fact could be used to support the O-protonation mechanism.

Perhaps the most compelling evidence for N-protonation, however, comes from comparison of the rates and temperature coefficients of the acid-catalyzed processes for carboxybiotin and carboxyimidazolidone.[8] Carboxybiotin shows an activation energy that is higher by 16 kcal/mol (although part of this effect may result from metal chelation in the low-temperature experiments, and not at 25 °C) and a calculated rate that is slower at 25 °C by a factor of 30. These differences imply a strong steric effect, but the O-protonation mechanism should involve little or no steric interference. N-Protonation, on the other hand, would be much more difficult with carboxybiotin, since N1′ must approach tetrahedral geometry in the transition state, and thus either the carboxyl group or the incoming proton would suffer steric interference from the syn proton on C9 of the sulfur-containing ring. Contributing to the difficulty of forming the tetrahedral transition state would be the necessary decrease in solvation of the carboxyl group caused by the steric crowding.

It is tempting to speculate that the fused-ring structure of biotin exists primarily to stabilize the carboxylated form against spontaneous decarboxylation. While carboxylases now involve bound biotin and the distance that biotin must move between the site where it is carboxylated and where it donates its carboxyl group is small (estimated to be ~7 Å for transcarboxylase[17]), this may not have been the case early in biological evolution. If carboxybiotin originally evolved as a free metabolite in the cell, there would be a great advantage to having it be more stable than simple imidazolidone derivatives. The same argument suggests that enzymatic carboxylation and decarboxylation of biotin do *not* involve N-protonation mechanisms, but rather involve polarization of the ureido oxygen by the close proximity of positively charged groups on the enzyme. Attwood et al. have recently concluded that pyruvate carboxylase involves the enol or enolate form of biotin as an intermediate and ruled out an N-protonation mechanism on the basis of the observed ^{13}C and D_2O solvent isotope effects.[18]

The pH-independent decarboxylation of carboxybiotin at pH values above 6.4 appears to produce the enolate as the product, and the ^{13}C isotope effect of 1.023 suggests a moderately early transition state. The near-equality of rates for carboxybiotin and carboxyimidazolidone[8] argues against an N-protonation mechanism, where a pronounced steric effect should be seen, as in the low-pH mechanism. The very low D_2O solvent isotope effect with a curved proton inventory presumably results from secondary isotope effects on the protons of water hydrogen bonded to the ureido oxygen as its negative charge increases. Similar effects on the protons of hydrogen-bonded waters are thought to be responsible for the low apparent fractionation factor of hydroxide relative to water of 0.48.[19] The failure to observe buffer catalysis by Hepes at pH 8 is consistent with this mechanism.

Note that in vivo carboxybiotin will decarboxylate spontaneously largely by the pH-independent path, since the crossover at 25 °C is at pH 6.4. The steric crowding caused by the fused-ring system thus has suppressed the acid-catalyzed pathway until it is not important physiologically but has little effect on the mechanism that simply involves spontaneous C–N bond cleavage.

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Interaction of Vanadate with Uridine and Adenosine Monophosphate. Formation of ADP and ATP Analogues

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Abstract: ^{51}V nuclear magnetic resonance has been used to study the interactions of vanadate with uridine and adenosine monophosphate (AMP). In addition to the vanadate esters formed with the hydroxyl groups of the ribose ring, two other products were formed. A major component of the reaction was a binuclear vanadate complex incorporating two ligands. This complex forms between the 2′- and 3′-hydroxyls of the ribose ring, and it was proposed that each vanadium has a trigonal-bipyramidal coordination. The formation of this product with either uridine or AMP is strongly favored. No evidence for existence of the corresponding monomeric compound was obtained. AMP readily forms an anhydride between vanadate and the phosphate group of AMP to yield an ADP analogue. An ATP analogue is similarly formed from AMP and divanadate. At pH 7.5 these ADP and ATP analogues have formation constants approximately 40 times larger than the formation constants normally observed for vandate esters but similar to those for the formation of anhydrides of vanadate with phosphate or pyrophosphate. Proton stoichiometries for the various reactions were determined from pH studies.

The role that vanadium oxyanions play in biochemical processes is not at all well understood. Although vanadium is thought to be an essential element, even this has not been unequivocally established.[1] There is no doubt, however, that vanadium ions have a dramatic impact on various enzymes either as activators or as inhibitors of the enzyme function.[2,3]

Activation of enzymes seems to occur because vanadate and possibly vanadyl act as phosphate analogues, spontaneously

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forming vanadium esters that are accepted as enzyme substrates in lieu of the normally phosphorylated substrate. It has recently been shown, for instance, that glucose 6-vanadate is accepted in place of glucose 6-phosphate by glucose 6-phosphate dehydrogenase for the oxidation of glucose to gluconic acid.[4] Similar behavior has been reported for arsenate esters, although in this case, ester formation is relatively slow.[5,6] Activation of enzyme catalysis by vanadate in conjunction with the dephosphorylated substrate may prove to provide a useful synthetic procedure for compounds otherwise difficult to obtain simply because their phosphorylated precursors are not available. The potential of this technique has been explored somewhat in arsenate-containing systems,[7] but may prove to be more useful with vanadate, where ester formation is ~5 orders of magnitude faster than with arsenate.[8]

In view of the fundamental importance of phosphate esters and anhydrides in biological systems, in particular adenosine mono-, di-, and triphosphate, AMP, ADP, and ATP, respectively, we have initiated a program of investigating the reactions of vanadate with phosphate and various phosphate derivatives, including pyrophosphate and phosphate esters.

