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# High Vanadate Interferes with the Fiske–Subbarow Determination of Inorganic Phosphate (42417)

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*Abstract.* We evaluated the possibility that oxyions of vanadium might react with molybdate and, in that manner, interfere with the Fiske–Subbarow colorimetric determination of inorganic phosphate. Phosphate ($P_i$) standard curves were prepared (0.03–0.30 μmole/ml) in the presence and absence of oxyvanadium solutions ($2 \times 10^{-4}$ *M*) prepared from ortho- and metavanadate. Molybdate prepared in 5 *N* sulfuric acid was added to each standard. Upon addition of a reducing agent to develop color of the phosphomolybdate complex, a less intense color was observed at any given $P_i$ concentration in the presence of oxyvanadium species. The slope of the regression line for the $P_i$ standard curve in the presence of $2 \times 10^{-4}$ *M* oxyvanadium species was markedly depressed. The effect of oxyvanadium was similar when solutions were prepared from ortho- and metavanadate, despite differences in pH of these solutions. In addition, in the final reaction the pH was similar in the presence and absence of oxyvanadium, independent of the source of vanadate used to prepare solutions. Thus, interference by oxyvanadium did not appear to be related to changes in pH of samples containing vanadium oxyions. Interference was concentration dependent and the minimal concentration of vanadium oxyions that interfered was $5 \times 10^{-5}$ *M*. The effects of oxyvanadium ($2 \times 10^{-4}$ *M*) on $Mg^{+2}$-dependent and $Na^+$–$K^+$-ATPase activities in a renal microsomal preparation were then evaluated through the measurement of inorganic phosphate generation. Enzyme activities were determined with and without correction for interference by oxyvanadium with the method of Fiske and Subbarow. A significant artifactual depression of $Mg^{+2}$ ATPase activity, but not $Na^+$–$K^+$-ATPase activity, was consistently observed when enzyme activities were not corrected for interference by oxyvanadium with the measurement of inorganic phosphate. These data indicate that when effects of high vanadate concentrations ($5 \times 10^{-5}$ *M*) on ATP hydrolyzing enzymes are evaluated through changes in $P_i$ generation, artifactual depression of enzyme activity may occur. 

The most commonly used method for the measurement of inorganic phosphate concentration is that of Fiske and Subbarow (1). This method is based on the reaction of phosphate with molybdate at acid pH to form a colorless phosphomolybdate complex. Addition of a reducing agent results in the development of molybdenum blue, the intensity of which varies as a function of inorganic phosphate concentration. Oxyions of some transition metals, including vanadium, however, are also known to react with molybdate in solution (2). An interaction of molybdate with these species might affect the measurement of inorganic phosphate. In the present study we evaluated the possibility that oxyions of vanadium, through their reaction with molybdate, might interfere with the colorimetric determination of phosphate by the method of Fiske and Subbarow. Such interference could affect the results of studies evaluating the effects of vanadate on ATP hydrolyzing enzymes, when enzyme activity is or has been assessed through direct measurement of inorganic phosphate generation.

**Materials and Methods.** Phosphate standards were prepared from potassium phosphate in deionized distilled water. One milliliter of each standard was diluted with an equal volume of water in glass cuvettes for determination of control standard curves. Additional aliquots of each standard were mixed with an aqueous solution prepared from sodium orthovanadate (Fisher Scientific, purified grade) at a concentration of $2 \times 10^{-4}$ *M* instead of water. The pH of all standards was then determined after which 100 μl of 2.5% ammonium molybdate (Certified ACS, Fisher



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Scientific) prepared in 5 $N$ sulfuric acid was added to each standard. Aminonaphtholsulfonic acid (50 $\mu$l of a 0.25% solution, Fisher Scientific) was then pipetted into each cuvette and the pH of all mixtures again determined. Ten minutes later the samples were read in a spectrophotometer at 660 nm wavelength, against water blanks containing molybdate and aminonaphtholsulfonic acid, as well as against similar blanks prepared with $2 \times 10^{-4}$ $M$ vanadate. Percentage transmittance was plotted against inorganic phosphate concentration on semilog charts. A total of six standard curves was obtained in the presence and absence of oxyvanadium. The slopes of resulting regression lines were determined by regression analysis. Comparison of slopes was carried out using analysis of covariance. The study was repeated using a $2 \times 10^{-4}$ $M$ solution of oxyvanadium ions prepared from metavanadate instead of orthovanadate. In addition, the possible concentration dependence of an oxyvanadium effect on the slope of phosphate standard curves was also determined.

