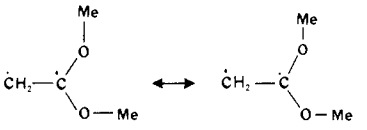
ester hydrolysis is subject to general-acid catalysis,[3] with proton transfer concerted with C–O bond breaking.[30] The negative $\Delta S^{\ddagger}$ values for cation hydration are consistent with an analogous picture in reverse eq 5, i.e. proton transfer concerted with C–O bond making. In fact, general-base catalysis of water addition to monoalkoxy carbocations has been suggested on the basis of trapping experiments.[31] Despite the mechanistic similarity, there is, however, no universal correlation in the rate constants for ortho ester hydrolysis and cation hydration (Figure 5). For example, $MeC^+(OEt)_2$ forms more rapidly and hydrates more slowly than $MeC^+(OMe)_2$. This is the behavior expected on the basis of cation stability, and there is a number of pairs that follow such a pattern. There are, however, equally many examples that do not. For example, the 2-methyl-1,3-dioxolan-2-ylium ion not only forms but also reacts more quickly than $MeC^+(OMe)_2$. In view of this, it may be unwise to interpret differences in rate constants for ortho ester hydrolysis in terms of differences in cation stabilities. Again, the explanation will probably involve the effect of the alkyl substituents on solvation and activation parameters.

Another comparison worth commenting upon involves di- and trialkoxy carbocations (Figure 5). The relative slowness with which the latter form from orthocarbonates was noted over 20 years ago,[29c] being attributed to the weaker basicity of the oxygen leaving group.[3] This effect, however, cannot be important in the hydration direction, where the order is much like one would expect on the basis of cation stability, $(EtO)_3C^+$, for example, reacting almost a 1000-fold more slowly[1] than $(EtO)_2C^+Et$. One explanation for this is that the transition state in eq 5 has proton transfer ahead of C–O bond breaking in the forward direction, so that basicity effects are important. This means that in the reverse direction C–O bond formation is ahead of proton transfer so that cation stability is important. However, the negative activation entropies show that proton transfer is not totally unimportant.

(f) Comparison with Aliphatic Radical Cations. Radical cations of a type that closely resemble α-dialkoxyalkyl carbocations have recently been described.[20] The acyclic radical cations assume in aqueous solution a *Z,E* conformation, and they decay by reaction with water or OH^- whereby OH^- attacks at C_α and the thus



formed hemi ortho ester radical eliminates alcohol to give, e.g. the ester radical $^{\bullet}CH_2CO(OMe)$.[20] What is interesting is the fact that the rate constants for reaction of the *radical* cations with water are 2–3 orders of magnitude *lower* than those for the cations, whereas the rate constants for reaction with OH^- are 1 order of magnitude *higher* for the *radical* cations than for the corresponding cations. This means that the two types of ion have a totally different selectivity in their reactions with the nucleophiles H_2O and OH^-. Another difference is that the *radical* cations are (kinetically) stabilized by increasing alkyl substitution at C_α/C_β, in contrast to the cations (see section d).

Summary and Conclusions

It has been shown that aliphatic α-dialkoxyalkyl carbocations can be produced in aqueous solution (i.e., under solvolytic conditions) from acetals and ortho esters as precursors by using the stepwise hydride-transfer method. The reaction of the cations with water was monitored via the production of H^+ by conductance techniques. On the basis of rate data obtained for 30 carbocations structure–activity relations could be established. For substitution in the alkoxyl group, the reactivity order is Me > Et > *i*-Pr with $\rho^* = +4.4$. For alkyl substitution at C_α, however, the usual steric/electronic order breaks down, and this is explained in terms of counteracting solvational effects. The reactivity order of the dialkoxyalkyl carbocations is discussed in connection with mechanistic schemes proposed for ester and ortho ester hydrolysis, and apparent inconsistencies between these areas are suggested to be due to neglect of solvational effects, the exact nature of which will still have to be established.

Registry No. **7**, 23012-06-8; **10**, 64950-82-9; **11**, 115094-90-1; **12**, 115094-91-2; **13**, 115094-92-3; **14**, 115094-93-4; **15**, 115094-94-5; **16**, 115094-95-6; **17**, 44650-88-6; **18**, 44813-30-1; **19**, 96349-96-1; **20**, 44897-27-0; **21**, 115094-96-7; **22**, 115094-97-8; **23**, 74146-10-4; **24**, 74146-12-6; **25**, 74146-24-0; **26**, 74146-26-2; **27**, 45380-51-6; **28**, 45676-01-5; **29**, 45731-09-7; **30**, 77428-96-7; **31**, 115094-98-9; **32**, 115094-99-0; **33**, 73777-06-7; **34**, 73777-08-9; **35**, 115095-00-6; **36**, 73777-04-5; **37**, 45592-74-3; **38**, 115095-01-7; **39**, 49873-29-2; **40**, 38091-69-9; **41**, 115095-02-8.