It has been shown that the reaction to form phosphate/vanadate anhydrides proceeds favorably, rapidly, and reversibly. Both phosphate and pyrophosphate have been shown to form phospho- and pyrophosphovanadate anhydrides, while pyrophosphate was also observed to form a cyclic vanadate derivative, which has been assigned an octahedral coordination about vanadate.[9] In addition to this, phosphate catalyzes the hydrolysis of vanadate esters and diesters, probably via an alkyl vanadophosphate intermediate.[10]

Vanadate, as mentioned, readily forms vanadate esters with hydroxyl groups[8,11] and in addition can form cyclic complexes when there are adjacent hydroxyls in the molecule.[11] Higher oxidation states of the ligand provide additional products.[12] It seems clear, then, that reaction of vanadate with AMP and with uridine allows for the formation of a variety of products including ADP and ATP analogues as well as 2′,3′-cyclic derivatives analogous, in the case of uridine, to the 2′,3′-cyclic phosphates. Complexes of this type have been used in investigations of the catalytic sites of ribonuclease A,[13,14] rabbit muscle myosin,[15-19] and the dynein ATPase from sea urchin sperm flagella.[20,21] They also have practical applications in recombinent DNA technology.[22] A detailed understanding of the chemistry of these complexes is essential to fully exploiting them as mechanistic probes and may lead to further applications of them.

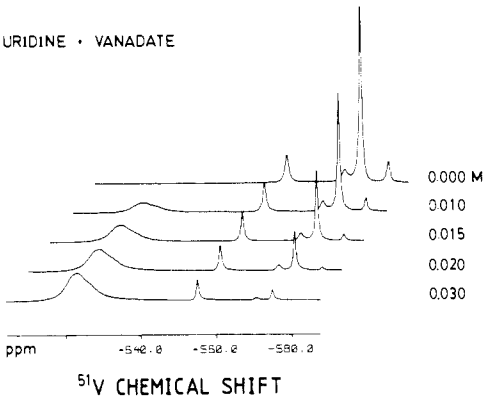

Figure 1. Stacked plot of ^{51}V NMR spectra showing the products of the reaction of vanadate with varying concentrations of added uridine at pH 7.5. A binuclear vanadium product formed from two vanadate ions and two uridine ligands gives rise to an NMR signal at −523 ppm. The signal at −555 ppm derives from monomeric vanadate T_1 and its uridine esters; the signal at −570 is from dimeric vanadate T_2 and its esters. The signal at −575 ppm arises from tetrameric vanadate, while that at −582 ppm is from pentameric vanadate. The solutions contained 3.0 mM total vanadate, 20 mM HEPES at pH 7.5, sufficient KCl to give 1.0 M ionic strength, and the indicated concentrations of uridine.

Experimental Section

Materials. Uridine, adenosine monophosphate (AMP), and HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid) buffer were obtained from Sigma Chemical Co. Vanadium(V) oxide (gold label, 99.999%) was obtained from Aldrich Chemical Co. All chemicals were used without further purification.

Solutions. All solutions were prepared at 1.0 M ionic strength by adding appropriate quantities of KCl. All pH measurements were made with a pH meter calibrated with freshly opened pH standards. Stock solutions of sodium vanadate were prepared by adding 0.5 mol equiv of vanadium pentoxide to a 1.0 M solution of NaOH and stirring until the orange solution became colorless (overnight). This solution was then diluted to 0.1 M with distilled water.

Stock solutions of 1.0 M HEPES and 2.0 M KCl were prepared in distilled water. For low ligand concentration studies a third stock solution of ligand was prepared, while for the higher concentration studies the ligand was added by direct weighing of either the uridine or the AMP.

Solutions for the NMR studies were prepared by combining appropriate quantities of the stock solutions for the desired final concentrations of vanadate, ligand, buffer, and KCl and diluting to near the final volume. The pH was adjusted to the desired value with a NaOH solution. This procedure avoided exposure of the vanadate solution to acid conditions and the subsequent formation of decavanadate. The volume was then adjusted by addition of distilled water, the pH was checked, and small adjustments were made when necessary by adding NaOH solution.

Spectroscopy. All NMR spectra were obtained by using the broadband facility of the Bruker WM-400 NMR spectrometer operating at ambient temperature. ^{51}V NMR spectra were obtained at 105 MHz, using 50° pulse widths, 40-kHz sweep widths, and 0.05-s acquisition times. The spectra were zero filled from 4K to 8K and a line-broadening factor of 35 Hz was applied to all spectra before Fourier transformation to the frequency domain. Signal intensities were measured by using the integration routine provided by the instrument manufacturer.

