After drying ($MgSO_4$), the chloroform solution was concentrated and the residue distilled to yield the products. **Diethyl α-acetoxy-β-acetamidoethylphosphonate** (**19**) [bp 160° (0.25 mm); ir (film) 3300, 3080 (NH), 1745 (ester C=O), 1660 (amide C=O), 1220 (P=O), 1158 (POC_2H_5), 1018 cm^{-1} (PO-alkyl)] was obtained from **17** (30% yield from **3a**) and **diethyl β-acetoxy-γ-acetamidopropylphosphonate** (**25**) [bp 162° (0.25 mm); ir (film) 3300, 3075 (NH), 1735 (ester C=O), 1682 (amide C=O), 1217 (P=O), 1170 (POC_2H_5), 1020 cm^{-1} (PO-alkyl)] was obtained from **24** (75% yield from **4**).

Registry No.—**3b**, 19462-37-4; **7a**, 19462-38-5; **7b**, 19462-39-6; **13**, 19462-40-9; **14**, 19462-41-0; **15**, 19462-42-1; **16**, 19462-43-2; **18**, 19462-44-3; **19**, 19462-45-4; **20**, 1866-28-0; **21**, 19462-47-6; **22**, 19462-48-7; **23**, 19462-49-8; **24**, 19462-50-1; **25**, 19462-51-2; **26**, 19462-52-3; **27**, 19462-53-4; **28**, 19462-54-5; **29**, 19462-45-4; **30**, 19462-56-7.

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The Action of Hydrogen Fluoride on Nucleotides and Other Esters of Phosphorus(V) Acids[1,2]

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The action of hydrogen fluoride, both liquid and aqueous, on a number of mono- and diesters of orthophosphoric acid, two cyclic phosphates, mono and diesters of polyphosphates, phosphorofluoridate esters, and inorganic phosphates was investigated. The particular reactions which take place are found to be a function of temperature (−50 to +25°), time (0.04–28 hr), and acid concentration. A comparison of the acid-catalyzed reactions of phosphate esters in aqueous solution with the behavior of such compounds toward 60% hydrofluoric acid brings out several interesting contrasts. First, the reactions in 60% hydrofluoric acid which are described here are fast compared with reactions observed with ordinary aqueous acids. Second, in the reactions with 60% hydrofluoric acid all of the evidence points toward the conclusion that phosphorus–oxygen, rather than carbon–oxygen, bond cleavage takes place exclusively. Third, this acid is a highly specific dephosphorylating agent compared with ordinary aqueous acids. These three features of the chemical properties of hydrogen fluoride in relation to phosphorus(V) esters are correlated with information concerning the characteristics of hydrogen fluoride both as an anhydrous liquid and in concentrated aqueous solution. Since extended exposure to 60% hydrofluoric acid causes no deamination of adenine, guanine, or cytosine, a method was devised for the base analysis of ribonucleic acid (RNA) by degradation with this reagent.

Hydrogen fluoride, either as anhydrous liquid or aqueous solution (hydrofluoric acid), has been used relatively little in nucleotide chemistry in comparison with other areas of organic chemistry.[3] The structure proofs of A-3′:5′-P[4,5] and of the diastereoisomeric 2′:3′-benzylidene ribonucleosides[6] were dependent, in part, on degradations carried out with liquid hydrogen fluoride and hydrofluoric acid, respectively. Furthermore, 5-iodouridine 5′-triphosphate has been degraded with 60% hydrofluoric acid to 5-iodouridine 5′-phosphate and then to 5-iodouridine.[7]

The principal objective of this study was to investigate, in detail, the action of hydrogen fluoride on ordinary mononucleotides. The study includes, however, a broader spectrum of phosphorus compounds. It covers a number of mono- and diesters of orthophosphoric acid, two cyclic phosphates, mono- and diesters of polyphosphates, phosphorofluoridate esters, and inorganic phosphates. That the present results are of rather general interest is attested to by the fact that they already have been utilized to obtain structural information concerning the teichoic acids.[8]

Results and Discussion

Various acid-catalyzed reactions have been observed for nucleoside monophosphates in aqueous solution.

(1) (a) This investigation was supported, in part, by Public Health Service Research Grant No. CA-03870 from the National Cancer Institute and, in part, by Grant No. GB-2980 from the National Science Foundation. (b) The major portion of this paper is based on the Ph.D. dissertation of J. W. Abrell, Washington University, St. Louis, Mo., 1965.

(2) For a brief summary of the results of this investigation, see D. Lipkin, J. W. Abrell, and B. E. Phillips, Abstracts, Seventh International Congress of Biochemistry, Tokyo, Aug 1967, No. B-57, p 631.

(3) K. Wiechert in "Newer Methods of Preparative Organic Chemistry," Interscience Publishers, New York, N. Y., 1948, pp 315–368.

(4) The abbreviations used follow: A-2′ (3′)-P, adenosine 2′(3′)-phosphate; A-5′-P, adenosine 5′-phosphate; A-2′:3′-P, adenosine 2′:3′- (cyclic) phosphate; A-3′:5′-P, adenosine 3′:5′- (cyclic) phosphate; A-5′-PF, adenosine 5′-phosphorofluoridate; ADP, adenosine 5′-diphosphate; APPA, P^1,P^2-diadenosine 5′-pyrophosphate; ATP, adenosine 5′-triphosphate; C-2′(3′)-P, cytidine 2′(3′)-phosphate; dA-5′-P, 2′-deoxyadenosine 5′-phosphate; DCC, dicyclohexylcarbodiimide; DNA, deoxyribonucleic acid; DPN, diphosphopyridine nucleotide; 2-dR-5-P, 2-deoxyribose 5-phosphate; G-2′(3′)-P, guanosine 2′(3′)-phosphate; Me A-5′-P, methyl ester of adenosine 5′-phosphate; Nam, nicotinamide; NR, nicotinamide riboside; NR-5′-P, nicotinamide riboside 5′-phosphate; NR-5′-PF, nicotinamide riboside 5′-phosphorofluoridate; RNA, ribonucleic acid; T-3′-P, thymidine 3′-phosphate; T-5′-P, thymidine 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; U-2′(3′)-P, uridine 2′(3′)-phosphate.

(5) (a) D. Lipkin, R. Markham, and W. H. Cook, *J. Amer. Chem. Soc.*, **81**, 6075 (1959); (b) D. Lipkin, W. H. Cook, and R. Markham, *ibid.*, **81**, 6198 (1959).

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(7) D. Lipkin, F. B. Howard, D. Nowotny, and M. Sano, *J. Biol. Chem.*, **238**, PC2249 (1963).

(8) L. Glaser and M. M. Burger, *ibid.*, **239**, 3187 (1964).

