(see Table I). Whether the small residual activity is significant or is instead merely a measure of the experimental error in the determinations is not clear. In any event, however, there can be no doubt that the only *important* mechanism for the rearrangement is purely intramolecular.

Acknowledgments.—It is a pleasure to thank Mr. William Saschek for many of the microanalyses reported above, and Professor W. G. Brown for the use of his counter and scaler. The radioactive carbon was obtained, in the form of $BaC^{14}O_3$, through the facilities of the Atomic Energy Commission.

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY]

Effect of Catalysts on the Hydrolysis of Acetyl Phosphate. Nucleophilic Displacement Mechanisms in Enzymatic Reactions[1]

BY DANIEL E. KOSHLAND, JR.[2]

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The effect of catalysts on the non-enzymatic hydrolysis of acetyl phosphate has been studied to provide a basis for interpreting the enzymatic mechanisms of this and related substrates. The *p*H-rate profile was determined over the *p*H range 0 to 12. Strong acid and base catalysis was shown at the extremes of *p*H with a broad flat minimum in the region of neutrality. From these data, it was concluded that nucleophilic attack by water on the phosphorus atom is the predominant reaction at neutrality whereas attack at the carbonyl carbon atom is the major pathway of hydrolysis in the strongly acidic and strongly basic solutions. Magnesium ion was found to catalyze the hydrolysis of the dinegative acetyl phosphate ion but not of the mononegative ion or of the uncharged acid. One mole of magnesium ion and one mole of acetyl phosphate are present in the activated complex. Pyridine was shown to catalyze the hydrolysis in neutral solutions and evidence is presented that the catalysis proceeds by formation of an acetylpyridinium ion. By analogy to the non-enzymatic reactions, it is suggested that nucleophilic displacements play an important role in the enzymatic reactions of acetyl phosphate and other substrates. A series of two successive displacements, each involving an inversion, is postulated to explain the over-all retention of configuration observed in certain enzymatic reactions which occur by attack at an asymmetric carbon atom. The biological evidence is shown to be consistent with these hypotheses.

To obtain a detailed picture of an enzymatic reaction, it is necessary to know the chemical identity of the active sites on the enzyme surface, their spatial arrangement and their effect on the substrate molecules. One approach to this problem is a study of the mechanism of the non-enzymatic reactions of the substrate molecules and the effect on these reactions of catalysts analogous to those active on the enzyme surface. In some cases the enzymatic and non-enzymatic mechanisms may be very similar; in others, the steric factors and concerted action of the groups on the enzyme surface may occasion qualitative as well as quantitative changes in mechanism. In either case, the non-enzymatic mechanism provides information essential to the interpretation of the enzymatic mechanism.

In the present study the effect of catalysts on the mechanism of hydrolysis of acetyl phosphate has been investigated. The catalysts, chosen because of their relationship to potential active groups on an enzyme, were: (a) acid and base as determined through the effect of *p*H, (b) a metal ion, magnesium and (c) a nucleophilic reagent, pyridine.

In addition to the specific information obtained about the individual catalysts, it was found that the non-enzymatic reactions of the acetyl phosphate all could be classed in the general category of nucleophilic displacement mechanisms.[3] This led to the suggestion that the same mechanisms might be operating in the biological reactions. The available information on the biochemistry of acetyl phosphate, and of some other substrates as well, was examined and found to be consistent with this hypothesis.

Experimental

The solution containing the appropriate buffer was allowed to stand in a 39.00 ± 0.02° thermostat until temperature equilibrium was attained. A few milligrams of powdered lithium acetyl phosphate were added, and the decrease in the concentration of acetyl phosphate followed as a function of time by removing two-ml. aliquots at appropriate intervals. The acetyl phosphate was assayed by a slight modification of the method of Lipmann and Tuttle.[4]

All solutions were brought to an ionic strength of 0.6 by adding the calculated amount of sodium perchlorate. The *p*H measurements were made at 25°, using a Beckman Model G instrument calibrated by the standard buffers recommended by Bates, Pinching and Smith.[5] All kinetic experiments to determine rate constants were run to greater than 75% disappearance of acetyl phosphate.

Results and Discussion

Effect of *p*H.—The rate of disappearance of acetyl phosphate was first order in all cases. Some typical results are shown in Fig. 1.

To clarify the effect of *p*H on the rate of hydrolysis, the first order rate constants were plotted as a function of *p*H. The results are shown graphically in Fig. 2. Each value of k_1 listed is the average of two kinetic runs which differed from each other by less than 5%.

It is seen that the hydrolysis is relatively unaffected by changes in *p*H in the region near neutrality but is greatly accelerated in strongly acidic and strongly basic solutions. The asymmetric hump in the *p*H 2–5 region resembles a titration curve and corresponds to the change in acetyl phosphate from the dinegative ion, $CH_3COPO_3^{--}$, to the mononegative ion, $CH_3COPO_3H^-$. The

(1) Presented at the Spring Meeting of the American Chemical Society, Boston, Mass., April, 1951.

(2) Brookhaven National Laboratory, Upton, N. Y.