Methods. The results were analyzed by casting the equations describing the various equilibria into the appropriate linear form as outlined in the text and then plotting the data to confirm the linearity. The least-squares program of a hand calculator was then used to obtain the best estimate of the slope and *y* intercept of the line. Errors in the slope and intercept were estimated from the graphs themselves unless indicated otherwise.

Results and Discussion

The reactions of uridine and of adenosine monophosphate can be expected to be somewhat similar. The ribose ring provides the possibility of forming two secondary esters and a 2′,3′-cyclic ester. In addition, uridine allows for the formation of a 5′-vanadate ester while AMP might form a 5′-phosphate/vanadate anhydride (RPV) or even a 5′-phosphate/divanadate anhydride (PPV_2). Thus, both ADP and ATP analogues might be formed.

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Table I. Concentrations of Vanadate Species Determined as a Function of Total Vanadate and Uridine Concentrations[a]

$[V_t]$	$[Ur_t]$	$[T_t(t)]$	$[T_2(t)]$	$[T_4]$	$[T_5]$	[-523]
3.0	0.0	0.471	0.225	1.966	0.338	0.000
3.0	5.0	0.471	0.229	1.774	0.286	0.240
3.0	10.0	0.429	0.202	1.368	0.202	0.798
3.0	15.0	0.387	0.144	0.835	0.107	1.527
3.0	20.0	0.337	0.097	0.485	0.051	2.031
3.0	25.0	0.294	0.074	0.241	0.025	2.366
3.0	30.0	0.265	0.054	0.133		2.530
3.0	40.0	0.180	0.032	0.041		2.747
0.10	20.0	0.054	0.002			0.045
0.05	20.0	0.035	0.001			0.014

[a] All concentrations are given in millimolar units and, except those for uridine, are vanadium atom concentrations. Conditions of the experiments: pH 7.50, 20 mM HEPES, 1.0 M ionic strength maintained with KCl, the indicated concentrations of vanadate and uridine. [b] Abbreviations: V_t, total vanadium atom concentration; Ur_t, total uridine concentration; $T_t(t)$, total concentration of tetrahedral vanadate species; $T_2(t)$, total concentration of divanadate species; T_4, tetrameric vanadate; T_5, pentameric vanadate; -523, vanadate product giving rise to the -523 ppm signal in the ^{51}V NMR spectrum.

The reactions of vanadate with uridine are very favorable, leading to several products.[13,14] Figure 1 shows ^{51}V NMR spectra of 3 mM vanadate in the presence of varying amounts of uridine at pH 7.5. The predominent new feature of the recorded spectra is the occurrence of a rather broad asymmetric signal at -522.9 ppm indicative of the presence of at least two compounds. A signal in this position was not observed for vandate in the presence of either 2',3'-isopropylideneuridine or 2'-deoxyuridine. In the presence of ethylene glycol[11] and lactate,[12] ^{51}V NMR signals have been observed near this chemical shift, and they have been assigned to pentacoordinate products of a possible trigonal-bipyramidal coordination geometry. A similar structural assignment has been made by other workers[14] for the uridine/vanadate product giving rise to this NMR signal, but their study was not sufficiently detailed to fully reveal the complexity of this system.

Figure 1 does not provide direct evidence for the occurrence of the simple vanadate esters. It is possible, however, to show that they occur and give rise to signals under the resonance from tetrahedral vanadate T_t. There are three hydroxyls available for ester formation, and only a sum of equilibrium constants can be determined for the three products. These products are designated collectively as $T\ell$, where ℓ refers to the ligand. Analogous products are formed from dimeric vanadate T_2, and these are designated as $T_2\ell$. The diesters $T\ell_2$ and $T_2\ell_2$ must also be formed. Table I gives the vanadium atom concentrations measured for the various products or, in the case of signal overlap, the sums of products.

Since the NMR signals from $T\ell$ and $T\ell_2$ lie under the T_t resonance, it is not possible to determine the concentrations of T_t directly from the set of superimposed signals. The vanadate tetramer T_4 does, however, provide a suitable reference from which the concentration of T_t can be determined, since T_4 gives rise to an isolated signal. The concentrations of the various vanadate species are related by eq 1-6.

$$4T_t \overset{K_0}{\rightleftharpoons} T_4 \qquad [T_t] = K_0^{-1/4}[T_4]^{1/4} \tag{1}$$

$$2T_t \overset{K_1}{\rightleftharpoons} T_2 \qquad [T_t] = K_1^{-1/2}[T_2]^{1/2} \tag{2}$$

$$2T_2 \overset{K_2}{\rightleftharpoons} T_4 \qquad [T_2] = K_2^{-1/2}[T_4]^{1/2} \tag{3}$$

$$T_t + \ell \overset{K_3}{\rightleftharpoons} T\ell \qquad [T_t][\ell]K_3 = [T\ell] \tag{4}$$

$$T\ell + \ell \overset{K_4}{\rightleftharpoons} T\ell_2 \qquad [T\ell][\ell]K_4 = [T\ell_2] \tag{5}$$

$$T_2 + \ell \overset{K_5}{\rightleftharpoons} T_2\ell \qquad [T_2][\ell]K_5 = [T_2\ell] \tag{6}$$

