hydrogen in the presence of 5 mg of platinum oxide catalyst. Purification of the product by tlc afforded tetrahydrovallesiachotamine (V) with an unchanged ultraviolet absorption spectrum and a mass spectrum, which was characterized by only six peaks of relative intensity over 15% in the range *m/e* 150–355: *m/e* 354 (17%) (M^+ calcd for $C_{21}H_{26}N_2O_3$: 354), 322 (24%), 282 (21%), 281 (100%), 221 (17%), and 156 (22%).

B. In Acetic Acid Solution. Vallesiachotamine (40 mg) was dissolved in 4 ml of glacial acetic acid by gentle warming and 300 mg of sodium borohydride was added in portions with stirring while cooling with tap water. After 15 min at room temperature, water was added, the solution was neutralized with sodium bicarbonate, and the product was extracted with methylene dichloride. Tlc purification (benzene–ethyl acetate–ethanol, 1:3:1) gave two products. The less polar one (8 mg, mp 261–264° after recrystallization from methanol) was the lactone **VII**, whose ultraviolet and mass spectra (M^+ calcd for $C_{20}H_{22}N_2O_2$: 322) are reproduced in Figures 1 and 4. The infrared spectrum (KBr) exhibited bands at 2.90 (indole NH) and 5.80 μ, while the nmr spectrum (d_5-pyridine) displayed the following diagnostically significant signals: δ 1.50 (doublets, J = 7 cps, CH_3CH=), 5.32 (quartet, J = 7 cps, CH_3CH=), 4.75 (singlet, =CCH_2O—), and 4.28 (multiplet, C-3 *H*).

Elution of the more polar band provided 25 mg of the unstable hydroxy ester X as a pale pink resin, which formed a methiodide, (mp 165–170° dec, after recrystallization from ethanol) upon standing overnight at room temperature in ether solution with methyl iodide. The base itself possessed the following spectral characteristics: λ_{max}^{EtOH} 226 mμ (log ϵ 4.45), 283 (3.76), and 290 (3.68); $\lambda_{max}^{CHCl_3}$ 2.85, 2.96, 5.80, and 5.99 μ; nmr signals at δ 1.53 (doublet, J = 7 cps, CH_3CH=), 5.62 (quartet, J = 7 cps, CH_3CH=), 4.11 (singlet, =CCH_2O—), 3.51 (singlet, CO_2CH_3), 4.43 (multiplet, C-3 *H*); mass spectral peaks at *m/e* 354 (10%) (M^+ calcd for $C_{21}H_{26}N_2O_3$: 354), 353 (8%), 322 (56%), 321 (47%), 184 (67%), 170 (20%), 169 (22%), and 156 (27%).

Reactions of the Lactone VII. A. Lithium Aluminum Hydride Reduction. Approximately 0.5 mg of the lactone was reduced with an excess of lithium aluminum hydride in ether solution (25° for 15 min) and after decomposition with aqueous sodium sulfate solution, the product was purified by tlc and subjected to mass spectral examination which confirmed that reduction of the lactone to the corresponding glycol had been effected: *m/e* 326 (57%) (M^+ calcd for $C_{20}H_{26}N_2O_2$: 326), 325 (46%), 295 (13%), 277 (6%), 253 (7%), 241 (9%), 235 (8%), 209 (16%), 184 (100%), 171 (17%), 170 (22%), 169 (20%), and 156 (28%).

B. Catalytic Hydrogenation. The lactone IV (3.5 mg) in ethyl acetate solution was shaken for 5 hr with 10 mg of prereduced platinum oxide catalyst in an atmosphere of hydrogen, whereupon the uptake of 2 molar equiv of hydrogen was complete. Filtration of the catalyst and evaporation of the filtrate left an amorphous solid, which was washed with chloroform to leave 2 mg of the amino acid VIII. The acid was homogeneous by tlc (methanol) and exhibited λ_{max}^{KBr} 2.90, 3.17, and 6.25 μ as well as mass spectral peaks at *m/e* 326 (100%) (M^+ calcd for $C_{20}H_{26}N_2O_2$: 326), 325 (82%), 282 (8%), 281 (7%), 269 (29%), 242 (16%), 241 (27%), 225 (17%), 223 (25%), 184 (39%), 171 (69%), 170 (72%), 169 (34%), and 156 (27%).

Methylation of 0.5 mg of the acid VIII in methanol solution with diazomethane followed by tlc (ethyl acetate) purification afforded the methyl ester (IX) as demonstrated by the mass spectrum: *m/e* 340 (100%)(M^+ calcd for $C_{21}H_{28}N_2O_2$: 340), 339 (78%), 283 (31%), 281 (13%), 256 (18%), 255 (15%), 253 (25%), 184 (15%), 170 (16%), 169 (7%), and 156 (4%).

Pyrolysis of Vallesiachotamine (I). A capillary tube containing 2 mg of vallesiachotamine was inserted into a sublimation furnace preheated to 250° and the temperature was raised to 290° while passing all effluent vapors into a saturated solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid. The precipitated hydrazone was centrifuged, washed with water, and then purified by tlc (ether–hexane 1:1). Identity of the principal spot with *n*-butyraldehyde 2,4-dinitrophenylhydrazone was demonstrated by the correspondence in tlc mobility and the complete identity of the respective mass spectra.

