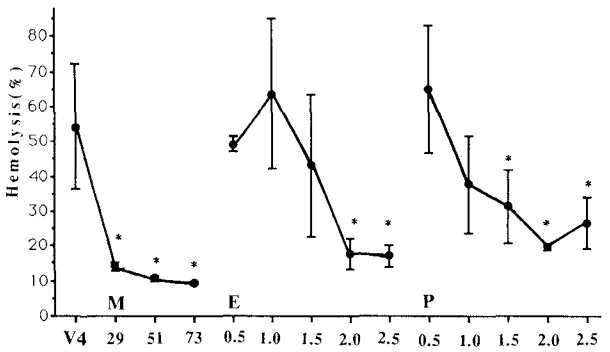

Figure 2. Effects of D-mannitol, ethanol and n-propanol on vanadyl-induced hemolysis of vitamin E-deficient erythrocytes. Final concentrations of D-mannitol (M), ethanol (E) and n-propanol (P) are shown by mM, % and % units, respectively. V4 shows mean and SD (bar) of vanadyl-induced hemolysis with no additions. Determinations were repeated 11 times for the baseline vanadyl-induced hemolysis and 3 times for other treatments. The values with an asterisk were significantly different ($p < 0.05$) from the V4 value. Final concentration of $VOSO_4$ was 1 mM and incubation time was 1.5 h.

Figure 2 shows the effects of hydroxyl radical scavengers such as D-mannitol, ethanol and n-propanol on vanadyl-induced hemolysis. In this experiment the mean vanadyl-induced hemolysis was 54.3%, which was lower than those in tables 1 and 2, because the animals did not become as severely vitamin E-deficient as those in tables 1 and 2. The addition of more than 29 mM D-mannitol, 2.0% ethanol or 1.5% n-propanol to Hepes buffer significantly prevented vanadyl-induced hemolysis, which suggests that hydroxyl radicals are involved in vanadyl-induced hemolysis. Hydroxyl radicals give rise to Hepes radicals, which might play an important role in hemolyzing the erythrocytes.

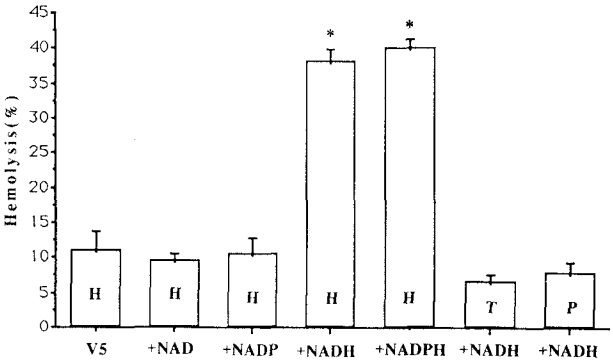

Figure 3. Effect of adding NAD(P) and NAD(P)H on metavanadate (V5)-induced hemolysis. The letters H, T and P indicate Hepes, Tris and Phosphate buffers. Final concentrations of $NaVO_3$, NAD(P) and NAD(P)H were 1 mM and incubation time was 2.5 h. Small bars show SD and asterisks show significant difference ($p < 0.05$) from the V5 value. Each determination was done in triplicate.

Figure 3 shows the effects of adding NAD(P) or NAD(P)H on metavanadate-induced hemolysis. Addition of NAD(P)H to metavanadate in Hepes buffer significantly increased the hemolysis of vaitamin E-deficient erythrocytes ($p < 0.05$), while adding NAD(P) did not. Metavanadate with NAD(P)H induced hemolysis in Hepes buffer, but this effect was not observed in Tris and phosphate buffers.

Figure 4 shows the effects of superoxide dismutase and vitamin E on vanadyl- and metavanadate plus NADH-induced hemolysis. Although superoxide dismutase did not prevent vanadyl-induced hemolysis, superoxide dismutase prevented metavanadate plus NADH-induced hemolysis, which suggested that superoxide anions are involved. Hydrogen peroxide, hydroxyl radicals and

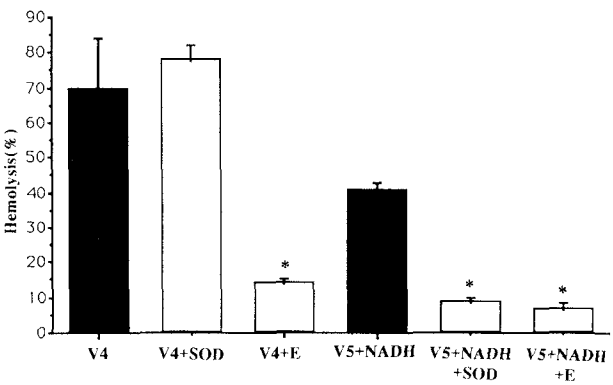

Figure 4. Effects of superoxide dismutase on vanadyl-induced and metavanadate-induced hemolysis of vitamin E-deficient or vitamin E-sufficient erythrocytes. $VOSO_4$, $NaVO_3$ and superoxide dismutase are indicated by V4, V5 and SOD, and vitamin E-sufficiency by E. Small bars show SD. Determinations were repeated 6 times for the hemolysis of vitamin E-deficient erythrocytes, and 3 times for vitamin E-sufficient erythrocytes. Asterisks show significant differences ($p < 0.05$) from the V4 or V5 value. Final concentrations of $VOSO_4$, $NaVO_3$ and NADH were 1 mM. Final concentration of SOD (human and dog erythrocytes) was 30 IU/ml. Incubation times of V4 and V5 were 1.5 and 2.5 h, respectively.

Hepes radicals were involved in vanadyl-induced hemolysis and superoxide anions were further involved in metavanadate plus NAD(P)H-induced hemolysis. Vitamin E prevented not only vanadyl-induced hemolysis but also metavanadate plus NAD(P)H-induced hemolysis.