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Mechanisms of Decarboxylation of Carboxybiotin

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Abstract: Carboxylation is decarboxylated by two different mechanisms at low pH, where the N1′ carboxyl is protonated (pK 4.25 at 2 °C) and at high pH where it is ionized. The low-pH mechanism has a rate constant of 5.9×10^{-3} min^{-1} at 2 °C (activation energy 33 kcal/mol), while the high-pH one has a rate constant of 4.6×10^{-3} min^{-1} at 25 °C, with the two mechanisms showing equal rates at 25 °C at pH 6.4. At pH 8 the D_2O solvent isotope effect is 1.1 with a curved proton inventory, while at pH 3.4 the value is 1.6 with a linear proton inventory. The ^{13}C isotope effect on decarboxylation is 1.023 at pH 8.2, but 1.012 for the low-pH reaction. The low-pH mechanism probably involves concerted protonation of N1′ and C–N bond cleavage, with an early transition state in which proton motion is ahead of C–N bond breaking. The high-pH mechanism has a moderately early transition state and gives the enolate as the product, with the small solvent isotope effect presumably arising from changes in the fractionation factors of protons in water hydrogen-bonded to the ureido oxygen.

The cofactor biotin serves as a carboxyl carrier in many enzymatic carboxylations, mediating between the carboxyl group donor, usually bicarbonate, and acceptor, usually an α-keto acid or acyl-CoA thioester. Biotin-dependent enzymes show ping-pong kinetics, consistent with the formation of a discrete carboxybiotin intermediate. Although the structure of carboxybiotin remained

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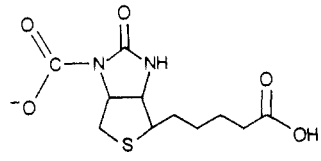
obscure for some time, the elegant studies of Lane[1,2] convincingly demonstrated that carboxybiotin is a carbamate, with the cofactor bearing the carboxyl group on N1′:



Model studies of carbamates have been directed toward gaining a better understanding of biotin chemistry, and in particular how biotin, which is a poor nucleophile, can so readily be carboxylated enzymatically[3-6] and how carboxybiotin is activated for carboxyl transfer.[7] Little work, however, has been done with carboxybiotin itself, although the decarboxylation of *N*-carboxy-2-imidazolidone has been studied.[8] As a preliminary to investigations of enzymatic biotin carboxylation, we have therefore explored the kinetics of decarboxylation of carboxybiotin and will show that two different mechanisms are involved when the carboxyl group on N1′ is protonated or ionized.

Experimental Section

Materials and Methods. Biotin was from Sigma, and biotin methyl ester was from Sigma or was synthesized from biotin.[9] (Methoxycarbonyl)biotin methyl ester was synthesized by the published procedure,[10] except that methyl chloroformate was added in three parts over 3 days. [^{14}C]$NaHCO_3$ was from Amersham. Biotin carboxylase was isolated from *Escherichia coli* B cells, obtained from Grain Processing Co., Muscatine, IA, as described by Tipton.[11]

Kinetic Studies. [^{14}C]Carboxybiotin was generated from [^{14}C]bicarbonate and biotin with biotin carboxylase under conditions based on those described by Guchhait et al.[1] Unreacted $H^{14}CO_3^-$ was removed from the solutions, and the rate of decarboxylation was determined by monitoring the residual ^{14}C content as a function of time. To generate carboxybiotin, 0.01 U of biotin carboxylase was incubated with 1 mL of ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM biotin, 10% (v/v) ethanol, and 9.6 mM $NaH^{14}CO_3$ (200 cpm/nmol) in 100 mM triethanolamine, pH 8.0, at room temperature for 1 h. Carboxybiotin synthesis was terminated by one of two methods, depending on the desired final pH of the solution. To monitor decarboxylation at pH values below the pK of bicarbonate, the reaction was quenched by dilution into two volumes of ice-cold water containing 2 drops/mL 1-octanol and 5 μL of a 20 mg/mL solution of carbonic anhydrase. The solution was bubbled with CO_2 for 30 min on ice to remove unreacted $H^{14}CO_3^-$. The pH was then adjusted to the desired value with HCl, and the solution was placed on ice to slow the decarboxylation sufficiently for it to be followed easily. Aliquots were removed periodically to scintillation vials containing 0.1 mL of 0.1 M NaOH, which served to stabilize the remaining carboxybiotin. Scintillation fluid was added, and the samples were counted immediately.

