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The Mechanism of Hydrolysis of Acetyl Dihydrogen Phosphate[1]

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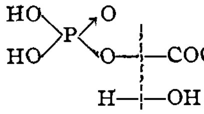
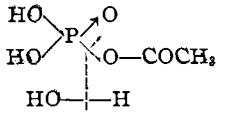
Acetyl dihydrogen phosphate is produced during the oxidation of pyruvic acid by certain bacteria,[3] and has been widely considered as a possible acetylating agent or as a possible phosphorylating agent in a variety of physiological systems.[4] In a previous paper[5] certain reactions of acetyl dihydrogen phosphate were recorded; it seemed desirable to investigate the hydrolytic cleavage of this substance and some of its derivatives to determine whether the split takes place between carbon and oxygen (I), or between phosphorus and oxygen (II). In the former case, it would be expected that acetyl dihydrogen phosphate would behave as an acetylating agent; in the second case, as a phosphorylating agent.


I


II

The cleavage was therefore studied with water enriched with the O^{18} isotope, a method which was first employed to study the alkaline hydrolysis of *n*-amyl acetate.[6] In case (I), O^{18} would be found in the acetic acid produced, and in case (II) in the phosphoric acid. As many organic and inorganic compounds exchange O^{18} atoms when dissolved in enriched water, it was first necessary to consider the possibilities of such exchange reactions. It has been shown, contrary to early reports,[7] that phosphate does not exchange oxygen atoms.[8] As, however, an analytical method for the estimation of O^{18} in phosphate was not readily available, only the acetate portion of the molecule was studied. A slow exchange of oxygen atoms in acetate had been observed in the presence of hydrochloric acid[9]; this exchange was further studied under the conditions to be used in the present hydrolytic experiments.

Experimental

All experiments were carried out in glass-stoppered (or sealed) Pyrex tubes, without exclusion of atmospheric carbon dioxide. (Carbon dioxide exchanges oxygen atoms, causing slight dilution of the water.)

Exchange Reactions.—Acetic acid or sodium acetate (1 mM. in 1 ml. of H_2O^{18}) was allowed to stand under the desired conditions of time and temperature. When an acid solution was used, the acetic acid and water were recovered by distillation in vacuum (1-3 mm.) at room temperature; a trap-type receiver cooled in an alcohol-carbon dioxide bath was used to ensure good recoveries. In cases where an alkaline reaction mixture was studied, the water was first distilled for recovery; the residue was re-dissolved in ice water, quickly acidified with sulfuric acid at 0°, and the acetic acid immediately distilled in vacuum. These operations were carried out as quickly as possible to minimize any exchange with the normal water. The acetic acid obtained by either process was treated with a slight excess of solid silver nitrate and the solution neutralized with dilute ammonium hydroxide to precipitate silver acetate. The washed and dried silver acetate was decarboxylated by heating *in vacuo*, and the carbon dioxide produced was led into a mass spectrometer for isotope analysis.

Hydrolytic Reactions.—In these experiments, from 100-150 mg. of disilver acetyl phosphate (or dibenzyl acetyl phosphate) was allowed to stand in 1-2 ml. of enriched water under the conditions specified. In some experiments the sodium salt was prepared by the initial addition of the equivalent amount of solid sodium chloride. When the effect of alkali was studied, solid potassium hydroxide was added to the mixture. In all cases, acetic acid was recovered and converted to silver salt for isotope analysis as in the exchange reactions.

Analysis of Water.—The amount of O^{18} present in the enriched water was determined by equilibration with normal carbon dioxide as described by Cohn and Urey.[10] This operation was conveniently carried out in a two-legged tube, fitted with a ground glass cap carrying a side arm which could thus be used as a stopcock.[11] The water to be analyzed was placed in one leg, anhydrous sodium carbonate added and the solution equilibrated for three days. After freezing the solution, the tube was evacuated. When it had regained room temperature the solution was tipped into the second leg containing anhydrous sodium bisulfate (the oxygen atoms of which do not exchange under these conditions[12]), and the liberated carbon dioxide analyzed in the usual way.

Results

The earlier observations on the exchange of the oxygen atoms of acetic acid had shown a slow exchange in the presence of hydrochloric acid at room temperature. Under the present experimental conditions, a very slow exchange was observed with acetic acid alone at room temperature, which increased with elevation of temperature, and was especially catalyzed by strong acid. Thus with *M* acetic acid in the presence of 1.4 *M* hydrochloric acid, almost complete exchange (91.6%) was obtained after six hours at 100°. In the presence of alkali (*M* sodium acetate in 0.25 *M* barium hydroxide), only 5.4% exchange was observed under the same conditions. These re-

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(3) F. Lipmann, *J. Biol. Chem.*, **155**, 55 (1944).

(4) F. Lipmann, *Advances in Enzymology*, **6**, 242, 257 (1946).

(5) R. Bentley, THIS JOURNAL, **70**, 2183 (1948).

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(8) E. R. S. Winter, M. Carlton and H. V. A. Briscoe, *J. Chem. Soc.*, 131 (1940).

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sults, summarized in Table I, are expressed as percentage of complete exchange.