Studies on Polynucleotides. LIV.[1] A Further Study of the Reaction of Nucleotide Pyrophosphates with Carboxylic Acid Anhydrides[2]

M. W. Moon and H. G. Khorana

Contribution from the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin. *Received November 13, 1965*

Abstract: In a further study of the previously observed degradation of disubstituted pyrophosphates (I) with an excess of acetic anhydride,[3] the reaction of P^1,P^2-di(3′-O-acetylthymidine-5′) pyrophosphate with varying amounts of acetic anhydride, benzoic anhydride, and benzoyl chloride in anhydrous pyridine has been investigated. The rate of the degradation reaction and amount of pyrophosphate surviving when equilibrium was attained were both determined and the influence of carboxylate anions and water on the reaction was also studied. Investigation of the reaction intermediates showed the rapid initial formation of polyphosphate species (*e.g.*, VII) followed by a slow step resulting in the predominant formation of the mixed anhydride between the mononucleotide and carboxylic acid (*e.g.*, VI).

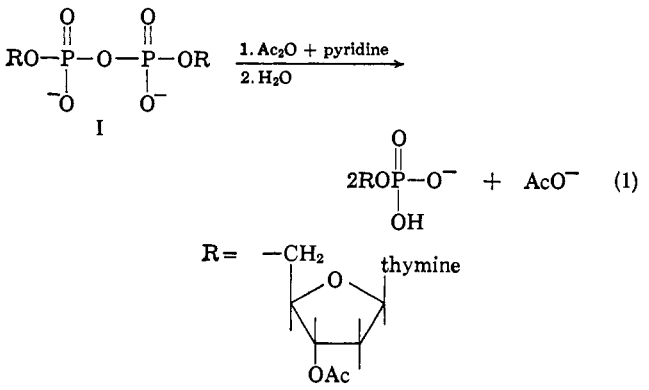
The reaction of a disubstituted pyrophosphate (*e.g.*, I) with an excess of acetic anhydride in pyridine followed by an aqueous treatment was shown earlier[3] to lead to the degradation of the pyrophosphate linkage (eq 1). This procedure for the cleavage of pyrophosphate bonds has been used extensively in work in the polynucleotide field[4] where pyrophosphates linking

(1) Paper LIII in this series: M. W. Moon, S. Nishimura, and H. G. Khorana, *Biochemistry*, **4**, 937 (1966).

(2) This work has been supported by grants from the National Science Foundation (Grant No. GB-3342), the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service (Grant No. CA-05178), and the Life Insurance Medical Research Fund (Grant No. G-62-54).

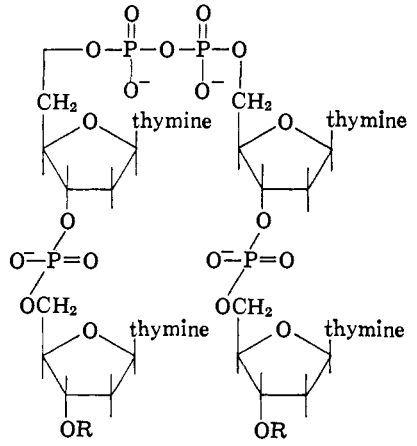
(3) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **81**, 4660 (1959).

(4) (a) H. G. Khorana, J. P. Vizsolyi, and R. K. Ralph, *ibid.*, **84**, 414 (1962); (b) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 1983 (1963); (c) A. Falaschi, J. Adler, and H. G. Khorana, *J. Biol. Chem.*, **238**, 3080 (1963); (d) G. Weimann, H. Schaller, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3835 (1963); (e) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3841 (1963); (f) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3857 (1963); (g) E. Ohtsuka, M. W. Moon, and H. G. Khorana, *ibid.*, **87**, 2956 (1965).



mono- and oligonucleotides through their phosphomonoester end groups are frequently encountered.[4,5] However, the method has not proven to be uniformly effective in completing the breakdown of the pyrophosphate linkages in oligo- and polynucleotides. Thus, for example, while in work with thymidine and deoxycytidine polynucleotides the method was effective, in the corresponding work with deoxyadenosine polynucleotides, a small amount of pyrophosphate bonds survived.[4a] Again, in the case of deoxyribopolynucleotides containing deoxycytidylate and deoxyguanylate units in alternating sequence, a considerable proportion of the pyrophosphate linkages survived.[4e]

As the synthetic work in the polynucleotide field advances, the utilization of suitably protected oligonucleotide blocks as starting materials assumes an ever increasing importance and in the separation and analysis of the polynucleotidic products, the complete removal of the pyrophosphate linkages (such as in II) continues to be of central importance. We have therefore studied in greater detail the previously observed reaction[3] of pyrophosphates with carboxylic anhydrides. The pyrophosphate used mostly was P^1,P^2-di(3′-O-acetylthymidine-5′) pyrophosphate (I), but some experiments were also carried out with the symmetrical pyrophosphate (II) of 3′-O-acetylthymidine dinucleotide. Again, while the bulk of the work has been carried out using benzoic anhydride, experiments using acetic anhydride and benzoyl chloride are also reported.



II, R = acetyl
III, R = H

The kinetic and equilibrium studies reported are of fundamental interest from the standpoint of the chemistry of nucleotides and phosphate esters even though the practical objective of defining uniformly applicable conditions for complete breakdown of pyrophosphates was not realized. An accompanying paper[6] describes related work in which the reaction of nucleotides with certain carboxylic acid anhydrides and chlorides, in fact, led to the synthesis of internucleotide bonds.

