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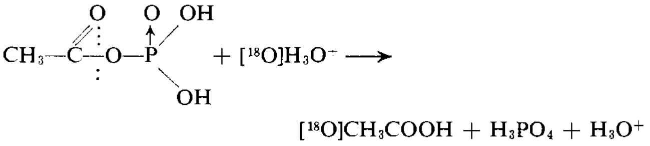
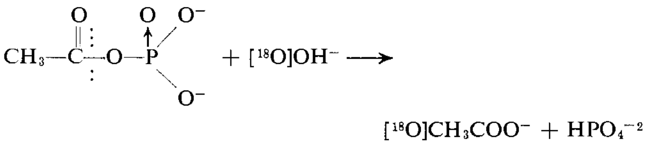
Oxygen-18 Studies to Determine the Position of Bond Cleavage of Acetyl Phosphate in the Presence of Divalent Metal Ions[*]

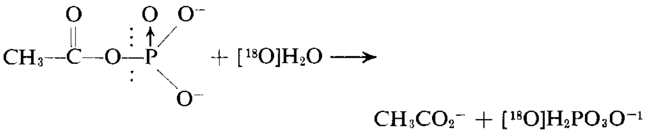
Judith Pollock Klinman[†] and David Samuel

ABSTRACT: The hydrolysis of acetyl phosphate has been carried out in $[^{18}O]H_2O$ at 39° in the presence of Mg^{2+} and Ca^{2+} in the pH range 7.4–8.2. The objective of this study was to determine the position of bond cleavage of metal-complexed acetyl phosphate for each of the rate terms contributing to the metal ion catalyzed hydrolysis. The breakdown of divalent metal ion complexed acetyl phosphate (AcPM) by water occurs by both P–O and C–O cleavage; P–O cleavage is the predominant mode for the Mg^{2+} system, whereas for the Ca^{2+} system C–O cleavage occurs to a greater extent than P–O cleavage. The breakdown of AcPM by MOH^+ occurs by C–O cleavage. The data obtained from this study are consistent with a C–O mode of cleavage of AcPM by OH^- in the case that values for K, the association constant for the formation of AcPM, are close to those originally reported; the effect of significantly larger values for K, which have been recently reported, on the interpretation of this mode is discussed.

This study was undertaken to determine the effect of divalent cations on the position of bond cleavage of acetyl phosphate in order to understand better the catalytic role of metal ions in the hydrolysis of acyl phosphates. The uncatalyzed hydrolysis of acetyl phosphate has been studied in considerable detail (Lipmann and Tuttle, 1944; Koshland, 1952; Kurz and Gutsche, 1960; DiSabato and Jencks, 1961). By isotope labeling experiments, acetyl phosphate has been shown to undergo C–O or P–O cleavage, depending on the pH (Bentley, 1949; Park and Koshland, 1958). Under acidic or basic conditions, C–O bond cleavage occurs. At more neutral pH values (where acetyl phosphate exists as either a mono- or dianion), P–O bond cleavage is the predominant pathway.







Oestreich and Jones (1966) describe the catalysis of the breakdown of the divalent anion of acetyl phosphate by divalent metal ions by eq 1.[1] The present paper describes the results of

$$v_{cat} = k_2[AcPM] + k_3[AcPM][OH^-] \qquad (1)$$

a determination of the amount of P–O and C–O cleavage contributing to each of the two rate terms in eq 1 for catalysis by Mg and Ca ions, utilizing the rate and equilibrium constants determined by Oestreich and Jones.

In a recent report by Briggs *et al.* (1970), these authors con-

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[*] From the Isotopes Department, Weizmann Institute of Science, Rehovoth, Israel. *Received September 15, 1970.*

[†] To whom all inquiries should be addressed. Supported by a Weizmann Institute of Science Fellowship. Current address: The Institute for Cancer Research, Philadelphia, Pa. 19111. We regret that there will be no reprints available.

[1] The following abbreviations are used: acetyl phosphate dianion, AcP^{2-}; acetyl phosphate monoanion, AcPH; divalent metal ion, M^{2+}; divalent metal ion complexed acetyl phosphate, AcPM; sum of all species of acetyl phosphate, AcP.