TABLE I

DEGRADATION OF NUCLEOSIDE MONOPHOSPHATES[a]

Substrate	Reaction conditions: Time, hr	Reaction conditions: Temp, °C	Separation method[b]	Reaction products: Nucleotide, %	Reaction products: Nucleoside, %	Reaction products: Base, %	Loss, %
A-3′-P[c]	1	−23	D	42[d]	58		
A-2′(3′)-P	0.5	0	A	4	84	10	2
	28[e]	25	A			94[f]	6
A-5′-P	1	−23	A	53	43		4
	0.5	0	A	13	73	7	7
	14[e]	25	A			102[f]	
A-5′-PF	0.25	0	B	70[g]	30	Trace	
Me A-5′-P	1	0	A	63[h]	26	4	7
C-2′(3′)-P	1	0	A		96		4
	17[i]	25	Borate			86	14
G-2′(3′)-P	0.5	0	Water[j]	Trace	76	13	<11
	14[e]	25	Water[j]			96	5
U-2′(3′)-P	0.25	25	A		97		3
	2.5[i]	25	A			96	4
NR-5′-P[k]	0.5	0	Phosphate	22	52	17	9
T-3′-P	0.5	0	A	15	86		
T-5′-P	0.5	0	Carbonate	14	78		8
	1	0	Carbonate	Trace	95		<5
	4	25	Carbonate			87	13
dA-5′-P[l]	0.25	−25	C	15		85	

[a] All degradations were performed with 60% hydrofluoric acid unless otherwise noted. [b] Solvent or buffer used for separation of the components of the reaction mixtures. [c] Pure A-3′-P isomer was used. [d] 38% was pure A-3′-P, while 4% was A-2′:3′-P. [e] Actually these degradations of purine ribonucleotides to free bases are essentially complete in 1 hr at 25°. [f] Electrophoresis in borate buffer showed no trace of hypoxanthine. [g] This was unreacted A-5′-PF; no A-5′-P was present. [h] Only unchanged Me A-5′-P. [i] Sample treated with 100% liquid hydrogen fluoride. [j] Descending paper chromatography in water adjusted to pH 10.0–10.8 with ammonium hydroxide. [k] In some of the NR-5′-P degradations, an unidentified product was detected in trace amounts. [l] 2-dR-5-P was recovered from this reaction in good yield.

One of these is hydrolysis of the N-glycosidic bond;[9-11] another is deamination of heterocyclic bases;[12,13] and a third is phosphate migration from one hydroxyl to another of the sugar.[14] Table I is a summary of the results obtained in a study of the action of hydrogen fluoride on nucleoside monophosphates. It is obvious that the most striking feature of 60% hydrofluoric acid as a reagent is its remarkable specificity, even though it it is a very acidic medium with a Hammett acidity function, H_0, of −6 (25°).[15] Except with a purine deoxyribonucleotide (dA-5′-P), reaction conditions can be controlled (0°, 0.5 hr) so that N-glycosidic bond cleavage is negligible and dephosphorylation of a nucleotide to a nucleoside is essentially the only reaction observed. Its specificity is further emphasized by the finding that pure A-3′-P, for example, is *ca.* half-dephosphorylated in 1 hr at −23° with no isomerization to the 2′phosphate, even though a small amount of A-2′:3′-P is formed. Furthermore, deamination of adenine, guanine or cytosine does not take place when nucleosides derived from these bases are exposed to 60% hydrofluoric acid for periods of up to 50 hr at room temperature.

The behavior of dA-5′-P toward 60% hydrofluoric acid is an interesting contrast. Even at −25° for only 15 min, essentially compete conversion takes place into adenine and 2-dR-5-P; only a trace amount of an isomeric deoxyribose phosphate is formed. In this particular case, therefore, the rate of cleavage of the very labile N-glycosidic bond is rapid compared with the rate of dephosphorylation and the rate of phosphate migration. It is possible, nevertheless, to cleave essentially quantitatively the N-glycosidic bond in other purine and pyrimidine nucleotides (preceded by dephosphorylation) by changing reaction conditions (Table I). One such change is the use of liquid hydrogen fluoride with pyrimidine ribonucleotides. The free bases can be isolated and, in the case of the ribosides or ribotides, the free sugar can be recovered as well. Using 60% hydrofluoric acid the yield of ribose is as high as 85–90%, but with liquid hydrogen fluoride the yields are somewhat lower owing to the formation of polyriboses. Free 2-deoxyribose, on the contrary, is rapidly decomposed by 60% hydrofluoric acid even at −50° (2 hr) and it is found among the degradation products from deoxyribosides or deoxyribotides in but very small amounts at most.

The highly specific behavior of 60% hydrofluoric acid was emphasized by a study of the same acid at concentrations less than 24%. As the concentration is decreased, the degradation of nucleotides take place in a manner similar to that observed with other dilute mineral acids,[16] *i.e.*, a slow disappearance of substrate at room temperature by nonselective hydrolysis of the phosphate ester and N-glycosidic bonds in purine nucleotides. Further contrast with the selective and rapid dephosphorylating action of 60% hydrofluoric acid toward nucleotides was provided by a study of the behavior of A-5′-P in trifluoroacetic acid solutions at room temperature. The slow rate of disappearance of

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(10) E. R. Garrett, *J. Amer. Chem. Soc.*, **82**, 827 (1960).
(11) A. M. Michelson, "The Chemistry of Nucleosides and Nucleotides," Academic Press, New York, N. Y., 1963, pp 26, 141.
(12) A. Bendich in "The Nucleic Acids," E. Chargaff and J. N. Davidson, Ed., Academic Press, New York, N. Y., 1955, Vol I, p 117.
(13) H. S. Loring in ref 12, pp 191–199.
(14) (a) W. E. Cohn, *J. Amer. Chem. Soc.*, **72**, 2811 (1950); (b) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 44 (1952).
(15) H. H. Hyman and J. J. Katz in "Non-Aqueous Solvent Systems," T. C. Waddington, Ed., Academic Press, London and New York, 1965, Chapter 2.
(16) Reference 11, p 142.

TABLE II

DEGRADATION OF MISCELLANEOUS PHOSPHATES[a]

Substrate	Registry no.	Reaction conditions: Time, min	Reaction conditions: Temp, °C	Phosphorus-containing reaction products[b]
$NH_4PO_2F_2$	15252-72-9	30	0	PO_3F^{2-}, $PO_2F_2^-$ [c]
$(NH_4)_2PO_3F$	14312-45-9	30	0	Only PO_3F^{2-}
K_2HPO_4	7758-11-4	10	−50	Only PO_4^{3-}
		30	−25	PO_4^{3-}, PO_3F^{2-} [d]
		30	0	PO_3F^{2-}, trace PO_4^{3-}
$CH_3PO_4Li_2$	19375-46-3	30	0	Only PO_3F^{2-}
$(CH_3)_2PO_4Li$	2870-40-8	30	0	Only $(CH_3)_2PO_4^-$
CH_3PO_3FLi	19375-30-5	30	0	$CH_3PO_3F^-$, PO_3F^{2-}, $PO_2F_2^-$ [e]
		60	0	Only PO_3F^-

[a] The reagent was 60% hydrofluoric acid. [b] The products were separated by electrophoresis and chromatography. Detection was by the use of Hanes and Isherwood spray (see ref 45). [c] PO_3F^{2-} and $PO_2F_2^-$ were present in *ca.* a 3:1 ratio. [d] PO_4^{3-} and PO_3F^{2-} were present in *ca.* equal amounts. [e] $CH_3PO_3F^-$, PO_3F^{2-}, and $PO_2F_2^-$ were present in a ratio of *ca.* 3:2:1.

A-5′-P is indicated by the following sets of data, in which the first percentage represents the acid concentration, the second number the reaction time in hours, and the third number represents the amount of A-5′-P which disappeared: 100%, 6 hr, 45%; 90%, 7.5 hr, 37%; 80%, 7.5 hr, 21%; and 60%, 7.5 hr, 13%.[17] The only reaction products found were adenine and ribose phosphate; the absence of ribose or adenosine clearly indicated that no dephosphorylation took place. One other observation of interest was made in trifluoroactic acid as the medium. A pure sample of A-2′-P dissolved in 80% trifluoroacetic acid was allowed to stand at room temperature for 1 hr. The 2′ and 3′ isomers in approximately equal amounts were the only ultraviolet-absorbing materials recovered (90% yield) from the solution.