Experimental Section

Materials and Methods. Pyridine was purified by fractional distillation and dried over calcium hydride. Diphenyl phosphoric acid was a commercial sample purified by recrystallization from chloroform–petroleum ether (bp 40–60°). Benzoic anhydride and benzoic acid were recrystallized before use. Acetic anhydride was purified by distillation. The preparation of the nucleotidic materials 3′-O-acetylthymidine-5′ phosphate[7] and P^1,P^2-di(3′-O-acetylthymidine-5′) pyrophosphate (I)[3] has been described previously. 5′-O-Acetylthymidylyl(3′→5′)-3′-O-acetylthymidine was prepared by acetylation of thymidylyl(3′→5′)thymidine with acetic anhydride in pyridine. The preparation of the thymidine dinucleotide pyrophosphate (II) is described below.

Paper chromatography was performed by the descending technique using Whatman No. 1 paper. The solvent systems used were: solvent A, isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v); solvent B, ethyl alcohol–0.5 *M* ammonium acetate, pH 3.8 (7:3, v/v).

Paper electrophoresis was carried out in a commercially available apparatus capable of giving a potential of 5000 v using potassium phosphate (0.03 *M*, pH 7.1) buffer.

Thymidine Dinucleotide Pyrophosphate (II). Triethylammonium 5′-O-phosphorylthymidylyl(3′→5′)-3′-O-acetylthymidine (200 mg, 0.23 mmole) was dissolved in pyridine (10 ml) and the solution was evaporated to a gum. Tri-*n*-butylamine (0.5 ml), pyridine (2 ml), and DCC (0.25 g) were added and the mixture was shaken at room temperature for 3 days. Water (5 ml) was added and the product was washed with ether (two 5-ml portions). The aqueous layer was rendered anhydrous by repeated addition and evaporation of pyridine; the residual gum was dissolved in pyridine (2 ml) and precipitated in ether (100 ml) to afford the pyrophosphate (190 mg), R_f in solvent B, 0.22 (R_f of the starting dinucleotide, 0.39).

Equilibrium Studies on the Degradation of P^1,P^2-Di(3′-O-acetylthymidine-5′) Pyrophosphate (I). General Procedure. Stock solutions of acetic anhydride, benzoic anhydride, and benzoyl chloride containing 0.2 mmole and 1.0 mmole/5 ml were prepared in anhydrous pyridine in graduated flasks. The pyrophosphate (0.5 mmole, tri-*n*-butylammonium salt) was dissolved in anhydrous pyridine, rendered anhydrous by evaporation, and transferred to a 5-ml graduated flask with anhydrous pyridine. Anhydrous stock solutions of pyridinium diphenyl phosphate, pyridinium acetate, and pyridinium benzoate in pyridine were prepared similarly. The reaction mixtures were prepared in a drybox, appropriate quantities of the reagents being added to the pyrophosphate (0.01 mmole) in 3-ml tubes which were kept sealed until work-up. The total volume and reaction times were as follows: all reactions involving acetic anhydride or benzoic anhydride, 0.3 ml, period of reaction 3 days; benzoyl chloride experiments, 0.2-ml volume, time 0.5 and 5.0 hr. The reaction mixture was then diluted with water (0.5–1.0 ml) and extracted with ether (2 ml). The aqueous phase was treated with 2 *N* NaOH (0.3 ml) for 10 min after which time Dowex-50 pyridinium resin was added to remove sodium ions, and an aliquot of the reaction mixture was analyzed in solvent A. After overnight development, the paper was dried, and ultraviolet absorbing spots corresponding to thymidine-5′ phosphate and its pyrophosphate were cut out and eluted to determine the product distribution.

Rate Studies on the Degradation of P^1,P^2-Di(3′-O-acetylthymidine-5′) Pyrophosphate (I). The pyrophosphate (0.05 mmole) and the required amount of benzoic acid or diphenyl phosphoric acid were dissolved in anhydrous pyridine and evaporated to a gum, which was dissolved in anhydrous pyridine (1.0 ml). While shaking the clear reaction mixture, solid benzoic anhydride was added. Samples (0.05 ml) were removed after appropriate time

(5) (a) A. F. Turner and H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 5641 (1959); (b) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961); (c) R. K. Ralph and H. G. Khorana, *ibid.*, **83**, 2926 (1961).

(6) M. W. Moon and H. G. Khorana, *ibid.*, **88**, 1805 (1966).

(7) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **83**, 675 (1961).

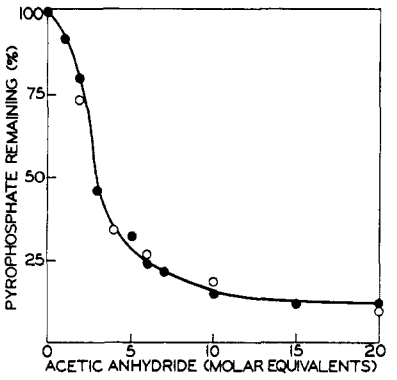


Figure 1. The reaction of P^1,P^2-di(3′-O-acetylthymidine-5′) pyrophosphate (II) with acetic anhydride in anhydrous pyridine. The amount of pyrophosphate shown is that surviving after a 3-day reaction period: ●, in absence of added acetate ion; ○, in presence of 20 molar equiv of pyridinium acetate. For further details see the Experimental Section.