TABLE I: Incorporation of ^{18}O into Inorganic Phosphate Resulting from the Hydrolysis of Acetyl Phosphate in the Presence of Mg^{2+} or Ca^{2+} in $[^{18}O]H_2O$.

Reaction	Metal Ion (M)	pH	% ^{18}O of Solvent	% ^{18}O of Phosphate[a]	Average of ^{18}O	
1	0.147 Mg^{2+}	7.6	6.21	0.469	0.495 ± 0.029	0.503 ± 0.016
				0.483		
				0.535		
2		7.6	6.21	0.493	0.511 ± 0.023	
				0.517		
				0.522		
3		7.4	6.21	0.586	0.592 ± 0.008	
				0.599		
				0.590		
4		7.9	5.92	0.351	0.356 ± 0.008	
				0.355		
				0.360		
5	0.240 Ca^{2+}	7.7	5.23	0.412	0.420 ± 0.017	
				0.418		
				0.431		
6	0.246 Ca^{2+}	8.2	6.21	0.321	0.322 ± 0.003	
				0.321		
				0.323		

[a] Each sample of $[^{18}O]$phosphate was prepared and converted to CO_2 as described in the Experimental Section. The samples of $[^{18}O]CO_2$ were determined in triplicate on the mass spectrometer for per cent enrichment.

clude that the rate expression for the metal ion catalyzed hydrolysis of acetyl phosphate is described by an expanded version of eq 1 to include a third term containing the concentration of divalent metal ion squared.

$$v_{\text{cat}} = k_2[\text{AcPM}] + k_3[\text{AcPM}][\text{OH}^-] + k_4[\text{AcPM}][\text{MOH}^+] \quad (2)$$

In addition, Briggs *et al.* report values of K, the association constant for the formation of AcPM, which are approximately an order of magnitude larger than those reported by Oestreich and Jones. The effect of the recent findings of Briggs *et al.* on the interpretation of the data presented in this paper is discussed.

Experimental Procedures

Materials. Acetyl phosphate was prepared as the disilver salt (Lipmann and Tuttle, 1944) which was converted to the dilithium salt (Lipmann and Tuttle, 1947). *Anal.* Calcd for $C_2H_3O_5PLi_2$: C, 15.8; H, 1.99; P, 20.4. Found: C, 15.4; H, 2.04; P, 19.91. Solutions of calcium perchlorate, hexahydrate (G. Frederick Smith Chem. Co., Columbus, Ohio), were analyzed spectrometrically for the concentration of Ca^{2+}. Solutions of magnesium perchlorate, anhydrous (G. Frederick Smith Chem. Co., Columbus, Ohio), were analyzed gravimetrically for the Mg^{2+} concentration. $[^{18}O]H_2O$ was supplied by Yeda (Rehovoth, Israel).

Hydrolysis. To a solution of either calcium or magnesium perchlorate in 30 ml of 5–7% $[^{18}O]H_2O$, which was thermostated at 39°, was added 35 mg of dilithium acetyl phosphate. The ionic strength of these solutions was 0.45–0.75. Constant pH was maintained throughout the course of the hydrolysis with a Radiometer titrigraph (type SBRz/SBU1).

Isotopic Analysis of Product Phosphate. At the end of each reaction, $BaCl_2$ was added to the reaction solution. The solution was centrifuged to precipitate $Ba_3(PO_4)_2$, which was converted to KH_2PO_4. The samples of KH_2PO_4 were dried and converted to CO_2 according to the method of Anbar and Guttman (1959). The CO_2 was analyzed by the method of ratios on a mass spectrometer for per cent ^{18}O enrichment.

Results

Based on the findings of Oestreich and Jones, the rate equation for the hydrolysis of acetyl phosphate in the presence of divalent metal ions can be written as follows.

$$v = k_1[\text{AcP}^{2-}] + k_{\text{OH}^-}[\text{AcP}^{2-}][\text{OH}^-] + k_2[\text{AcPM}] + k_3[\text{AcPM}][\text{OH}^-] \quad (3)$$

