acid was placed in the reactor and allowed to come to bath temperature. The stirrer was started and a mixture of 4.50 g. of 70% nitric acid and 5.20 g. of 96% sulfuric acid was added dropwise through the side-arm during 30 min. After the reaction mixture had stirred an additional 5 min., about 20 ml. of cold water was added. The quenched reaction mixtures from three such nitrations were poured into 400 ml. of water and the mixture was distilled through a 12-in. Berl-saddle-packed column provided with a Dean–Stark trap for removing the heavier than water layer. No toluene was recovered. (In some nitrations unreacted toluene distilled with the first few milliliters of water and easily was separated from the nitrotoluenes which distilled later.) The mixed nitrotoluenes steam distilled, leaving any di- and trinitrotoluenes in the residue. The nitrotoluenes were extracted into ether, the ether solution was dried with magnesium sulfate and the solution was evaporated on a steam-cone to leave 19.0 g. (92%) of mixed nitrotoluenes.

A 3.11-g. sample of the mixed nitrotoluenes was diluted with 8.09 g. of pure (inactive) *m*-nitrotoluene. This mixture was distilled through the Podbielniak column and the following fractions were collected at 30/1 reflux ratio: 1, 2.38 g., b.p. 137.0–146.9° (90 mm.); 2, 0.77 g., b.p. 146.9–147.3°; 3, 1.31 g., b.p. 147.8°. Fraction 3 corresponded to pure *m*-nitrotoluene, according to previous trial distillations. To a 2.31-g. sample of fraction 3 was added 3.88 g. of sodium dichromate and 8.50 g. of water; 9.70 g. of concd. sulfuric acid was then added dropwise during 8 min. The mixture was heated under reflux and stirred for 45 min. At the end of this time, the reaction mixture was cooled in an ice-bath for two hours. The solid material which separated was collected on a sintered-glass filter and then boiled with benzene, which extracted the organic material away from the inorganic salts. After separating these by filtration, the benzene solution was extracted with 5% sodium hydroxide solution using a gentle swirling motion of the separatory funnel so as to avoid emulsification. The aqueous solution was acidified with sulfuric acid and the crystals of *m*-nitrobenzoic acid were collected on a filter (80% yield of crude material). The acid was recrystallized twice from 1% hydrochloric acid and radioassayed. The average of four assays (10- to 14-mg. samples) was 0.0357 ± 0.0030 μc./mmole, corresponding to 3.43% *m*-nitrotoluene among the nitrotoluenes. The *m*-nitrobenzoic acid was recrystallized once more and re-assayed; the average of two assays (10- and 11-mg. samples) was 0.0356 ± 0.0003μc./mmole. The *m*-nitrobenzoic acid melted at 141.2°.

The nitrations at the other temperatures were carried out similarly on the same amount (5 g.) of radioactive toluene-1-C^{14}, except in the 60° nitration only 2.32 g. of toluene was used and the amounts of the other materials were cut down proportionately. Other details and results of these experiments are given in Table I.

Separation of *m*-Nitrotoluene from *p*-Nitrotoluene by Distillation.—A mixture was prepared which contained 3.02 g. of *o*-nitrotoluene, 3.00 g. of *m*-nitrotoluene and 6.26 g. of *p*-nitrotoluene-1-C^{14} (0.32 μc./mmole). This mixture was distilled through the Podbielniak column and a cut of 0.80 g. was taken at the *m*-nitrotoluene plateau. This material was oxidized by dichromate–sulfuric acid as described above and a 51.7-mg. sample of the *m*-nitrobenzoic acid obtained was radioassayed. The electrometer reading was not significantly above background. Since a 1% contamination by *p*-nitrobenzoic acid would have given a reading seven times background from this size sample, it was concluded that the separation by fractional distillation was satisfactory.

Separation of *m*-Nitrotoluene from *o*-Nitrotoluene by Oxidation.—A mixture of 2.00 g. of *m*-nitrotoluene and 0.05 g. of *o*-nitrotoluene-1-C^{14} (0.09 μc./mmole) was prepared and oxidized by dichromate in sulfuric acid as described above. The *m*-nitrobenzoic acid obtained was recrystallized twice and a 53.4-mg. sample was radioassayed. The electrometer reading was not significantly above background. Since a 1% contamination by *o*-nitrobenzoic acid would have given a reading twice background from this size sample, it was concluded that the *o*-nitrotoluene was satisfactorily separated in the oxidation procedure.