The inorganic phosphorus recovered from the adenylic acid degradations was identified as phosphorofluoridate. Unfortunately this observation does not contribute evidence toward any particular mechanism for the hydrogen fluoride degradation of phosphate esters, since it was observed that inorganic orthophosphate is converted rapidly into phosphorofluoridate by means of 60% hydrofluoric acid (Table II). This latter observation is particularly interesting in view of the fact that nucleotide recovered from reactions of 60% hydrofluoric acid with the adenylic and the thymidylic acids, which were interrupted short of completion, do not contain nucleoside phosphorofluoridates. Related to this latter set of observations is the behavior of A-5′-PF when treated with 60% hydrofluoric acid. A 30% yield of adenosine is obtained in 15 min at 0°, while the remainder of the substrate is recovered unchanged. It is important to note that no A-5′-P, which would result from acid-catalyzed hydrolysis of A-5′-PF, was found in the reaction mixture.

Me A-5′-P[18] is more resistant to 60% hydrofluoric acid than the corresponding monester, A-5′-P. The reaction products do not contain any A-5′-PF, methyl phosphorofluoridate, or A-5′-P; the only phosphorus-containing product which was recovered was inorganic phosphorofluoridate. Even if formed initially, they undoubtedly would have been further degraded in 1 hr at 0° (Tables I and II). The comparative stability of Me A-5′-P toward hydrofluoric acid parallels the relative kinetic stabilities of the conjugate acids of dialkyl and monoalkyl phosphates, $R_2H_2PO_4^+$ and $RH_3PO_4^+$, toward nucleophilic attack by water, even though these latter reactions involve mostly carbon–oxygen bond cleavage.[19]

Monomethyl phosphate, dimethyl phosphate, and methyl phosphorofluoridate were treated with 60% hydrofluoric acid (Table II). The qualitative results of these reactions are in agreement with the results obtained with the corresponding nucleoside derivatives. Obviously the substitution of a nucleoside residue for a methyl group does not appreciably affect the behavior of the phosphorus moieties toward 60% hydrofluoric acid.

The behavior of the two cyclic phosphates, A-3′:5′-P and A-2′:3′-P, on treatment with 60% hydrofluoric acid, is summarized in Table III. The behavior of the 3′:5′- (cyclic) phosphate was most surprising in view of the finding that the cyclic phosphate isolated from the reaction run at −23 or 0° was entirely the 2′:3′ isomer. This isomerization takes place, in all likelihood, through the conversion of A-3′:5′-P into A-3′-PF and this to A-2′:3′-P. It is obvious from the data in Table III that the C_5'-O-P structure is more rapidly attacked than the C_3'-O-P moiety, analogous to the results which were obtained in the acid-catalyzed hydrolysis of A-3′:5′-P.[5b] Also unexpected was the behavior of A-2′:3′-P. In view of the marked instability of A-2′:3′-P toward acid-catalyzed hydrolysis,[20] it was surprising to find *ca.* 50% of it still present in the reaction mixture after 1 hr. This may be due to the low concentration of the nucleophile H_2O in 60% hydrofluoric acid (see below) and to the fact that the equilibrium between A-2′:3′-P and A-2′- (and 3′-) PF, which would be formed by ring opening of A-2′:3′-P by HF, is far toward this cyclic phosphate. This latter statement is supported by three observations. First, condensation of adenosine and inorganic phosphorofluoridate by means of DCC leads to a considerable yield of A-2′:3′-P instead of A-2′- (and 3′-) PF. Second, attempted preparation of A-3′-PF by the reaction of 2,4-dinitrofluorobenzene with A-3′-P resulted in the formation of A-2′:3′-P.[21] Third, the aforementioned isomerization of A-3′:5′-P to A-2′:3′-P.

(17) The Hammett acidity function, H_0, for aqueous solutions of trifluoroacetic acid have been determined by J. E. B. Randles and J. M. Tedder [*J. Chem. Soc.*, 1218 (1955)]. They reported the following values (the first figure is the per cent concentration of acid; the second is the corresponding H_0 value): 90%, −2.3; 80%, −1.2; and 60%, −0.6.

(18) Me A-5′-P was prepared by the method described by M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Amer. Chem. Soc.*, **80**, 6204 (1958).

(19) J. R. Cox, Jr., and O. B. Ramsay, *Chem. Rev.*, **64**, 317 (1964).

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TABLE III

DEGRADATION OF ADENOSINE CYCLIC PHOSPHATES[a]

Substrate	Reaction conditions: Time, min	Reaction conditions: Temp, °C	Reaction products[b]: Cyclic phosphate, %	Reaction products[b]: A-2′-P + A-3′-P, %	Reaction products[b]: A-5′-PF, %	Reaction products[b]: Adenosine, %	Loss, %
A-2′:3′-P	60	−23	57[c]	8		35	
A-3′:5′-P	60	−50	91[d]				9
	60	−23	48[c]	9	22	26	
	15	0	25[c]	8	14	50	3

[a] The degradations were carried out with 60% hydrofluoric acid. [b] All reaction mixtures were subjected to chromatography in solvent C for separation of the products. [c] A-2′:3′-P was the only cyclic phosphate present. This was demonstrated by hydroxide ion hydrolysis at room temperature. [d] 15% was A-2′:3′-P and 76% was A-3′:5′-P.

TABLE IV

DEGRADATION OF ADP AND ATP[a]

Substrate	Reaction time, min	Reaction products: ATP, %	Reaction products: ADP, %	Reaction products: A-5′-P, %	Reaction products: Adenosine, %	Loss, %
ADP	30			81	2	17
ATP	2[b]	50	31	22		
ATP	10[b,c]		47	36		15
ATP	60			84	4	9

[a] All reactions were carried out at −50° using 60% hydrofluoric acid. Reaction mixtures were separated by electrophoresis in citrate buffer. [b] Sodium hydroxide (1 *N*) was used for quenching the reaction mixture. [c] A trace component was present in this reaction mixture which behaved like A-5′-PF upon electrophoresis in borate buffer and chromatography in solvents B and C.

TABLE V

DEGRADATION OF APPA AND DPN[a]

Substrate	APPA	DPN	DPN
Reaction time, hr	0.5	0.5	0.5
Reaction temp, °C	−23	−25	0
Separation method	Solvent A	Citrate–borate, solvent B[c]	Phosphate, solvent B[c]
Recovery of reaction products, %[b]			
Substrate	13	18	
A-5′-P	37	30	12
NR-5′-P		35	15
A-5′-PF	39	38	29
NR-5′-PF		29	26
Adenosine	12	8	64
NR			34
Nam			13
Loss, %			
Adenine moiety		6	
Nicotinamide moiety		18	12

[a] The reagent was 60% hydrofluoric acid. [b] Recoveries for the DPN degradations were calculated separately for the adenine and nicotinamide moieties. [c] Some of the bands obtained in the electrophoretic separations were mixtures of reaction products. These were separated into pure components by subsequent chromatography of each such band in solvent B.

The behavior of polyphosphate esters on treatment with 60% hydrofluoric acid is summarized in Tables IV and V. If ADP were degraded by random nucleophilic attack of fluoride ion on either phosphorus atom, then adenosine and/or A-5′-PF would be major reaction products. Neither of these, both of which are stable under the conditions of the ADP degradation, was found in significant amount. Another possibility worth considering, however, is that the reaction may occur by elimination.[22] In this case, monomeric inorganic metaphosphate rather than "metapyrophosphate" must be the leaving group. Elimination of the latter from ADP would give adenosine and, as already indicated, this was found in only small amount.