TABLE I

EXCHANGE OF OXYGEN ATOMS OF ACETIC ACID

Correction has been made for the dilution of the H_2O^{18} by the normal oxygen atoms of the acetic acid.

Reagent	Time, hr.	Temp., °C.	Atom % excess of water used	Atom % excess of isolated AgAc	% Exchange
Acetic acid[a]	1	25	1.03	0.012	1.2
Acetic acid	16	25	1.03	.047	4.6
Acetic acid	62	25	1.03	.129	12.5
Acetic acid	3	100	1.03	.866	87.4
NaAc HCl[b]	6	00	1.03	.765	91.6
NaAc $Ba(OH)_2$[c]	6	100	1.03	.056	5.4

[a] 1 mM. per 1 ml. [b] Sodium acetate, 1 mM.; H_2O^{18}, 0.8 ml.; 12 *N* HCl, 0.2 ml. Further correction made for dilution by the normal oxygen atoms in aqueous HCl. [c] Sodium acetate, 1 mM.; H_2O^{18}, 1.0 ml.; anhyd. $Ba(OH)_2$, 0.25 mM.

When the disilver salt and dibenzyl ester of acetyl dihydrogen phosphate (150 mg. in each case) were hydrolyzed separately for eighteen and seventy two hours in 1-ml. portions of H_2O^{18} (1.03 atom % excess O^{18}) containing, respectively, 95 and 140 mg. of potassium hydroxide, O^{18} was found in the acetic acid subsequently isolated. Isotope analysis showed the atom % excess O^{18} of the silver acetate (from the disilver salt) to be 0.453, indicating that 45.3% of the oxygen atoms were derived from the water; in the case of the dibenzyl ester, the atom % excess O^{18} of the isolated silver acetate was 0.450 indicating that 45.7% of the oxygen atoms were so derived. (In both cases correction has been made for the dilution of the H_2O^{18} by the normal oxygen atoms of the potassium hydroxide.) If the split had taken place as in (I), 50% of the oxygen atoms in the acetic acid would have been derived from the water. Under the strongly alkaline conditions of these hydrolyses, the exchange reaction would not have taken place to any appreciable extent (see Table I). Since the observed values were 45.3 and 45.7% it is concluded, therefore, that under alkaline conditions the carbon–oxygen bond preferentially undergoes rupture as in (I).

When disodium and disilver acetyl phosphate were treated with enriched water alone, the solutions became acid as the hydrolysis proceeded; when the silver salt was used, the final *p*H was 3.5, and with the sodium salt, 6.0. When the disilver salt (150 mg.) in 1-ml. portions of H_2O^{18} (1.03 atom % excess O^{18}) was hydrolyzed for two hours at 85° and seventy-two hours at 25°, the atom % excess O^{18} of the isolated silver acetate was respectively 0.300 and 0.337; under these conditions, therefore, 29.1 and 32.7%, respectively, of the oxygen atoms were derived from the water. When the disodium salt was prepared by the addition of sodium chloride (50 mg.) to the same quantities of reactants, hydrolysis for sixteen and seventy-two hours at 25° in water with the same enrichment of O^{18} gave silver acetate 0.072 and 0.088 atom % excess O^{18}. When disilver acetyl phosphate (100 mg.), sodium chloride (33 mg.), and H_2O^{18} (1 ml.) were heated for two hours at 85°, the isolated silver acetate had 0.078 atom % excess O^{18}. In these three experiments, 7.0, 8.5 and 7.6% of the oxygen atoms of the acetic acid were derived from water. If rupture of the carbon–oxygen bond had occurred under these conditions, isotope analysis would have been expected to show that more than 50% of the oxygen atoms were derived from the water, since the acid conditions would also promote the exchange reaction. As there was always less than 50% of the O^{18} in the acetic acid formed under these conditions of acid hydrolysis it is concluded that the phosphorus–oxygen bond is split (case II). The sharp difference between the O^{18} content of the acetic acid produced when the sodium salt was used instead of the silver salt is probably due to the higher *p*H of the solution formed with the sodium salt. This higher *p*H is due to a buffering effect as shown by the following equations.

$$3C_2H_3O_5PAg_2 + 5OH^- \longrightarrow 2Ag_3PO_4 + 3CH_3COO^- + HPO_4^= + 2H_2O$$

$$C_2H_3O_5PNa_2 + OH^- \longrightarrow HPO_4^= + CH_3COO^- + 2Na^+$$

Confirmation of the rupture of the phosphorus–oxygen bond under slightly acid conditions was provided by carrying out the hydrolysis of dilithium acetyl phosphate with an acetylphosphatase preparation (very kindly provided by Dr. F. Lipmann). With this enzyme, hydrolysis was complete in one hour at 37°, the solution becoming acid. Owing to the greatly increased velocity of hydrolysis, the exchange reaction was very much reduced; using dilithium acetyl phosphate (100 mg.), acetylphosphatase (20 mg.) in 1 ml. of enriched water (1.03 atom % excess O^{18}), only a very small amount of O^{18} was found in the isolated acetic acid (0.025 atom % excess).

