Interaction of vanadate with phenol and tyrosine: Implications for the effects of vanadate on systems regulated by tyrosine phosphorylation

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ABSTRACT   The interaction of vanadate with phenol and *N*-acetyltyrosine ethyl ester in aqueous solution has been studied by using ^{51}V nuclear magnetic resonance spectroscopy. On the basis of these studies, it has been concluded that vanadate rapidly esterifies the hydroxyl group of the aromatic ring to yield a phenyl vanadate. For phenol, the equilibrium constant for this reaction in terms of the convention that the activity of liquid water is 1.0 is $K_1' = $ [phenyl vanadate]/[phenol][vanadate] $= 0.97 \pm 0.02$. This value is well over 4 orders of magnitude larger than estimates from the literature for the corresponding equilibrium constant for the esterification of phenol by phosphate. The equilibrium constant for esterification of the phenol moiety of *N*-acetyltyrosine ethyl ester is similar to that for esterification of phenol. The relevance of these observations to processes that are regulated by reversible phosphorylation/dephosphorylation of tyrosine residues is discussed, in particular the insulin-like effect of vanadate.

Vanadate has been reported to have an insulin-like effect on animals (1), on cell cultures (2, 3) and on purified insulin receptors (3). Both insulin and vanadate activate phosphorylation of a tyrosine residue of the insulin receptor (3–6). The vanadate- and insulin-activated phosphorylation of the insulin receptor have been observed with highly purified insulin receptor (3), an observation that led to the conclusion that the effects were not because of inhibition of phosphotyrosine phosphatase but rather activation of a tyrosine kinase activity that resides in the insulin receptor itself. It has been reported that the phosphorylated insulin receptor is active as a tyrosine kinase, even in the absence of insulin (4).

These reports, along with our observations that vanadate rapidly esterifies hydroxyl groups to yield vanadate esters that are accepted as substrates by some enzymes whose physiological substrates are the corresponding phosphate esters (7, 8), has led us to the hypothesis that the insulin-like effect of vanadate is the result of the esterification of tyrosine by vanadate. As a first step in testing the feasibility of this hypothesis, we have studied the interaction of phenol and *N*-acetyltyrosine ethyl ester with vanadate in aqueous solution, using ^{51}V NMR spectroscopy.
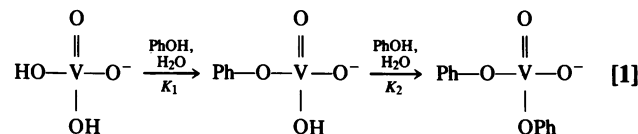
EXPERIMENTAL PROCEDURES

Materials. Solutions were prepared and ^{51}V NMR spectra were obtained exactly as described (7). Conditions and solute concentrations were as indicated on the figures and in the figure legends. Aqueous acetone solutions were prepared by measuring out a volume of acetone equal to 50% of the desired final volume, adding the desired weight of phenol,

adding H_2O to bring the volume to near the final volume, adding Tris base to give the desired final concentration, adjusting the pH to slightly below the desired value by adding HCl, adding Na_3VO_4 to give the desired concentration, adding H_2O to give the final volume, and finally adjusting the pH to the desired value by using a few drops of NaOH solution. The mass of the solution was then determined so that the molar H_2O concentration could be calculated for use in Fig. 2. This procedure also avoided adding acid to the solution containing vanadate, which causes decavanadate formation.

Methods. ^{51}V NMR spectra were obtained at 105 MHz from a Bruker 400 MHz NMR spectrometer operating at ambient temperature in the Fourier transform mode. Spectral widths of 20 kHz, 0.05-sec acquisition times, and 45° pulse-widths were used throughout. A line-broadening factor of 20 Hz was applied to all spectra, and integrations were done by using the instrument manufacturers' software.