Equations 1, 4, and 5 can be combined to give eq 7. A plot of the ratio on the left of this equation versus $[\ell]$ would be expected to give a line of intercept $K_0^{-1/4}$ and upward curvature. When

$$\frac{[T_t] + [T\ell] + [T\ell_2]}{[T_4]^{1/4}} = K_0^{-1/4} + K_0^{-1/4}K_3[\ell] + K_0^{-1/4}K_3K_4[\ell]^2 \tag{7}$$

the experimental parameters were plotted, no upward curvature in the line was observable. This means that the diester $T\ell_2$ was not formed in high enough concentration to be observable by this procedure. An unfortunate consequence of this type of analysis is that comparatively high proportions of the diester must be formed for upward curvature to be observable. This problem is eased considerably when the product gives rise to an isolated signal. From the above plot the intercept gave $K_0^{-1/4} = (3.19 \pm 0.05) \times 10^{-3}$ $M^{3/4}$, and $K_3 = 0.6 \pm 0.3$ M^{-1} was calculated from the slope of the line. The large error in K_3 arises because quite low ligand concentrations were used in this study. Use of higher concentrations provided no advantage since the added ligand shifted the equilibrium away from $T\ell$ to the products giving rise to the -523 ppm signals. The value obtained for K_0 [$(9.7 \pm 0.6) \times 10^9$ M^{-3}] is very close to previous determinations of this value,[23] while K_3, taking into consideration that it is the sum of the equilibrium constants for esterification of the three hydroxyls of uridine, is very close to the value of 0.19 M^{-1} measured for the formation of ethyl vanadate[8] and the value of 0.4 M^{-1} determined for 2-hydroxyethyl vanadate,[11] or $\sim$0.2 M^{-1} per hydroxyl group.

The reaction of uridine with T_2 can be expected to proceed similarly to that with T_t but here again ligand concentrations may not be sufficiently high to form significant proportions of $T_2\ell_2$. Combination of eq 3 with eq 6 leads to eq 8. When the experimental results were plotted according to eq 8, the line obtained had no observable slope, mainly because at high enough concentrations of uridine to form significant proportions of $T_2\ell$ much of the divanadate was incorporated into other products. As a consequence of this, the experimental error was comparable to effects resulting from formation of $T_2\ell$ and a value for K_5 was not obtainable. It is to be expected, however, that this value is comparable to K_3, as has been observed for other systems.[10,23] The value obtained for the intercept of the graph ($K_2^{-1/2}$) was $(5.0 \pm 0.4) \times 10^{-3}$ $M^{-1/2}$ so that $K_2 = (4.0 \pm 0.4) \times 10^4$ M^{-1}.

$$([T_2] + [T_2\ell])/[T_4]^{1/2} = K_2^{-1/2} + K_2^{-1/2}K_5[\ell] \tag{8}$$

From the changes in the relative intensities of the NMR signals that occur in the ^{51}V NMR spectrum as vanadate and uridine concentrations are varied it is clear that the major products giving rise to the NMR signals at -523 ppm are binuclear in vanadium and have two uridine ligands. These products are thus isomers, probably stereoisomers, and they will maintain a constant proportionality to each other as vanadate or uridine concentration is varied. The observable asymmetry in the signal at -523 ppm is attributed to the presence of these isomers, which will be observed when there is little preference for a particular orientation of the critical ligand in the complex. This behavior has been clearly demonstrated for the analogous product formed with 1,2-propanediol.[11] The formation of these binuclear products can be written as a two-step process involving in the first step the for-

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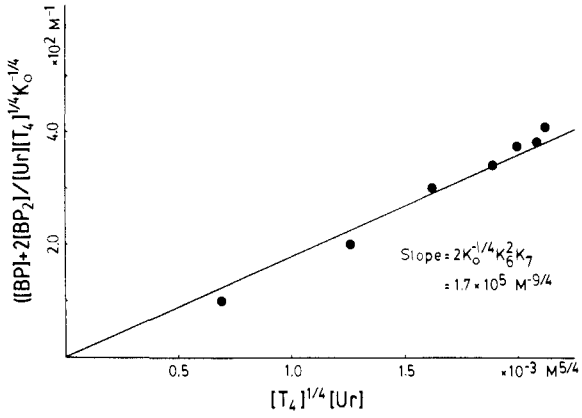


Figure 2. The linear relationship expressed by this graph shows that the formation of the binuclear vanadium product BP_2 formed with uridine is highly favored. This graph provides no evidence for the formation of the monomeric product BP.