AUSTIN 12, TEXAS

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF BUFFALO SCHOOL OF MEDICINE]

Fluoroacetyl Phosphate; Preparation and Properties[1]

BY ABRAHAM MARCUS[2] AND W. B. ELLIOTT

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Fluoroacetyl phosphate has been synthesized and compared with acetyl phosphate. The rate of hydrolysis of fluoroacetyl phosphate, like that of acetyl phosphate, is first order with respect to substrate but in contrast to that of acetyl phosphate is minimal at *p*H 2. Both reactions are increased at neutral *p*H by Mg^{++}, Ca^{++} or pyridine, but pyridine catalysis is greater with fluoroacetyl phosphate. At acid *p*H, Mg^{++} and Ca^{++} accelerate the hydrolysis of fluoroacetyl phosphate, whereas acetyl phosphate hydrolysis is unaffected. Fluoroacetyl phosphate is much more susceptible to mammalian and bacterial acyl phosphatase than is acetyl phosphate. Possible explanatory mechanisms are discussed.

In a previous report,[3] it was shown that fluoroacetate was enzymatically activated by pigeon liver extracts and could function in condensation reactions which involve both the carboxyl and methyl groups. The latter reactions have also been demonstrated with synthetic fluoroacetyl-coenzyme A.[3,4] During this study it was noted[5] that both FAc-CoA[6] and fluoroacethydroxamic acid (prepared from fluoroacetic anhydride) were much more unstable than the respective acetyl compounds. To obtain additional insight into the chemical and biological properties introduced by the α-F atom, it appeared desirable to seek another fluoroacetyl compound which could be prepared in quantity sufficient for detailed study. The present communication is concerned with the synthesis of fluoroacetyl phosphate and comparison of its chemical properties with those of acetyl phosphate.

Results

Synthesis of Fluoroacetyl Phosphate.—In seeking a convenient method for the preparation of fluoroacetyl phosphate, several reactions were studied for suitability in preparing acetyl phosphate. It was found that Ac-P could be prepared by reaction of either acetic anhydride or a mixed anhydride of acetic acid and ethyl chlorocarbonate[7] with

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(2) Research Fellow of the National Heart Institute, Public Health Service. This work represents a portion of a dissertation submitted by Abraham Marcus to the Graduate School of the University of Buffalo, May, 1956, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(3) A. Marcus and W. B. Elliott, *J. Biol. Chem.*, **218**, 823 (1956).

(4) R. O. Brady, *ibid.*, **217**, 213 (1955).

(5) A. Marcus and W. B. Elliott, unpublished observations.

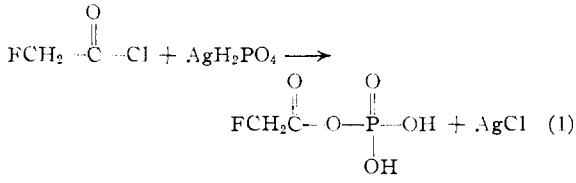
(6) The following abbreviations are used: FAc-CoA, S-fluoroacetyl coenzyme A; Ac-P, acetyl phosphate; FAc-P, fluoroacetyl phosphate; tris, tris-(hydroxymethyl)-aminomethane.

(7) T. Wieland and L. Rueff, *Angew. Chem.*, **65**, 186 (1953).

potassium phosphate by a procedure analogous to that described by Kaufman for succinyl phosphate.[8] In a typical reaction with 10 mmoles of mixed anhydride, 4.44 mmoles of dilithium Ac-P were obtained (44% yield) containing 3.0 μmoles of Ac-P per mg. (45% purity based on a molecular weight of 153). The preparation was purified by ethanol and acetone precipitation yielding a product of 92% purity, free of inorganic phosphate.

In attempting to prepare FAc-P by similar procedures, no acyl phosphate was formed by the reaction with fluoroacetic anhydride, while preparations of 10 to 14% purity were obtained from reactions of the mixed anhydride of fluoroacetic acid. This material could not be purified further.

