Hydrolysis of Acetyl Dimethyl Phosphate, a Reactive Acyl Phosphate†

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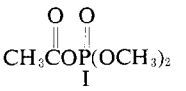
ABSTRACT: The hydrolysis of acetyl dimethyl phosphate was investigated in an attempt to measure the reactivity of an uncharged derivative of acetyl phosphate in the pH region where acetyl phosphate exists in anionic forms. Methanolysis of acetyl dimethyl phosphate occurs with cleavage of the carbon to oxygen anhydride bond. Hydrolysis results from a water reaction (k = 1.75 min^{-1}, 21°) and a hydroxide reaction (k = 2.90 × 10^4 M^{-1} min^{-1}, 21°). The magnitude of the water reaction is attributed to the good leaving ability of dimethyl phosphate and is consistent with rates of hydrolysis of other substituted acetates. The implications of these findings on the mechanism of certain enzymic reactions of acyl phosphates are discussed.

Acetyl phosphate is an important mediator for the transfer of "high-energy phosphate" (Lipmann, 1946) and an important biological acetylating agent (Stadtman, 1952) whose nonenzymatic hydrolysis has received considerable study. Koshland (1951) investigated the rate of hydrolysis of acetyl phosphate as a function of pH and observed catalysis of the hydrolysis by magnesium(II) chloride (1952). DiSabato and Jencks (1961) showed that the spontaneous hydrolysis reactions of the anions of acetyl phosphate involve initial elimination of a metaphosphate ion. Oestreich and Jones (1966) have measured the catalytic effects of various metal ions on the hydrolysis rate of acetyl phosphate. Briggs *et al.* (1970) have provided improved kinetic data on those effects. Klinman and Samuel (1971) have determined the position of cleavage of acetyl phosphate in magnesium(II)- and calcium(II)-catalyzed reactions. Some fundamental questions concerning the enzymatic reactivity of acetyl phosphate include the following. (1) How does acetyl phosphate become more reactive on binding? (2) How do different enzymes control the portion of the anhydride molecule that is subject to nucleophilic attack? In regard to the second question, for example, acyl phosphatase (Lipmann, 1946; Bentley, 1949) catalyzes cleavage of the phosphorus–oxygen anhydride bond of acetyl phosphate while phosphotransacetylase (Stadtman, 1952) and (oxidized) glyceraldehyde-3-phosphate dehydrogenase (Park and Koshland, 1958) catalyze attack at the carboxyl carbon atom.

There are several ways that a charged substrate, upon binding to an oppositely charged site on an enzyme, might become more reactive due to the electrostatic consequences of that binding. These include (1) the substrate becomes susceptible to attack by a species that normally would be repelled by the charged substrate, (2) the electron density at the point of attack changes so that the adding species can combine more easily, and (3) the leaving ability of a departing group is improved. More complex consequences of such binding, specifically to metals, have been considered by Selwyn (1968) and Farrell *et al.* (1969). Since acetyl dimethyl phosphate (I) contains an acetic acid–phosphoric acid anhydride bond, a determination of the reactivity of the diester can provide a basis for estimating the reactivity of acyl phosphates whose charge has been neutralized by binding to cations. An indication of the compound's acetylating ability and its sensitivity to water have been provided by qualitative experiments on a crude preparation by Avison (1955) and on the pure diethyl ester by Cramer and Gartner (1958).



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Materials and Methods

Materials. Acetyl chloride was purchased from the Allied Chemical Co. Trimethyl phosphate (97%) was supplied by the Aldrich Chemical Co. The titrant used for pH-Stat kinetic experiments was Fisher 0.1 N sodium hydroxide. All inorganic materials used were reagent grade.

