[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL]

Studies on Polynucleotides. VII.[1] Approaches to the Marking of End Groups in Polynucleotide Chains: The Methylation of Phosphomonoester Groups[2]

BY H. G. KHORANA

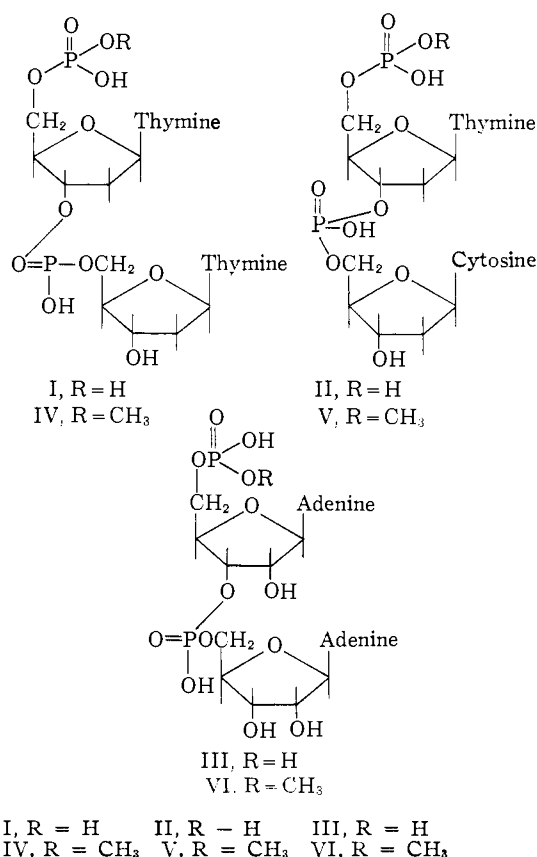
RECEIVED MARCH 13, 1959

A general procedure for the selective methylation of the phosphomonoester end groups in a variety of ribo- and deoxyribo-oligonucleotides is described. The potentialities of this technique in work on the end group and sequential analysis of polynucleotides are discussed.

Little has so far been accomplished in the general field of end group and sequential analysis of the nucleic acids. Using synthetic oligonucleotides of completely defined structures, possible approaches to these problems are now being investigated in this Laboratory. The methods developed with the smaller polymers will, it is hoped, be adapted to the problems of handling biologically active macromolecular nucleic acids. It also seems clear at the outset of these investigations that maximum progress will accrue from a combination of chemical and enzymic approaches as has been amply demonstrated in the protein field.[3] Both types of approaches are therefore being investigated concurrently and some results of enzymic studies from this Laboratory have already been reported.[4,5] Chemically, our efforts are being devoted initially to developing methods for the marking of end groups in polynucleotide chains. The distinguishing features of end nucleotides will, obviously, be either a free hydroxyl group[6] or a phosphomonoester group. Acetylation of the end hydroxyl groups was suggested recently[7] as a possible approach. Selective methylation of the phosphomonoester groups to form monomethyl esters is now proposed as a means of labeling those ends of the polynucleotide chains which bear such groups.

The present procedure for the selective methylation of phosphomonoester groups is based on work recently reported by Smith, Moffatt and Khorana.[8] There it was shown that while the trialkylammonium salts of diesters of phosphoric acid are completely inert toward dicyclohexylcarbodiimide (DCC) at room temperature, the corresponding salts of monoesters of phosphoric acid undergo reaction readily. Using dilute (under 0.1 molar) solution of monoesters of phosphoric acid (*e.g.*, adenosine-5′ phosphate) in methyl alcohol, quantitative yields of the corresponding monomethyl esters were obtained.