Discussion

Vanadyl and not vanadate triggers peroxidation of linoleic acid[20]. Vanadyl is readily oxidized to metavanadate in neutral Hepes buffer containing atmospheric oxygen and the oxidation of vanadyl is catalyzed by traces of metal ions which can be removed by chelating agents[21].

Our experiments show that free vanadyl is effective in hemolyzing vitamin E-deficient hamster erythrocytes but a complex of vanadyl with EDTA is not. The autoxidation of vanadyl generated H_2O_2 by the reactions: $V(IV) + O_2 = V(V) + O_2^-$ and $O_2^- + O_2^- + 2H^+ = H_2O_2 + O_2$. The addition of superoxide dismutase did not prevent vanadyl-induced hemolysis (table 2 and fig. 4), which suggests that superoxide anion formation is not rate-limiting[22]. Catalase prevented vanadyl-induced hemolysis (table 2), which suggests that H_2O_2 was generated by the spontaneous dismutation reactions shown above. Added H_2O_2 became a hemolyzing agent at much higher concentration (5 mM) than that (< 1 mM) generated during vanadyl autoxidation. So, H_2O_2 alone could not become a hemolyzing species in vanadyl-induced hemolysis. The H_2O_2 generated could oxidize remaining vanadyl molecules and contributed to the generation of hydroxyl radicals (HO$^\bullet$) by the following reaction that is similar to Haber-Weiss reaction[23]: $V(IV) + H_2O_2 = V(V) + HO^- + HO^\bullet$.

Hydroxyl radicals are extremely reactive and potentially severely damaging in living systems because they can remove an electron from almost any neighbouring organic molecule[24]. However, the hydroxyl radical itself might not induce membrane lipid peroxidation and subsequent hemolysis because of its relatively short half-life. Hydroxyl radicals were quickly contained and eliminated by surrounding Hepes molecules since Hepes is an effective hydroxyl radical scavenger[25]; however, in the process a Hepes radical[25,26] is generated that contributes to membrane lipid fraction oxidation. Copper ions and H_2O_2 also stimulate free radical generation from Hepes[27]. Tris is also a hydroxyl radical scavenger[23] but in contrast to Hepes does not produce Tris radicals. So in Tris-buffer vanadyl-induced hemolysis did not occur.

Vanadate markedly stimulates the rate of NADH oxidation in erythrocyte membranes. Superoxide dismutase eliminates this effect[15], reflecting an O_2^--dependent pathway. Vanadate functions as a catalyst by the following free radical chain reactions[26,28,29]. When V(IV) is limited and V(V) abundant, the reduction of V(V) by O_2^- may produce additional V(IV). The following reactions are involved. $V(V) + O_2^- = V(IV)\text{-}OO^\bullet$ (**1**). With NAD(P)H the product V(IV)-OO$^\bullet$ can oxidize NAD(P)H and produces V(V) and H_2O_2 as follows: $V(IV)\text{-}OO^\bullet + NAD(P)H = V(IV)\text{-}OOH + NAD(P)^\bullet$ (**2**) and $V(IV)\text{-}OOH + H^+ = V(V) + H_2O_2$ (**3**). Without NAD(P)H the V(IV)-OO$^\bullet$ apparently dissociates, yielding V(IV) by the reaction: $V(IV)\text{-}OO^\bullet = V(IV) + O_2$ (**4**). However, in phosphate-free media (Hepes buffer) the V(IV) that is generated by the direct V(V)-dependent oxidation of NADH forms a complex with V(V) by the reaction: $V(V) + V(IV) = V(V)\text{-}V(IV)$ (**5**). Phosphate becomes an inhibitor for the reaction **5**.

In the present experiment metavanadate-induced hemolysis occurred only with NAD(P)H in Hepes buffer. In this case the increase in O_2^- production stimulated V(IV)-OO$^\bullet$ production by reaction **1**. With NAD(P)H reactions **2** and **3** proceeded and in those reactions the vanadium oxidation state did not change. However, after oxidative depletion of NAD(P)H the reduction of V(IV)-OO$^\bullet$ to V(IV) via the reaction **4** occurred. Accordingly V(IV), a true hemolyzing species, accumulated in the incubation medium. The extent of metavanadate plus NADH-induced hemolysis was less than that of vanadyl-induced hemolysis (fig. 4), because some of the V(IV) produced may form a complex with V(V) by reaction **5**.

Long-term oral ingestion of metavanadate causes damage in the peripheral blood and hemopoietic system of the rat[30]. Deferoxamine mesylate, an iron-chelator, also strongly chelates vanadium[27,31]. Tiron has also been suggested to alleviate the in vivo vanadium toxicity[32]. Previously, using the chick embryo system, the abilities of various chelating or reducing compounds to prevent vanadium accumulation in the leg bones and to alleviate vanadium toxicity have been determined and following results obtained[33]: deferoxamine mesylate is the most effective; EDTA-2Na, xylenol orange and basophenanthrolinesulfonic acid effective; Tiron, PDTS, L-ascorbic acid, Variamine Blue B and D-penicillamine slightly effective; Thorin and acetylacetone not effective; and dithiothreitol and zephiramine toxic. The addition of chelating agents such as deferoxamine mesylate and EDTA in vitro to vanadyl-induced hemolysis showed a protective effect (table 2). Intracellularly, vanadium is present as vanadyl complexes that protect generation of harmful radicals from free vanadyl. However, absorbed vanadium tends to be accumulated by the kidneys of adult animals[10–12]. The results suggest that deferoxamine mesylate may be one of the best antidotes against increased renal accumulation of vanadium.

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