Since preliminary data revealed that oxyvanadium interfered with the measurement of inorganic phosphate by the method of Fiske and Subbarow, the possibility that such interference might lead to artifactually depressed values of ATP hydrolyzing enzyme activities was then evaluated. Total and $Mg^{+2}$-dependent ATPase activities of rat renal microsomal fractions ($N = 8$) were determined as described elsewhere (3). Assay tubes contained a final concentration of 100 m$M$ NaCl, 20 m$M$ KCl, 6 m$M$ $MgCl_2$, 10 m$M$ imidazole, and 3 m$M$ ATP (disodium salt, vanadium-free) at a pH of 7.4, in a final assay volume of 5.0 ml. The assays were carried out in the presence and absence of $2 \times 10^{-4}$ $M$ oxyvanadium (prepared from sodium orthovanadate) at 37°C in a shaking water bath. Final pH of assay tubes was not affected by the presence of oxyvanadium under these conditions. Sodium and potassium were omitted from those assay tubes prepared for the measurement of $Mg^{+2}$-dependent ATPase activities. Reactions were initiated by the addition of ATP. After 10 min, reactions were stopped by the addition of 1.0 ml ice-cold 30% trichloroacetic acid (TCA). Following protein precipitation, inorganic phosphate concentration was measured in resulting supernatants from each assay tube to determine the rate of inorganic phosphate generation. In addition to standard assay blank tubes used to assess spontaneous ATP hydrolysis, separate blank tubes were assayed in the absence of ATP but in the presence of both enzyme fractions and $2 \times 10^{-4}$ $M$ oxyvanadium. Following protein precipitation, supernatants from these blanks were used to prepare phosphate standard curves in the presence of that oxyvanadium concentration recovered from assays of each enzyme fraction tested. Thus, a phosphate standard curve was prepared for each microsomal fraction assayed, in addition to the preparation of a control phosphate standard curve. All samples containing oxyvanadium were then read not only against the control standard curve, but also against the standard curve containing that concentration of oxyvanadium recovered when assaying the respective enzyme fraction (corrected values). This was done because initial studies revealed that the recovery of oxyvanadium following protein precipitation with TCA varied somewhat with variations in the protein concentrations of microsomal fractions. ATPase activities were expressed as $\mu$mole $P_i$/mg protein · hr, and $Na^+$–$K^+$-ATPase activity was expressed as the difference between total and $Mg^{+2}$-dependent ATPase activities.

**Results.** The presence of $2 \times 10^{-4}$ $M$ oxyvanadium ions prepared from sodium orthovanadate markedly decreased the slope of the phosphate standard curve (Fig. 1). The same decrease in the slope of the standard curve was observed when phosphate standards containing oxyvanadium species were read against blanks having the same vanadium oxyion concentration. This effect did not appear to be pH related since the final pH was similar ($<1.0$) in all phosphate standards in the presence and absence of oxyvanadium ions. The pH of phosphate standards prior to the addition of molybdate, however, was increased by the presence of oxyvanadium. Solutions prepared from ortho- and metavanadate produced a similar effect when used at the same concentration (Fig. 1), whereas initial pH's of the solutions were different. These data provide further evidence that the interference by ovyvanadium ions with determination of phosphate cannot be attributed to increased