Equation 3 can be rewritten, utilizing the relationship, $K = [\text{AcPM}]/[\text{AcP}^{2-}][\text{M}^{2+}]$

$$v = k_{\text{obsd}}[\text{AcP}] = k_{\text{T}}[\text{AcP}^{2-}] \quad (4)$$

where $k_{\text{T}} = k_1 + k_2K[\text{M}^{2+}] + k_3K[\text{M}^{2+}][\text{OH}^-]$. The contribution of k_{OH^-} to k_{T} can be ignored in the pH range studied. It is possible to define the fraction of the total concentration of product, $[\text{P}]_i/[\text{P}]_{\text{T}}$, which arises as a result of each of the reaction pathways contributing to eq 4.

$$\frac{[\text{P}]_1}{[\text{P}]_{\text{T}}} = k_1' = \frac{k_1}{k_{\text{T}}};\quad \frac{[\text{P}]_2}{[\text{P}]_{\text{T}}} = k_2' = \frac{k_2K[\text{M}^{2+}]}{k_{\text{T}}};\quad \frac{[\text{P}]_3}{[\text{P}]_{\text{T}}} = k_3' = \frac{k_3K[\text{M}^{2+}][\text{OH}^-]}{k_{\text{T}}} \quad (5)$$

TABLE II: Summary of Literature Values for k_1, k_2, k_3, and K^a for Hydrolysis of Acetyl Phosphate in the Presence of Mg and Ca Cations at 39°, $\mu = 0.6$.

Metal Ion	$k_1 \times 10^3$ min^{-1}	$k_2 \times 10^2$ min^{-1}	$k_3 \times 10^{-4}$ M^{-1} min^{-1}	K M^{-1}
Mg^{2+}	4.4	1.4	17.0	5.7
Ca^{2+}	4.4	2.8	3.7	7.7

[a] From the data of Oestreich and Jones (1966).

The expression which relates the observed per cent ^{18}O of product phosphate to the relative amounts of P–O and C–O bond cleavage for each of the rate terms is then written as follows

$$(\%\ ^{18}O)_{obsd} = k_1'[X(0.204) + (1 - X)(I)] + k_2'[Y(0.204) + (1 - Y(I)] + k_3'[Z(0.204) + (1 - Z)(I)] \quad (6)$$

where X = the fraction of C–O bond cleavage occurring along the reaction coordinate represented by k_1, $(1 - X)$ = the fraction of P–O bond cleavage occurring and likewise for Y and $(1 - Y)$ and Z and $(1 - Z)$. The number, 0.204, represents the per cent natural abundance of ^{18}O, and I is the per cent ^{18}O of product phosphate one would observe in the case of complete P–O bond cleavage ($X = Y = Z = O$).

The experimental results for the incorporation of ^{18}O into product inorganic phosphate for the hydrolysis of acetyl phosphate in the presence of Mg^{2+} and Ca^{2+} are presented in Table I. In Table II, the literature values at 39° for the kinetic and equilibrium constants, k_1, k_2, k_3, and K, for the hydrolysis of acetyl phosphate in the presence of Mg and Ca ions are tabulated. The calculated values for the parameters, k_1', k_2', k_3', and I, which have been defined above, are given in Table III.

With the data in Tables I–III and eq 6, one can write the following equations for the Mg system

$$0.592 = (0.08)(1.70) + 0.23a + 0.69b \quad (7a)$$

$$0.503 = (0.06)(1.70) + 0.16a + 0.78b \quad (7b)$$

$$0.356 = (0.03)(1.63) + 0.09a + 0.88b \quad (7c)$$

where $a = [Y(0.204) + (1 - Y)(I)]$ and $b = [Z(0.204) + (1 - Z)(I)]$. Since it has been demonstrated that the uncatalyzed hydrolysis of AcP^{2-} occurs predominantly by P–O bond fission, the value of $X = 0$ has been assumed in solving for Y and Z.

TABLE III: Values for Parameters k_1', k_2', k_3', and I from Equations 7 and 8 and as Defined in the Text.

Reaction[a]	I	k_1'	k_2'	k_3'
1 and 2	1.70	0.06	0.16	0.78
3	1.70	0.08	0.23	0.69
4	1.63	0.03	0.09	0.88
5	1.46	0.05	0.57	0.38
6	1.70	0.03	0.31	0.66

[a] Refers to the reaction numbers of Table I.

Similarly, from the data at two pH values for the Ca system, two simultaneous equations can be written.

$$0.420 = (0.05)(1.46) + 0.57a + 0.38b \quad (8a)$$

$$0.322 = (0.03)(1.70) + 0.31a + 0.66b \quad (8b)$$

Solutions of Y and Z for the Mg and Ca systems are given in Table IV.