Reactions above pH 5 were carried out at room temperature. The enzymatic generation of carboxybiotin was quenched by the addition of EDTA to a final concentration of 80 mM. The solution was then adjusted to the desired pH with HCl. Aliquots were removed periodically and placed on ice, and unreacted $H^{14}CO_3^-$ was removed by bubbling with CO_2 for 30 min after addition of carbonic anhydrase. The samples were then counted as above. Proton inventories were carried out by these procedures, but 200 mM Hepes was used in place of triethanolamine.

^{13}C Isotope Effects. Carboxybiotin was generated by saponifying (methoxycarbonyl)biotin methyl ester. A 190-mg portion (0.6 mmol) was dissolved in 6 mL of methanol with warming. After solution, 12 mL of water was added and the atmosphere above the solution was purged with nitrogen. The solution was cooled on ice, and 0.2 mL of saturated CO_2-free KOH was added. After 4 h, contaminating bicarbonate was precipitated by addition of 0.9 mL of 2 M $BaCl_2$/0.1 M $Ba(OH)_2$, and excess barium was precipitated by addition of 3.6 mL of 0.5 M K_2SO_4. The solution was centrifuged at 4 °C at 15000*g* for 10 min, and aliquots of CO_2-free carboxybiotin were then removed from the supernatant to nitrogen-flushed vessels with use of a syringe equipped with a stopcock.

Reaction vessels for low-conversion samples were similar to those described by O'Leary[12] but were equipped with a very fine glass frit over an outlet that could be attached to an aspirator or sealed off. The decarboxylation reactions were quenched by addition of 50 μL of saturated CO_2-free KOH and 0.15 mL of 2 M $BaCl_2$/0.1 M $Ba(OH)_2$. The solution was then filtered through the glass frit, and the soluble carboxybiotin was discarded, leaving the $BaCO_3$ from the decarboxylation trapped on the frit. This $BaCO_3$ was dissolved in 5 mL of 2 M H_3PO_4, the CO_2 was collected and distilled, and its isotopic content was determined as described by O'Leary.[12] The extent of reaction was determined by manometric measurement of the CO_2 produced.

Data Analysis. Rate constants for decarboxylation of carboxybiotin were calculated from the slopes of ln cpm vs time. The pH variation of the rate constants below pH 5 was fitted to eq 1 by the HBBELL program of Cleland.[13] The pH variation of the rate constants above pH 5 was fitted to eq 2, and ^{13}C isotope effects were calculated from eq 3, where R_f is the isotopic composition of the product of the reaction at partial conversion f and R_0 is the isotopic composition of the starting material.

$$\log k = \log [C/(1 + K_1/H)] \quad (1)$$

$$\log k = \log [C(1 + H/K_2)] \quad (2)$$

$$k_{12C}/k_{13C} = \frac{\log (1 - f)}{\log [1 - f(R_f/R_0)]} \quad (3)$$

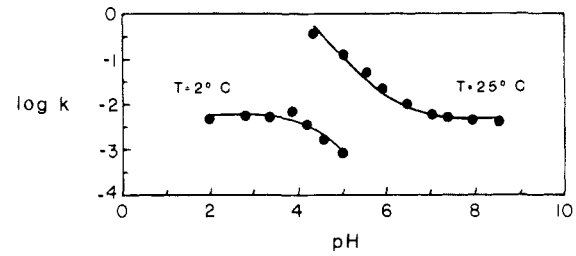


Figure 1. pH variation of the rate constant for decarboxylation of carboxybiotin. The data at 2 °C were fitted to eq 1, while the data at 25 °C were fitted to eq 2.