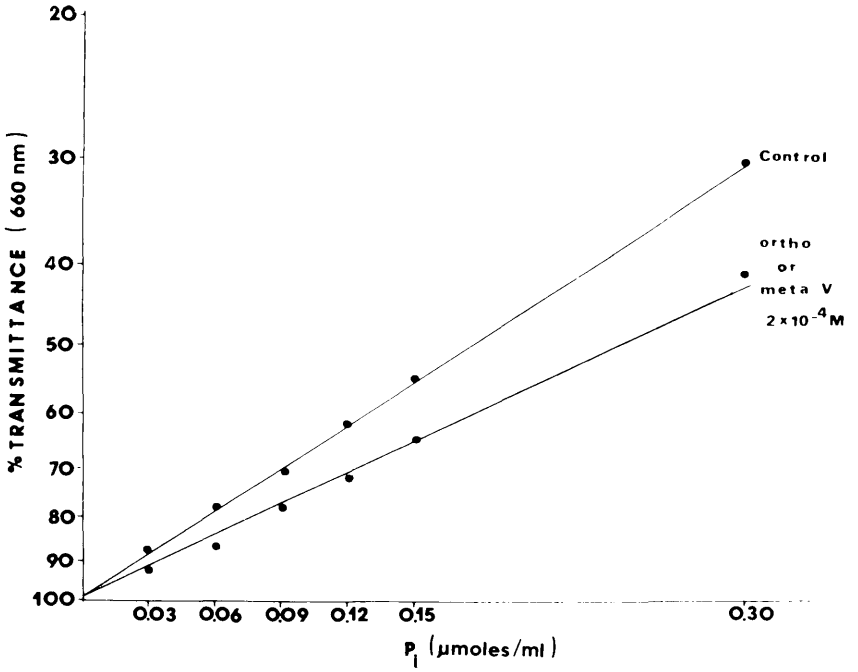


FIG. 1. Phosphate standard curve based on the reaction with molybdate in 5 *N* sulfuric acid in the absence (control) or presence of $2 \times 10^{-4}$ *M* ortho- or metavanadate.

pH of phosphate standards due to the addition of oxyvanadium solutions. The degree to which oxyvanadium species decreased the slope of phosphate standard curves was directly related to the concentration of oxyvanadium species (Fig. 2). The minimum concentration of vanadium oxyions that produced a significant reduction in the slope of standard curves was $5 \times 10^{-5}$ *M*. Vanadate effects on $Mg^{+2}$-dependent and $Na^+$–$K^+$-ATPase activities, with and without correction for interference by oxyvanadium with measurements of inorganic phosphate are shown in Table I. Oxyvanadium ($2 \times 10^{-4}$ *M*) almost completely inhibited $Na^+$–$K^+$-ATPase activity in rat renal microsomal fractions. The degree of $Na^+$–$K^+$-ATPase inhibition by oxyvanadium was similar when values were corrected for oxyvanadium influence on the measurement of inorganic phosphate. Thus, interference by oxyvanadium with measurements of inorganic phosphate did not artifactually alter values of $Na^+$–$K^+$-ATPase activity. In contrast, artifactual inhibition of $Mg^{+2}$-dependent ATPase activity by oxyvanadium was observed since activity of this enzyme was not affected by vanadium oxyions when values were corrected for oxyvanadium interference with measurement of inorganic phosphate.

**Discussion.** The development of a yellow color when molybdate in acid is mixed with phosphate in the presence of oxyvanadium species was described years ago in a seldom used method for the determination of inorganic phosphate (4). That method, however, was not based on the use of a reducing agent for color development, and consequently, interference by oxyvanadium species with measurements of inorganic phosphate by the widely used method of Fiske and Subbarow was not detected. In the present study we have described this interference. The specific oxyion of vanadium that reacts with molybdate, however, cannot be determined from our data. This issue is difficult to resolve due to the unusually complex aqueous chemistries of both vanadium (5) and molybdenum (6). Whatever the case, the present data demonstrate that oxyvanadium ion concentrations of $5 \times 10^{-5}$ *M* or greater result in a significant depression of the normal phosphate standard curve using the method of Fiske and Subbarow. These