(3) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 131.

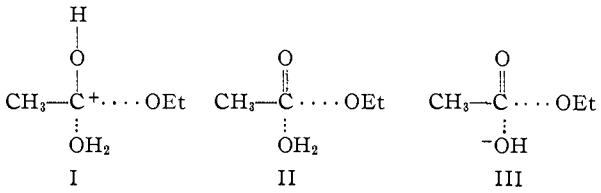
(4) F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **159**, 21 (1945).

(5) R. G. Bates, G. D. Pinching and E. R. Smith, *J. Research Natl. Bur. Standards*, **45**, 418 (1950).

curve appears to be shifted slightly to lower *p*H's as compared to the titration curve of Lipmann and Tuttle,[6] but this might be expected in view of the increased ionic strength of the solution. A similar increased rate of hydrolysis is observed on converting ethylphosphoric acid from the dinegative to the mononegative ion.[7]

At very low *p*H's the rate of reaction is proportional to the hydrogen ion concentration and at high *p*H's, it is proportional to the hydroxide ion concentration. The values for k_{H^+} and k_{OH^-} so determined are 0.008 and 5.4 liters mole^{-1} minutes^{-1}, respectively.

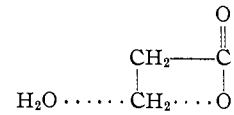
In order to interpret the *p*H-rate profile of acetyl phosphate hydrolysis, it is necessary to compare it with the corresponding graphs obtained in the hydrolysis of carbonyl compounds, such as ethyl acetate and β-butyrolactone, whose mechanisms of hydrolysis are known. The kinetics in the hydrolysis of ethyl acetate can be separated into three independent components: (a) an acid-catalyzed hydrolysis in which water attacks the conjugate acid of ethyl acetate (I), (b) a "spontaneous" hydrolysis in which water attacks the neutral ester (II), and (c) a base-catalyzed hydrolysis in which ester and hydroxide ion are the reacting species (III). Dotted lines are used to indicate the bonds which are either being formed or broken.



I II III

The observed rate of hydrolysis at a given *p*H is obtained by adding the contribution of each component at that hydrogen ion concentration as shown in Fig. 3. Using the measured values of the rate constants[8] to determine the positions of the lines representing the respective components, the *p*H-rate curve represented by the dotted line A in Fig. 3 is obtained. (This curve does not correspond exactly to that observed experimentally because the contributions of general acid and general base catalysis by the buffer ions have not been included. Since the latter corrections are small, and do not affect the conclusion in this work, they have been omitted to simplify the discussion.) It is to be noted that the approach to the minimum point is steep and that the spontaneous (k_0) reaction makes an appreciable contribution to the total rate only over a small range of hydrogen ion concentration.

The same three kinetic components give a curve of very different shape, however, if the value of *p*H independent reaction is increased by a large factor, *e.g.*, 10^4. The observed rates in the latter case, represented in Fig. 3 by the dotted line B, give a *p*H-rate profile which has a broad flat minimum. Curves of this type have been obtained in the hydrolysis of β-butyrolactone and β-propiolactone.[9,10] The difference in shape of the two curves is due to the alternative mechanism of hydrolysis available to the lactones but not to the straight chain esters. In the case of ethyl acetate, all three pathways of hydrolysis involve a nucleophilic attack on the carbonyl carbon atom with cleavage of the bond between the carbonyl carbon and the ether oxygen (I, II and III). In the case of the β-lactones, the acid- and base-catalyzed reactions are analogous to those for ethyl acetate, but the water reaction goes by an entirely different mechanism, *i.e.*, a nucleophilic attack by a water molecule on the alkyl carbon atom with cleavage of the alkyl carbon–ether oxygen bond (IV). This reaction, which is independent of *p*H, is so rapid compared to the normal attacks on the carbonyl atom in approximately neutral solutions that it is a major pathway of hydrolysis over a wide range of hydrogen ion concentration.



IV

The *p*H-rate profile for acetyl phosphate is seen to be very similar to that of the β-lactones and unlike that of ethyl acetate. The acetyl phosphate curve is somewhat more complicated due to the changing ionic species in solution, but it exhibits a broad flat minimum and the *p*H independent reaction is much more rapid than one would expect for