The data for the ATP degradations are insufficient to determine whether phosphate is lost sequentially from the triphosphate chain or whether there is random attack at the central and unesterified terminal phosphorus atoms. It is certain, however, that the esterified phosphorus is not involved in the initial reaction, since little adenosine or A-5′-PF is found in the reactions run for 2, or even 10 min. Hood and Lange[23] suggested that liquid hydrogen fluoride removed phosphoryl groups sequentially from complex ethyl polyphosphates.

The reactions of APPA and DPN with 60% hydrofluoric acid (Table V) each lead to 1 mol of 5′-phosphate and 1 mol of 5′-phosphorofluoridate. This behavior is analogous to that observed when P^1,P^2-diethyl pyrophosphate was treated with liquid hydrogen fluoride.[23] These reactions are presumed to involve direct hydrofluorinolysis of the pyrophosphate bond.

No reaction takes place when A-5′-P is treated with 4 *M* potassium bifluoride solution at 0° for 0.5 hr, nor does ADP react with the saturated bifluoride in 0.5 hr at either 0° or room temperature. The highly nucleophilic character of fluoride ion[24] toward phosphorus-

(22) M. Tetas and J. M. Lowenstein, *Biochemistry*, **2**, 350 (1963).

(23) A. Hood and W. Lange, *J. Amer. Chem. Soc.*, **72**, 4956 (1950).

(24) Potassium bifluoride solution (4 *M*) contains a high concentration of fluoride ion since the equilibrium constant for the reaction, $HF_2^- \rightleftarrows HF + F^-$, is of the order of 10^{-1} (see ref 15, p 63).

TABLE VI
BASE COMPOSITION OF RNA[a]

	Yeast RNA		Calf liver RNA	
	HF degradation[b]	Methanolysis[c]	HF degradation[b]	Methanolysis[c]
Adenine	26 ± 2[d]	24.2	17 ± 1[e]	16.6
Guanine	32 ± 2	30.8	38 ± 1	36.7
Cytidine	20 ± 1	20.4	32 ± 1	31.7
Uridine	21 ± 1	22.8	13 ± 1	14.2
Unidentified		2.1		0.9

[a] Compositions are given as mole per cent of total amount of ultraviolet-absorbing material recovered. [b] These were carried out at 25° using 60% hydrofluoric acid. [c] Reference 28. [d] Average of three determinations. [e] Average of three determinations.

(V)[25-27] and these bifluoride experiments, as well as others already mentioned with hydrofluoric acid of relatively low concentrations, emphasize the point that the rapid degradation of ADP by 60% hydrofluoric acid at −50° (Table IV) is due to the highly acidic nature of the reaction medium.

A simple and rapid method was developed for determining the base composition of a polyribonucleotide by degradation of the polymer with hydrofluoric acid to purine bases and pyrimidine nucleosides. The results of these RNA degradations compare favorably with the base compositions obtained by Lipkin, *et al.*, on the same two RNA samples (Table VI) using methoxide ion catalyzed methanolysis.[28] It should be emphasized that the present analytical method has a decided advantage over others because of the fact that the hydrofluoric acid degradation is rapid and it does not bring about any detectable deamination of the heterocyclic bases over extended periods of time at room temperature. The common methods of degrading ribonucleic acids by hydrolysis with the usual acids or bases cause appreciable deamination of both aminopurines and -pyrimidines.[12,13]

DNA is degraded by 60% hydrofluoric acid in *ca.* 20 hr at room temperature to the four heterocyclic bases. In 0.5 hr, the ultraviolet-absorbing products are the two purine bases and the two pyrimidine nucleosides.

Wittmann[21] has indicated that nucleoside 5′-phosphorofluoridates are substrates for snake venom diesterase. The diesterase from *Crotalus adamanteus* effectively catalyzes the hydrolysis of A-5′-PF and NR-5′-PF to the corresponding 5′-phosphates.[29] Toward this enzyme then, which does not attack inorganic phosphorofluoridates, A-5′-PF and NR-5′-PF behave like typical phospho diesters. On the other hand, A-5′-PF does not act like a mixed acid anhydride in an appropriate system containing polynucleotide phosphorylase.[30] A-5′-PF is inactive as a substrate for this enzyme.

Mechanistic Considerations.[31]—A comparison of the acid-catalyzed reactions in aqueous solution of phosphate esters in general, and of nucleotides in particular, with the behavior of such compounds toward 60% hydrofluoric acid brings out several interesting contrasts. First, the reactions in hydrofluoric acid which are described here are fast compared with reactions observed with ordinary aqueous acids. Second, in the reactions with 60% hydrofluoric acid all of the available evidence points toward the conclusion that phosphorus–oxygen, rather than carbon–oxygen, bond cleavage takes place exclusively,[32] in contrast to the results obtained with the common aqueous acids.[19] Third, as indicated previously, 60% hydrofluoric acid is a highly specific reagent compared with ordinary aqueous acids.

Bunton, *et al.*,[33] have pointed out that methyl phosphate is hydrolyzed by halogen acids much more rapidly than by equivalent concentrations of perchloric or sulfuric acid and that this is due to the incursion of bimolecular reactions at carbon involving halide ions and the neutral or conjugate acid species. It should not have been too surprising to find, therefore, that phosphate esters are rapidly degraded by hydrogen fluoride. Although fluoride is a poor nucleophile toward saturated carbon, it is an unusually powerful nucleophile toward phosphorus(V) as already mentioned. Furthermore, because 60% hydrofluoric acid is a very strongly acidic medium,[15] it is an effective catalyst for the degradation. Regardless of the particular entity, or entities, responsible for the acidity, it is likely that in solutions of phosphate esters in 60% hydrofluoric acid there are present a variety of multiply protonated species or species hydrogen bonded to two or more HF molecules.

It is interesting to make a more quantitative comparison of the rates of degradation of phosphate esters by 60% hydrofluoric and 10 *M* perchloric acids, since both have approximately the same H_0.[34] The half-life of methyl phosphate in 10 *M* perchloric acid at 100° is *ca.* 9×10^3 sec.[33] By contrast, the half-life of A-5′-P in 60% hydrofluoric acid is *ca.* 4×10^3 sec at −23° (Table I). At 0°, furthermore, the half-life of A-5′-P, T-3′-P, or T-5′-P is calculated to be *ca.* 6×10^2 sec, assuming disappearance of phosphate ester by a first-order reaction. These data serve to emphasize the rapidity of the dephosphorylating action of 60% hydrofluoric acid on phosphate monoesters.

The fact that only phsophorus–oxygen bond cleavage

(25) I. Dostrovsky and M. Halmann, *J. Chem. Soc.*, 508 (1953).

(26) G. DiSabato and W. P. Jencks, *J. Amer. Chem. Soc.*, **83**, 4393 (1961).

(27) (a) W. Feldman, *Z. Anorg. Allg. Chem.*, **338**, 235 (1965); (b) R. E. Mesmer, *J. Inorg. Nucl. Chem.*, **28**, 691 (1966).

(28) D. Lipkin, J. S. Dixon, and P. T. Talbert, *J. Amer. Chem. Soc.*, **83**, 4772 (1961).

(29) A diesterase from Russell Viper venom (CalBiochem, Los Angeles) also catalyzes the hydrolysis of A-5′-PF to A-5′-P.