mation of the monomeric product (eq 9) from which the dimeric product is formed (eq 10). This process provides a test to ascertain

$$T_i + \ell \overset{K_6}{\rightleftharpoons} BP_1 \qquad [BP_1] = K_6[T_1][\ell] \qquad (9)$$

$$2BP_1 \overset{K_7}{\rightleftharpoons} BP_2 \qquad [BP_2] = K_7[BP_1]^2 \qquad (10)$$

whether mononuclear products are also formed. The symbol BP is adopted since it is thought that these products[14] and similar ones formed from ethylene glycol[11] have a trigonal-bipyramidal coordination geometry. Addition of eq 9 to eq 10 and incorporation of eq 1 leads to eq 11, where the factor of 2 is taken into

$$\frac{[BP_1] + 2[BP_2]}{K_0^{-1/4}[T_4]^{1/4}[\ell]} = K_6 + 2K_0^{-1/4}K_6^2K_7[T_4]^{1/4}[\ell] \qquad (11)$$

the equation since only vanadium atom concentrations are measured. Upon plotting the appropriate parameters of eq 11 as shown in Figure 2, a line with a least-squares intercept that is actually slightly negative was obtained (-31 ± 27 M^{-1}). The intercept cannot be negative; however, from the statistical error in the value of the intercept, an upper limit (two standard deviations) of 23 M^{-1} can be placed on K_6. Much lower levels for the formation constant of a monomeric product have been obtained from studies of 1,1,1-tris(hydroxymethyl)ethane, where a value of 0.023 ± 0.024 M^{-1} was determined.[23] With zero intercept, the slope determined was $(1.7 \pm 0.2) \times 10^5$ $M^{-9/4} = 2K_0^{-1/4}K_6^2K_7$ from which $K_6^2K_7 = K_8 = (2.8 \pm 0.3) \times 10^7$ M^{-3}. This value, K_8, is the formation constant for the formation of BP_2 from two vanadate ions and two uridine ligands and is almost 5 orders of magnitude larger than the analogous value previously determined for ethylene glycol.[9] That this reaction is so highly favorable presumably is an indication that the 2'- and 3'-hydroxyls of the ribose ring, which are cis to each other, are in an optimum geometric relationship for condensation to occur.

Adenosine Monophosphate Plus Vanadate. It is evident from Figure 3 that the reaction of vanadate with AMP is similar to its reaction with uridine (Figure 1). The spectra in this figure differ from those of Figure 1 in two important aspects. The chemical shift of the signal at −551 ppm (T_i in the absence of AMP) shows a significant broadening and a change in chemical shift as the concentration of AMP is increased. This type of behavior is also observed for vanadate in the presence of phosphate,[9] and it is a result of an exchange process that leads to an averaged chemical shift for the signals from vanadate and AMPV. Similar behavior is exhibited by the signal from divanadate but to a lesser extent. The experimental conditions for the spectra of Figure 3 were similar to those for the spectra of Figure 1 except that the pH was 8.0 instead of 7.5. The analysis of the spectra proceeds identically in both cases. The form of the equilibria, eq 4–6, does not change; however, the equilibrium constants now represent formation of vanadate esters in the 2'- and 3'-positions

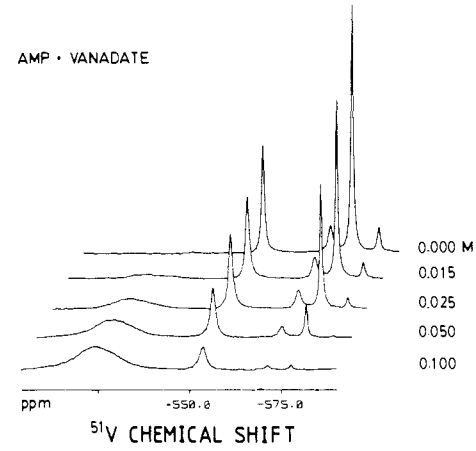


Figure 3. Stacked plot of ^{51}V NMR spectra showing the products formed from the reaction of vanadate with varying proportions of adenosine monophosphate at pH 8.0. A binuclear vanadate complex with two ATP ligands gives rise to a ^{51}V NMR signal at −523 ppm. The signal at −551 ppm arises from vanadate T_i, the esters formed with the 2'- and 3'-hydroxyls of the ribose ring, and the anhydride AMPV, formed with the 5'-phosphate. The substantial broadening and change in chemical shift of this signal arises from the equilibration process as AMPV forms and hydrolyzes. The signal at −569 ppm arises from divanadate T_2, its esters, and also from $AMPV_2$. The signals at −575 and −582 ppm are from tetravanadate and pentavanadate, respectively. Conditions were similar to those given with Figure 1, except that the pH was 8.0.