Discussion

The calculation of Y and Z from eq 6 was based on the rate equation of Oestreich and Jones (1966) and utilized the kinetic and thermodynamic constants of these authors. As a result of the recent conclusions of Briggs *et al.* (1970), that rate eq 2 better describes the metal ion catalyzed hydrolysis of acetyl phosphate, we have evaluated the effect of a kinetic term containing the concentration of divalent metal ion squared on the absolute values of Y and Z. In addition, due to the discrepancy between values of K reported by Oestreich and Jones and Briggs *et al.*, we have examined the effect of changes in K on Y and Z.

A reevaluation of the data of Oestreich and Jones at 39° according to rate eq 2 was carried out to give new values for all constants; the kinetic constants reported by Briggs *et al.* were determined at 25° and are not directly applicable to the data presented in this paper. A description of the equations used for this reevaluation follows. Expansion of eq 2 to contain the noncatalytic term contributing to the hydrolysis of acetyl phosphate in the pH range studied and expression of acetyl phosphate as the concentration of uncomplexed material gives

$$V = k_{obsd}[AcP] = [AcP^{2-}](k_1 + k_2K[M^{2+}] + k_3K[M^{2+}][OH^-] + k_4KK_2[M^{2+}][OH^-]) \quad (9)$$

where $K_2 = [MOH^+]/[M^{2+}]\,[OH^-]$. A careful examination of the data of Oestreich and Jones reveals that, as predicted by eq 9, plots of $k_{obsd}[AcP]/[AcP^{2-}]$ as a function of divalent metal ion concentration at pH 7.7 for Mg^{2+} and pH 8.8 for Ca^{2+} indicate a greater than first-order dependence on metal ion concentration. If one substitutes $1/(1 + [M^{2+}]K) = [AcP^{2-}]/[AcP]$ into eq 9, and rearranges terms, one obtains

$$k_{obsd} - k_1/[M^{2+}] = k_C = k_2K + k_3K[OH^-] + k_4KK_2[M^{2+}][OH^-] - k_{obsd}K \quad (10)$$

where by definition $K^* = k_2K + k_3K[OH^-]$. Plots of k_C as a function of $[M^{2+}]$ at a single pH were carried out by Oestreich and Jones; according to these authors intercepts of such plots were equal by K^*. According to eq 10, and as pointed out by Briggs *et al.*, intercepts of such plots equal $(K^* - k_1K)$. Rewriting eq 10 in terms of K^*, adding k_1K to both sides of the equation, and rearranging terms gives

$$(K^* - k_1K)[M^{2+}] = \{K[1 - k_4K_2[OH^-][M^{2+}]/(k_{obsd} - k_1)]\} \times (k_{obsd} - k_1)[M^{2+}] + k_{obsd} - k_1 \quad (11)$$

Plots of $(K^* - k_1K)[M^{2+}]$ *vs.* $(k_{obsd} - k_1)[M^{2+}]$ were carried out

TABLE IV: Position of Bond Fission in the Hydrolysis of Acetyl Phosphate in the Presence of Mg and Ca Ions.

Metal Ion	Equations Solved	Y^a	Z^b
Mg^{2+}	7a and 7b	0.45	0.93
	7b and 7c	0.0	1.02
	7a and 7c	0.21	1.01
		Av = 0.22 ± 0.15	Av = 0.99 ± 0.04
Ca^{2+}	8a and 8b	0.76	1.04

[a] Y = fraction of C–O bond fission for metal-catalyzed hydrolysis. [b] Z = fraction of C–O bond fission for metal-catalyzed attack of hydroxide ion.