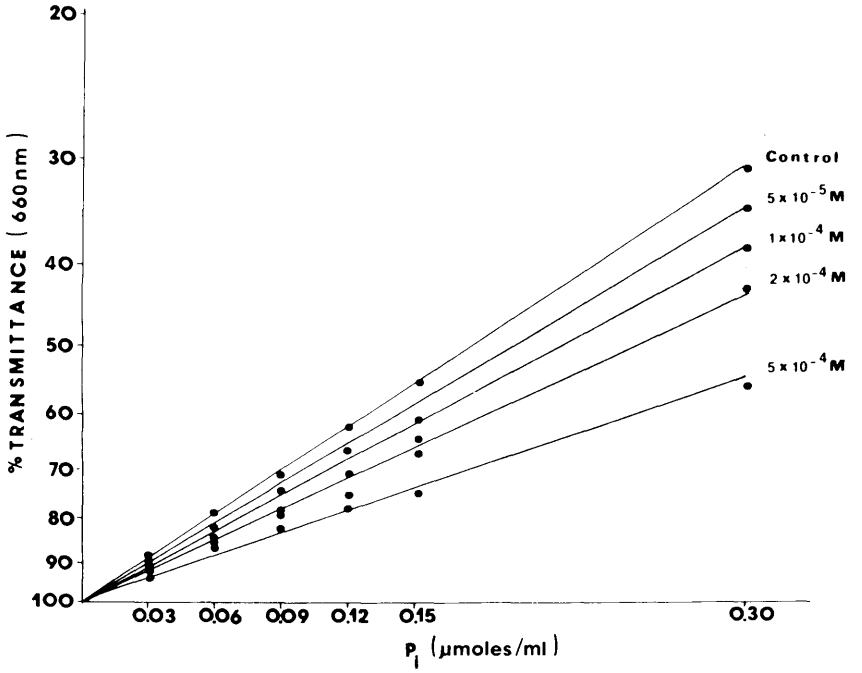


FIG. 2. Phosphate standard curve based on the reaction with molybdate in 5 *N* sulfuric acid in the absence (control) or presence of oxyvanadium in concentrations ranging from $5 \times 10^{-5}$ to $5 \times 10^{-4}$ *M*.

oxyvanadium concentrations are considerably greater than those frequently used to evaluate the effects of vanadium oxyions on ATP hydrolyzing enzymes (7–9). In addition, enzymatic activities in numerous studies performed to examine the effect of vanadate have been determined using linked enzyme systems rather than direct measurement of inorganic phosphate generation (7, 8). Nevertheless, several studies of vanadate effects of ATP hydrolyzing enzymes have used the method of Fiske and Subbarow to determine inorganic phosphate generation, and have also used vanadate concentrations greater than $5 \times 10^{-5}$ *M* (10, 11). For example, in one study of the effect of vanadate on rat brain $Na^+$–$K^+$-ATPase activity, an inhibitory effect was not observed at vanadate concentrations less than $10^{-6}$ *M* (10). Since $Na^+$–$K^+$-ATPase activity was determined through the measurement of

TABLE I. EFFECTS OF OXYVANADIUM ($2 \times 10^{-4}$ *M*) ON $Mg^{+2}$-DEPENDENT AND $Na^+$–$K^+$-ATPase ACTIVITIES WITH AND WITHOUT CORRECTION FOR OXYVANADIUM INTERFERENCE WITH THE MEASUREMENT OF INORGANIC PHOSPHATE

| | $Mg^{+2}$-ATPase (μmole $P_i$/mg protein · hr) | $Na^+$–$K^+$-ATPase (μmole $P_i$/mg protein · hr) |
|---|---|---|
| Control | 156.0 ± 6.9 | 103.7 ± 7.8 |
| Oxyvanadium (uncorrected) | 128.5 ± 7.4[a] | 6.9 ± 4.0[a] |
| Oxyvanadium (corrected) | 158.9 ± 7.6[b] | 6.9 ± 4.3[a] |

*Note.* All values are expressed as the arithmetic mean ± SEM.