RESULTS

Fig. 1 shows ^{51}V NMR spectra of 0.5 mM vanadate in 50% aqueous acetone solution at various concentrations of phenol. The resonances that occurred at -559, -574, and -579 ppm in the absence of phenol were assigned to monomeric inorganic vanadate and its dimeric and tetrameric forms, respectively (9, 10). With increasing concentrations of phenol, two new resonances at -566 and -571 ppm appeared, which were assigned to phenyl vanadate and diphenyl vanadate, respectively, as shown in Eq. 1, where PhOH is phenol.

$$\underset{\text{OH}}{\overset{\text{O}}{\underset{|}{\overset{\|}{\text{HO}-\text{V}-\text{O}^-}}}} \xrightarrow[K_1]{\overset{\text{PhOH,}}{\text{H}_2\text{O}}} \underset{\text{OH}}{\overset{\text{O}}{\underset{|}{\overset{\|}{\text{Ph}-\text{O}-\text{V}-\text{O}^-}}}} \xrightarrow[K_2]{\overset{\text{PhOH,}}{\text{H}_2\text{O}}} \underset{\text{OPh}}{\overset{\text{O}}{\underset{|}{\overset{\|}{\text{Ph}-\text{O}-\text{V}-\text{O}^-}}}} \quad [1]$$

These assignments are consistent with the changes induced in relative intensities of the three peaks at -559, -566, and -571 ppm with increasing phenol concentration. Thus, the esterification of phenol with vanadate is qualitatively similar to the esterification of ethanol with vanadate (7). The equilibrium constants K_1 and K_2, defined in Eqs. 2 and 3, were determined by plotting the relevant ratios as shown in Fig. 2. The values obtained were $K_1 = 45.0 \pm 4.0$ and $K_2 = 27.0 \pm 1.0$. In this regard, it should be noted that the points in Fig. 2 should probably be fitted to curves rather than straight lines. At the relatively high phenol concentrations used in this study, the nature of the solvent may change significantly as phenol concentration is increased. In that event, K_1 and K_2 need not necessarily remain constant. The effect of solvent change on equilibria has previously been discussed for the formation of ethyl vanadate (7). The curvature, if any, in Fig. 2 is small, so that fitting the data with a straight line produces minimal error in the equilibrium constant. For the studies of
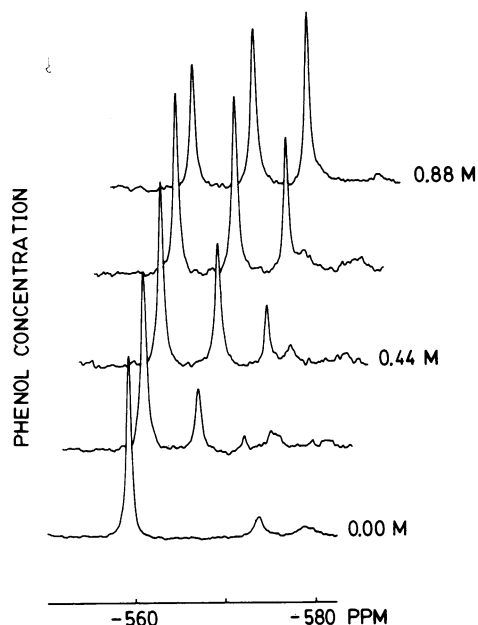
FIG. 1. ^{51}V NMR spectra of vanadate in 50% aqueous acetone and the indicated concentrations of phenol. Solutions contained 20 mM Tris chloride buffer at pH 7.5 as determined by using a combination pH electrode standardized in aqueous buffers, 0.5 mM Na_3VO_4, and the indicated concentrations of phenol.

aqueous phenol solutions, the phenol concentrations were much lower and considerably better agreement was obtained.

$$K_1 = \frac{[PhOVO_3H^-][H_2O]}{[PhOH][H_2VO_4^-]} \qquad [2]$$

$$K_2 = \frac{[(PhO)_2VO_2^-][H_2O]}{[PhOH][PhOVO_3H^-]} \qquad [3]$$

Fig. 3 shows the ^{51}V NMR spectrum of a solution of 0.5 mM vanadate and 0.19 M N-acetyltyrosine ethyl ester in 50% aqueous acetone. The resonance at -564 ppm and the weaker resonance at -574 ppm were assigned to tyrosine vanadate and dityrosine vanadate, respectively, by analogy with the results from the phenol experiments. The resonance at -559 ppm corresponds to the free vanadate. The relative intensities of the vanadate ester peaks in Fig. 3 are consistent with values of K_1 and K_2 for esterification of tyrosine with
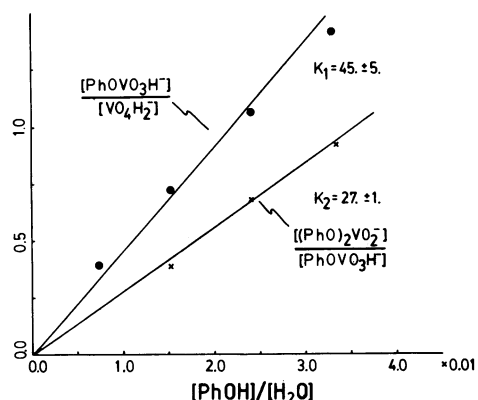