for Mg^{2+} at pH 6.8 and 7.7, and for Ca^{2+} at pH 6.8 and 7.8, using the data of Oestreich and Jones to obtain values for $(K^* - k_1K)[M^{2+}]$ and $(k_{obsd} - k_1)[M^{2+}]$. These plots for Ca^{2+} are illustrated in Figure 1; it can be seen that the dependence of these slopes on $[M^{2+}]/(k_{obsd} - k_1)$ is small in the pH range and the range of concentration of metal ion covered. The values obtained for these slopes were 6.9 M^{-1} at pH 6.8 and 5.4 M^{-1} at pH 7.7 for Mg^{2+}; the values for Ca^{2+} were 10.9 M^{-1} at pH 6.8 and 8.4 M^{-1} at pH 7.8. From the pH dependence of these slopes, using average values of $[M^{2+}]/(k_{obsd} - k_1)$ at each pH, K and k_4K_2 were calculated to be 8.2 M^{-1} and 16×10^4 M^{-2} min^{-1}, respectively, for Mg^{2+} and 12 M^{-1} and 7.8×10^4 M^{-2} min^{-1}, respectively, for Ca^{2+}. The values obtained for K are extremely close to those originally obtained by Oestreich and Jones (Table II).

A reevaluation of k_2 and k_3 was carried out graphically, as described by Oestreich and Jones, using the values of K and k_4K_2 given above. Values obtained for the pH-independent term, k_2, were 1.0×10^{-2} min^{-1} for Mg^{2+} and 2.4×10^{-2} min^{-1} for Ca^{2+}. The pH-dependent term, which is a sum of terms $(k_3 + k_4[M^{2+}])$ was found to be 16×10^4 M^{-1} min^{-1} for $Mg^{2+} = 0.2$ M, and 3.1×10^4 M^{-1} min^{-1} for $Ca^{2+} = 0.1$ M; k_3 was calculated to be 13×10^4 M^{-1} min^{-1} for Mg^{2+} and 2.3×10^4 M^{-1} min^{-1} for Ca^{2+}.

In the calculation of Y and Z from eq 6 based on rate eq 1, it was assumed that Z represented the fraction of C–O cleavage for a single rate term described by k_3. From the above discussion, it is concluded that the value originally ascribed to k_3 for Mg^{2+} and Ca^{2+} can be attributed to a sum of two terms $[k_3 + k_4K_2[M^{2+}])$. If one corrects for the contribution of $k_4K_2[M^{2+}]$ to $(k_3 + k_4K_2[M^{2+}])$ at $Mg^{2+} = 0.15$ M and $Ca^{2+} = 0.24$ M, which are the concentrations of M^{2+} at which the oxygen-18 experiments reported in this paper were carried out, one obtains $(k_3 + k_4K_2[M^{2+}])$ equal to 15×10^4 M^{-1} min^{-1} for Mg^{2+} and 4.2×10^4 M^{-1} min^{-1} for Ca^{2+}. Using these values for the hydroxide-dependent terms and the newly calculated values of k_2 and K, Y and Z were recalculated. The values obtained for Mg^{2+} were $Y = 0.06 \pm 0.04$ and $Z = 0.98 \pm 0.07$; the values obtained for Ca^{2+} were $Y = 0.69$ and $Z = 1.04$. The agreement of these numbers with those given in Table IV can be seen to be good, with the exception of Y for the Mg system which is still statistically greater than zero, although less than one-third the value originally calculated for this parameter.

The values of K determined by Briggs *et al.* for Mg^{2+} by direct measurement were 30 M^{-1} at 4° and 75 M^{-1} at 25°; a value of K for Ca^{2+} was estimated to be 50 M^{-1} at 25°. Using the observed temperature dependence of K for Mg^{2+} to calculate values for K at 39° for both Mg^{2+} and Ca^{2+}, one obtains 140 M^{-1} for Mg^{2+} and 95 M^{-1} for Ca^{2+}. These values for K are surprisingly large and almost identical at 25° with those obtained for the formation of $MHPO_4$. Smith and Alberty (1956) found at 25°, $\mu = 0.2$, that $K = 76$ M^{-1} for the formation of $MgHPO_4$ and $K = 50$ M^{-1} for the formation of $CaHPO_4$. Due to the electron-withdrawing properties of the acetyl group of AcPM, one would expect the association constants for AcPM to be less than those for $MHPO_4$ (Kosower, 1962). However, since the error in the values of K for AcPM, obtained from the treatment of the data of Oestreich and Jones according to rate eq 2, is considered large, we have determined the effect of large values for K on the data presented in this paper. The data of Oestreich and Jones were evaluated graphically using $K = 140$ M^{-1} for Mg^{2+} and $K = 95$ M^{-1} for Ca^{2+} to determine values for k_2 and $k_3 + k_4K_2[M^{2+}]$, which were found to be 0.85×10^{-2} min^{-1} and 9.7×10^4 M^{-1} min^{-1}, respectively, for Mg^{2+}, and 1.6×10^{-2} min^{-1} and 1.7×10^4 M^{-1} min^{-1} for Ca^{2+}. An approximate value for k_4K_2 was calculated from the increase in $(k_{obsd}[AcP]/[AcP^2] - k_1)/[M^{2+}]$ (eq 9) with increasing concentration of divalent metal ion at pH 6.8, 7.8, and 8.8 for Ca^{2+} and pH 6.8 and 7.7 for Mg^{2+}. Multiplication of these values by $Mg^{2+} = 0.2$ M and $Ca^{2+} = 0.1$ M gave values for $k_4K_2[M^{2+}]$ of $9.9 \pm 0.3 \times 10^4$ M^{-1} min^{-1} for Mg^{2+} and $3.0 \pm 2.0 \times 10^4$ M^{-1} min^{-1} for Ca^{2+}.