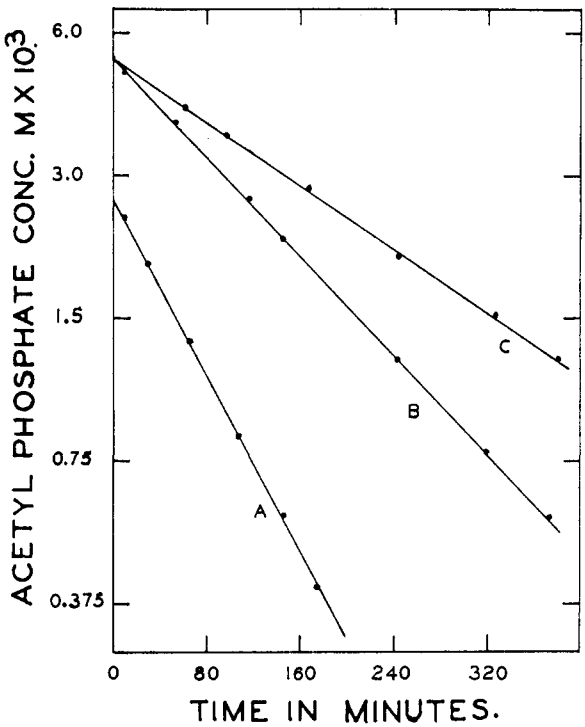


Fig. 1.—Hydrolysis of acetyl phosphate at 39.00° in: (A) 0.05 *M* bicarbonate buffer, *p*H 7.7, 0.1 *M* magnesium chloride; (B) 0.05 *M* phosphate buffer, *p*H 7.2, 0.248 *M* pyridine; (C) 0.05 *M* phosphate buffer, *p*H 7.2.

(6) F. Lipmann and L. C. Tuttle, *Arch. Biochem.*, **13**, 373 (1947).
(7) A. Desjobert, *Bull. soc. chim., France*, 809 (1947).
(8) H. M. Dawson and W. Lowson, *J. Chem. Soc.*, 2444 (1927).
(9) A. R. Olson and R. J. Miller, THIS JOURNAL, **60**, 2687 (1938); A. R. Olson and J. L. Hyde, *ibid.*, 2459 (1941).
(10) F. A. Long and M. Purchase, *ibid.*, **72**, 3267 (1950).

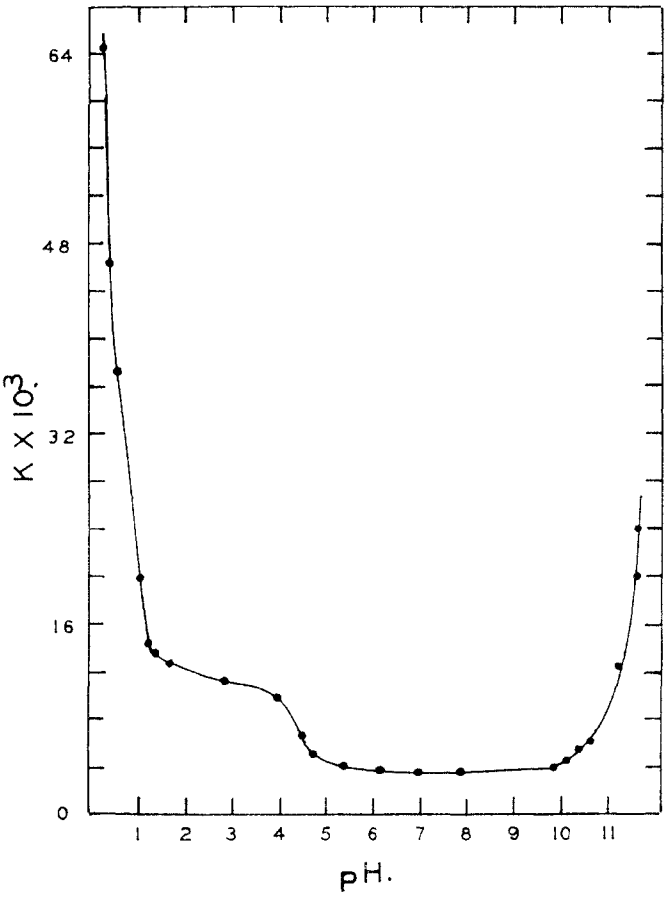
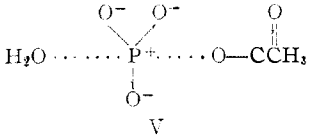


Fig. 2.—Effect of *p*H on rate of hydrolysis of acetyl phosphate: (k in liters moles^{-1} minutes^{-1}).

an attack by water at the carbonyl carbon. It appears, therefore, that the strong acid and strong base reactions are the usual nucleophilic attacks on the carbonyl carbon atom, whereas an alternative mode of hydrolysis is the predominating pathway at moderate *p*H's.

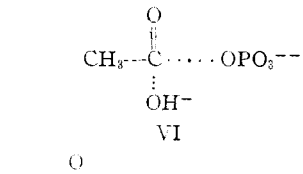
The alternative possibility in the case of acetyl phosphate is a nucleophilic attack by water on the phosphorus atom with displacement of the acetate ion (V). Displacements on the phosphorus atom



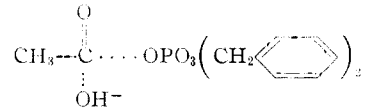
V

of phosphate esters[11] and the sulfur atom of sulfate esters[12] have been demonstrated, and Bentley[13] has established by isotopic experiments that a phosphorus–oxygen cleavage occurs in the hydrolysis of acetyl phosphate itself. Since the phosphorus–oxygen cleavage was observed under acidic conditions, Bentley assigned this splitting to the "acid" reaction. Re-examining his results in the light of the kinetic data, it is seen that the experiments were performed at *p*H's (4–6) at which the phosphorus displacement mechanism rather than the acid catalyzed reaction would be expected to be predominant. His results are, therefore, consistent with and support the explanation derived from the *p*H-rate data.