Synthesis of Acetyl Dimethyl Phosphate. The general method described by Whetstone (1953) for the synthesis of mixed anhydrides of dialkyl esters of phosphoric acids and carboxylic acids was followed for the synthesis of acetyl dimethyl phosphate. Trimethyl phosphate (140 g, 1.0 mole) and acetyl chloride (60 g, 0.77 mole) were refluxed for 12 hr. After rotary evaporation (50°, 25 Torr) to remove considerable amounts of unreacted acetyl chloride, the solution was distilled through a 100-mm vacuum-jacketed Vigreaux column. The fraction boiling at 60–67° (0.1 Torr) was collected, redistilled, and collected at 51–52° (0.05 Torr). Lower boiling fractions contained mostly trimethyl phosphate. The yield was 8.0 g (0.05 mole) of acetyl dimethyl phosphate. *Anal.* Calcd for $C_4H_9O_5P$: C, 28.65; H, 5.40; P, 18.44. Found (Gailbraith Laboratories, Knoxville, Tenn.): C, 28.57; H, 5.35; P, 18.21.

Nuclear Magnetic Resonance Spectrum. Peaks observed for a carbon tetrachloride solution (in parts per million downfield from internal Me_4Si): 2.2 (3 H doublet, J_{P-H} = 1.5 Hz, acetyl methyl), 3.75 (6 H doublet, J_{P-H} = 11.0 Hz, methyl ester).

Infrared Spectrum. The infrared spectrum of acetyl dimethyl phosphate was recorded as a liquid film with major absorbance peaks (cm^{-1}) at 2950, 2850, 1780, 1440, 1380, 1170, 900, 840, 790, and 740.

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TABLE I: Hydrolysis of Acetyl Dimethyl Phosphate.

Salt	Concn (M)	pH	Temp (°C)	k_{obsd} (min^{-1})
KCl	0.1	1.5	21.0	1.97, 1.68
KCl	0.1	2.0	21.0	1.73
KCl	0.1	2.5	21.0	1.57
KCl	0.1	3.0	21.0	1.68, 1.77
KCl	0.1	3.5	21.0	1.68, 1.65
KCl	0.1	4.0	21.0	1.65, 1.77
KCl	0.1	4.5	21.0	1.68, 1.87
KCl	0.1	5.0	21.0	1.65, 1.92
KCl	0.1	5.5	21.0	1.61, 1.68
KCl	0.1	6.0	21.0	1.57, 1.68, 1.97, 1.77
KCl	0.1	6.5	21.0	1.86, 1.77, 1.68
KCl	0.1	7.0	21.0	1.77, 1.87, 1.92, 1.82
KCl	0.1	7.5	21.0	1.77, 1.77, 1.97, 2.16
KCl	0.1	8.0	21.0	1.82, 1.82, 2.03, 2.23
KCl	0.1	8.5	21.0	1.77, 1.77, 1.82, 2.03, 2.23
KCl	0.1	9.0	21.0	1.92, 1.92, 2.10
KCl	0.1	9.5	21.0	2.23, 2.47, 2.56
KCl	0.1	9.6	21.0	2.47
KCl	0.1	10.0	21.0	3.46, 4.94
KCl	0.1	10.5	21.0	6.29, 7.69
KCl	0.1	6.0	11.0	0.58, 0.58
KCl	0.1	6.0	15.0	0.96, 0.96
KCl	0.1	6.0	23.0	2.16
KCl	0.1	6.0	25.0	2.30, 2.77
KCl	0.1	6.0	30.0	4.07
KCl	0.1	6.0	34.0	5.54, 5.54
KCl	0.5	6.0	23.0	2.16
KCl	1.0	6.0	23.0	2.23, 2.23
$NaClO_4$	0.1	6.0	23.0	2.10
$MgCl_2$	0.05	5.0	21.0	1.57, 1.50, 1.47
D_2O	pD	6.0	21.0	1.11, 1.10, 1.25