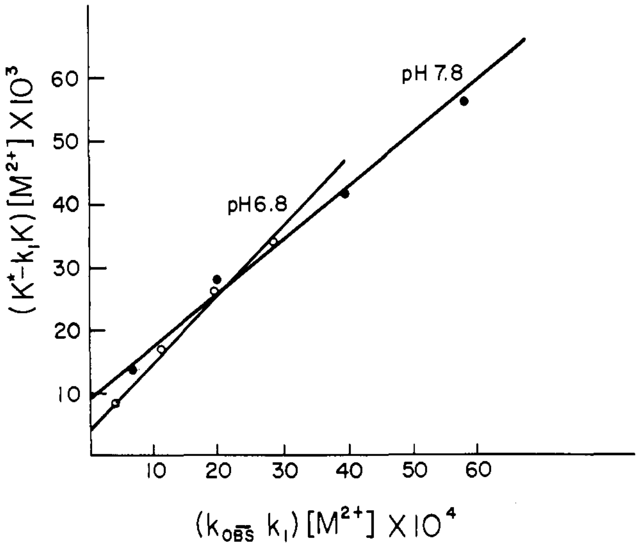


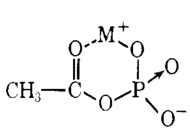
FIGURE 1: Plots of eq 11 for the calcium system at pH 6.8 and 7.8 to determine K and k_4K_2, as described in the text.

A comparison of these values for $k_4K_2[M^{2+}]$ with the values obtained graphically for $k_3 + k_4K_2[M^{2+}]$ indicates that $k_4K_2[M^{2+}] \gg k_3$. Therefore, if K is large, the calculation of Z from eq 6 gives rise to a value which represents the fraction of C–O cleavage for a single rate term described by $k_4K_2[M^{2+}]$. Upon correction of the value of this term, reported as k_3 by Oestreich and Jones at $Mg^{2+} = 0.2$ M and $Ca^{2+} = 0.1$ M, for $Mg^{2+} = 0.15$ M and $Ca^{2+} = 0.24$ M, and using the values for k_2 obtained under conditions where K is large, Y and Z were recalculated: for Mg^{2+}, $Y = 0.21 \pm 0.06$ and $Z = 0.99 \pm 0.02$; and for Ca^{2+}, $Y = 0.64$ and $Z = 0.99$. These values are extremely close to those originally calculated (Table IV).

From the above discussion, one concludes that the absolute values of Y and Z are not significantly different after treatment of the data to include an additional kinetic term. Also, large changes in K have a small effect on Y and Z. The reason for this insensitivity to changes in K appears to be due to the method of calculation of Y and Z, which involves obtaining differences between ratios of rate constants.