Discussion

Apart from a number of kinetic studies (more especially of simple carboxylic anhydrides) there have apparently been no investigations of the mechanism of anhydride hydrolysis. However, by the use of ethanol labelled with O^{18}, it has been shown that the alcoholysis of acetic anhydride takes place as follows, the hydrogen–oxygen bond of the ethanol being split as in esterification.[13]

$$CH_3CO\boxed{—O—COCH_3 + H—}O^{13}C_2H_5 \longrightarrow CH_3COO^{18}C_2H_5 + CH_3COOH$$

Carboxylic ester hydrolysis has been extensively studied, and it is known that the hydrolysis

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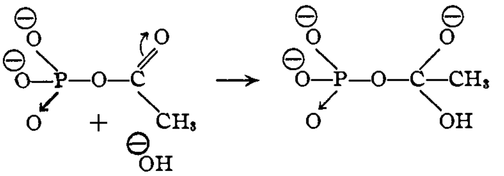
(13) N. I. Dedusenko and A. I. Brodskiĭ, *Acta Physicochim. U.S.S.R.*, **17**, 314 (1942).

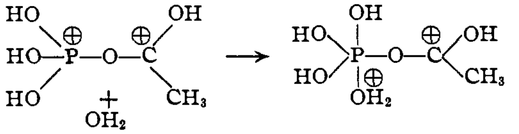
proceeds by different mechanisms depending on the *p*H of the solution.[14a,b]

A somewhat similar behavior has been observed with some lactones[15] and in the hydrolysis of trimethyl orthophosphate.[16]

It is tentatively suggested that the mechanism of hydrolysis of acetyl dihydrogen phosphate can be expressed in terms similar to those which have been proposed for ester hydrolysis. In alkaline solution, the attack is by the nucleophilic hydroxyl ion on the ionized molecule. In view of the negatively charged oxygen atoms surrounding the phosphorus atom the carbon atom will be more readily attacked, and the initial stage of alkaline hydrolysis may be pictured as



Under acid conditions, again due to the influence of the hydroxyl groups (now un-ionized), the phosphorus atom will have a greater capacity for sharing electrons, and the primary reaction will be of the type indicated below.



It seems likely that a similar *p*H effect would be observed with the mixed anhydrides of carboxylic acids, and there is already some evidence for this. Mixed anhydrides are known to react in different ways, depending on the reactant and the conditions. Thus, for example, the mixed anhydride of acetic and formic acids reacts with alcohols to form formic esters, but with phenols, the main products are the phenol esters of acetic acid.[17] It has also been shown that when this anhydride reacts with nitro-alcohols, etc., at 50–60°, formic esters are obtained. At higher temperatures, or in the presence of sulfuric acid, the acetates are produced.[18] It is possible that acetyl dihydrogen phosphate, under suitable conditions, could be used as a phosphorylating agent. No evidence has yet been obtained for non-enzymatic phosphorylations with this anhydride.[5]

Acknowledgment.—The author wishes to express his gratitude to Dr. D. Rittenberg for constant encouragement and advice during the course of the work described in this, and the earlier paper[5]; and to Mr. I. Sucher for the isotope analyses.

Summary

1. On hydrolysis under alkaline conditions acetyl dihydrogen phosphate and acetyl dibenzyl phosphate are split between the carbon and oxygen bond. Under acid conditions the phosphorus–oxygen bond is ruptured.

2. In the enzymic hydrolysis with acetyl phosphatase, the phosphorus–oxygen bond is split.

3. The probable primary reactions are discussed.

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[17] A. Béhal, *Ann. chim. phys.*, [7] **20**, 419, 426 (1900).

[18] C. D. Hurd, S. S. Drake and O. Fancher, THIS JOURNAL, **68**, 789 (1946).

[19] Original manuscript received January 13, 1948.

[CONTRIBUTION NO. 16 FROM THE THERMODYNAMICS LABORATORY, PETROLEUM EXPERIMENT STATION, BUREAU OF MINES, BARTLESVILLE, OKLA.]

Thermodynamic Properties of Three Isomeric Pentenes[1]

BY D. W. SCOTT, GUY WADDINGTON, J. C. SMITH[2] AND H. M. HUFFMAN

In continuation of the program of the Bureau of Mines for studying the thermodynamic properties of hydrocarbons and related compounds, vapor heat capacities, heats of vaporization, and vapor pressures have been measured for three of the isomeric pentenes, namely, 1-pentene, 2-methyl-1-butene and 2-methyl-2-butene. The entropies of vaporization derived from these measurements, when added to the entropies of the liquid substances found in a previous investigation in this laboratory,[3] have yielded values for the entropies of the three compounds in the vapor state. The calorimetric data for 2-methyl-2-butene have been correlated and extended to other thermodynamic functions and to higher temperatures by means of statistical mechanical calculations based on spectroscopic and molecular structure information.

Methods and Results

Vapor Heat Capacities and Heats of Vaporization.—Measurements of vapor heat capacities and heats of vaporization were made with the flow calorimeter and cycling vaporizer described

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[2] Present address, Army, Chemical Center, Edgewood, Md.

[3] Todd, Oliver and Huffman, THIS JOURNAL, **69**, 1519 (1947).