This interpretation also clarified an apparent anomaly in the previously suggested explanation for the shift from phosphorus–oxygen cleavage under acid conditions to carbon-oxygen cleavage in base. Bentley felt that the change was caused by electrostatic repulsion between the hydroxide ion and the phosphate group causing the hydroxide ion to react preferentially at the more distant atom, *i.e.*, the carbonyl carbon (VI). This was difficult to reconcile with his observation[13] that the acetyl dibenzyl phosphate also cleaves between the carbon and oxygen atoms in strong base (VII). It now appears that the attack by hydroxide ion is another example of the well-known reactivity of carbonyl compounds with strongly nucleophilic



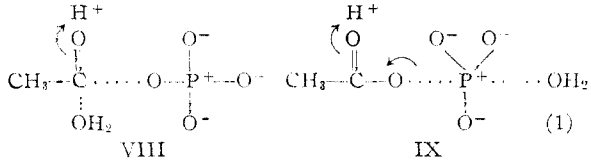
VI



VII

reagents and hence cleavage of the carbon–oxygen bond for both the dinegative acetylphosphate ion and the uncharged acetyldibenzyl phosphate is understandable.

Bentley also observed that the acetyl phosphatase catalyzed hydrolysis caused cleavage of the phosphorus–oxygen bond.[13] One might be tempted to conclude that the strong acid type of catalysis is not active on the enzyme surface since it catalyzes a carbon–oxygen split in the absence of enzyme. In theory, however, acid might be expected to aid either the carbon–oxygen (VIII) or the phosphorus–oxygen cleavage (IX). (The arrows indicate the shift in electrons under the polar influence of the proton.) In aqueous solution the former is evidently favored. On the protein, however, the approach of water to the carbonyl carbon atom might be hindered and the alternative phosphorus displacement might then be predominant. It can



VIII IX (1)

be concluded, therefore, that the enzymatic action is either not of the strong acid type or else it combines with some other factor, such as steric hindrance, to prevent reaction at the carbonyl atom.

Magnesium Ion Catalysis.—Lipman and Tuttle[14] showed that the hydrolysis of acetyl phosphate was accelerated by addition of 0.5% calcium chloride,

(11) E. Blumenthal and J. B. M. Herbert, *Trans. Faraday Soc.*, **41**, 611 (1945).

(12) G. N. Burckhardt, W. G. K. Ford and E. Singleton, *J. Chem. Soc.*, 17 (1936).

(13) R. Bentley, THIS JOURNAL, **71**, 2765 (1949).

(14) F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **153**, 571 (1944).

and Lehninger[15] has reported that dialyzed acyl phosphatase was activated by magnesium ion. In the present work, the effect of magnesium ion on the non-enzymatic reaction was studied and the results are summarized in Table I.

TABLE I

EFFECT OF MAGNESIUM IONS ON THE RATE OF HYDROLYSIS OF ACETYL PHOSPHATE

*p*H	Buffer	Mg^{++} concn. (*M*)	Mg salt added	Observed first order rate constant (minutes^{-1})	Catalytic constant, $k_{Mg^{++}}$ (l. moles^{-1} min.$^{-1}$)
7.7	Bicarbonate	...	.....	0.0039	...
7.7	Bicarbonate	0.050	Sulfate	.00754	0.073
7.7	Bicarbonate	.100	Sulfate	.0106	.067
7.7	Bicarbonate	.100	Chloride	.0108	.069
2.7	Formate	...	.....	.0112	...
2.7	Formate	.10	Chloride	.0112	...
0.63	Perchloric acid	...	.....	.033	...
0.63	Perchloric acid	.073	Sulfate	.033	...

At *p*H 7.7, the magnesium ion catalyzes the reaction markedly and the catalyzed reaction is first order in magnesium ion as well as first order in acetyl phosphate. Both a molecule of magnesium and a molecule of acetyl phosphate are, therefore, present in the transition state. A representative curve showing the first order disappearance of acetyl phosphate is shown in Fig. 1. The catalytic constants were calculated by assuming (a) that the metal ion catalyzed reaction and the straight hydrolysis proceed simultaneously and independently and (b) that the catalysis is first order in magnesium ion, *i.e.*, $k_{obsd} = k_u + k_{Mg^{++}}(Mg^{++})$ where k_u is the constant for the uncatalyzed reaction. The constancy of the $k_{Mg^{++}}$ values obtained with changing magnesium ion concentrations indicates that the assumptions are valid.

At *p*H 2.7 and 0.63, no change in rate greater than the experimental error is observed on adding magnesium ion to the buffer solutions. At *p*H 0.63 the *p*H in the magnesium-catalyzed kinetic run was calculated from the known concentrations of perchloric acid, magnesium sulfate, and lithium acetyl phosphate using the published values for the acid constants of bisulfate ion[16] and acetyl phosphoric acid.[4] The observed rate constant of 0.0332 differs slightly from the interpolated value in the absence of magnesium sulfate (0.0328) when the values are calculated to three figures. In view of the uncertainty in the *pK* values and the rapid change in rate of hydrolysis with a small change in hydrogen ion concentration in this region, the observed difference in the presence and absence of magnesium ion cannot be considered significant. Allowing for the maximum error, the catalytic constant in the more acid solutions is at least an order of magnitude less than at *p*H 7.7.