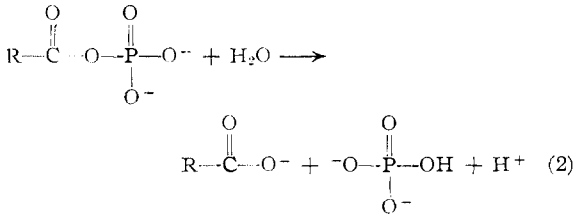
The reaction of fluoroacetyl chloride with silver phosphate (equation 1) by a modification of the



(1)

procedure of Lipmann and Tuttle[9] was more successful. The procedure involved treating fluoroacetyl chloride with silver phosphate in ether, neutralizing with lithium hydroxide, removal of insoluble silver salts and fractional precipitation with ethanol. Preparations of 50 to 60% purity (based on a molecular weight of 171 for the dilithium salt) were consistently obtained in 6% yield.

Analysis and Identification of Fluoroacetyl Phosphate.—The usual method for determining FAc-P involved incubation of a weighed quantity of solid dilithium salt directly with a solution of hydroxylamine and determining the formed hydroxamic acid as indicated in the Experimental section. Data obtained within 1 week after preparation indicated a maximum purity of about 60% with less than 1 part of inorganic phosphate to 1 part of FAc-P. Approximately 12% decomposition occurred when the preparations were stored at −16° for 3 months. Since there was some uncertainty regarding the quantitative determination of FAc-P by this method, (see "experimental") determinations of the organic phosphorus content and acid liberated by hydrolysis (equation 2) were made.



(2)

Comparison of the data (Tables I and II) indicates that the values obtained by phosphate determination are in approximate agreement with those obtained from hydroxamic acid determinations, while titration values were approximately 8% lower with Ac-P and 35% higher with FAc-P than the corresponding hydroxamic acid values. The low values with Ac-P may be due to volatilization of acetic acid during the hydrolysis. The higher values obtained with FAc-P would indicate that the hydroxamic acid method as employed, did not determine FAc-P quantitatively. The values for FAc-P reported in the ensuing experiments are based on hydroxamic acid determination and represent minimum values.

TABLE I

ANALYSIS OF FLUOROACETYL PHOSPHATE BY PHOSPHATE ANALYSIS[a]

Expt. no.	Hydroxamic acid, μmoles	Inorganic phosphate, μmoles	Total phosphate, μmoles	Phosphate as FAc-P, %
1	9.90		18.60	53.5
2	4.80		9.30	51.5
3		7.44	15.40	52.0
4		4.56	10.40	56.0

[a] Phosphate as FAc-P is hydroxamic acid/total phosphate or total phosphate minus inorganic phosphate/total phosphate.

TABLE II

ANALYSES OF ACETYL AND FLUOROACETYL PHOSPHATE BY TITRATION

Sample	Hydroxamic acid, μmoles	Alkali required, μmoles	A/H
Ac-P	20.5	18.9	0.92
	22.3	20.4	0.91
FAc-P	18.3	25.0	1.37
	24.0	32.5	1.35

A/H is alkali required/hydroxamic acid.

For qualitative identification, samples of acetyl and fluoroacetyl phosphate were converted to the hydroxamic acids and chromatographed on paper in phenol–water (3:1). Each substance gave only one spot when sprayed with $FeCl_3$. The R_f values were identical with those of the hydroxamic acids prepared from acetic and fluoroacetic anhydrides.[3] When chromatographed directly, acyl phosphate spots could be detected for Ac-P but not for FAc-P. In phenol–water, however, FAc-P gave two spots when sprayed with the phosphate reagents,[10,11] one with R_f = 0.04 and the other with R_f = 0.13. The first spot is apparently inorganic phosphate (inorganic phosphate alone gave R_f = 0.04) while the second spot is due to the fluoroacetyl phosphate which decomposed prior to spraying.