The values of Y for the Mg and Ca systems lie between 0 and 1, indicating mixed pathways for the water attack on AcPM. The uncatalyzed hydrolysis of acetyl phosphate dianion has been shown previously to occur by greater than 90% P–O bond fission (Bentley, 1949; Park and Koshland, 1958). The role of the metal ion in this hydrolysis can be examined in terms of the observed rate accelerations by Mg^{2+} and Ca^{2+} on P–O cleavage. If it is assumed that there is no C–O bond cleavage in the absence of metal ion these accelerations, which are equal to $k_2K[M^{2+}](1 - Y)/k_1$, represent a lower limit. The acceleration for Mg^{2+} is calculated to be 2.6 when $K = 8.2$ M^{-1} and 32 when $K = 140$ M^{-1}; the acceleration for Ca^{2+} is 4.8 when $K = 12$ M^{-1} and 30 when $K = 95$ M^{-1}. The accelerations by Mg^{2-} and Ca^{2+}, which are calculated at the lower values of K, are small and comparable to the threefold acceleration observed in going from the dianion of acetyl phosphate to the monoanion (DiSabato and Jencks, 1961). This similarity suggests that structure Ia is the catalytically active one for P–O cleavage of AcPM, in analogy to structure Ib which was suggested by DiSabato and Jencks (1961) to explain the rate acceleration of $AcPH^-$ relative to AcP^{2-}.

The P–O cleavage of acetyl phosphate dianion and monoanion has been described as a unimolecular process involving the formation of an intermediate metaphosphate ion (DiSabato and Jencks, 1961). On the assumption that P–O cleavage of AcPM is also unimolecular, structure II would be considered unlikely as the catalytically active complex between AcP^{2-} and M^{2+}: although structure II might be expected to accelerate a bimolecular P–O cleavage (Farrell *et al.*, 1969) it would inhibit metaphosphate ion formation.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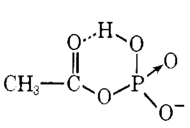
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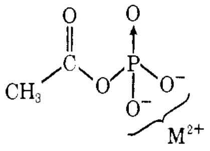
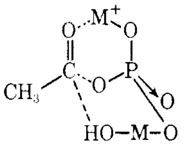

II

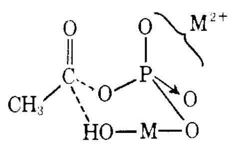
The values of Z for the Mg and Ca system are 1. The interpretation of Z depends on the value of K. On the assumption that K is small, Z represents the position of bond cleavage for the sum of two kinetic terms. One calculates the relative contribution of $k_4K_2[M^{2+}]$ to $(k_3 + k_4K_2[M^{2+}])$ to be 16% at $Mg^{2+} = 0.15$ M and 45% at $Ca^{2+} = 0.24$ M, indicating that the contribution of both kinetic terms is significant under the conditions of the oxygen-18 experiments. Therefore, one can conclude that C–O bond cleavage is the preferred pathway for cleavage of AcPM by both $OH^-(k_3)$ and $MOH^+(k_4)$.

If, however, K is as large as indicated by Briggs *et al.*, Z is interpreted to represent the position of bond cleavage for a single kinetic term described by $k_4K_2[M^{2+}]$. Independent of the value of K, one concludes from Z that the cleavage of AcPM by MOH^+ occurs by C–O bond cleavage. The interpretation of the data presented in this paper to indicate that the cleavage of AcPM by OH^- occurs by C–O cleavage is only valid in the case that the values described for K by Oestreich and Jones are not appreciably in error.

The metal ion catalyzed hydrolysis of esters and amides had been demonstrated to depend on the concentration of divalent metal ion to the first power (Conley and Martin, 1965a,b). The appearance of a pH-dependent kinetic term containing the concentration of divalent metal ion squared in the hydrolysis of acetyl phosphate dianion suggests the formation of a complex between AcPM and MOH^+, the large negative charge on the phosphate moiety of acetyl phosphate facilitating the formation of this complex.

The catalytically active form of such a complex could be represented either by structure III or IV, depending on whether the rate-limiting step for the C–O cleavage involves a rate-limiting formation of a tetrahedral intermediate (structure III) or the breakdown of this intermediate (structure IV).