At *p*H 7.7, the acetyl phosphate exists in solution as the dinegative ion, $CH_3COPO_3^{--}$, whereas at *p*H 2.7 it is predominantly present as the mononegative ion, $CH_3COPO_3H^-$, and at *p*H 0.63, as the uncharged acid, $CH_3COPO_3H_2$. The added electrostatic attraction between the dinegatively charged acetyl phosphate and the positively charged magnesium ion appears to be a major factor in the strong catalysis. This is consistent with the observation of Tabor and Hastings[17] that magnesium ion combines with the HPO_4^{--} ion to a considerable extent ($K = 0.16$), whereas the association with the mononegative ion, $H_2PO_4^-$, is negligible. In the decarboxylation of dimethyloxalacetic acid, Steinberger and Westheimer[18] demonstrated that metal ions catalyze the reaction of the anionic form of the acid (X) but not of the monoester (XI). They concluded that a chelate ring including the metal ion and the charged gamma-carboxyl was present in the transition state. In the present case which has some similar features, a chelate ring of the type shown below (XII) may be active in the catalyzed reaction.

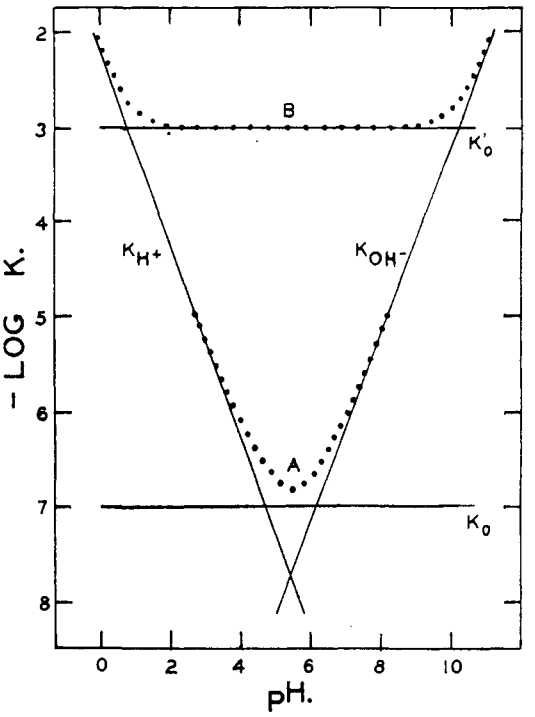
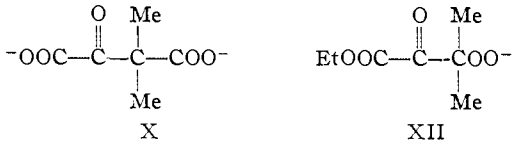


Fig. 3.—Components in the *p*H-rate profile for some hydrolysis reactions. Dotted line A corresponds to values for $k_{obsd.} = k_{H^+}(H^+) + k_{OH^-}(OH^-) + k_0 = 0.006\ (H^+) + 6.5\ (OH^-) + 10^{-7}$. Dotted line B corresponds to values for $k_{obsd.} = k_{H^+}(H^+) + k_{OH^-}(OH^-) + k_0' = 0.006\ (H^+) + 6.5\ (OH^-) + 10^{-3}$.



The role of metal ions in the enzymatic reactions of phosphorylated compounds is not yet clear.[19]

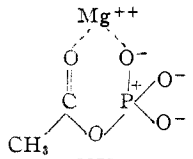
(15) A. L. Lehninger, *J. Biol. Chem.*, **162**, 340 (1946).

(16) I. M. Kolthoff, "Gebrauch der Farbenindikatoren," Springer, Berlin, **43**, 207 (1923).

(17) H. Tabor and A. B. Hastings, *J. Biol. Chem.*, **148**, 627 (1943).

(18) R. Steinberger and F. H. Westheimer, THIS JOURNAL, **73**, 429 (1951).

(19) A. L. Lehninger, *Physiol. Rev.*, **30**, 393 (1950).



XII

They might be expected to aid in binding the substrates to the protein surface by electrostatic attraction. Although the magnesium ion concentration used in these experiments is larger than the average values in tissues, the observed catalytic action in aqueous solution in the absence of enzymes suggests that the metal ions may in some cases have the added function of polarizing the electrons in the substrate molecules.