Hydrolysis of Fluoroacetyl Phosphate.—Initial experiments indicated that the rate of hydrolysis was first order with respect to substrate at all *p*H values and the following procedure was employed in determining the effect of *p*H on the rate of hydrolysis. Several milligrams of the acyl phosphate were dissolved in cold water and 0.2-ml. aliquots were added to the tubes containing buffer (0.6 ml. at 35°) and another 0.2 ml. aliquot was added immediately to a tube containing 0.3 ml. (900 μmoles) of neutral hydroxylamine. Tubes containing buffer were then incubated at 35° for 30 min. for FAc-P and 60 min. for Ac-P. At the end of the incubation period, 0.3 ml. (900 μmoles) of neutral hydroxylamine was added and the hydroxamic acid determined. The curve for Ac-P is in close agreement with data of Koshland[12] indicating maximum stability at neu-

(8) S. Kaufman, *Arch. Biochem. Biophys.*, **50**, 506 (1954).
(9) F. Lipmann and C. Tuttle, *J. Biol. Chem.*, **153**, 571 (1944).
(10) R. S. Bandurski and B. Axelrod, *ibid.*, **193**, 405 (1951).
(11) H. E. Wade and D. M. Morgan, *Nature*, **171**, 529 (1953).
(12) D. Koshland, THIS JOURNAL, **74**, 2286 (1952).

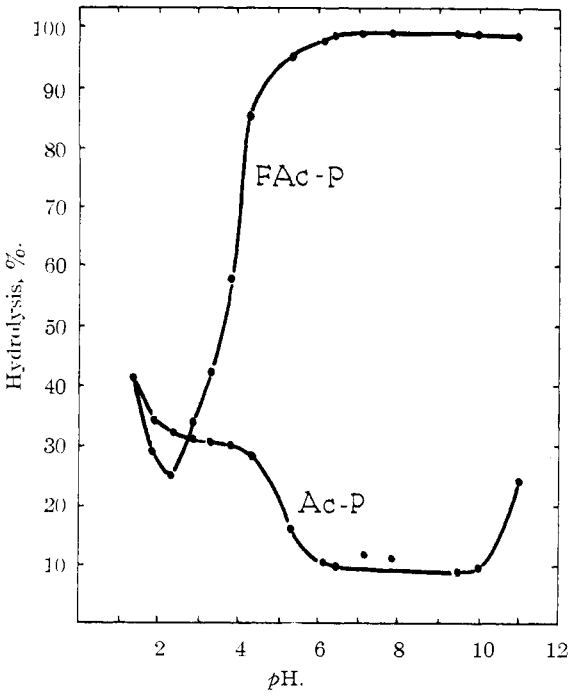


Fig. 1.

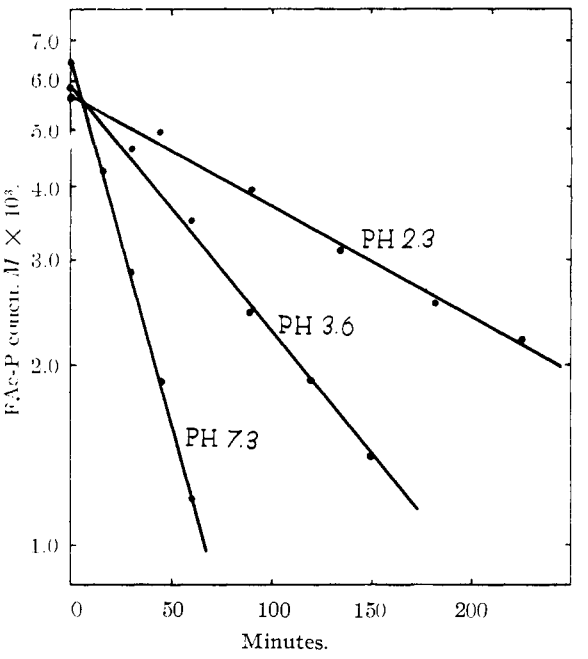


Fig. 2.

tral *p*H, less stability from about *p*H 4.5 to 1.5 and rapid destruction below *p*H 1.5 (Fig. 1). In contrast, FAc-P shows maximum stability at about *p*H 2 with marked instability at neutral *p*H. Inorganic phosphate added to Ac-P in excess of the maximum contamination of the FAc-P, showed no effect on the rate of hydrolysis. Experiments at a lower temperature showed that FAc-P was affected by alkaline *p*H in a manner similar to Ac-P, in that a sharply increased rate of hydrolysis was noted between *p*H 10 and 11.