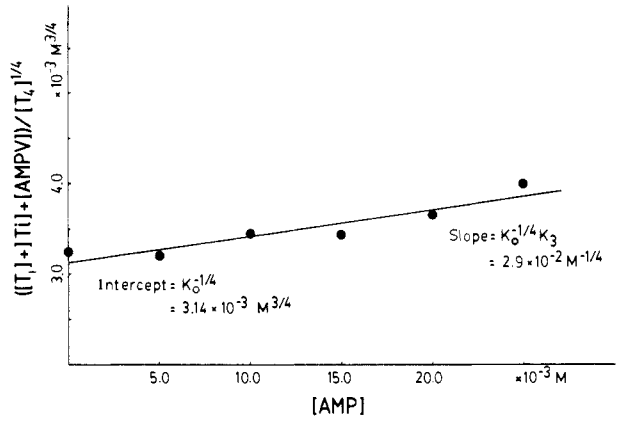


Figure 4. The upward slope of this graph indicates the formation of AMP-vanadate. The intercept is dependent on the formation of tetrameric vanadate T_4.

of the ribose ring as well as phosphate/vanadate anhydrides formed with the phosphate group at the 5'-position of the ribose moiety. K_3 of eq 4 then represents a sum of three equilibrium constants, two for ester formation and one for anhydride formation. Table II gives the vanadium atom concentrations of the various products measured as a function of concentration and pH. A plot of the relevant data of Table II according to eq 7 provided lines of no observable curvature, as can be seen in Figure 4 where the pH 7.5 data are plotted. The slope and intercept then provided the values $K_0 = (10.4 \pm 0.6) \times 10^{10}$ M^{-3} and $K_3 = 9.2 \pm 0.7$ M^{-1}. This latter value is much larger than that determined for the case of uridine, $K_3 = 0.6$ M^{-1}, also at pH 7.5. The main contribution to the value of 9.2 M^{-1} apparently derives from formation of the anhydride between the phosphate of AMP and vanadate. Taking into account the number of hydroxyls in uridine and in AMP, and assuming that the equilibrium constants for esterification of all of the hydroxyls are equal, the equilibrium constant for the formation of the phosphate/vanadate anhydride is 8.8 ± 0.7 M^{-1}. The formation of this anhydride is thus very favorable, about a factor of 40 more favorable than ester formation and very close to that (11.7 M^{-1}) for anhydride formation with phosphate at pH 7.5.[9] It is clear then that a solution of AMP with vanadate produces as a major product the AMP-vanadate derivative, which is an ADP analogue.

Table II. Concentrations of Vanadate Species Determined as a Function of Adenosine Monophosphate Concentration[a]

pH	[AMP]	$[T_i(t)]$	$[T_2(t)]$	$[T_4]$	$[T_5]$	[-523]
6.53	0.0	0.354	0.15	1.30	0.191	
6.50	5.0	0.385	0.13	1.27	0.178	0.030
6.50	10.0	0.394	0.09	1.13	0.121	0.264
6.50	15.0	0.424	0.08	0.96	0.083	0.415
6.51	20.0	0.487	0.887		0.087	0.539
6.97	0.0	0.385	0.17	1.26	0.182	
6.93	5.0	0.379	0.18	1.24	0.202	
6.95	10.0	0.401	0.19	1.11	0.161	0.137
6.95	15.0	0.398	0.13	1.02	0.153	0.305
6.96	20.0	0.417	0.12	0.98	0.142	0.429
6.97	25.0	0.417	0.10	0.76	0.105	0.624
7.48	0.0	0.431	0.165	1.235	0.168	
7.45	5.0	0.421	0.180	1.193	0.170	0.020
7.46	10.0	0.445	0.183	1.099	0.159	0.114
7.47	15.0	0.432	0.163	1.013	0.139	0.252
7.46	20.0	0.446	0.189	0.884	0.097	0.384
7.48	25.0	0.463	0.165	0.726	0.083	0.562
7.97	0.0	0.583	0.213	1.055	0.130	
7.97	5.0	0.585	0.224	1.028	0.136	
7.97	10.0	0.579	0.212	0.994	0.126	0.068
7.98	15.0	0.596	0.220	0.886	0.099	0.174
7.97	20.0	0.591	0.181	0.789	0.092	0.319
7.99	25.0	0.591	0.182	0.682	0.079	0.466

[a] All concentrations are millimolar and, except for those of AMP, are given as vanadium atom concentrations. Conditions of the experiments: 2.0 mM total vanadate, 20 mM HEPES buffer, 1.0 M ionic strength maintained with KCl, the indicated pH's and ligand concentrations. Abbreviations are as for Table I except that AMP refers to adenosine monophosphate.

The vanadate dimer T_2, in a reaction analogous to that of the monomer, can reasonably be expected to form a phosphate/divanadate anhydride. Examination of the results in Table II according to eq 8 provided the formation constants $K_2^{-1/2} = 4.8 \pm 0.4$ M^{-2} and $K_5 = 12.1 \pm 1.4$ M^{-1} for the pH 7.5 study. The value for K_5 when subjected to a small correction factor of $\sim$0.4 M^{-1} for $T_2\ell$ formation is comparable to that for the formation of AMP-vanadate and strongly supports the hypothesis that AMP-divanadate, which is an ATP analogue, is formed.

It can be concluded from this study that both ADP and ATP analogues are favored products of the spontaneous reaction of vanadate with AMP.