E_{act} = 17 kcal/mole (for the 11.0°, 15.0°, 23.0°, 25.0°, 30.0°, and 34.0° entries)

pH-Stat Kinetics. The rate of hydrolysis of acetyl dimethyl phosphate at various pH's was determined in a pH-Stat assembly. Rates were of sufficient magnitude that no provision was necessary to prevent carbon dioxide absorption from having an effect on the observations in the alkaline region. A 15-ml 14/20 standard taper pear-shaped flask with side arm was modified to have a flat bottom 12 mm in diameter and also was modified to have an extra side arm. Sodium hydroxide solution was added from a Radiometer ABU11 buret through polyethylene tubing *via* one side arm. Addition was controlled by a Radiometer TTT1 automatic titrator. The volume of titrant (0.1 N sodium hydroxide) added was recorded as a function of time on a Radiometer SBR2 titrigraph. A Radiometer GK202B combined pH electrode was secured through the center hole of the flask. The flask was provided with a magnetic stirrer and was immersed in a jacketed beaker through which thermostated water was circulated. Temperature was maintained to within 0.1° by a Cole Parmer Versa-Therm proportional controller. Acetyl dimethyl phosphate (usually 5 μl) was added from a Hamilton syringe *via* the vacant side arm. All reported runs gave good first-order plots (pseudo-first-order conditions) for at least four half-times.

Product Analysis. Analysis of the products of hydrolysis in a large-scale run in deuterium oxide (0.1 g in 0.5 ml of D_2O) was done with the aid of nuclear magnetic resonance spectroscopy. The initial rapid reaction produced peaks corresponding to acetic acid and dimethylphosphoric acid (acetic acid singlet, δ 2.1 relative to external Me_4Si, dimethylphosphoric acid doublet, J = 10 Hz, δ 3.8). No peak corresponding to the methyl group singlet absorption of methanol appeared. Addition of a drop of methanol caused the appearance of a new singlet (at δ 3.4).

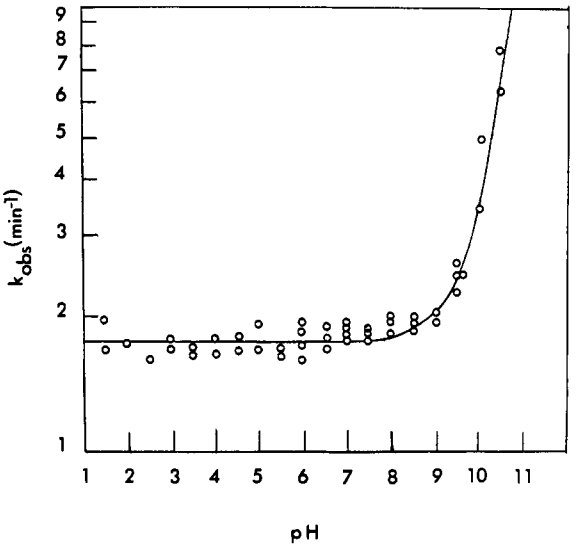

FIGURE 1: pH–rate profile for the hydrolysis of acetyl dimethyl phosphate (0.003 M) at 21° in 0.1 M KCl solution. The plotted points correspond to values in Table I. The curve follows the expression k_{obsd} = 1.75 min^{-1} + 2.90 × 10^{-4} l. $mole^{-1}$ min^{-1} × (OH^-).

Methanolysis of Acetyl Dimethyl Phosphate. Nuclear magnetic resonance spectroscopy was utilized to determine the products of methanolysis of acetyl dimethyl phosphate. Acetyl dimethyl phosphate (13.4 mg) was added to 0.4 ml of a solution containing 10% (by volume) deuterium oxide (Diaprep, Inc.) and 90% (by volume) methyl alcohol-d_4 (Thompson-Packard, Inc.). The nuclear magnetic resonance spectrum of the solution obtained after 1 hr at 25° showed peaks coincident with the acetyl methyl proton absorption of methyl acetate (95%) and acetic acid (5%) in the δ 2 region. The assignments were confirmed by addition of the known compounds. In the region where methyl ester absorption peaks would be expected, a doublet (δ 3.3, J = 11 Hz) appeared which was assigned to dimethylphosphoric acid. (Addition of trimethyl phosphate produced a second doublet, δ 3.4, J = 11 Hz).