More accurate comparisons of the rates of hydrolysis of acetyl and fluoroacetyl phosphate were made at 24° by a similar procedure in which aliquots of the reaction mixture were transferred at intervals to 0.3 ml. of freshly prepared neutral hydroxylamine. Typical plots of concentration against time (Fig. 2) demonstrate that the rate of hydrolysis of FAc-P is first order with respect to substrate concentration. Rate constants were determined, from half-life or by calculation from the first-order kinetic equation. These values, together with half-life values and the ratio (K_F/K_A) which is a comparison of the rates of hydrolysis of acetyl and fluoroacetyl phosphate are given in Table III. At *p*H 2.3 FAc-P is approximately twice as unstable as Ac-P, while at *p*H 7.3 it is 63 times as unstable as Ac-P.

Catalysis of the Hydrolysis of Acetyl and Fluoroacetyl Phosphate.—Lipmann and Tuttle[9] showed that the hydrolysis of Ac-P was accelerated by Ca^{++} while Koshland[12] has reported similar effects of Mg^{++} and pyridine at neutral *p*H. The effects of these catalysts were studied. Initial experiments showed that the hydrolysis of FAc-P was accelerated under conditions such that Ac-P was not hydrolyzed appreciably even in the presence of the catalysts. Accordingly, comparisons were made under conditions allowing significant hydrolysis of both compounds, the effect of the catalyst being expressed as % increase in hydrolysis relative to an uncatalyzed control (Table IV). The catalysts were 0.05 *M*, Mg^{++} and Ca^{++} and 0.2 *M* pyridine. There are several noteworthy points: (a) at neutral *p*H Ca^{++} accelerates the hydrolysis more than

TABLE III

RATE CONSTANTS OF ACETYL AND FLUOROACETYL PHOSPHATE HYDROLYSIS AT 24°

*p*H	Substrate	$K \times 10^4$, min.$^{-1}$	$t_{1/2}$, hr.	K_F/K_A[a]
2.3	Ac-P	20	5.8	
	FAc-P	46	2.5	2.3
3.7	Ac-P	21	5.6	
	FAc-P	92	1.3	4.5
7.3	Ac-P	4.6	25.1	
	FAc-P	290	0.4	63.0

[a] K_F/K_A = the rate constant for FAc-P divided by the rate constant for Ac-P at the given *p*H.

Mg^{++}. That it is not due to removal of inorganic phosphate is shown by the fact that added inorganic phosphate does not retard the hydrolysis (see section on hydrolysis of fluoroacetyl phosphate). (b) Pyridine is at least twice as effective in catalyzing FAc-P hydrolysis as with Ac-P. (c) Mg^{++} and Ca^{++} accelerate the hydrolysis of FAc-P at acid *p*H but have no effect on Ac-P.

Enzymatic Hydrolysis of Fluoroacetyl Phosphate.—Lipmann[13] reported that Ac-P was rapidly broken down by mammalian and bacterial extracts due to acyl phosphatase activity. Accordingly, experiments were carried out to determine the susceptibility of FAc-P to both mammalian and bacterial phosphatase. As a source of mammalian enzyme, a 10% extract of pig heart acetone powder in 0.02 *M* $NaHCO_3$ was used while the data on bacterial activity were obtained with *E. coli* extract. In the presence of pig heart extract, more than

(13) F. Lipmann, *Advances in Enzymol.*, **6**, 231 (1946).

TABLE IV

EFFECT OF Mg^{++}, Ca^{++} AND PYRIDINE ON THE HYDROLYSIS OF ACETYL AND FLUOROACETYL PHOSPHATE

	Conditions[a]	Additions	Amount hydrolyzed, μmoles	Incr. in hydr., %
1.	13.2 μmoles FAc-P	.....	4.6	..
	30 min., 21°	0.05 *M* Mg^{++}	6.8	48
	*p*H 7.3	.05 *M* Ca^{++}	7.6	65
		.2 *M* pyridine	6.6	43
2.	16.5 μmoles Ac-P	.....	6.4	..
	30 min., 51°	0.05 *M* Mg^{++}	9.4	47
	*p*H 7.3	.05 *M* Ca^{++}	10.9	70
		.2 *M* pyridine	7.8	17
3.	16.6 μmoles FAc-P	.....	7.2	..
	35 min., 35°	0.05 *M* Mg^{++}	8.0	11
	*p*H 1.3	.05 *M* Ca^{++}	8.0	11
4.	13.2 μmoles Ac-P	.....	3.2	..
	30 min., 35°	0.05 *M* Mg^{++}	3.2	0
	*p*H 1.3	.05 *M* Ca^{++}	3.2	0