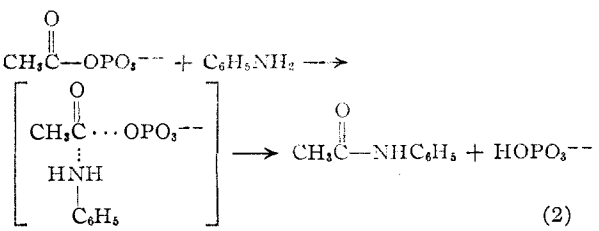
Catalysis by Pyridine.—The increased rate of disappearance of acetyl phosphate in buffered solutions containing pyridine shows that pyridine catalyzes the reaction (*cf.* Table II). The hydrolysis of acetyl phosphate was first order in all cases (*cf.* Fig. 1 for a typical run). Making assumptions analogous to those used in the determination of the catalytic constant for magnesium ion, the catalytic constant for pyridine, k_p, was calculated from the expression $k_{obsd} = k_u + k_p(\text{pyridine})$. The constancy of the k_p values on changing the pyridine concentration from 0.149 *M* to 0.496 *M* demonstrates that the assumptions cited are valid for this system and that the catalysis is first order in pyridine.

TABLE II

CATALYSIS BY PYRIDINE OF THE HYDROLYSIS OF ACETYL PHOSPHATE

Pyridine concentration (moles/liter)	Observed first order rate constant (minutes^{-1})	Calculated second order catalytic constant k_p(liters moles^{-1} min.$^{-1}$)
0.149	0.00523	0.0089
.248	.00602	.0086
.496	.00822	.0087

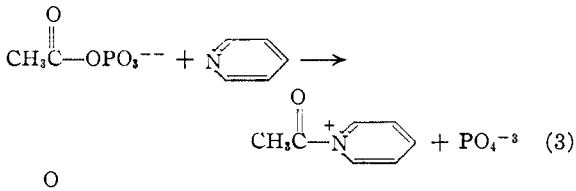
In order to clarify the mechanism, the pyridine catalysis was compared to the reaction of acetyl phosphate with aniline, which had previously been shown to involve a nucleophilic attack by the nitrogen of the aniline on the carbonyl carbon atom of the acyl phosphate.[20] Since pyridine and aniline would be expected to have similar steric effects in such a reaction, the second order rate constant



(2)

for the formation of acetanilide and the catalytic constant for pyridine, k_p, should be in the approximate ratio of their respective basicities if they react by the same mechanism. This is seen to be the case. The ratio of the basicities, $K_{pyridine}/K_{aniline}$, is 5.0 and the ratio of the rate constants is 3.0, using the previously determined value for aniline.[20] The ratio of the rate constants is slightly less than that of the basicities, but the nucleophilic reactivity of bases is not precisely proportional to the base strengths. The Brönsted catalytic coefficient, for example, usually has values between 0.4 and 0.8.[21] The corresponding exponent in this case is 0.69.

It can be concluded, therefore, that the pyridine attacks the carbonyl carbon of the acyl phosphate, forming a nitrogen to carbon bond with displacement of the phosphate group (Equation (3)). The acetylpyridinium ion thus formed then reacts rapidly with water to form acetic acid and regenerate the pyridine catalyst (Equation (4)). The ultimate fates of the phosphate and hydrogen ions produced depend on the *p*H of the buffer solution.



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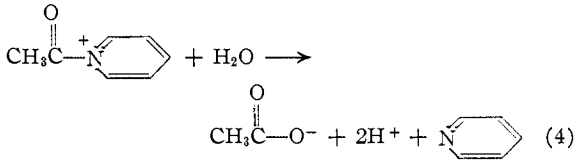


(4)

At *p*H 7, for example, they will in large part combine to form HPO_4^{--} and $H_2PO_4^-$.

If the above two-step mechanism is to agree with the observed kinetics, the rate of hydrolysis of the acetylpyridinium ion must be very rapid with respect to its rate of formation. To test this, solid acetylpyridinium chloride was prepared and one portion was added directly to a 2 *M* solution of hydroxylamine. Quantitative measurement of the acetylhydroxamic acid produced showed that 65% of the acetylpyridinium ion had reacted with the hydroxylamine and the remaining 35% had hydrolyzed. A second portion of the solid was added to a phosphate buffer solution at *p*H 7; it was then immediately poured into a 2 *M* solution of hydroxylamine. No detectable amount of acetylhydroxamic acid was observed in this case. The acetylpyridinium chloride had decomposed completely in the few seconds between addition of the solid to the buffer solution and the transfer of the buffer solution to the analytical reagent. Since the half-life for hydrolysis of the pyridinium intermediate is of the order of a second or less whereas the half-life for the over-all reaction in, for example, 0.149 *M* pyridine is about 130 minutes, the independently determined rate of decomposition of the acetylpyridinium ion is consistent with the observed kinetics.

The acetylpyridinium ion is a true intermediate which has a finite, although small, half-life and an appreciable energy of activation for reaction with water or hydroxylamine. This is indicated by the fact that the solid acetylpyridinium chloride compound can be prepared and also by the selectivity of the reaction with hydroxylamine. A compound which reacts without any appreciable activation

(20) D. E. Koshland, Jr., THIS JOURNAL, **73**, 4103 (1951).

