ophage lambda (*8*) and a recombinationless mutant of a lambda-ϕ80 hybrid, ϕtde120R (*9*), were tested for multiplicity reactivation on *E. coli* strains AB1886 *uvr*−, *rec*+, and AB-2480 *uvr*−, *rec*− (*10*).

Bacteriophage suspended in dilution buffer ($10^{-2}M$ tris (hydroxymethyl) aminomethane, *p*H 7.4; $10^{-2}M$ $MgSO_4$; 0.01 percent gelatin) were irradiated with ultraviolet by a germicidal lamp (General Electric). Samples removed at various times were assayed for survivors by adsorbing to the appropriate bacterial strains at multiplicities of much less than one (monocomplexes) or greater than one (multicomplexes). The infected bacteria were then assayed by the soft-agar technique (*11*) for infective centers on *uvr*−, *rec*− bacteria to avoid multiplicity reactivation on the plate.

The ratio of multicomplex survival to monocomplex survival (B/A) increases with ultraviolet dose when either the bacteriophage or the host or both are *rec*+ (Table 1). But when both host and bacteriophage are *rec*−, the ratio of multicomplex to monocomplex survival remains constant and essentially the same as the multiplicity of infection.

This indicates that the bacterial and bacteriophage recombination systems are involved in multiplicity reactivation. Thus multiplicity reactivation becomes another method for detecting the presence of recombinational systems in bacteria and bacteriophage and does not require the use of genetic markers.

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Metal Ion Activation of Phosphate Transfer by Bidentate Coordination

Abstract. *The hydrolysis of methyl phosphate bound to the triethylenetetraminecobalt(III) ion is much faster than the hydrolysis of either dimethyl phosphate bound to the same cation or methyl phosphate bound to the pentamminecobalt-(III) ion. The rate enhancement is attributed to bidenate coordination of the methyl phosphate. This feature suggests a pseudorotation mechanism analogous to that proposed by Westheimer for the hydrolysis of ethylene methyl phosphate. Stabilization of bidentate coordination might play a role in metal ion activation of phosphate-transfer enzymes.*

Divalent metal ions are required by most enzymes which catalyze phosphate-transfer reactions (*1*). Using fluorine nuclear magnetic resonance, Mildvan *et al.* (*2*) have demonstrated direct interaction between Mn^{2+} bound to pyruvate kinase and fluorophosphate, which is the product of the fluorokinase reaction. In none of the enzymes which take part in phosphate transfer has the mode of coordination of the metal ion activator been elucidated. We report here on a model system which may be of relevance.

For studies of mechanisms, cobalt(III) complexes offer the advantage of a well-defined coordination sphere, in which ammine ligands are quite inert to substitution. We have studied the hydrolysis of methyl phosphate esters bound to the complex ions $Co(trien)^{3+}$ ("trien" is an abbreviation for triethylenetetramine) and $Co(NH_3)_5^{3+}$. In the former case, two coordination sites are available to the phosphate; in the latter case, only one site is available.

A $0.5M$ solution of $[Co(trien)(PO_4CH_3)]NO_3$ in D_2O was prepared by treating $\beta[Co(trien)CO_3]NO_3$ (*3*) with $H_2PO_4CH_3$ (*4*). Its nuclear magnetic resonance (NMR) spectrum showed a doublet for the methyl protons, whose resonance is split by the phosphorus nuclear spin, at 3.47 and 3.65 parts per million relative to tetramethylsilane. The intensity of the doublet diminished when the solution was heated, and a new peak, attributable to methanol, arose at 3.27 ppm. On the basis of the NMR peak areas, the kinetics were determined to be first order, with a rate constant of 2.7×10^{-4} sec^{-1} at 78°C. The rate was reduced by half for a temperature decrease of approximately 10°C. For $HPO_4CH_3^-$ (*5*), the species with the highest rate of hydrolysis in the aqueous methyl phosphate system, the rate constant at 100°C is 8.23×10^{-6} sec^{-1}, about 130 times slower than the value for $Co(trien)PO_4CH_3^+$ obtained by extrapolation to the same temperature.

A $0.6M$ solution of $[Co(NH_3)_5(PO_4CH_3)]NO_3$ in D_2O, prepared by treating $[Co(NH_3)_5CO_3]NO_3$ (*6*) with $H_2PO_4CH_3$, was heated in a sealed NMR tube at 68°C. From the equilibrium constant for dissociation of $Co(NH_3)_5(PO_4H)^+$, calculated from the results of Schmidt and Taube (*7*), we estimate that the extent of dissociation of $Co(NH_3)_5(PO_4CH_3)^+$ was less than 1 percent. After 48 hours, no trace of a methanol NMR peak could be observed and the methyl phosphate doublet maintained its original intensity. The rate of hydrolysis is at least two orders of magnitude less than that for $Co(trien)(PO_4CH_3)^+$.

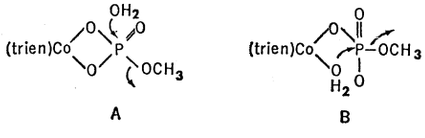
Dimethyl phosphate was also investigated. A $0.5M$ solution of $[Co(trien)(PO_4(CH_3)_2)](NO_3)_2$ was prepared from $\beta[Co(trien)CO_3]NO_3$, $HPO_4(CH_3)_2$ (*8*), and HNO_3 (1:1:1 mole ratio). As with methyl phosphate bound to pentamminecobalt(III), no methanol production could be observed by NMR after extended heating at 68°C. Schmidt and Taube (*7*) have failed to detect methanol in solutions of $\{Co(NH_3)_5[PO_4(CH_3)_2]\}^{2+}$.