[a] All tubes contained either 120 μmoles of tris buffer *p*H 7.3 or 120 μmoles of citrate HCl buffer *p*H 1.3 and other additions as indicated to a final volume of 0.9 ml.

75% of the FAc-P was hydrolyzed in 5 minutes whereas less than 8% of the Ac-P was broken down under corresponding conditions (Table V). The *E. coli* extract catalyzed the hydrolysis of FAc-P to about 85% completion in comparison with only 18% in the case of Ac-P.

TABLE V

ENZYMATIC HYDROLYSIS OF ACETYL AND FLUOROACETYL PHOSPHATE[a]

	Substrate	Additions	Amount hydrolyzed, μmoles
1.	7.8 μmoles	.....	0.0
	Ac-P	Enz. (pig heart)	.6
	7.7 μmoles	.....	.8
	FAc-P	Enz. (pig heart)	5.8
2.	1.41 μmoles	.....	0.03
	Ac-P	Enz. (*E. coli*)	.25
	3.00 μmoles	.....	.76
	FAc-P	Enz. (*E. coli*)	2.50

[a] The complete system contained 100 μmoles of tris buffer *p*H 7.3, in both experiments, 6.4 μmoles of glutathione in experiment 2 and 0.1 ml. of the respective enzyme preparation. Incubation was for 5 min. in experiment 1 and 20 min. in experiment 2, both in a total vol. of 1.0 ml.

Discussion

Since the mechanism of Ac-P hydrolysis has been elaborated by Koshland[12] on the basis of a series of experiments similar to those described, it appears possible to explain some of the finding with FAc-P by analogy. Koshland proposed that the hydrolysis is a nucleophilic displacement reaction involving the OH^- ion at alkaline *p*H and HOH at neutral *p*H. At strongly alkaline *p*H (>10) the rate controlling attack by the hydroxide ion might be expected to affect both acetyl and fluoroacetyl phosphate similarly. However, at neutral *p*H, the reaction is promoted primarily by the relative electron deficit at the carbonyl carbon. At this *p*H the effect of the electronegative α-fluorine is most apparent, establishing a marked deficiency of electrons at the carbonyl carbon, thus facilitating a nucleophilic attack. As the *p*H falls below 5, the rate of hydrolysis gradually decreases presumably due to the decrease of nucleophilic attacking reagents.

This explanation further allows that FAc-P hydrolysis is more susceptible than Ac-P hydrolysis to pyridine catalysis. The acceleration by pyridine has been shown by Koshland to involve a nucleophilic attack on the carbonyl carbon forming an acetyl pyridinium ion which decomposes almost instantaneously. With FAc-P, this reaction would be augmented by the increased electron deficit at the carbonyl carbon.

At acid *p*H (<2), continuing by analogy to Ac-P, the mechanism involves a primary attack by a proton on the carbonyl oxygen. As is shown in Fig. 1, the α-fluorine does not appear to exert any effect in this type of reaction.

The remaining unexplained phenomenon is the catalysis by Mg^{++} and Ca^{++}. At neutral *p*H there appears to be no essential differences between acetyl and fluoroacetyl phosphate with regard to the catalysis. The mechanism suggested by Koshland for Mg^{++} catalysis involves formation of a Mg^{++} chelate polarizing the electrons in the substrate. In the experiments reported (Table V) it was noted that Ca^{++} is a more effective catalyst than Mg^{++}. This would appear to be due to the formation of a more stable chelate in Ca^{++} than with Mg^{++}.