(30) We are indebted to Dr. Audrey Stevens, University of Maryland Medical School, Baltimore, Md., for a sample of this enzyme.

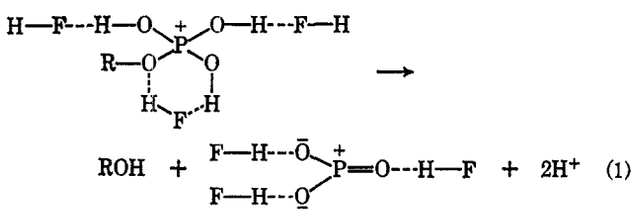
(31) Mechanistic interpretations are based on rate comparisons which are usually only single points on rate curves. The limitations inherent in this approach are realized, but it is believed that the conclusions reached are of significance.

(32) A compound such as a 5′-fluoro-5′-deoxynucleoside, which would be produced if there were carbon–oxygen bond cleavage, was not found in any of the reaction mixtures resulting from the action of 60% hydrofluoric acid on nucleotides. Furthermore, it has been demonstrated[5b] that dephosphorylation of A-2′(3′)-P by means of liquid hydrogen fluoride does not cause a change in the configuration of $C_{2'}$ (or $C_{3'}$) in the ribose moiety of the nucleotide.

(33) C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, 3574 (1958).

(34) H_0 for 10 *M* perchloric acid is *ca.* −6.2 [K. Yates and H. Wai, *J. Amer. Chem. Soc.*, **86**, 5408 (1964)]. Sulfuric acid has an H_0 (−6) equivalent to that of 60% hydrofluoric acid at a concentration of 72% [H. H. Hyman, M. Kilpatrick, and J. J. Katz, *ibid.*, **79**, 3668 (1957)].

is observed in the hydrofluoric acid reactions also may be explained by the highly nucleophilic character of fluoride toward phosphorus(V) compared with carbon. In 5 *M* perchloric acid solutions, the rate constant for the hydrolysis of methyl phosphate by phosphorus–oxygen cleavage is about one-half the constant for the carbon–oxygen cleavage reaction.[33] It is reasonable to assume that in the presence of fluoride the rate of the carbon–oxygen cleavage reaction becomes relatively negligible. However, another possible explanation of the observations that phosphate esters in 60% hydrofluoric acid solution always react by phosphorus–oxygen cleavage and that the reactions are rapid is that unimolecular deomposition of the highly protonated and hydrogen-bonded phosphate takes place to give monomeric inorganic metaphosphate (eq 1). The



solvated metaphosphate is then rapidly converted by fluoride into phosphorofluoridate. Monomeric inorganic metaphosphate has been suggested as an intermediate in reactions of monoesters of phosphoric acid,[19,22,33,35–37] but not in reactions under highly acidic conditions. The HF-solvated monoester on the left-hand side of the above equation is analogous to the water-solvated structure proposed by Westheimer, *et al.*,[35] as an intermediate species in the rapid hydrolysis of the monoanion, $RHPO_4^-$, *via* a metaphosphate intermediate.

In contrast, it is presumed that reactions which would lead to the formation of a monomeric metaphosphate *ester*, $ROPO_2$, as an intermediate are not favored and are slow compared with direct nucleophilic attack on phosphorus by fluoride. This statement is supported by facts such as the following: (1) A-5′-P is converted by 60% hydrofluoric acid into adenosine and inorganic phosphorofluoridate, but not to A-5′-PF; (2) diesters of phosphoric acid such as Me A-5′-P or dimethyl phosphate are degraded slowly by 60% hydrofluoric acid compared with the corresponding monoesters A-5′-P and methyl phosphate; and (3) ADP and ATP are degraded more rapidly than APPA or DPN by 60% hydrofluoric acid.

There remains unexplained the unusual specificity of the interaction of nucleotides with 60% hydrofluoric acid. As pointed out previously, dephosphyorylation in this highly acidic medium obviously has become a rapid reaction compared with such reactions as hydrolysis of N-glycosidic bonds (except in the purine deoxyribosides); deamination of heterocyclic bases; and phosphate migration. It is worth noting that 60% hydrofluoric acid is 37 *M* in HF and only 27 *M* in H_2O, in contrast to 10 *M* perchloric acid which is 32 *M* in H_2O. Furthermore, HF exerts a powerful effect in reducing the thermodynamic activity of H_2O in HF–H_2O mixtures.[38,39] Some of this specificity, therefore, could be explained on the basis of equilibria. On the other hand, the explanation may lie, in large part, in the fact that, since the concentration of the nucleophile H_2O is very small in the strongly acidic medium, 60% hydrofluoric acid, hydrolytic reactions consequently are relatively slow.

(35) (a) W. W. Butcher and F. H. Westheimer, *J. Amer. Chem. Soc.*, **77**, 2420 (1955); (b) J. Kumamoto and F. H. Westheimer, *ibid.*, **77**, 2515 (1955).

(36) D. M. Brown and N. K. Hamer, *J. Chem. Soc.*, 1155 (1960).

(37) G. DiSabato and W. P. Jencks, *J. Amer. Chem. Soc.*, **83**, 4400 (1961).

Experimental Section

Whatman No. 1 or No. 3MM filter paper was used in descending paper chromatography. The solvent systems used for developing chromatograms, prepared on a volume basis, follow: 1-butanol–acetic acid–water, 4:1:1 (solvent A);[40] isopropyl alcohol–water, 80:20 (solvent B); isopropyl alcohol–water, 70:30, with an ammonia atmosphere (solvent C);[41] *t*-butyl alcohol–water, 80:20 (solvent D); water saturated with ammonium sulfate–1 *M* sodium acetate–isopropyl alcohol, 80:20:2 (solvent E).[42]

Paper electrophoreses were performed using apparatus and techniques that already have been adequately described.[43] The following buffers were used: 0.1 *M* ammonium formate–formic acid, pH 3.5; 0.1 *M* sodium acetate–acetic acid, pH 5.1; 0.05 *M* citric acid plus 0.01 *M* sodium borate, adjusted to pH 5.1 with 5 *M* sodium hydroxide; 0.05 *M* sodium citrate–citric acid, pH 5.2; 0.1 *M* sodium metabisulfite, adjusted to pH 5.5 with 5 *M* sodium hydroxide;[44] 0.05 *M* sodium phosphate, pH 7.2; 0.05 *M* sodium borate, pH 9.2; 0.05 *M* potassium bicarbonate, adjusted to pH 9.2 with 5 *M* potassium hydroxide; 0.05 *M* sodium carbonate–sodium bicarbonate, pH 10.2; and 0.1 *M* ethylenediamine plus 0.005 *M* sodium borate, adjusted to pH 11 with 5 *M* sodium hydroxide.

Ultraviolet-absorbing materials on paper chromatograms and electrophoretograms were detected visually and photographically by a method which already has been described.[5a] Three sprays also were used as needed: the Hanes and Isherwood reagent for phosphates;[45] periodate and benzidine sprays for vicinal glycol groups;[46] and a cysteine–sulfuric acid solution to detect the deoxyribose moiety.[47]

The components of reaction mixtures generally were identified by appropriate combinations of paper chromatography, paper electrophoresis, and ultraviolet (uv) spectroscopy.[48] R_f and M_R values not readily available in the literature are summarized in Table VII. Special chemical methods which were needed to complete identification of some products are indicated where necessary.