FIG. 2. Determination of the equilibrium constants K_1 and K_2 for formation of phenyl vanadate and diphenyl vanadate. The molar concentration ratios [phenyl vanadate]/[vanadate] and [diphenyl vanadate]/[phenyl vanadate], obtained from the relative peak intensities of the spectra shown in Fig. 1, are plotted against the concentration ratio [phenol]/[H₂O]. The slopes give K_1 and K_2, as shown in Eqs. 2 and 3.

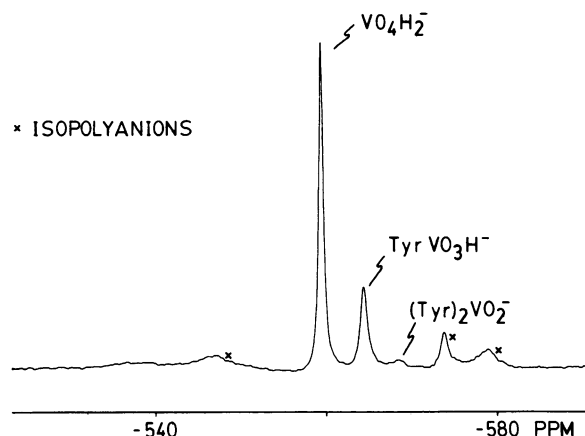FIG. 3. The ^{51}V NMR spectrum of 0.5 mM Na_3VO_4 in the presence of 0.19 M N-acetyltyrosine ethyl ester and 20 mM Tris chloride (pH 7.5) in 50% (vol/vol) aqueous acetone.

vanadate similar to those for esterification of phenol. Because of solubility problems, somewhat lower phenol concentrations were obtained when water without acetone was used as solvent. Fig. 4 shows the ^{51}V NMR spectra of vanadate in the presence of the specified concentrations of phenol in aqueous solution. The resonance appearing at -554 ppm in these spectra was assigned to phenyl vanadate. This assignment is consistent with the effect of change in phenol concentration on the relative intensities of the resonances at -557 ppm and -554 ppm. The plot of the ratio of the areas of the peaks assigned to phenyl vanadate and vanadate is shown in Fig. 5. The slope of this plot is equal to K_1 (defined in Eq. 2) for this solvent. This value of K_1 ($K_1 = 54.0 \pm 1.0$) is very close to the corresponding value determined in 50% aqueous acetone ($K_1 = 45.0 \pm 4.0$). No resonance is apparent in Fig. 4 that can be assigned to diphenyl vanadate. If K_2 in aqueous solution were equal to K_2 in 50% acetone, a reasonable first approximation in view of the similarity of the K_1 values in these two solvents, then the concentration ratio [diphenyl vanadate]/[phenyl vanadate] would be expected to
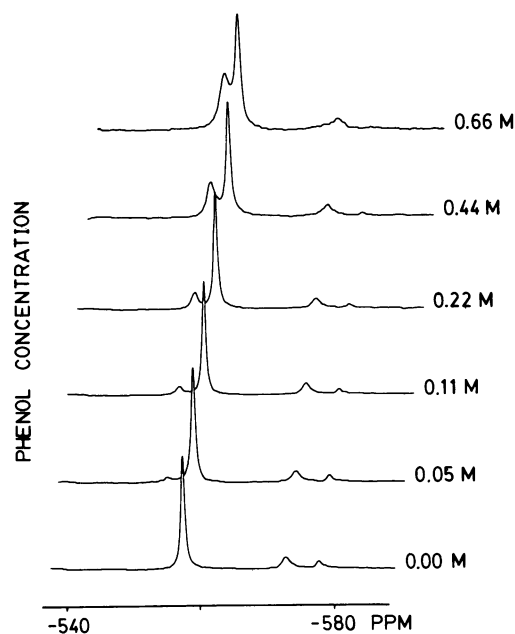