At acid *p*H, Ac-P hydrolysis is unaffected by Mg^{++} or Ca^{++}. Under similar conditions, FAc-P hydrolysis is catalyzed to a significant degree. In explanation, it appears possible that FAc-P may form a five-membered chelate at acid *p*H involving one link to the fluorine atom. Such a chelate would be different in nature than that postulated at neutral *p*H (which involves chelation through two oxygen atoms) thus accounting for the equal effectiveness of both Mg^{++} and Ca^{++} at acid *p*H with FAc-P.

With regard to the rapid enzymatic hydrolysis of FAc-P, it would appear, by analogy to the nonenzymatic reaction, that the active site of the enzyme is nucleophilic in nature thus possessing stronger affinity for the α-fluoro compound. By fractionating the *E. coli* extract with ethanol, preparations have been obtained that are devoid of phosphatase activity toward Ac-P, while retaining ability to hydrolyze FAc-P. Such separation of activity is consistent with a report by Stadtman and Barker[14] indicating separate phosphatases for Ac-P and higher acyl phosphates.

Experimental

Synthesis of Acyl Phosphates.—Acetyl phosphate was prepared by two procedures. In the first procedure, 8 mmoles of acetic anhydride (as determined by the hydroxamic acid reaction) was added to a mixture of 20 ml. of 1 *M* potassium phosphate *p*H 7.4 and 40 ml. of 1 *M* $KHCO_3$. The formed acetyl phosphate was isolated as the dilithium salt according to Kaufman.[8] The yield was 31% (based on anhydride) and the purity 18%. The product was brought easily to 76% purity with two precipitations between 33 and 85% ethanol (v./v.). In the second procedure, 5 g. of sodium acetate[15] was dissolved in 8 to 10 ml. of water, acidified with 2 ml. of concd. HCl and extracted four times by

(14) E. R. Stadtman and H. A. Barker, *J. Biol. Chem.*, **184**, 769 (1950).

(15) The sodium salt was used to establish conditions for an analogous reaction with sodium fluoroacetate.

shaking with 15-ml. portions of ether. Three and one-half ml. of cold ethyl chlorocarbonate was added to the ether extract and while the solution was stirred mechanically, 2.9 ml. of pyridine was added dropwise. After stirring for 3 min., the ether phase was decanted and concentrated *in vacuo* to a vol. smaller than 3 ml.[16] The 10.5 mmoles of mixed anhydride so obtained, was treated with potassium phosphate as in the first procedure yielding a product of 45% purity in 44% yield. The product was then purified as above except that acetone was used in place of ethanol with a final purity of 92%.

Fluoroacetyl phosphate was prepared by a procedure based on that of Lipmann and Tuttle.[9] Five ml. of fluoroacetyl chloride[17] (approximately 50 mmoles) mixed with 5 ml. of cold anhydrous ether was added dropwise to a homogeneous suspension of 8 g. of silver phosphate (19 mmoles) and 2.75 ml. of 85% phosphoric acid as described[9] for the preparation of acetyl phosphate. After the addition was completed, the bottle was stoppered and shaken vigorously at room temperature for 20 min. to give a milk white viscous suspension. The suspension was cooled to 3° and 20 ml. of cold water (3°) was added followed by careful addition of 30 ml. of cold 4 *N* lithium hydroxide while stirring. The insoluble material was filtered and the ether layer removed in a cold separatory funnel. The water solution was then brought to the first purple shade with *m*-cresol purple by careful addition of 4 *N* lithium hydroxide and the precipitate was removed by filtration and discarded. To the filtrate, cold (−16°) 95% ethanol was added to a final ethanol concentration of 23% (v./v.) followed by lithium hydroxide to bring the indicator color back to the first purple shade. The precipitate was filtered off and discarded. To the filtrate, cold ethanol was added slowly with stirring to an ethanol concentration of 85% (v./v.). The precipitate was collected on a buchner funnel, washed with cold ethanol and ether and dried at 3° *in vacuo* over P_2O_5. The material obtained was about 50% pure (based on a molecular weight of 170 for dilithium fluoroacetyl phosphate) with a yield of about 12%. An optional purification involved dissolving the material in cold water to give a 10% solution, addition of 4 *N* lithium hydroxide to the purple shade with *m*-cresol purple and repetition of the alcohol precipitation as above. The material was now about 60% pure with a yield of 6% (*i.e.*, a 50% loss during the purification) and contained about equal parts of inorganic phosphate and FAc-P.