Results

The pH *vs.* log rate constant profile for the hydrolysis of acetyl dimethyl phosphate at 21.0° in 0.1 M potassium chloride solution (5 μl of acetyl dimethyl phosphate in 7.5 ml of solution) is shown in Figure 1. The plotted points correspond to values listed in Table I. The lowest pH where the hydrolysis was studied is pH 1.5 (pH 1 and 2 were calibrated with 0.1 and 0.01 N hydrochloric acid, respectively). Below pH 1.5 the dissociation of dimethylphosphoric acid is reduced to the point where the pH-Stat method is not accurate at the substrate concentration used at higher pH. The curve in Figure 1 has been plotted according to the equation k_{obsd} = k_{H_2O} + k_{OH}-(OH^-). No acid catalysis was observed. Values of 1.75 min^{-1} for k_{H_2O} and 2.90 × 10^4 M^{-1} min^{-1} for

k_{OH^-} have been used to obtain the curve. Rates of the water reaction at a variety of temperatures, ionic strengths and alternate ions are also given in Table I. Activation energy from the appropriate temperature versus rate plot is included. The water reaction can be seen to be insensitive to variation in potassium chloride concentration and change to magnesium chloride or sodium perchlorate in the medium. Methanolysis experiments, as described under Materials and Methods, reveal that attack by methanol in the mixed water–methanol system occurs at carboxyl carbon to give methyl acetate.

Discussion

The rate of hydrolysis of acetyl dimethyl phosphate is about 40,000 times that observed for the hydrolysis of acetyl phenyl phosphate (DiSabato and Jencks, 1961) extrapolated to a common temperature. The two hydrolyses are subject to similarly directed solvent isotope effects and methanolysis in both cases leads to the production of methyl acetate, suggesting that a common hydrolytic mechanism may be involved. The relative basicities of the anion of dimethylphosphoric acid ($pK_{a'}$ = 1.29; Kumler and Eiler, 1943) and the dianion of phenylphosphoric acid ($pK_{a'}$ = 5.88; Chanley and Feageson, 1955), by the ratio of the dissociation constants of the conjugate acids, are identical with the ratio of hydrolysis rates of the respective monoanhydrides with acetic acid. The apparent sensitivity of the overall rate to the basicity of the leaving group suggests that the slowest step of the mechanism involves breaking of the anhydride bond and is consistent with Kirsch and Jencks' observations (1964) for acetate esters. Mechanisms proposed for the hydrolytic reactions of substituted neutral acetate esters should also be valid for the case of the mixed anhydride. The high degree of the sensitivity of the hydrolysis rate to the leaving group's basicity suggests that the transition state for departure is far along the reaction coordinate (Marcus, 1969; Cohen and Marcus, 1968).

The evidence we have presented indicates the extent to which a neutral acetyl phosphate derivative is a more reactive acetylating agent than is an anionic acetyl phosphate derivative. The reason for the enhanced reactivity is the conversion of the phosphate moiety to a better leaving group. Binding of a charged acyl phosphate to a cationic site on an enzyme should result, by electrostatic neutralization of the charge of the anion, in improvement of that substrate's acylating ability. This should facilitate cleavage of acetyl phosphate, for example, between carbon and oxygen in reactions such as those catalyzed by glyceraldehyde-3-phosphate dehydrogenase (Park and Koshland, 1958) and phosphotransacetylase (Stadtman, 1952). Consideration of special modes of binding of acyl phosphates to enzymes which may enhance rates of phosphorylation, analogous, for example, to that of phosphate to metal cations found by Farrell *et al.* (1969), should take into account the tendency of such binding to accelerate the competing acylation reaction. Our further studies are aimed at arriving at an understanding of other factors involved in determining the reactive position of acyl phosphates in enzymatic reactions.

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