Charcoal, acid washed as previously described,[49] was partially deactivated with 2-octanol.[50] This charcoal will be referred to as charcoal A. For some experiments charcoal A was further deactivated by neutralization with 6 *N* ammonium hydroxide. The neutralized charcoal was then washed five times with water

(38) (a) J. H. Simons in "Fluorine Chemistry," J. H. Simons, Ed., Academic Press, New York, N. Y., 1950, p 249; (b) P. A. H. Wyatt *Discussions Faraday Soc.*, **24**, 167 (1957).

(39) Chemical evidence in support of the view that the thermodynamic activity of H_2O in 60% hydrofluoric acid is very low is provided by the fact that H_2PO_3F, A-5′-PF, and methyl phosphorofluoridate are not hydrolyzed to H_3PO_4, A-5′-P, and methyl phosphate, respectively, in 60% hydrofluoric acid (see Tables I and II). The half-life of PO_3F^{2-} in 0.4 *M* HCl at 39° is 18 min.[26] Nevertheless, the thermodynamic activity of H_2O cannot be negligible, since $PO_2F_2^-$ is converted rather rapidly into PO_3F^{2-} in 60% hydrofluoric acid at 0° (Table II).

(40) G. D. Dorough and D. L. Seaton, *J. Amer. Chem. Soc.*, **76**, 2873 (1954).

(41) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(42) R. Markham and J. D. Smith, *Nature*, **168**, 406 (1951).

(43) R. Markham in "Modern Methods of Plant Analysis," K. Paech and M. V. Tracey, Ed., Springer-Verlag, Berlin, 1955, Vol. IV, pp 278–288.

(44) Addition of a small amount (0.01–0.1%) of sodium (ethylenedinitrilo)-tetraacetate to this buffer increases considerably its stability toward air oxidation.

(45) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(46) M. Viscontini, D. Hoch, and P. Karrer, *Helv. Chim. Acta*, **38**, 642 (1955).

(47) J. G. Buchanan, *Nature*, **168**, 1091 (1951).

(48) Details of the methods used for the separation and unambigous identification of specific components of reaction mixtures are discussed by J. W. Abrell.[1b] They are too extensive to be repeated here.

(49) D. Lipkin, P. T. Talbert, and M. Cohn, *J. Amer. Chem. Soc.*, **76**, 2871 (1954).

(50) C. J. Threlfall, *Biochem. J.*, **65**, 694 (1957).

TABLE VII
CHROMATOGRAPHIC AND ELECTROPHORETIC MOBILITIES[a]

Compound	Chromatography				Electrophoresis				
	Solvent A	Solvent B	Solvent C	Solvent D	Solvent E	Formate, pH 3.5	Acetate, pH 5.1	Phosphate, pH 7.2	Bicarbonate, pH 9.2	Borate, pH 9.2
A-5'-PF	0.22	0.96	0.69	0.43	2.20	1.00	0.69	0.86	0.66	0.84
NR-5'-PF			0.39				0.21			0.53
H_2PO_2F	1.15	1.39	0.98	1.09		1.24	1.38		1.11	1.18
HPO_2F_2	4.02	1.43	0.97	5.60		1.46	1.38		1.00	1.20
$(CH_3)H_2PO_4$	1.84	1.81	0.98	1.79		1.06	1.01		0.97	
$(CH_3)HPO_2F$	2.87	3.08	2.35	4.95		1.24	1.01		0.87	
$(CH_3)_2HPO_4$		2.79	1.80			1.12	1.01		0.79	

[a] Chromatographic mobilities for A-5'-PF and NR-5'-PF are given relative to adenosine. The electrophoretic mobilities for these compounds are given relative to A-5'-P. All other mobilities are given relative to inorganic orthophosphate.

and dried in air at 80° (16 hr). This charcoal will be referred to as charcoal N.

The 60% aqueous hydrofluoric acid was a technical grade obtained from Baker and Adamson. Its freezing point was *ca.* −55 to −60°. Whenever liquid hydrogen fluoride was used as a reagent, it was drawn directly from a tank as a liquid into a polyethylene centrifuge tube chilled in an ice bath. No particular precautions were taken to keep the liquid anhydrous.

Hydrofluoric Acid Degradations. A. General Procedures.—A general procedure was developed for the degradation of various substrates by 60% hydrofluoric acid. The weighed substrate was transferred to a polyethylene centrifuge tube of appropriate size (2 ml for 2–15-mg samples). Hydrofluoric acid, cooled to the temperature[51] at which the reaction was to be run, was added to the sample by means of a polyethylene capillary pipet. All substrates dissolved immediately on addition of the acid (150–300 μl). The reaction was quenched by addition of sufficient saturated lithium hydroxide solution (*ca.* 5 *M*) to neutralize 75–80% of the hydrofluoric acid. Care was taken to avoid an appreciable rise in the temperature of the reaction mixture during this neutralization step. The reaction mixture then was brought to pH ~7 with solid lithium carbonate. After removal of the supernatant, the lithium fluoride precipitate was washed four times with small volumes of water. The supernatant and washings were combined and concentrated to *ca.* 0.5 ml. This solution was subjected to either paper chromatography or paper electrophoresis. The uv-absorbing bands which developed were eluted. In some experiments non-uv-absorbing areas were eluted for the inorganic phosphate or sugar. After spectroscopic properties of the eluents were determined, the samples were concentrated to *ca.* 0.5 ml for further examination.

Modifications of the above general procedure which were used are as follows. First, the guanylic acid degradations were quenched and neutralized with calcium hydroxide and calcium carbonate, respectively. The calcium fluoride precipitate then was washed with 0.1 *N* sodium hydroxide. Second, with the thymidylic acids, 1 *N* ammonium hydroxide washes were substituted for the water washes. Third, in all degradations involving the NR moiety, special care was taken during the neutralization to ensure that the pH did not exceed 6.5. Fourth, the neutralization procedure for some of the ATP degradations was changed to avoid coprecipitation of lithium fluoride and the substrate or ADP. The cold reaction mixture was partially neutralized with 850 μl of cold 5 *M* sodium hydroxide. After 50 mg of charcoal A was added, the mixture was brought to pH 7 with solid sodium carbonate. The charcoal then was washed four times with 0.5-ml portions of water. The combined supernatant and washings accounted for less than 5% of the original uv absorbance of the substrate. The charcoal was eluted with ten 1-ml portions of 50% ethanol. This eluate was concentrated to 0.5 ml prior to separation of the reaction products.

A general procedure for the degradation of nucleotides by liquid hydrogen fluoride already has been reported.[5b]

B. Ribonucleic Acids.—A 9–11-mg sample was treated with 200 μl of hydrofluoric acid for 3 hr at room temperature, cooled in an ice bath, quenched with 2 ml of saturated lithium hydroxide solution, and neutralized to pH 7.5 with lithium carbonate. After the supernatant was decanted, the lithium fluoride precipitate was washed four times with 0.5–0.75-ml portions of water. The residual solid is solid I. Washings and supernatant were combined and evaporated to dryness. The resulting residue (solid II) was washed five times with 0.25–0.5-ml portions of water. The washings were transferred to a 2-ml volumetric flask and diluted to the mark with water.

The following operations were done without interruption. A 500-μl portion of the above solution of reaction products was subjected to electrophoresis (ethylenediamine buffer, 2.25 hr at a gradient of *ca.* 40 V/cm) on a 20 × 60 cm doubly acid-washed paper (S. and S. Green Ribbon, Grade 589). Relative to adenine, the other three products have the following mobilities: guanine, 1.25; cytidine, 1.55; and uridine, 2.29. After electrophoresis the paper was dried in an oven at 80°. It was removed from the oven as soon as it was dry because continuing exposure to heat causes the paper to turn yellow. The four products, as well as two blanks, were eluted into 10-ml volumetric flasks—guanine and one blank with 0.1 *N* hydrochloric acid, the other three components and the second blank with water.