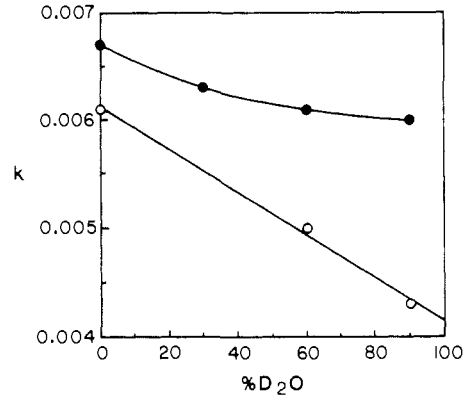


Figure 2. Proton inventories for decarboxylation of carboxybiotin. Open circles for decarboxylation at pH 3 and 2 °C; closed circles are for decarboxylation at pH 8 and 25 °C.

Results

At pH 8.2 and 2 °C, no decomposition of carboxybiotin was detected over 12 h. At higher temperatures and/or lower pH's, however, the decarboxylation proceeds at measurable rates. The pH dependence of the rate constant is shown in Figure 1. Rate constants below pH 5 were determined at 2 °C, and those above

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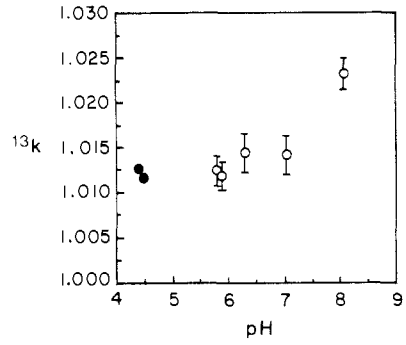


Figure 3. ^{13}C isotope effects on decarboxylation of carboxybiotin. Open circles represent average of two or more determinations; closed circles are for single determinations.

pH 5, at 25 °C. The pH profile shows a plateau below pH 4 with a rate constant of 5.9 ± 0.8 × 10^{-3} min^{-1} at 2 °C and a lower plateau above pH 7 with a rate constant of 4.6 ± 0.5 × 10^{-3} min^{-1} at 25 °C, indicating that two different mechanisms operate at the pH extremes. The break at low pH defines a pK of 4.25 ± 0.13, which we assign to the *N*1′-carboxyl group of carboxybiotin. At pH 6.4 ± 0.07 the two pathways have equal rates at 25 °C. If we assume that the p*K* of carboxybiotin is the same at 2 °C as at 25 °C, the difference in rates at the two temperatures gives an activation energy of 33 kcal/mol for the low-pH mechanism.

Proton inventories (Figure 2) support the conclusion that different pathways operate at the pH extremes. At low pH the D_2O solvent isotope effect is 1.6, and the linear proton inventory suggests that only one proton is in flight in the transition state. At high pH the D_2O solvent isotope effect is about 1.1, but the proton inventory appears curved, suggesting that more than one proton has a decreased fractionation factor in the transition state. Varying the concentration of the Hepes buffer from 50 to 500 mM at pH 8 had no effect on the rate of decarboxylation, so the high-pH mechanism does not show buffer catalysis.

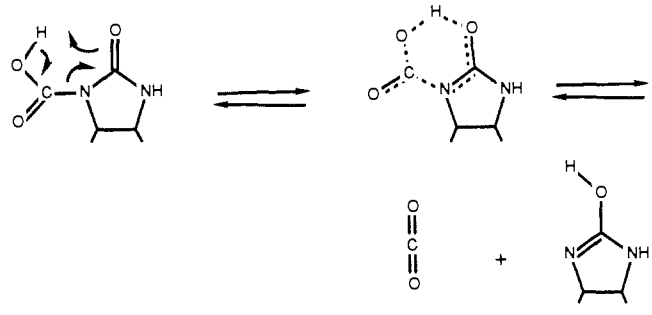
Carbon isotope effects on the decarboxylation of carboxybiotin were measured in order to determine the extent to which C–N bond scission is rate limiting relative to proton transfer. In order to measure the ^{13}C isotope effect accurately, it was necessary quantitatively to separate residual carboxybiotin from the CO_2 produced by decarboxylation without inducing any isotopic fractionation. This was accomplished by precipitation of barium carbonate at high pH, with carboxybiotin remaining in solution. Tests with ^{14}C-labeled bicarbonate showed that less than 1% of the label remained in the supernatant after addition of barium ions and filtration, and in a similar experiment with unlabeled material the redissolved $BaCO_3$ had the same $^{13}C/^{12}C$ ratio as the original bicarbonate. When carboxybiotin was generated from (methoxycarbonyl)biotin methyl ester and immediately treated with Ba^{2+}, no CO_2 was subsequently detected manometrically. Therefore, carboxybiotin is soluble and stable under the conditions of the experiments.