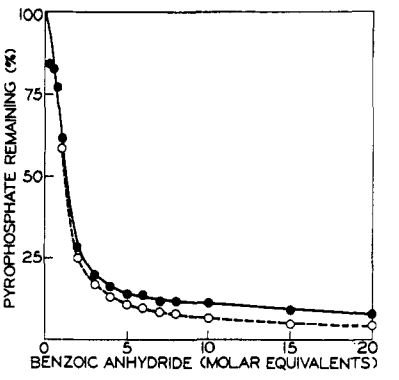


Figure 2. The reaction of P^1,P^2-di(3′-O-acetylthymidine-5′) pyrophosphate (II) with benzoic anhydride in anhydrous pyridine. The amount of pyrophosphate shown is that surviving after a 3-day reaction period: ●, in absence of added benzoate ion; ○, in presence of 5 molar equiv of pyridinium benzoate. For further details see the Experimental Section.

intervals and these were precipitated in petroleum ether (about 3 ml). Two methods of analysis were used.

Method A. The precipitate was extracted into water (0.5 ml) and the aqueous phase was separated. The aqueous layer was then treated with alkali and analyzed as described above for the equilibrium studies.

Method B. The precipitate obtained was collected by centrifugation, dissolved in aqueous pyridine, and immediately subjected to paper electrophoresis at pH 7.1. The bands were eluted with water and the products in water (about 0.3 ml) were treated with pyridine (0.1 ml) for 30 min. Following hydrolysis with concentrated ammonia solution (0.3 ml) for 1 hr, the total product was applied in solvent A to obtain the product distribution.

Experiments where limited amounts of water were added were carried out in a similar way except that in setting up the reaction mixtures pyridine (1 ml) containing a measured amount of water was used in place of anhydrous pyridine to dissolve the anhydrous pyrophosphate gum.

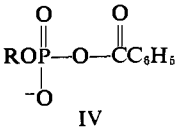
Equilibrium Studies on the Degradation of Thymidine Dinucleotide Pyrophosphate (II). The pyrophosphate (90 mg, 0.05 mmole) was dissolved in pyridine and rendered anhydrous. The gum was dissolved in 1 ml of anhydrous pyridine. Portions (0.2 ml) of this stock solution were treated with the appropriate amount of benzoic anhydride added from a stock solution in anhydrous pyridine. The mixture was sealed for 3 days and then added to water (0.5 ml) and extracted with ether (2 ml). The aqueous layer was treated with 2 *N* NaOH solution (0.2 ml) for 20 min, after which time Dowex-50 pyridinium resin was added to remove sodium ions, and an aliquot (about 40 optical density units, 267 mμ) of the solution was chromatographed in solvent B. The spots corresponding to the dinucleotide (R_f 0.29) and its pyrophosphate (R_f 0.09) were eluted and the proportions of these products were determined spectrophotometrically. The results are in Table III.

Equilibrium Studies Using the Dinucleotide 5′-O-Phosphorylthymidylyl(3′→5′)-3′-O-acetylthymidine. The dinucleotide (0.04 mmole) was rendered anhydrous and was dissolved in anhydrous pyridine (1.0 ml). The solution was divided into four equal parts: one was treated with 20 equiv of acetic anhydride, and the others were treated with 20 equiv of benzoic anhydride in the presence of 0, 10, and 20 equiv of pyridinium benzoate. The reaction mixtures were sealed for 3 days after which time water (0.5 ml) was added. After ether extraction the aqueous phase was hydrolyzed with 2 *N* NaOH solution and, after neutralization, an aliquot was chromatographed in solvent B. In every case, the formation of the dinucleotide pyrophosphate (III) was observed. The amounts as determined spectrophotometrically were: acetic anhydride experiment, 38.4%; benzoic anhydride experiments, no benzoate ion added, 31.6%; 10 equiv of benzoate ion added, 31.7%; 20 equiv of benzoate ion added, 32.2%.

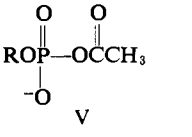
Results

Degradation of I with Acetic Anhydride. The previously reported study[3] of this reaction was repeated and extended. Treatment of I with an increasing excess of acetic anhydride in pyridine for 3 days followed by an aqueous pyridine treatment gave the results shown in Figure 1. These results are generally similar to those obtained earlier except that a little of the pyrophosphate was found to survive. (In the present work, analysis was performed by applying a much larger quantity of nucleotidic material on paper chromatograms than was used previously.) Also shown in Figure 1 are the results obtained when acetate ions (20 molar equiv as based on I) were provided in the reaction mixture. Acetate ions were implicated[3] as a reactant species in the degradative process and, furthermore, they might easily be generated in the reaction mixtures from hydrolysis of acetic anhydride if the removal of moisture is incomplete. In fact, this interpretation was previously advanced[4a,4e] to account for the variation in the effectiveness of the acetic anhydride–pyridine treatment for the breakdown of the pyrophosphate linkages. As seen in Figure 1, the deliberate addition of relatively large amounts of acetate ions made little difference so far as the final position of equilibrium was concerned.

Degradation of I with Benzoic Anhydride. Mixed anhydrides of the type IV are far more stable than are the corresponding anhydrides (V) between acetic acid[8] and phosphate esters. Since in pyrophosphate



IV



V

degradation species of the type V were considered to be the probable end products in anhydrous pyridine (ref 3, and see below), it seemed possible that benzoic anhydride would be more effective than acetic anhydride in the cleavage of the pyrophosphate linkage. The bulk of the present studies have in fact been carried out with this carboxylic anhydride. Figure 2 shows the results of a study[9] comparable to that shown in Figure

(8) For example, while compounds of the type V will hydrolyze completely at room temperature in aqueous pyridine in 1–2 hr, IV would be essentially unaffected over a period of many days under these conditions. See *e.g.*, ref 5b.