III


IV

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Selective Inhibition of Serine Proteases by Alkyldimethylbenzylammonium Chloride[*]

Elliot Feldbau and Christian Schwabe[†]

ABSTRACT: The interaction of benzalkonium chloride (a powerful bacteriostatic agent) with trypsin and chymotrypsin has been examined. Trypsin and the ligand interact after preincubation at low pH values (pH 3.0) while no inhibition could be detected after incubation at pH values above 5.0. Trypsin, inhibited at low pH, can be reactivated by high concentrations of benzylarginine ethyl ester or tosylarginine ethyl ester under assay conditions. α-Chymotrypsin, similarly inactivated at low pH, is ten times more sensitive to benzalkonium chloride and is not reactivated by substrate at neutral pH. Reactivation of α-chymotrypsin can be effected only after partial denaturation in 6 M guanidine hydrochloride. Measurement of the remaining activity, following incubation in 0.1 M benzalkonium chloride, suggests strongly that the interaction of the enzyme and the ligand depends on the protonation of a carboxyl group. Several serine peptidases tested under identical experimental conditions show a similar behavior while non-serine peptidases are not inhibited by benzalkonium chloride. It is thus suggested that the protonation of a side chain carboxyl group permits benzalkonium to slip into the hydrophobic pocket of the enzyme which contains the active site. It is further suggested that serine peptidases (mammalian and bacterial) might have similar active site environments in spite of differences in the primary structure. Non-serine peptidases possess generally an active site environment which does not permit close association of the ligand. Active-site-directed dye binding studies, cinnamoylimidazole titrations, and optical rotatory dispersion spectra show that benzalkonium occludes the active site while no drastic changes in tertiary structure occur. Experiments involving ^{14}C-labeled cetyldimethylbenzylammonium chloride suggest that 1 mole of inhibitor is bound per mole of α-chymotrypsin.

Since the proposal by Hartley (1960) that proteases fall into four "catalysis classes" the search for similarities, particularly within the "serine protease" group, has been intense. An evolutionary relation was suggested for serine proteases on the basis of homology in amino acid sequence (Hartley, 1964, 1970) and the tertiary structures (Birktoft *et al.*, 1970) as determined by X-ray crystallography. Similarly, numerous reports have been published relating serine proteases on the basis of similar interaction with ligands in solution (Glazer, 1968), specificity determinants (Inagami, 1964), and the thermodynamics of active-site-specific hydrophobic interaction (Mares-Guia and Figueiredo, 1970).

We have studied the interaction of trypsin, α-chymotrypsin, and other serine proteases with benzalkonium chlorides, molecules possessing a partially shielded permanent positive charge, surrounded by two hydrophobic areas (a benzene ring and an alkyl radical C_8 to C_{18}). These quaternary ammonium compounds are powerful bacteriocidal agents and appear to interact specifically with certain proteins. While this property has been utilized imperically for the fractionation of proteins (Schwabe, 1969), evidence for the specific interaction of benzalkonium chlorides with serine protease is presented in this paper.

Materials and Methods

Enzymes. Trypsin (3.4.4.4, lyophilized, twice recrystallized, salt free) was obtained from Worthington Biochemical Co. (Lot No. TRL 86 N). Stock solutions were prepared in 0.001 M HCl and protein concentrations determined spectrophotometrically from the optical density at 280 mμ ($E_{1\%}^{1\,\mathrm{cm}}$ 15.4). The α-chymotrypsin (3.4.4.5) used was a crystalline, salt-free preparation obtained from C. C. Boehringer & Soehne, Mannheim, Germany (Lot No. 6145213). Stock solutions were prepared in 10^{-3} M HCl and protein concentrations were determined at 280 mμ ($E_{1\%}^{1\,\mathrm{cm}}$ 20.4). The active enzyme was determined by spectrophotometric titration with *N*-transcinnamoylimidazole (Schonbaum *et al.*, 1961) to be 88%. Lyophilized crystalline subtilisin (Carlsberg) was purchased from Schwarz–Mann Research Laboratory (Lot No. +4853). Subtilisin solutions were prepared by weight in 10^{-3} M HCl. Aspergillopeptidase (chromatographically homogeneous, Subramanian and Kalnitsky, 1964) was a gift from Dr. George Kalnitsky. Solutions of this enzyme were prepared by weight in 10^{-3} M HCl and used immediately after preparation. Stock solutions of elastase (courtesy of Dr. E. R. Blout) were prepared by weight in 10^{-2} M $CaCl_2$. A sample of chymotrypsinogen was purchased from Boehringer & Soehne, Mannheim.

Crystalline preparations of chymopapin and pepsin were obtained from Worthington Biochemical Co. The leucine aminopeptidase and cathepsin samples used were purified in

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[†] To whom to address correspondence.