FIG. 4. ^{51}V NMR spectra of 0.5 mM Na_3VO_4 in the presence of the indicated concentrations of phenol in aqueous solution. Conditions were as in Fig. 1 except that no acetone was present.
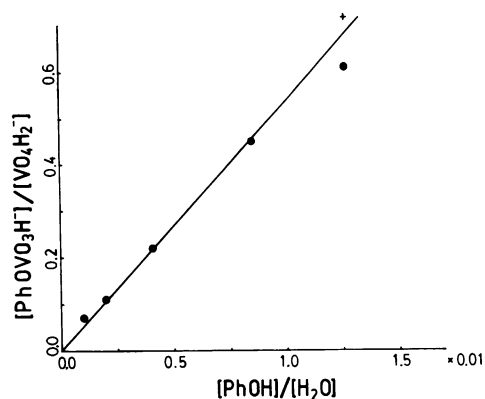
FIG. 5. Determination of the equilibrium constant K_1 for formation of phenyl vanadate in aqueous solution. The molar concentration ratio [phenyl vanadate]/[vanadate], obtained from the relative peak intensities of the spectra shown in Fig. 4, are plotted against the concentration ratio [phenol]/[H$_2$O]. The slope is equal to K_1, defined in Eq. 2, for this solvent.

be equal to 0.34 at the highest phenol concentration used in the series shown in Fig. 4. The fraction of the total vanadate that would be expected to exist as diphenyl vanadate would then be 0.12. It is not surprising, therefore, that we obtained little evidence for the existence of diphenyl vanadate in the experiments shown in Fig. 4, particularly in view of the possibility that, under the conditions of these experiments, diphenyl vanadate, if formed, might have a resonance in the ^{51}V NMR spectrum that would not be resolved from the vanadate or phenyl vanadate resonances. In fact, if it is assumed that the diphenyl vanadate resonance occurs at the same position as the inorganic vanadate resonance, then from the above estimated proportion of diphenyl vanadate, the point marked with an *x* on Fig. 5 is obtained. This point lies much nearer the line than the original, giving credence to the supposition that the two resonances are superimposed. It was not possible to achieve phenol concentrations much higher than those used in Fig. 4 without changing to partially organic solvents, such as the 50% aqueous acetone solvent used previously.

Vanadate has an ionizable proton with a pKa value of about 8.3 (9, 10). Phenyl vanadate also has an ionizable proton, which should have a pKa value similar to that of inorganic vanadate. A change in ionization state is reflected in the NMR spectrum by a change in chemical shift. This change can be related to the pKa by Eq. 4,

$$pH = pKa + \log \frac{|\delta_1| - |\delta|}{|\delta| - |\delta_h|} \qquad [4]$$

where δ_1 is the limiting shift at low pH, δ_h is the limiting shift at high pH, and δ is the observed value. This equation assumes $|\delta_h| < |\delta_1|$, the situation obtained here. A plot of pH versus the log term in Eq. 4 gives a line of unit slope and a *y* intercept equal to the pKa value. Fig. 6 shows the behavior of the chemical shifts of vanadate and phenyl vanadate as pH was varied from 6.5 to 10.0 under conditions of 0.22 M phenol, 20 mM Tris chloride, and 0.5 mM vanadate. The pKa values obtained were 8.0 and 8.4 for phenyl vanadate and vanadate, respectively. Phenyl vanadate monoanion has a slightly lower pKa than does vanadate monoanion, whereas the reverse is true for ethyl vanadate monoanion. This difference is consistent with the greater electron withdrawing ability of the phenyl derivative compared with the ethyl group, as indicated by the pKa values of 10 and 16 for phenol and ethanol, respectively (11).

The value of K_1 determined in Fig. 5 for formation of phenyl vanadate exceeds by a factor of *ca.* 5 the value of the equilibrium constant for formation of ethyl vanadate (7). This
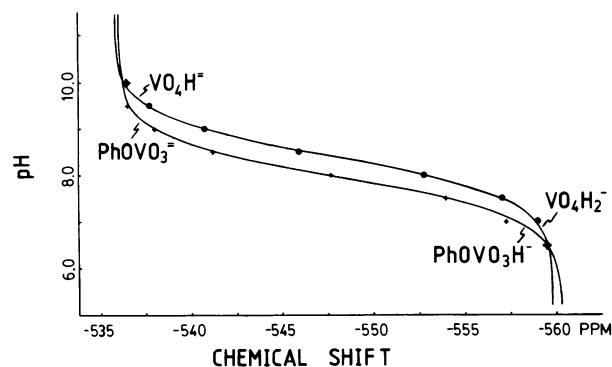