(9) It should be added that the alkaline treatment given during work-up would ensure hydrolysis of the mixed anhydride IV to the nucleotide.

Table I. Effect of Addition of Various Salts on the Rate of Degradation of P^1,P^2-Di(3′-O-acetylthymidine-5′) Pyrophosphate (I)[a]

Time	No addition	+5 equiv of pyridinium benzoate	+20 equiv of pyridinium benzoate	+5 equiv of pyridinium diphenyl phosphate	+4 equiv of pyridinium diacetyl thymidylylthymidine
5 min	51.0	55.8	78.8	28.0	27.3
10 min	49.0	45.5	68.5	18.9	
15 min	46.5	44.7	62.8	19.3	11.9
0.5 hr	45.5	33.8	49.1	18.7	11.7
1.0 hr	40.0	22.5	34.7	18.0	13.2
2.0 hr	35.5	14.2	21.4	15.7	12.1
4.0 hr	27.2	9.9	10.6		12.8
6.0 hr	21.0	7.0	8.6		
8.0 hr	17.2	5.3	6.7		
24.0 hr	11.0	6.4	5.9		

[a] By benzoic anhydride (10 molar equiv). The experiments were set up as in the general procedure described for equilibrium studies and for rate studies (method A) in the Experimental Section. The numbers in each column refer to the percentage of pyrophosphate surviving at different time intervals. For details see the Experimental Section.

1 for acetic anhydride. The results of Figure 2 show that, at equal concentrations, benzoic anhydride was slightly more effective than acetic anhydride but that again a small amount of pyrophosphate survived. Figure 2 also shows the effect of addition of benzoate ions on the final position of equilibrium. Under these conditions, the amount of the pyrophosphate surviving at equilibrium was further reduced.

Kinetics of Degradation of I with Benzoic Anhydride. The experiments of Figure 2, where only the position of equilibrium was measured, were followed by the kinetic study shown in Figure 3 carried out using three levels of benzoic anhydride. In all cases, an initially rapid reaction (left part of Figure 3 with an expanded scale of time) reduced the amount of the pyrophosphate to about 50% of its original value. The subsequent reaction leading to equilibrium required a much longer time (right part of Figure 3). The amounts of the pyrophosphate surviving at equilibrium in these experiments are in agreement with those found in the experiments of Figure 2 where a 3-day reaction period was used.

Effect of Benzoate and Phosphodiester Anions on the Kinetics of Pyrophosphate Degradation Using Benzoic Anhydride. The influence of added pyridinium benzoate on the kinetics of degradation of the pyrophosphate I, using benzoic anhydride, is shown in Table I. Thus, two opposing effects were evident: the first rapid reaction was slowed down while the second slower process (Figure 3) was accelerated. Further, in agreement with the results of Figure 2, at equilibrium more pyrophosphate was broken down in the presence of benzoate ions than in their absence.

In model experiments to ascertain the effect of an internucleotidic linkage on the kinetics of pyrophosphate degradation, pyridinium diphenyl phosphate and pyridinium 5′-O-acetylthymidylyl(3′→5′)-3′-O-acetylthymidine were added to the standard reaction mixtures and the results are shown in Table I. As is seen, these anions too accelerated the rate of degradation of the pyrophosphate, but the final position of equilibrium was insignificantly effected. Thus, in an experiment in which 5 equiv of benzoic anhydride was used, 13.5% of the total pyrophosphate survived in the absence of diphenyl phosphate whereas in the presence of 20 equiv of the phosphodiester anions the pyrophosphate surviving amounted to 9%.

Effect of Addition of Water on the Kinetics of Pyrophosphate Degradation Using Benzoic Anhydride. Limited amounts of water accelerated the rate of degradation markedly as is seen in the results of Figure 4. With 10 molar equiv of benzoic anhydride best results were obtained in the presence of 5–10 equiv of water, pyrophosphate breakdown being virtually complete in 1–2 hr. When the products were examined at this time by paper electrophoresis without the alkaline treatment, the predominant product was the mixed anhydride of benzoic acid and 3′-O-acetylthymidine-5′ phosphate (VI), a trace of the pyrophosphate I being present as the only other nucleotidic product. It is noteworthy that with longer reaction periods in this experiment (see also Figure 4), the amount of the pyrophosphate slowly increased.

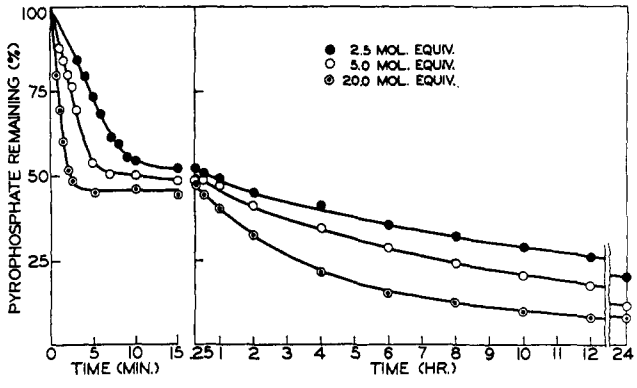


Figure 3. The rate of degradation of P^1,P^2-di(3′-O-acetylthymidine-5′) pyrophosphate (II) by benzoic anhydride in anhydrous pyridine. The left part of the figure shows the first 15 min of reaction on an expanded time scale, while in the right part the reaction is followed to a time of 24 hr: ●, 2.5 molar equiv of benzoic anhydride; ○, 5.0 molar equiv of benzoic anhydride; ⊙, 20 molar equiv of benzoic anhydride. For further details see the Experimental Section.