Adenosine monophosphate, like uridine, forms the cyclic product BP_2. Here, as for the considerably more intensively studied uridine system, no evidence for the existence of a monomeric pentacoordinate product was obtained. The formation constant, K_8, for BP_2 from $2T_i$ and two ligands as given by eq 11 was found to be $(3.4 \pm 0.2) \times 10^6$ M^{-3} at pH 7.5. This value is a factor of 10 smaller than the corresponding value determined for uridine. The difference in value may reflect different conformational preferences for the ribose rings of the two compounds or electrostatic effects due to the phosphate groups of the AMP complex.

In an effort to gain more information about the equilibrium reactions of AMP with vanadate, the equilibrium constants for formation of the various products were determined at various pH values. The results are shown in Table III.

The hydrogen ion concentration has its effect on the observed equilibria through the K_a's of the ligand, the vanadate, and the product as described by eq 12 for the formation of the anhydride

$$\begin{array}{ccccc} VO_4H_2^- & + & AMP^- & \overset{K_3'}{\rightleftharpoons} & AMPV^{2-} \\ \Big\downarrow\!\!\uparrow K_{a2}\,H^+ & & \Big\downarrow\!\!\uparrow K_{a\ell}\,H^+ & & \Big\downarrow\!\!\uparrow K_{a3}\,H^+ \\ VO_4H^{2-} & & AMP^{2-} & & AMPV^{3-} \end{array} \qquad (12)$$

AMP-vanadate. The equilibrium constants K_{a2} and $K_{a\ell}$ have the values $(6.2 \pm 0.4) \times 10^{-9}$ M^{-1} and $(4.0 \pm 0.5) \times 10^{-7}$ M^{-1}, respectively. K_{a2} was determined from the chemical shift dependence on pH of the NMR signal from T_i as defined by eq 13,[8] where δ_l and δ_h are the limiting shifts at low and high pH, respectively. The shift dependence observed was as follows: pH

$$pH = pK_{a2} + \log [(\delta_l - \delta)/(\delta - \delta_h)] \qquad (13)$$

6.03, -560.2 ppm; pH 6.94, -559.2 ppm; pH 7.48, -556.2 ppm; pH 7.97, -551.1 ppm; pH 8.03, -550.9 ppm, to give the measured $pK_{a2} = 8.21 \pm 0.03$ and the values $\delta_l = -560.4$ ppm and $\delta_h = -535.5$ ppm for the 1.0 M ionic strength conditions of this study. The $K_{a\ell}$ for AMP^- was determined by titration as $pK_{a\ell} = 6.4$, which agrees very well with the reported value of 6.45 albeit obtained under slightly different conditions.[24]

Table III. Equilibrium Reactions and the Corresponding Equilibrium Constants for the Vandate/AMP System Determined as a Function of pH

pH	equilibrium reaction	equilibrium constant
6.51	$T_i + AMP \rightleftharpoons AMPV$	19.6 ± 1.8 M^{-1}
6.96		12.2 ± 0.7 M^{-1}
7.47		9.2 ± 0.7 M^{-1}
7.97		5.4 ± 0.4 M^{-1}
7.47	$T_2 + AMP \rightleftharpoons AMPV_2$	12 ± 2 M^{-1}
7.97		1 ± 2 M^{-1}
6.51	$2T_i + 2AMP \rightleftharpoons BP_2$	$(8.5 \pm 1.0) \times 10^6$ M^{-3}
6.96		$(5.1 \pm 0.4) \times 10^6$ M^{-3}
7.47		$(3.4 \pm 0.2) \times 10^6$ M^{-3}
7.97		$(1.3 \pm 0.1) \times 10^6$ M^{-3}
7.47	$2T_2 \rightleftharpoons T_4$	$(4.3 \pm 0.4) \times 10^4$ M^{-1}
7.97		$(2.2 \pm 0.2) \times 10^4$ M^{-1}
6.51	$4T_i \rightleftharpoons T_4$	$(2.1 \pm 0.1) \times 10^{10}$ M^{-3}
6.96		$(1.8 \pm 0.2) \times 10^{10}$ M^{-3}
7.47		$(1.0 \pm 0.1) \times 10^{10}$ M^{-3}
7.97		$(2.4 \pm 0.2) \times 10^9$ M^{-3}

The observed equilibrium constants of Table III, top entry (K_3), when corrected for ester formation, are related to the pH and the unknown equilibrium constant K_{a3} by eq 14 where K_3' is defined

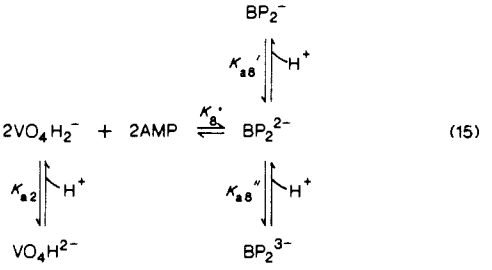
$$K_{corr}(1 + K_{a2}/[H^+])(1 + K_{a\ell}/[H^+]) = K_3' + K_3'K_{a3}/[H^+] \qquad (14)$$

by eq 12 and K_{corr} is the formation constant for the anhydride AMPV. Upon plotting the term on the left of this equation versus $1/[H^+]$, a line of intercept $K_3' = 37 \pm 3$ M^{-1} and slope $K_3'K_{a3} = (2.8 \pm 0.3) \times 10^{-6}$ M^{-2} was obtained from which $K_{a3} = (7.6 \pm 1.2) \times 10^{-8}$ M^{-2} or $pK_{a3} = 7.1 \pm 0.1$. This pK_a is similar to that of adenosine diphosphate, $pK_a = 6.88$, although the latter was measured at 0.2 M ionic strength.[24] Unfortunately, it did not prove possible to obtain a pK_a for AMP-divanadate as the formation constant of the compound could not be determined for the lower pH's of this study. It should be recalled that the