FIG. 6. The effect of pH on the resonance position of the peaks in the ^{51}V NMR spectrum assigned to inorganic vanadate and phenyl vanadate. Na$_3$VO$_4$ was present at 0.5 mM, phenol at 0.22 M, and Tris chloride buffer at 20 mM, and the pH was as indicated. The points were taken from the chemical shift values of the resonances in the ^{51}V NMR spectra (not shown) assigned to inorganic vanadate (●), and phenyl vanadate (+). The lines were calculated from Eq. 4 by using for inorganic vanadate the values pKa = 8.40, δ_1 = −559.8, and δ_h = −535.8 and using for phenyl vanadate the values pKa = 7.95, δ_1 = 560.3, and δ_h = −536.0.

is somewhat surprising because formation of esters of phosphoric and acetic acid tends to be less favorable with more acidic alcohols (12). The stability of phenyl vanadate relative to ethyl vanadate is possibly due in part to an energetically favorable interaction between the π electrons in the phenyl group with the *d* orbitals of the vanadium atom. If the value of K_1 determined in Fig. 5 is divided by 55.5, the molar concentration of liquid water, one obtains the value of the equilibrium constant for formation of phenyl vanadate from phenol and vanadate in terms of the convention that the activity of liquid water is equal to 1.0. We shall define this value as K_1'. Thus, $K_1' = K_1/55.5$ M = 0.97 ± 0.02 M^{-1}. This value is about 4 orders of magnitude larger than the available equilibrium constants for phenyl phosphate formation (13).

DISCUSSION

If vanadate esterification of phenol and the phenol side chain of tyrosine proceeds rapidly with an equilibrium constant much larger than that for the corresponding phosphate esterification reactions, then an obvious and interesting explanation arises for the action of vanadate on systems that involve activation of enzymes by phosphorylation on tyrosine. Studies using the enzymes glucose-6-phosphate dehydrogenase and α-glycerophosphate dehydrogenase have yielded strong support for the hypothesis that vanadate esters are accepted by these enzymes as substrates in lieu of the corresponding phosphate esters (8). Therefore, it is not unreasonable to hypothesize that an enzyme that is activated by phosphorylation of a given amino acid side chain is similarly activated by spontaneous esterification with vanadate. This provides an attractive explanation for the observation that vanadate activates phosphorylation of the highly purified insulin receptor by ATP in the absence of insulin (3). Since the insulin receptor, when phosphorylated on the tyrosine residue, has tyrosine kinase activity in the absence of insulin (4), vanadate esterification of the tyrosine residue might reasonably be expected to activate the receptor as a catalyst of phosphorylation of other receptors on tyrosine by ATP.

In a living animal, it may not be possible for one insulin receptor to catalyze phosphorylation of another insulin receptor, but any kinase subject to activation by phosphorylation on tyrosine can be activated by vanadate via the mechanism outlined above. If this mechanism is to be

responsible for any of the effects of vanadate *in vivo*, then the relative values of the equilibrium constants for esterification of tyrosine by phosphate and vanadate are of primary importance. An estimate for the equilibrium constant for formation of phenyl phosphate can be obtained from published values of the equilibrium constants for hydrolysis of ethyl phosphate, *p*-nitrophenyl phosphate, and 2,4-dinitrophenyl phosphate and their pK_a values (13). The reasonable assumption can be made that phenyl phosphate follows the same linear free energy relationship as do the above three phosphate esters, in which case a value of 1.2×10^{-5} M^{-1} is calculated for the equilibrium constant for formation of phenyl phosphate from phenol and phosphate at pH 7.5, using the convention that the activity of liquid water is 1.0. The corresponding value calculated for ethyl phosphate formation is 3.8 M^{-1}. From this, it is seen that formation of ethyl phosphate is favored over formation of ethyl vanadate by a factor of 20 (7), while formation of phenyl vanadate is favored by about 5 orders of magnitude over formation of phenyl phosphate. There appears to be no report of a measurement of the equilibrium constant for formation of tyrosine phosphate, although the free energy of hydrolysis of one phosphoprotein phosphorylated on tyrosine has been determined (14).