In further experiments using different amounts of benzoic anhydride for the degradation of the pyrophosphate I, limited amounts of water were added *after* the reactions had attained equilibrium. The results are shown in Table II. More pyrophosphate breakdown was observed after the water treatment than without it.

However, pyrophosphates of the type I would be completely stable to this treatment.

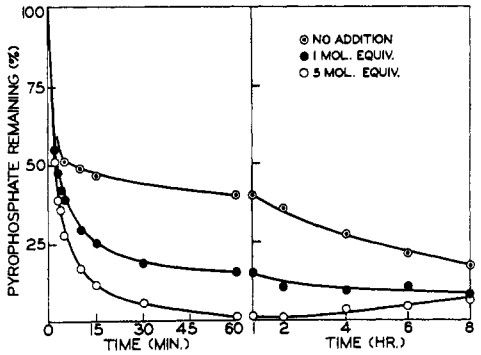
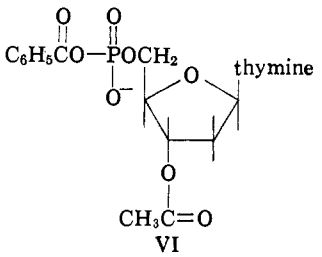


Figure 4. The rate of degradation of P^1,P^2-di(3′-O-acetylthymidine-5′) pyrophosphate (II) by benzoic anhydride (10 molar equiv) in pyridine containing traces of water: ●, 1 molar equiv of water; ○, 5 molar equiv of water; ⊙, reaction in absence of water.

When a 10–20-fold excess of benzoic anhydride was used, the degradation was virtually complete shortly (about 1 hr) after the addition of water.



VI

Polyphosphate as Intermediate in the Reaction between the Pyrophosphate I and Benzoic Anhydride. Analysis of the products in the above experiments (*e.g.*, that of Figure 2) was carried out after an alkaline treatment (method A in the Experimental Section)

Table II. The Effect of Water on the Degradation of P^1,P^2-Di(3′-O-acetylthymidine-5′) Pyrophosphate (I) with Benzoic Anhydride[a]

	Excess of benzoic anhydride used				
	1.25	2.5	5.0	10.0	20.0
Equilibrium position after 24 hr of reaction	39.9	19.8	11.1	7.6	7.7
Water treatment given after attaining equilibrium	38.9	15.3	3.5	0.6	0.6

[a] The pyrophosphate was treated with varying amounts of benzoic anhydride as shown for 24 hr. One-half of the reaction solution was removed and analyzed following alkaline treatment. The other half was treated with water (10 molar equiv as based on the pyrophosphate) for 1 hr and the alkaline work-up was then given. For further details see the Experimental Section. Numbers shown refer to per cent of pyrophosphate surviving using different excesses of acetic anhydride.

which would hydrolyze labile products except the disubstituted pyrophosphate I. When the alkaline step was omitted (method B in the Experimental Section) and the products, after a rapid work-up, were examined directly by paper electrophoresis at neutral pH, three main products were observed in addition to a small amount of the free mononucleotide. These were the unchanged pyrophosphate (I), the benzoyl phosphate (VI), and a third product with mobility 0.80 of that of the pyrophosphate I. This product was identified as P^1,P^2,P^3-tri(3′-O-acetylthymidine-5′) triphosphate

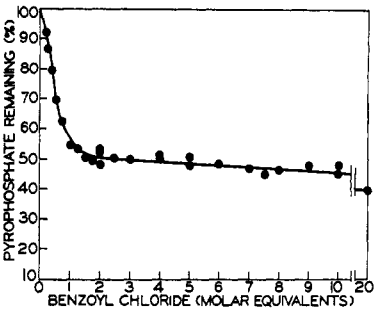
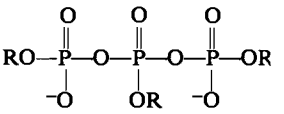


Figure 5. The reaction of P^1,P^2-di(3′-O-acetylthymidine-5′) pyrophosphate (II) with benzoyl chloride in anhydrous pyridine. The amount of pyrophosphate shown is that surviving after 0.5 hr. For further details see the Experimental Section.

(VII), its chromatographic properties and hydrolytic behavior being identical with that described previously for this compound.[10]



VII, R = 3′-O-acetylthymidine

In the experiment of Figure 2, when 10 equiv of benzoic anhydride was used and the products were examined after a reaction period of only 5 min, 26% of the total nucleotidic material was present as the benzoyl phosphate (VI), 16% as the unchanged pyrophosphate (I), 8% as the mononucleotide, and 50% as the triphosphate (VII). Examination of an identical reaction mixture after attainment of the equilibrium showed the nucleotidic products to be the benzoyl phosphate (VI) and the pyrophosphate (I), no triphosphate being detected.