(24) Alberty, R. A. *J. Biol. Chem.* **1969**, *244*, 3290-3302.

measured values of K_3 are actually the sums of the equilibrium constants for anhydride and acyclic ester formation. The K_{corr} utilized in eq 14 were estimated from the various K_3's by assuming ester formation is essentially pH independent in the range of pH utilized here. Actually, ester formation probably decreases slightly, since the pK_a values for esters tend to be slightly larger than for vanadate itself.[25]

The formation of the 2′,3′-cyclic product BP_2 proceeded favorably at all pH's utilized in this study, as is evident from Table III. The presence of two ionizable phosphate groups as well as the two vanadate centers in the BP_2 presents problems in analyzing the results. The analysis is considerably simplified if it is assumed that the pK_a's of the phosphate groups are the same in AMP and in BP_2 and thus do not affect the formation of BP_2 because they are distant from the vanadate moieties and from each other and on the opposite side of the furan ring. Accepting this, the protonation state of the phosphate groups in AMP and BP_2 can be ignored, and the change in formation constant with change in pH reflects only changes in protonation state of the vanadate moieties of BP_2 and of T_i. The pentacoordinate product has positions available for either loss or uptake of a proton with change in pH. On detailed examination of the data it became evident that the ionizations to be considered are those expressed in eq 15. Re-



(15)

organization of eq 15 into an equilibrium equation relating the observed equilibrium constants (K_{obsd}) of Table III, 3rd entry, to K_8' of eq 15 provides eq 16. Examination of the experimental

$$K_{obsd}(1 + K_{a2}/[H^+])^2 = K_8'(1 + [H^+]/K_{a8}' + K_{a8}''/[H^+]) \quad (16)$$

results showed that the term on the left of this equation increased linearly with $[H^+]$, not with $[H^+]^{-1}$, indicating that BP_2^{2-} can incorporate a single proton. A plot of $K_{obsd}(1 + K_{a2}/[H^+])^2$ vs $[H^+]$ gave a line of intercept $K_8' = (3.6 \pm 0.4) \times 10^6$ M^{-3} and slope $K_8'/K_{a8}' = (1.7 \pm 0.2) \times 10^{13}$ M^{-2} from which $K_{a8}' = (2.1 \pm 0.5) \times 10^{-7}$ M^{-1} or $pK_{a8}' = 6.67 \pm 0.10$. No evidence for gain of two protons was obtained. The results did not indicate that BP_2^{2-} can lose a proton at the higher pH of this study although there are protons in the product BP_2^{2-} that might be ionizable, as indicated in eq 15. These results show that formation of BP_2^{2-} from two $VO_4H_2^-$ and two AMP proceeds readily and favorably. The product can gain a proton at the lower pH of this study, but formation of the product does not require uptake of a proton.

(25) Tracey, A. S.; Galeffi, B.; Mahjour, S. *Can. J. Chem.*, in press.

Conclusions

Vanadate ion reacts readily and spontaneously with the hydroxyl groups of uridine to form vanadate esters. The formation constants of these esters are similar to those for the formation of ethyl vanadate[8] and 2-hydroxyethyl vanadate.[11] In addition to the esters, binuclear complexes containing two uridine ligands are formed. These products are proposed to be stereoisomers resulting from different relative orientations of the two uridine molecules, each complexed to a pentacoordinate vanadate ion that is bonded to the second vanadate moiety by way of an oxygen linkage.

Products similar to the above are also formed from the condensation of vanadate with adenosine monophosphate. In addition, vanadate forms an anhydride with the phosphate group of the AMP to give AMP-vanadate. The formation of this adenosine diphosphate (ADP) analogue is favored by a factor of ~40 over that for vanadate ester formation. AMP also reacts readily with divanadate to form the adenosine triphosphate (ATP) analogue AMP-divanadate.

Vanadate is known to readily form a linear pyrophosphatovanadate[9] from pyrophosphate and vanadate so, although the reaction has not specifically been investigated, the condensation of adenosine diphosphate with vanadate to form the ATP analogue ADP-vanadate is expected to be a favorable reaction. On this basis a choice of AMP plus vanadate or ADP plus vanadate allows for the formation of AMP-vanadate, AMP-divanadate, or ADP-vanadate. These vanadate-containing analogues of ADP and ATP may be very useful products for studies that investigate the function of ADP- and ATP-utilizing enzymes.

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