(21) R. P. Bell, in Schwab, "Handbuch der Katalyse," Springer, Vol. II, 229, 1940.

energy should form products purely on the basis of the relative concentrations of reactants. In a solution containing two moles per liter of hydroxylamine and 55 moles of water, the amount of reaction with hydroxylamine would be only (2/2 + 55) or 3.7%. On the other hand, compounds which have a significant energy of activation do not react purely on a probability basis and the nucleophilic properties of the reagents are of importance in determining the ratio of the products. Acetyl phosphate, for example, reacts quantitatively with aqueous hydroxylamine to yield the hydroxamic acid. The acetylpyridinium ion evidently is less selective than the acetyl phosphate but still requires very appreciable activation. The calculated k_{NH_2OH}/k_{H_2O} ratio, which would be 1.0 on a collision probability basis, is greater than 3000 for acetyl phosphate and about 50 for acetylpyridinium ion.

It should be emphasized that pyridine catalyzes the reaction by changing the entire mechanism of the hydrolysis. In the absence of pyridine at *p*H 7, cleavage of the phosphorus–oxygen bond occurs with displacement of acetate. At the same *p*H, the pyridine-catalyzed reaction involves a carbon–oxygen split with displacement of phosphate. Thus, the pyridine changes the acetyl phosphate from a phosphorylating to an acetylating agent.

Nucleophilic Displacement Reactions in Enzymatic Processes.—In the previous discussion, evidence has been presented for some nucleophilic displacement mechanisms in the non-enzymatic reactions of acetyl phosphate. It may be worthwhile to consider whether the enzymatic reactions of this compound and analogous ones may also involve this type of mechanism.

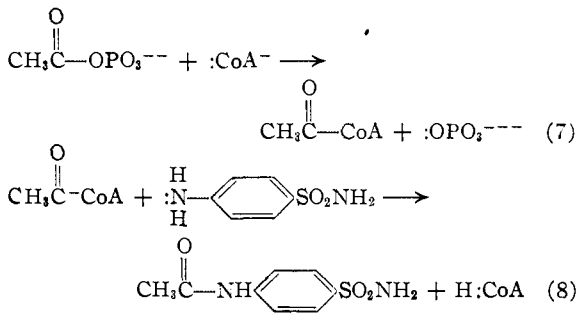
The displacement reaction, which is symbolized in Equation (5), is probably the most generally observed mechanism in organic chemistry.[3] The

$$A + B{-}C \longrightarrow A{-}B + C \qquad (5)$$

letters A, B and C may represent single atoms or groups of atoms and may be charged or uncharged. The reactions discussed in this paper are all of the more restricted class of ionic nucleophilic displacements,[22] in which the entering group, A, has an unshared pair of electrons which is attracted to a nucleus, and the departing group, C, is ejected with its pair of electrons (*cf.* Equation (6)).

$$A^{-}\!: + B\!:\!C \longrightarrow A\!:\!B + :C^{-} \qquad (6)$$

The pyridine catalysis mechanism is notably similar in form to the coenzyme A catalyzed acetylations of acetyl phosphate.[23] Both steps in the pyridine catalysis are nucleophilic displacements on carbon, the pyridine being the reagent with the unshared electron pair in the first step (Equation (3)) and the water being the nucleophilic reagent in the second (Equation (4)). It seems highly probable that the coenzyme A catalyzed acetylations also proceed by a series of nucleophilic displacements on carbon, first by the coenzyme A (Equation (7)) and then by the acetyl acceptor molecule, *e.g.*, sulfanilimide (Equation (8)). The recent evidence that the acetylated coenzyme A is a thioester[24] fits this hypothesis nicely since mercaptans are strong nucleophilic reagents and thioesters readily undergo nucleophilic attack at the carbonyl carbon atom.



Displacements on the phosphorus atom of acetyl phosphate appear to occur in several of the enzymatic reactions of this compound. The isotopic evidence of phosphorus–oxygen cleavage indicates this type of mechanism in the acetyl phosphatase hydrolysis. The reaction with adenosine diphosphate to yield the triphosphate[25] would seem to involve a displacement on phosphorus by an oxygen on the terminal phosphate group of the ADP. The possibility of a phosphorylated coenzyme A, also, is consistent with a nucleophilic displacement on the phosphorus atom, in this case, by the sulfhydryl group of the coenzyme, and is supported by the known existence of thiophosphates.

Nucleophilic displacement mechanisms probably occur in many other enzymatic reactions involving substrates other than acetyl phosphate. Esterases, phosphatases and phosphotransferases are a few of the examples which appear to fit in this category. In the case of sucrose phosphorylase, the available data are in good agreement with a mechanism involving two successive substitutions. The pertinent facts in the formation of sucrose from glucose-1-phosphate are: (a) that the C-1 carbon atom in both the initial substrate, glucose-1-phosphate, and the final product, sucrose, are in the alpha configuration[26,27]; (b) a glucose–enzyme intermediate is formed during the reaction[28]; and (c) cleavage in both the forward and reverse reactions occurs at the carbon–oxygen bond of the C-1 atom of the glucose.[29] All these observations are explained by the mechanism outlined in Equations (9) and (10). In the first step, the electron sharing

$$\alpha\text{-glucose-1-phosphate} + :\text{enzyme} \longrightarrow \beta\text{-glucose-1-enzyme} + :\text{phosphate} \qquad (9)$$

$$\beta\text{-glucose-1-enzyme} + :\text{fructose} \longrightarrow \alpha\text{-glucose-1-fructose (sucrose)} + :\text{enzyme} \qquad (10)$$

(22) In this article the term nucleophilic displacement will be used to include both a stepwise displacement of the carbonyl addition type and the single step displacement of the classical Walden inversion mechanism.