Reaction of I with Benzoyl Chloride. The results of experiments in which the pyrophosphate I was treated with varying amounts of benzoyl chloride are shown in Figure 5. The amounts of the pyrophosphate were similar when short (30 min) or long (5 hr) reaction periods were used, indicating a rapid attainment of equilibrium. Even with an excess as large as 20 molar equiv of benzoyl chloride about 50% of the pyrophosphate (I) survived. Direct examination of the reaction mixtures by paper electrophoresis showed the presence of the linear triphosphate (VII) and other products as observed in the experiments with benzoic anhydride.

Degradation of the Pyrophosphate of Thymidine Dinucleotide (II) with Benzoic Anhydride. While the effect of addition of phosphodiester anions has been described above, a study was carried out with the pyrophosphate (II) so as to see the effect of internucleotide bonds present within the same molecule. Table III shows the results of an experiment in which this pyrophosphate was treated with varying amounts of benzoic anhydride. It is seen that a considerable amount of the pyrophosphate survived. In a further experiment (see the Experimental Section), when the parent dinucleotide, 5′-O-phosphorylthymidylyl(3′→5′)-3′-O-acetylthymidine, was treated with an excess of benzoic anhydride in the presence of benzoate ions, the formation of a consid-

(10) G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 4329 (1962).

Table III. Action of Benzoic Anhydride on Dinucleotide Pyrophosphate (II)[a]

Benzoic anhydride (molar equiv)	1	2	5	10	20
Pyrophosphate remaining (%)	69.5	45.5	29.0	25.0	25.2

[a] Amount of pyrophosphate shown is that surviving after a 3-day reaction period. For further details see the Experimental Section.

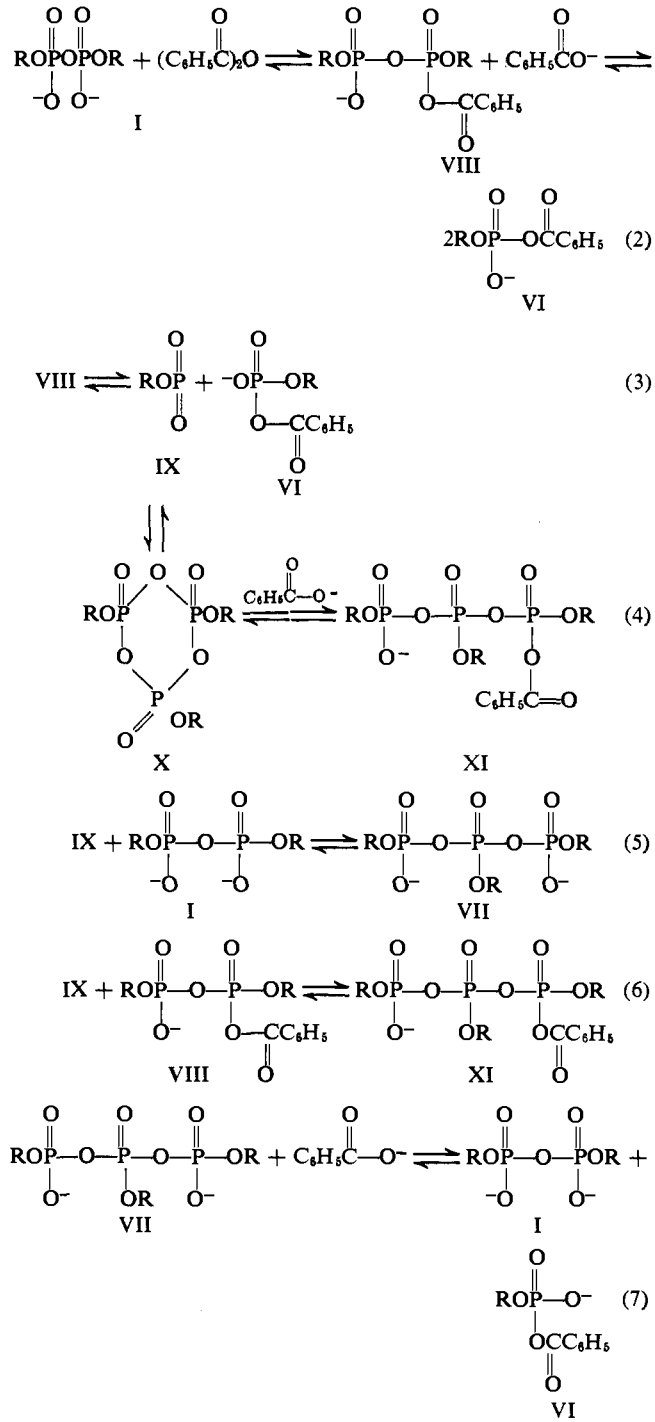
erable amount (32.2%) of the corresponding pyrophosphate was observed.

Discussion

The mechanism proposed previously[3] for the degradation of the disubstituted pyrophosphates (*e.g.*, I) with acid anhydrides is shown in eq 2 for benzoic anhydride. Thus, the first step was postulated to be the formation of acyl pyrophosphate (VIII) and benzoate ion. In the next step the benzoate ion attacked VIII to form the acyl anhydride of the nucleotide (VI). Both of these steps were postulated to be reversible and the position of the equilibrium was shown to be influenced by the amount of the carboxylic anhydride used.[3] While the steps in eq 2 must be involved, the present work has shown that this simplified version of the mechanism is deficient. The most significant accomplishment of the present investigation of the reactive intermediates has been the demonstration of the formation of polyphosphate species. The formation of these species constitutes an initial rapid step leading to the degradation (after a work-up involving an alkaline treatment) of about 50% of the original pyrophosphate and this is followed by a second slow process which reduces the amount of the pyrophosphate further. While normally the whole process comes to an equilibrium in about 24 hr, the rate may be influenced by the addition of different salts and water.