(51) Temperatures below 0° were maintained (±1°) by addition of Dry Ice to an acetone or propanol-2 bath.

Guanine was recovered quantitatively from solid I and the residue remaining after the washing of solid II by solution and reprecipitation of the lithium fluoride. The residues, in a polyethylene test tube, were dissolved in 6 *N* hydrochloric acid and precipitated by neutralization with 5 *N* ammonium hydroxide. Solution of the lithium fluoride and its reprecipitation were repeated four times. All supernatants were placed in a 25-ml flask and diluted to the mark with water. Spectrophotometric measurements were made on all solutions.

The average recovery of optical density units from the hydrofluoric acid degradations was 80%. In order to calculate this value, the optical density units per milligram of RNA sample were determined by subjecting a sample to complete hydrolysis with 1 *N* potassium hydroxide and then measuring the optical density of the neutralized hydrolysate at 260 mμ.

C. 2-Deoxyribose 5-Phosphate from Deoxyadenosine 5′-Phosphate.—A sample of dA-5′-P (162 mg, 465 μmol) was degraded with 2 ml of hydrofluoric acid (−25°, 15 min). The reaction tube was cooled in a Dry Ice bath before neutralization to pH 8 with 20 ml of a saturated lithium hydroxide solution. After centrifugation, the supernatant was decanted and the lithium fluoride precipitate was washed five times with 5-ml portions of water. The supernatant and washings were combined and concentrated to approximately 10 ml. To the concentrate 4.5 g of charcoal N was added to adsorb adenine and unchanged dA-5′-P. This mixture was centrifuged and, after removal of the supernatant, the charcoal was washed three times with 2-ml portions of water. The supernatant and washings were combined and concentrated. A sample of this solution, when subjected to electrophoresis in formate buffer, was shown to contain one major and three minor components which did not absorb in the uv. The major component was identified as 2-dR-5-P, while two of the three minor components were identified as 2-deoxyribose and inorganic phosphorofluoridate. The other component, which was present in trace amounts moved 1.17 times as fast as 2-dR-5-P in the formate buffer and was developed by both the deoxyribose and phosphate sprays.

To the concentrated solution containing 2-dR-5-P, 2-deoxyribose, inorganic phosphorofluoridate, and the trace amount of an isomerized deoxyribose phosphate, 1 ml of a saturated barium acetate solution was added. The precipitate which formed immediately was discarded after it was washed twice with cold water. The supernatant and washings were combined and mixed with an equal volume of methanol. The resulting precipitate was collected, washed three times with methanol, and dried in a vacuum desiccator. This solid contained 2-dR-5-P and the other deoxyribose phosphate. A portion of this solid (7 mg) in 1 ml of water was mixed with 3 mg of sodium sulfate. The resulting supernatant was combined with 400 μl of 0.1 *M* sodium metaperiodate. The mixture was allowed to react for 1.5 hr in the dark and then sodium borohydride (114 mg, 300 μmol) was added to the oxidized solution. After 16 hr at room temperature, the reaction mixture was brought to pH 6 by the addition of 3 *N* sulfuric acid. The residue obtained after evaporation of this mixture to dryness was washed three times with 0.5-ml portions of cold water. A saturated solution of barium acetate was added dropwise to the combined washings until precipitation ceased. The supernatant obtained by centrifugation was passed through a column containing 4 ml of Dowex 50 (H^+). The effluent from the column was concentrated and subjected to chromatography, together with an authentic sample of β-hydroxyethyl phosphate,[52] in solvents A and C and to electrophoresis in bicarbonate, formate, and acetate buffers. The behavior of the major reaction product was identical with that of known β-hydroxyethyl phosphate in all of the systems.

D. Nicotinamide Riboside 5′-Phosphorofluoridate from Diphosphopyridine Nucleotide.—A sample of DPN (351 mg, 502 μmol) was treated with 3 ml of hydrofluoric acid at −20° for 0.5 hr. The mixture was partially neutralized with 16 ml of saturated lithium hydroxide solution and then neutralized to pH 6 with lithium carbonate. The supernatant was decanted from the lithium fluoride precipitate, which in turn was washed four times with 3-ml portions of cold water. The combined supernatant and washings were added to 3–4 g of cellulose powder. This mixture was evaporated to dryness and then the residue was suspended in 10–20 ml of 2-propanol–water, 9:1 (v/v). This slurry was loaded onto a cellulose powder column (Whatman, ashless, standard grade) 8.1 cm^2 cross section and 60 cm long. Elution was first carried out using 3 l. of 2-propanol–water, 9:1; this was followed by 2.5 l. of 2-propanol–water, 85:15. Fractions of 20–24 ml were collected at a flow rate of 3 ml/min. The elution was followed by optical density determinations at 260, 266, and 280 mμ.

Nam, adenosine, and A-5′-PF were collected in that order in fractions 1–150, inclusive. The fractions (170–220) corresponding to the elution peak containing both A-5′-P and NR-5′-PF were pooled and evaporated to dryness. The resulting solid was dissolved in 10 ml of water and poured through a column containing 3 ml of Dowex-2 (formate). The effluent contains only NR-5′-PF, which is not adsorbed by the resin since it is a zwitterion. The phosphorofluoridate was adsorbed from the effluent by adding charcoal N. The charcoal was separated by centrifugation and it was washed three times with 5-ml portions of water. Elution from the charcoal was achieved with five 20-ml washings with a 50% ethanol solution. The eluate was evaporated to dryness. The resulting residue was dissolved in 5 ml of water and filtered to remove traces of charcoal. The filtrate contained 100 μmol of NR-5′-PF.

This NR-5′-PF has the same uv-absorption spectrum as NR-5′-P. The presence of vicinal hydroxyl groups in the compound was demonstrated by oxidation with 0.1% sodium metaperiodate followed by electrophoresis in bisulfite buffer. Its electrophoretic mobility was compared with that of an unoxidized sample. NR-5′-PF, when incubated with whole *Crotalus adamanteus* venom in 0.1 *M* Tris buffer, pH 8.5, was hydrolyzed to NR.

The fluoride in NR-5′-PF was determined by the same procedure as that described below for A-5′-PF. Triplicate fluoride determinations, using the venom diesterase, gave values of 65, 55, and 55% of theoretical based on the optical density of the NR-5′-PF solution used for the determinations. Electrophoresis in borate buffer of a small aliquot of each enzyme digest showed that complete hydrolysis of NR-5′-PF to NR-5′-P had occurred.

Adenosine 5′-Phosphorofluoridate.—This compound was isolated in low yield from a reaction mixture containing adenosine, tri-*n*-butylammonium phosphorofluoridate, DCC, and tri-*n*-butylamine in a dioxane–dimethylformamide solvent. The sample was purified by paper chromatography in solvent D. Recently a number of good general methods have become available for the preparation of ribo- and deoxyribonucleoside 5′-phosphorofluoridates.[53]

The uv-absorption spectrum of the compound is identical with that of A-5′-P. Vicinal hydroxyls were demonstrated by the same procedure as that used above for NR-5′-PF. The compound yields A-5′-P as the only uv-absorbing product when treated with 1 *N* potassium hydroxide (16 hr, room temperature) or saturated barium hydroxide solution (8 hr, room temperature).