Analytical Methods.—Acetyl and fluoroacetyl phosphate were analyzed by two modifications of the hydroxamic acid reaction of Lipmann and Tuttle.[18] That of Beinert, *et al.*.[19] (method I) was used when necessary to overcome previously added buffer, while that described by Jones and Lipmann[20] (method II) was employed when small amounts of buffer were present. When converted to the hydroxamic acid, Ac-P gave the same values by both methods. With FAc-P, method I uniformly gave 51% of the value obtained with method II. Accordingly all values obtained by method I with FAc-P have been multiplied by a factor of 1.95. Since this method was used primarily in kinetic studies, the correction is not of primary significance. That the two methods of analysis yield different results with FAc-P may account for the finding by Brady[4] that FAc-CoA gave different values by hydroxamic acid determination and spectrophotometric analysis. Experiments in which various dilutions of hydroxylamine were employed indicated that the reaction with hydroxylamine was quantitative and that the variation depended upon the determination of the formed hydroxamic acid.

Total phosphate was determined according to Fiske and SubbaRow.[21] Inorganic phosphate in the presence of acyl phosphate was determined by dissolving a few mg. of the acyl phosphate preparation in a known volume of ice cold 0.1 *M* tris buffer *p*H 8.1. One aliquot was analyzed for total phosphate while another aliquot was added to 1.5 ml. of ethanolic calcium chloride and the inorganic phosphate determined according to Lipmann and Tuttle.[9] The centrifugation time was 1.5 minutes at 1650 × *g*. The difference between total and inorganic phosphate was taken to be organic phosphate.

The analysis of acyl phosphate by titration was carried out by the following procedure. A sample of acyl phosphate was dissolved in 6 ml. of ice-cold water. Two tenths of a ml. was transferred rapidly to a hydroxylamine solution for hydroxamic acid analysis. A 5.0-ml. aliquot was rapidly brought to the first pink shade by the addition of phenolphthalein and 0.1 *N* NaOH from a micrometer type buret. The solutions were then heated in a steam-bath to obtain complete hydrolysis, 40 min. being allowed for Ac-P and 10 min. for FAc-P. The tubes were cooled, brought to the original volume and titrated with 0.1 *N* NaOH to the original pink color.

Enzyme Preparations.—Pig heart acetone powder was prepared according to Kaplan and Lipmann.[22] The dried powder was extracted by making a 10% suspension in 0.02 *M* potassium bicarbonate and centrifuging off the insoluble material at 1650 × *g*.

E. coli 4157 (obtained from the National Type Culture Collection) was grown in Kolli flasks or Roux bottles on nutrient agar[23] (Difco) for 18 hr. at 37°. The cells were washed off the agar with 0.4% NaCl, centrifuged, washed with 0.4% NaCl, suspended in a minimal volume of distilled water, lyophilized and stored at −16°. The cells were extracted by grinding in a cooled mortar with an equal weight of alumina-A301 (Aluminum Company of America) with gradual addition of buffer (0.02 *M* potassium phosphate *p*H 6.6–0.1 *M* KCl) to give a 5% (w./v.) suspension. The suspension was centrifuged at 23,000 × *g* for 20 minutes and the supernatant fluid was frozen at −16°.

(16) In experiments in which the ether vol. was larger than 3 ml., poor yields were obtained.

(17) W. E. Truce, THIS JOURNAL, **70**, 2828 (1948).

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

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Amine Boranes. I. The Preparation of Pyridine Arylboranes

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The pyridine complexes of a series of arylboranes have been prepared by the lithium aluminum hydride reduction of the corresponding ethyl or *n*-butyl boronates at low temperatures in ethyl ether solution and in the presence of pyridine. These materials are air-stable crystalline solids which exhibit reactions characteristic of the boron–hydrogen bond.

In a preliminary communication[1] the preparation and properties of the pyridine complexes of various aryl- and diarylboranes were described. The present paper presents further data regarding the former compounds.

Although various alkyl derivatives of diborane have been reported in the chemical literature, the

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