[a] Values significantly different ($P < 0.01$) from corresponding control values.

[b] Value significantly different from that observed in the presence of oxyvanadium, ($2 \times 10^{-4}$ *M*) but without correction for oxyvanadium interference with the measurement of inorganic phosphate.

inorganic phosphate concentration, it is possible that the effects of vanadate in that study may have been artifactual. In another study the influence of vanadate on cardiac $Ca^{+2}$-ATPase in the presence of high calcium levels was not observed at vanadate concentrations less than $10^{-5}$ *M* (11). ATPase activity was again evaluated through direct measurement of inorganic phosphate concentration, and consequently the reported enzyme inhibition may reflect the influence of vanadium oxyions on the measurement of phosphate as reported herein. Data from the present study is directly related to the interpretation of such findings. In fact, these data illustrate that interference by high oxyvanadium concentrations with the measurement of inorganic phosphate can lead to false values of ATP hydrolyzing enzyme activities. Thus, artifactual inhibition of $Mg^{+2}$-dependent ATPase was observed when values were not corrected for oxyvanadium interference with the method of Fiske and Subbarow. Although artifactual inhibition of $Na^+$–$K^+$-ATPase activity was not observed, this may be explained by the fact that $Na^+$–$K^+$-ATPase is expressed as the difference between total and $Mg^{+2}$-dependent ATPase activities, both of which were artifactually depressed by oxyvanadium. Whatever the case, our results suggest that the determination of inorganic phosphate in any sample containing high oxyvanadium ion concentrations should be read against standard curves obtained in the presence of the same oxyvanadium concentration to avoid artifactually depressed values.

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1. Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. J Biol Chem **66**:375–400, 1925.
2. Cotton FA, Wilkinson G. Advanced Inorganic Chemistry, 4th ed. New York, Wiley, p708, 1980.
3. Rodriguez-Sargent C, Cangiano JL, Opava-Stitzer S, Martinez-Maldonado M. Renal $Na^+$-$K^+$-ATPase in Okamoto and Dahl hypertensive rats. Hypertension **3**:II86–91, 1981.
4. Quinlan KP, De Sesa MA. Spectrophotometric determination of phosphorus as molybdovanadophosphoric acid. Anal Chem **27**:1626–1629, 1955.
5. Rubinson KA. Concerning the form of biochemically active vanadium. Proc R Soc London **212**:65–84, 1981.
6. Cotton FA, Wilkinson G. Advanced Inorganic Chemistry, 4th ed. New York, Wiley, p844, 1980.
7. Cantley LC, Josephson L, Warner R, Yanagisawa M, Lechene C, Guidotti G. Vanadate is a potent (Na, K)-ATPase inhibitor found in ATP derived from muscle. J Biol Chem **252**:7421–7423, 1977.
8. Hobbs A, Froelich JP, Albers RW. Inhibition by vanadate of the reactions catalyzed by the ($Na^+$-$K^+$) stimulated ATPase: A transient state kinetic characterization. J Biol Chem **255**:5724–5727, 1980.
9. Bond GH, Hudgins PM. Dog kidney ($Na^+$, $K^+$)-ATPase is more sensitive to inhibition by vanadate than human red cell ($Na^+$, $K^+$)-ATPase. Biochim Biophys Acta **646**:479–482, 1981.
10. Phillis JW, Wu PH. Failure of vanadate contamination in ATP to account for noradrenaline stimulation of $Na^+$, $K^+$-ATPase in rat brain homogenates. J Pharmacol **31**:556–558, 1979.
11. Wierichs R, Hagenmeyer A, Bader H. Influence of $Ca^{2+}$ and $Mg^{2+}$ on the vanadate inhibition of the $CA^{2+}$-ATPase from pig heart sarcoplasmic reticulum. Biochem Biophys Res Commun **92**:1124–1129, 1980.

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