The diesterase from *Crotalus adamanteus* venom (activity, 750 μmol of nucleotide released/hr by 1 ml of solution at 37°),[54] which catalyzes the hydrolysis of A-5′-PF to A-5′-P,[21] was adapted for a quantitative determination of fluoride in A-5′-PF. The reaction mixtures contained 1 ml of 0.1 *M* Tris buffer, pH 8.5, 10 μl of the diesterase solution, and the substrate. Substrates for five assays were A-5′-P (5.7 μmol), Me A-5′-P (6.9 μmol), and A-5′-PF (triplicate determinations with 6.9 μmol each). A-5′-PF is completely stable in 0.1 *M* Tris buffer, pH 8.5, at 37° for 22 hr. After incubation at 37° for 16 hr, 0.3 g of charcoal N was added to each assay. The supernatants and charcoal washings (five each with 2.5 ml of water) from each assay were passed through Dowex 50 (H^+) columns containing *ca.* 1 ml of resin. The eluents, collected in 100-ml volumetric flasks, were then subjected to a quantitative fluoride analysis using thorium chloranilate as the colorimetric reagent.[55] The A-5′-P and Me A-5′-P samples gave readings identical with those of the fluoride blank. The average value for the three A-5′-PF samples was 78% of the theoretical fluoride, based on the optical density of the A-5′-PF solution used for the determinations.

Methyl Phosphorofluoridate.—This compound was made by two different procedures. The first was that of Hood,[56] while

(52) F. R. Atherton, H. I. Openshaw, and A. R. Todd, *J. Chem. Soc.*, 382 (1945). The negative temperature coefficient of solubility of the barium salt of this compound is taken advantage of in its purification.

(53) D. Lipkin and B. S. Pitzele, unpublished results.

(54) This enzyme was a gift from Dr. John Josse, Syntex Institute for Molecular Biology, Stanford Industrial Park, Palo Alto, Calif. 94304.

(55) This colorimetric procedure is described in Technical Data Sheet TD 138 of the Fisher Scientific Co.

(56) A. Hood, U. S. Patent 2,712,548 (1955).

the second involved the use of DCC and tri-*n*-butylammonium phosphorofluoridate in an excess of methanol. Neither synthesis was completely satisfactory, although a pure sample was finally obtained by paper chromatography in solvent E. The sample was homogeneous as shown by electrophoresis in bicarbonate and formate buffers and by chromatography in solvents A and D. The sample when treated with 1 *N* hydrochloric acid at room temperature for 36 hr yielded monomethyl phosphate.

Anal. Calcd for $CH_3FLiO_3P \cdot 2H_2O$: P, 19.9. Found: P, 20.6.

Alkaline Hydrolysis of A-2′:3′-P, A-3′:5′-P, and A-5′-PF.—Three sets of conditions were employed: (1) 1 *N* potassium hydroxide at room temperature for 16 hr; (2) saturated barium hydroxide solution at room temperature for 8 hr; and (3) saturated barium hydroxide solution at 100° for 20 min. Conditions 1 and 2 are sufficient to hydrolyze A-2′:3′-P and A-5′-PF to A-2′- (and 3′-) P and A-5′-P, respectively, but not A-3′:5′-P. Condition 3 is necessary for hydrolysis of A-3′:5′-P to a mixture of A-3′-P and A-5′-P.[5b]

Trifluoroacetic Acid Degradations.—Samples of A-5′-P (4–7 mg) were dissolved in 0.4-ml portions of aqueous trifluoroacetic acid (60–100%). After a predetermined reaction time, one of the solutions was frozen in a Dry Ice bath and lyophilized. A solution of the residue in a small amount of water was subjected to paper chromatography in solvent A and to paper electrophoresis in both borate and formate buffers.

Registry No.—Hydrogen fluoride, 7664-39-3; A-3′-P, 84-21-9; A-2′-P, 130-49-4; A-5′-P, 61-19-8; A-5′-PF, 19375-33-8; MeA-5′-P, 13039-54-8; C-2′-P, 85-94-9; C-3′-P, 84-52-6; G-2′-P, 130-50-7; G-3′-P, 117-68-0; U-2′-P, 131-83-9; U-3′-P, 84-53-7; NR-5′-P, 1094-61-7; T-3′-P, 2642-43-5; T-5′-P, 365-07-1; dA-5′-P, 653-63-4; A-2′:3′-P, 634-01-5; A-3′:5′-P, 60-92-4; ATP, 56-65-5; ADP, 58-64-0.

Studies on Phosphorylation. IV. Selective Phosphorylation of the Primary Hydroxyl Group in Nucleosides[1]

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Nucleoside 5′-phosphates of a number of naturally occurring and synthetic purine and pyrimidine ribonucleosides and their 2′-deoxy and 2′-O-methyl derivatives were prepared in good yields by direct phosphorylation of their corresponding unblocked nucleosides with pyrophosphoryl chloride in *m*-cresol or *o*-chlorophenol. Similar treatment of purine and pyrimidine arabino- and gluconucleosides, and aristeromycin resulted in the selective phosphorylation of the primary hydroxyl groups to give the corresponding phosphates. α-Guanosine and 2′-deoxyadenosine gave the 5′-phosphates in relatively low yield. The 5′-phosphate and 3′,5′-cyclic phosphate were obtained from 9-β-D-xylofuranosyladenine. Acetonitrile, benzonitrile, ethyl acetate, methyl acrylate, ethyl benzoate and nitrobenzene, when used as solvents, gave satisfactory results in the direct phosphorylation reaction.

A new method was reported[2] from our laboratories for the preparation of naturally occurring ribonucleoside 5′-phosphates by protecting the 2′,3′-*cis*-glycol system of the corresponding nucleosides with borate followed by phosphorylation with pyrophosphoryl chloride. Several attempts to phosphorylate primary hydroxyl groups selectively without blocking secondary alcoholic functions of nucleosides have failed.[3–5] We now report on a new method for direct phosphorylation of unblocked nucleosides at the 5′ position.[6–8] When inosine was suspended in *m*-cresol and was treated with pyrophosphoryl chloride[9,10] in the absence of metaboric acid or boric anhydride followed by hydrolysis, inosine 5′-phosphate was obtained in almost quantitative yield. This method was then applied to many other nucleosides and a number of the corresponding 5′-phosphate derivatives were selectively obtained.

A general procedure is as follows. A nucleoside, suspended in *m*-cresol or *o*-chlorophenol (15–80-fold by weight), is treated with pyrophosphoryl chloride (2–15 molar excess) at 0–10° for 2–4 hr and then diluted with an ice–water mixture followed by extraction with ethyl ether or benzene. The nucleotide is adsorbed onto charcoal and the aqueous layer discarded. After elution from the charcoal the nucleotide is subjected to ion exchange chromatography (Dowex 1). The identification of the nucleotide thus obtained is made as follows: (i) elementary analyses and ultraviolet absorption spectra, (ii) comparison of its mobility on paper electrophoresis and on paper chromatography with authentic samples, (iii) treatment of the nucleotide with bull semen 5′-nucleotidase to give quantitative liberation of phosphoric acid, (iv) treatment with periodic acid, and (v) chemical shifts of the 5′-proton resonances.

Adenosine, inosine, 2-chloroinosine, 6-thioinosine, uridine, and cytidine gave the corresponding 5′-phosphates in 55–85% yield (Table I). In the case of guanosine, a larger quantity of solvent was necessary

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