Solutions of $[Co(trien)PO_4CH_3]NO_3$ have a *p*H of ~ 3.7. Titration with acid or base produced well-defined, reversible buffer regions at about *p*H 2.4 and 6.7, respectively. The titration curve (Fig. 1) reflects protonation and deprotonation of a monodentate aquo complex, $[Co(trien)(PO_4CH_3)(H_2O)]^+$. The pK_a of this species, ~ 6.7, may

be compared (*9*) with the value 6.75 for $[Co(en)_2(PO_4H)(H_2O)]^+$ ("en" is an abbreviation for ethylenediamine). Lincoln and Stranks (*9*) showed that for HPO_4^{2-} bound in a bidentate linkage to $Co(en)_2^{3+}$, ring opening is facile; the same is evidently true for $PO_4CH_3^{2-}$ bound to $Co(trien)^{3+}$.

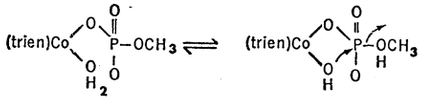
The effect of the protonation-deprotonation equilibria on the rate of methanol production is shown qualitatively in Fig. 1. Precise *p*H control was not maintained because we were unable to find buffers which did not destroy the complex at the high concentration and temperature required. Fortunately, the system is fairly well self-buffered and the *p*H drifts never exceeded 0.5 *p*H unit. The rate of hydrolysis decreases on addition of acid or base. This effect cannot be due to phosphate dissociation from the complex. The NMR chemical shifts of bound and free methyl phosphate would permit detection of free methyl phosphate, and none was observed.

The rate profile can be interpreted with respect to two likely mechanisms for hydrolysis: (A) external attack by water on bidentate methyl phosphate; and (B) internal attack on monodentate methyl phosphate by coordinated water [a mechanism which has been suggested for the catalysis of adenosine triphosphate (ATP) hydrolysis with lanthanon hydroxides (*10*)], namely



A B

Mechanism A is consistent with the observed decrease in rate upon addition of acid or base. Either protonation or deprotonation of $[Co(trien)(PO_4CH_3)(H_2O)]^+$ would shift the equilibrium between bidentate and monodentate forms and reduce the concentration of bidentate methyl phosphate. For mechanism B, one might expect an increase in rate upon addition of base, since coordinated hydroxide should be a better nucleophile than water. However, we cannot rule out the possibility that the leaving methoxide group requires prior protonation and that mechanism B should be written



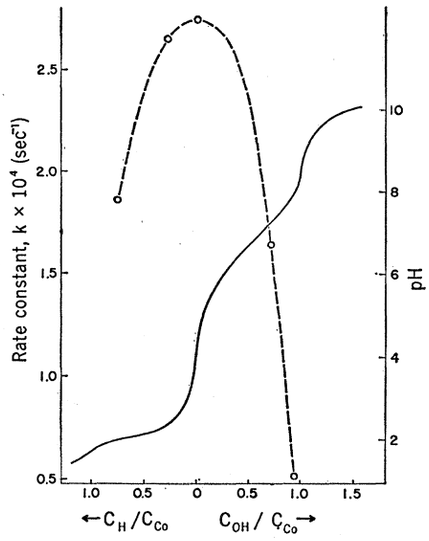


Fig. 1. Titration curve (solid line) for $[Co(trien)(PO_4CH_3)]NO_3$ and first-order rate constants (○) for methanol production in D_2O at 78°C; C_{Co}, concentration of the cobalt complex (0.5*M*); C_H and C_{OH}, concentrations of added HNO_3 or NaOH, respectively.

In this case either protonation or deprotonation might lead to a decrease in rate.

Even though the evidence is not conclusive, it appears likely that bidentate coordination of methyl phosphate by $Co(trien)^{3+}$ produces a pronounced activation of hydrolysis. For the remaining complexes tested and found to be inactive, bidentate coordination is precluded. For $[Co(NH_3)_5PO_4CH_3]^+$ all but one of the coordination sites are blocked, whereas for $[Co(trien)PO_4(CH_3)_2]^{2+}$ binding through the phosphoryl oxygen must be very weak {as may be inferred from the instability of $[Co(NH_3)_5PO_4(CH_3)_3]^{3+}$ (*7*)}.

Previous discussions of the role of metal ions in phosphate activation have focused on the polarization of phosphate electrons by the metal ion and the likelihood that the phosphorus is thereby rendered susceptible to nucleophilic attack or to elimination of a metaphosphate intermediate (*10*, *11*). For all of the systems under consideration, however, the cation is Co^{3+}; although polarization is no doubt somewhat greater for bidentate than for monodentate coordination of the phosphate, the dramatic differences in hydrolysis rates can scarcely be explained in this way. Rather the "bidentate effect" may have a steric basis. The constraint of the O-P-O angle produced by bidentate coordination of methyl phosphate should be similar to that in ethylene methyl phosphate. Hydrolysis of the methyl group in this cyclic phosphate is a million times faster than hydrolysis of trimethyl phosphate (*12*). To explain this difference, Westheimer (*12*) has presented arguments for a pseudorotation mechanism induced by the strain associated with the O-P-O angle in ethylene phosphate. A metal ion could replace the ethylene moiety with similar consequences.

We suggest that bidentate activation may play a role in the mechanism of enzymatic phosphate transfer. The molecular environment of the active site of the enzyme could stabilize bidentate coordination of the metal ion to the transferring phosphate group. Such a structure might not be favored in equilibrium binding in the absence of the enzyme. In the case of ATP, for example, divalent metal ions coordinate two or all three phosphate groups (*13*) in aqueous complexes. The relative concentrations of structures in which two oxygens of the terminal phosphate are bound are probably quite small. ATP utilizing enzymes may stabilize this kind of binding because of favorable geometric features at the binding sites. Phosphate activation by bidentate coordination could thereby be effected.

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