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site on the enzyme surface attacks the C-1 carbon atom, replacing the phosphate group with cleavage of the carbon–oxygen bond and formation of the glucose–enzyme intermediate. In the second step, the fructose molecule is the nucleophilic reagent which attacks the C-1 atom, displacing the enzyme and forming the final product, sucrose. The transglucosidase action of this enzyme,[30] the catalysis of phosphate exchange in glucose-1-phosphate[28] and fructose exchange in sucrose[31] are readily explained by analogous mechanisms.

There is a substantial amount of theoretical and experimental evidence to indicate that all single step nucleophilic displacements at a saturated carbon atom require "backside" attack by a nucleophilic reagent.[3,32] This appears to be true of "carbonium ion" mechanisms, which might be expected in the cleavage of an acetal type compound, as well as in the Walden inversion type of mechanism. The nucleophilic site on the enzyme would, therefore, make a backside attack leading to a glucose–enzyme intermediate of inverted configuration at the C-1 carbon atom. The fructose molecule would then displace the enzyme, again with inversion, to produce a final product, sucrose, having the same configurations as the initial glucose-1-phosphate substrate.

This mechanism leads to the suggestion that other enzymatic reactions which result in retention of configuration, *e.g.*, some glucosidase-catalyzed hydrolyses, may actually proceed in two steps, each involving an inversion. In all these cases a nucleophilic site on the enzyme surface takes direct part in the reaction to form an intermediate of greater or lesser stability depending on the particular system involved.

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[CONTRIBUTION FROM THE UNIVERSITY OF COLORADO]

Directed Chlorination of Fluorinated Aliphatic Ethers[1]

BY J. D. PARK, D. M. GRIFFIN[2] AND J. R. LACHER

A study was made of the photochemical chlorination of fluorinated aliphatic ethers of the type $ROCF_2CX_2H$ (R = CH_3– or C_2H_5–, X = chlorine, fluorine or hydrogen). Henne and co-workers had previously found that attack by chlorine on fluorinated aliphatic hydrocarbons is directed away from the hydrogen atom linked to a carbon alpha to a $-CF_2-$ or CF_3- group. The chlorination of the fluorinated aliphatic ethers was found to proceed in accord with the observations made by Henne and co-workers on the fluorinated aliphatic hydrocarbons. In all the ethers studied, chlorination was found to be directed away from the carbon atom adjacent to the $-CF_2-$ group of the fluorinated ethyl radical. The chloromethyl ethers were remarkably stable toward water, acids, methylmagnesium bromide and sodium. Physical properties determined for the chlorinated fluoroethers included boiling point, freezing point, refractive index, density, ultraviolet absorption spectrum and infrared absorption spectrum.

In a series of studies on the photochemical chlorination of fluorinated straight-chain aliphatic hydrocarbons, Henne and co-workers[3-8] found that methyl and methylene groups adjacent to CF_3- or $-CF_2-$ groups were protected from attack by chlorine. The lack of reactivity of hydrogen atoms adjacent to CF_3- and $-CF_2-$ groups was explained by Henne and Zimmerschied on the basis of the inductive effect of fluorine. Such an inductive effect was thought to increase the protonic character of hydrogen atoms adjacent to the CF_3- and $-CF_2-$ groups by a shift of C–H bonding electrons toward the carbon atom.

In the present work, a study was made of the photochemical, liquid-phase chlorination of the fluorinated ethers and their respective products

Parent compound	Chloro derivative
CH_3OCF_2CFHCl[9]	$ClCH_2OCF_2CFHCl$
$CH_3OCF_2CFH_2$[10]	$ClCH_2OCF_2CFH_2$
$CH_3OCF_2CF_2H$[11]	$ClCH_2OCF_2CF_2H$
$CH_3OCF_2CH_2Cl$[12]	$ClCH_2OCF_2CH_2Cl$
$CH_3OCF_2CHCl_2$[13]	$ClCH_2OCF_2CHCl_2$
$C_2H_5OCF_2CFHCl$[9]	$ClCH_2CH_2OCF_2CFHCl$ and $CH_3CCl_2OCF_2CFHCl$

in order to determine whether a directing effect on the entry of chlorine into the molecule might be observed. The chlorinations were carried out using a deficiency of chlorine to suppress the formation of polychlorinated products.

In each case, chlorination was found to take place only in the unsubstituted radical of the ether. Thus, only chloromethyl ethers of the type CH_2Cl-

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(2) Atomic Energy Commission predoctoral Fellow, 1948–1949.

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