The ^{13}C isotope effects on decarboxylation of carboxybiotin are shown as a function of pH in Figure 3. At pH 8.1 the value was 1.023 ± 0.002, while at pH 5.9 it was 1.012 ± 0.002; C–N bond scission is thus partially rate limiting at all pH values.

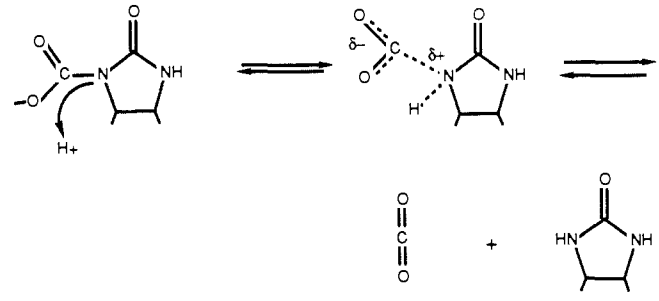
Discussion

Caplow[5] reported that the decarboxylation of *N*-carboxy-2-imidazolidone, an analogue of the ureido ring of carboxybiotin, was acid catalyzed and later found that the anion decomposed by a pH-independent pathway with a half-life of 4 h at 25 °C.[8] These workers did not study the reaction below pH 5, however, and thus did not observe the plateau at low pH seen in the present work (Figure 1). Their rate constant for acid catalysis of 5 × 10^4 M^{-1} min^{-1} at 6 °C is considerably higher than the value of 105 M^{-1} min^{-1} at 2 °C seen for carboxybiotin in the present work. Only a small part of this comes from the difference in temperature (their value for carboxyimidazolidone was 7-fold higher at 25 °C than at 6 °C), but part may come from the effect of metal ions

Scheme I



Scheme II



(which inhibit decarboxylation), since no EDTA was present in our experiments at 2 °C. The dissociation constant of Mn^{2+} from carboxyimidazolidone was reported as 3–4 mM,[8] and our reaction mixtures contained 1.67 mM Mg^{2+}, but the dissociation constant for carboxybiotin may be lower than that for the analogue. In any case, carboxybiotin is ~30-fold more stable in acid at 25 °C than carboxyimidazolidone, if the p*K*s of the carboxyls are the same in the two compounds. By contrast, the decarboxylation of the two compounds at high pH and 25 °C have similar rate constants (2.5 × 10^{-3} min^{-1} for carboxyimidazolidone vs 4.6 × 10^{-3} min^{-1} for carboxybiotin). The change from the acid-catalyzed to pH-independent pathway of decarboxylation of carboxyimidazolidone occurs at a higher pH (>8) than for carboxybiotin (6.4).

Several mechanism can be envisaged for decarboxylation of protonated carboxybiotin. Proton transfer can be intramolecular, as shown in Scheme I. This mechanism is consistent with the D_2O solvent isotope effect of 1.6 and linear proton inventory, which shows that only one proton is in flight in the transition state. The rather low ^{13}C isotope effect of 1.012 suggests an early transition state in which proton motion leads C–N bond breaking. This mechanism leads to the enol of biotin as the product, and it could be argued that such a decarboxylation is not energetically favorable. Tautomerization of enol biotin to the keto form would be very rapid once CO_2 diffused away, however.

Other alternative mechanisms involve protonation of the dianion of carboxybiotin on N1′, as in Scheme II, instead of on the ureido oxygen. This protonation cannot be a preequilibrium process, since the fractionation factor of a proton on N1′ would be the same as that of water and thus there would be no D_2O solvent isotope effect on the reaction. If protonation were concerted with decarboxylation, however, there would be a D_2O solvent isotope effect as observed. This mechanism is energetically much more favorable than the one in Scheme I, since it leads to the stable ureido form of biotin instead of the enol, and is similar to the one proposed by Jencks for cleavage of carbamates of weakly basic amines.[14] In favor of this mechanism is the fact that the rate of decarboxylation of carboxyimidazolidone follows the same dependence of rate on p*K* of the carbamate as is seen for decomposition of other carbamates, which necessarily decarboxylate after N-protonation.[15] However, the protonation of biotin in strong acid occurs on the ureido oxygen rather than on a nitrogen,[16] so this

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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.

Acknowledgment. We thank Dr. William P. Jencks for useful discussions. This work was supported by a grant from the NIH (GM 18938). P.A.T. was a trainee on NIH Grant 5T32-GM 07215.

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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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