Plausible steps leading to the formation of the polyphosphate(s) are shown in eq 2–6 of Chart I. The benzoyl pyrophosphate (VIII) initially formed could collapse to generate the transient monomeric metaphosphate (IX) and the mixed anhydride VI as shown in eq 3. The monomeric metaphosphate (IX) could polymerize to the trimetaphosphate (X) which would rapidly be attacked by any of the ions present in solution. For example, attack of X by the benzoate ion would give benzoyl triphosphate (XI; eq 4) whereas attack by unreacted I or VIII could lead to higher linear polyphosphates. It is more likely that the monomeric metaphosphate (IX) would be directly attacked either by unreacted I to give the linear triphosphate (VII; eq 5) or by unreacted VIII to give the benzoyl triphosphate (XI; eq 6). It should be emphasized that species such as X, XI, or higher polyphosphates, would probably break down very rapidly during the work-up and would not be detected. It is very probable that, particularly in the reaction of I with acid chlorides such as benzoyl chloride, species much more reactive than VII are formed. Indirect evidence for this postulate comes from the utilization of this reaction in the synthesis of internucleotide bonds.[6] (Species of the type VII would not be expected to cause significant phosphorylation of the 3′-hydroxyl group of a nucleoside.)

Chart I. Possible Intermediates in the Reaction of Dialkyl Pyrophosphates (I) with Benzoic Anhydride



The formation of the polyphosphates postulated in Chart I was demonstrated to be rapid and it would appear that in the presence of a large excess (tenfold or higher) of benzoic anhydride the equilibrium in this initial step lies well over to the formation of the polyphosphate (*e.g.*, VII). Thus an examination of the products present after an 8-min reaction period showed 50% of the nucleotidic material as VII, 24% as the benzoyl nucleotide VI, and smaller amounts of the free mononucleotide (8%) and the original pyrophosphate (I, 16%) were present. The last two products almost certainly arose from hydrolysis of the triphosphate (VII) or its equivalent even though the work-up was rapid. It is difficult otherwise to explain the

formation of the free mononucleotide. If this reasoning is correct then it follows that the initial steps lead rapidly to the formation of the benzoyl nucleotide (VI), representing about 25% of the original nucleotide material, and the remainder (75%) is converted to a polyphosphate, VII, or its derivative.

The second slow step in the degradative process involves the breakdown of the triphosphate (and/or other polyphosphates), and direct paper electrophoresis of the reaction mixtures run for prolonged periods showed, in fact, the mixed anhydride VI to be the predominant species. The breakdown of the polyphosphates is postulated to occur by attack of the benzoate anion as shown in eq 7. The original pyrophosphate (I) thus formed would enter the cycle of reversible steps.

As benzoate ion is given a key role in these reactions, a study was made of the effect of added benzoate ions on the degradative process. The kinetic study (Table II) showed two opposing effects as would be expected from the reaction steps postulated in Chart I. Thus the slowing down of the initial step may be explained by inhibition of the formation of VIII according to eq 2. On the other hand, once the triphosphate species are formed, the degradation should be more rapid in the presence of added benzoate ions (eq 7). This would also account for the observed more rapid attainment of equilibrium in the presence of benzoate ions. Further evidence for the importance of the benzoate ion in pyrophosphate degradation is provided by the study involving benzoyl chloride (Figure 5). In this case, no benzoate ions are present and only about half of the pyrophosphate is converted to mononucleotide.

The presence of small amounts of water in the reaction mixture greatly accelerated the degradation reaction. The effect cannot be explained by assuming generation of benzoate ions, for the effect observed with water was much more marked than the effect produced by added benzoate ions. A more plausible explanation is the rapid degradation of the polyphosphate species (VII and/or XI) to the free pyrophosphate (I) and the benzoyl phosphate (VI) or free mononucleotide. (The latter would rapidly react with benzoic anhydride to give VI and benzoate ion.) It is also noteworthy that water then would provide perhaps the only irreversible step in all of the postulated reactions.

While in the case of the nucleotide pyrophosphate (I) it was possible, with the addition of small quantities of water, to bring about virtually complete and rapid breakdown to mononucleotide, the corresponding study with the dinucleotide pyrophosphate (II) showed the survival of a considerable amount of the pyrophosphate. The total experience of the present and the earlier work[3,4] shows that the position of the equilibrium in the pyrophosphate–carboxylic anhydride reactions is influenced by the nature of the pyrophosphates and that to date a uniformly effective method for the complete breakdown of pyrophosphates by the use of carboxylic anhydrides is not available. More recent work in this laboratory, however, has shown that when aromatic sulfonyl chlorides are used as the condensing agents in place of dicyclohexylcarbodiimide in polynucleotide synthesis, pyrophosphate linkages survive only to a small extent and it therefore may be unnecessary to include any extra step for pyrophosphate cleavage during the work-up of the polynucleotidic products.

Finally, it seemed possible that the polyphosphate species proposed above as intermediates in pyrophosphate degradation are sufficiently activated to be of use in the synthesis of the internucleotide bond. This possibility was investigated with considerable success and the results are reported in the accompanying paper.[6]