It has been pointed out recently that the lowest possible ratio of the phosphorylated to the nonphosphorylated form of an enzyme that is regulated by reversible phosphorylation/dephosphorylation is determined by the equilibrium constant for the dephosphorylation reaction and the existing concentrations of the reactants and products of the dephosphorylation reaction (15). In the case where the dephosphorylation reaction is simply hydrolysis to yield the dephosphorylated enzyme and phosphate, the equilibrium constant for the reaction is the reciprocal of that for esterification by phosphate. The concentration of phosphate and the value of this equilibrium constant then determine the lowest possible phosphorylation level of the enzyme that can be achieved when the protein kinase is totally inactive. Thus, it is apparent that, in order for esterification by vanadate to activate an enzyme that is normally activated by phosphorylation, the ratio of the equilibrium constants for esterification by vanadate and phosphate must exceed the ratio of the phosphate and vanadate concentrations. It would not otherwise be possible to achieve fractional levels of vanadate esterification in excess of the lowest possible level of phosphorylation of the regulated enzyme, and activation by vanadate would not be possible by the mechanism under consideration.

In the normal biological system, this may well be true because vanadium levels are typically less than 1 μM (2). For physiological phosphate concentrations of a few millimolar, it can be estimated that approximately equal levels of tyrosine esterification by phosphate and vanadate are obtained with a vanadate concentration of about 0.2 μM. It seems very probable that in vanadium-fed animals, concentrations considerably higher than this will be encountered.

It should be mentioned, however, that the above constraint imposed by ratios of equilibrium constants and of concentrations would be removed in systems where the dephosphorylation reaction is driven by an energy-yielding reaction such as ATP hydrolysis. No cases of such dephosphorylation mechanisms have yet been identified, but they may exist. Mg·ATP-dependent phosphoprotein phosphatases exist (16), and it has been proposed that the Mg·ATP could be used to activate the phosphatase by causing it to become dephosphorylated (ref. 16, p. 203). ATP-requiring proteolytic enzymes are known for which there is strong evidence that ATP hydrolysis is required to drive the protein hydrolysis reaction (17). There are also well-known cases in which the demodification reaction of an enzyme that is regulated by

reversible modification-demodification is not a simple hydrolysis reaction. Thus, although the proposal of an energy-driven dephosphorylation reaction is speculative, it is not a possibility which should be considered to be remote. An energy-driven dephosphorylation reaction would result in a minimum ratio of phosphorylated-to-nonphosphorylated enzyme much lower than that predicted by the equilibrium constant for esterification by phosphate because nonenzymic phosphorylation by phosphate is much too slow to be physiologically significant (18, 19).

Although the hypothesis being discussed provides a reasonable explanation for the effect of vanadate on the phosphorylation of the insulin receptor in experiments using cell-free systems, there remains considerable doubt concerning the extent to which the chemistry of vanadium(V) is relevant to the effects of vanadium *in vivo*. It has been found that vanadium(V) is reduced to vanadium(IV) inside erythrocytes (20), and it is reasonable to expect that this occurs to some extent in most cells. However, there appears to be no report of studies of the effect of vanadium on live mammals in which the oxidation state of the vanadium in the animal was monitored, and the fact that mechanisms exist for oxidation as well as for reduction cannot be ignored.

It is also possible that the vanadium(V) chemistry that we report here occurs with vanadium(IV) as well. Vanadium(IV) and vanadium(V) have similar inhibitory effects on ribonuclease (21) and on the Na^+,K^+-ATPase (22), possibly via similar mechanisms.

Although we have discussed vanadate esterification of tyrosine in terms of its relevance to the insulin mimetic effect of vanadium, it may also be relevant to other systems that involve tyrosine phosphorylation, such as the action of growth factors (23) and the tyrosine kinase oncogene products (24). Vanadate is known to mimic and potentiate the effects of growth factors (25-27).

CONCLUSION

We conclude, on the basis of a ^{51}V NMR study of vanadate in the presence of phenol or *N*-acetyltyrosine ethyl ester in aqueous solution, that vanadate rapidly esterifies phenol and tyrosine. The equilibrium constants for these reactions are several thousand times larger than those for the corresponding esterifications by phosphate. This conclusion may be relevant to the known effect of vanadate on systems that are regulated by reversible phosphorylation of tyrosine residues, in particular the insulin mimetic effect of vanadate.

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