In the present work methylation was first studied with dinucleotides. Thus, thymidylyl-(5′ → 3′)-thymidylic-(5′) acid[9] (I), deoxycytidylyl-(5′ →3′) thymidylic-(5′) acid[9] (II) and adenylyl-(5′ → 3′)-adenylic-(5′) acid[9] (III) were all converted quantitatively to the corresponding methyl esters (IV, V and VI, respectively) when these substances were treated with an excess of DCC in methyl alcohol according to the general procedure described in the Experimental Section. The formation of methyl esters was first evident by the increase in mobility of



I, R = H II, R – H III, R = H
IV, R = CH3 V, R = CH3 VI, R = CH3

the products over the starting materials on paper chromatograms developed in the isopropyl alcohol–ammonia–water system. Their structures were con-

(1) Paper VI, A. F. Turner and H. G. Khorana, THIS JOURNAL, **81**, 4651 (1959).

(2) This work has been supported by grants from the National Research Council of Canada and the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

(3) H. G. Khorana, *Quart. Revs.* (*London*), **6**, 340 (1952); F. Sanger, *Adv. in Protein Chem.*, **7**, 1 (1952).

(4) W. E. Razzell and H. G. Khorana, THIS JOURNAL, **80**, 1770 (1958); H. G. Khorana, G. M. Tener, W. E. Razzell and R. Markham, *Federation Proc.*, **17**, 253 (1958).

(5) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2114 (1959).

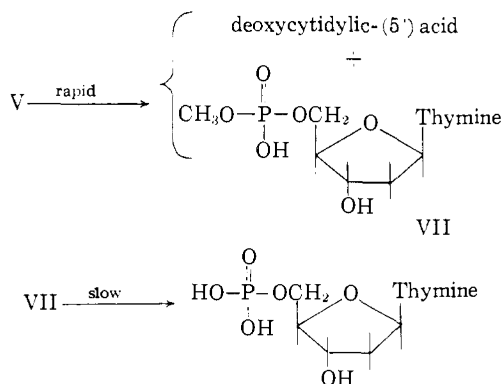
(6) In the ribopolynucleotides, a 2′,3′,-*cis* pair of hydroxyl groups may be present. Methods for stepwise degradation based on the selective periodate oxidation of such a pair of hydroxyl groups have been proposed; P. R. Whitfield, *Biochem. J.*, **58**, 390 (1954); D. M. Brown, M. Fried and A. R. Todd, *J. Chem. Soc.*, 2206 (1955); J. D. Smith, unpublished work.

(7) G. M. Tener, P. T. Gilham, W. E. Razzell, A. F. Turner and H. G. Khorana, *Ann. N. Y. Acad. Sci.*, in press (1958).

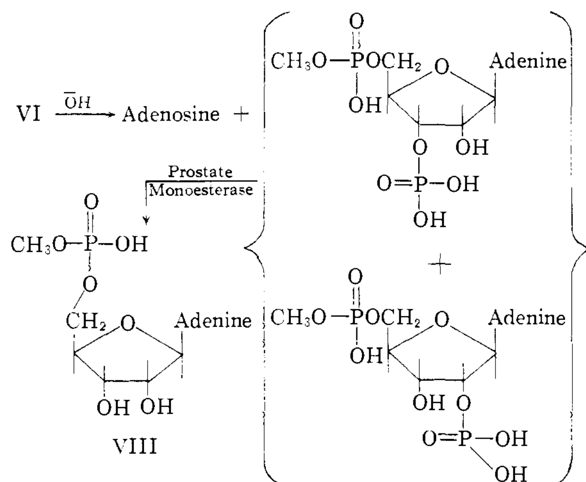
(8) M. Smith, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **80**, 6204 (1958).

(9) The systematic nomenclature used is that which was described in paper I of the present series (P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958)).

firmed by hydrolysis with venom diesterase. Thus, for example, degradation of V occurred, as illustrated in Scheme 1, to form first equimolar amounts of the free mononucleotide cytidylic-(5′) acid and methyl thymidylic-(5′) acid (VII); the latter was subsequently hydrolyzed very slowly to thymidylic-(5′) acid. In the case of the methyl ester VI, further confirmation was obtained by combined chemical and enzymic degradation as shown in Scheme 2; VIII was identical chromatographically with the sample synthesized earlier.[8]



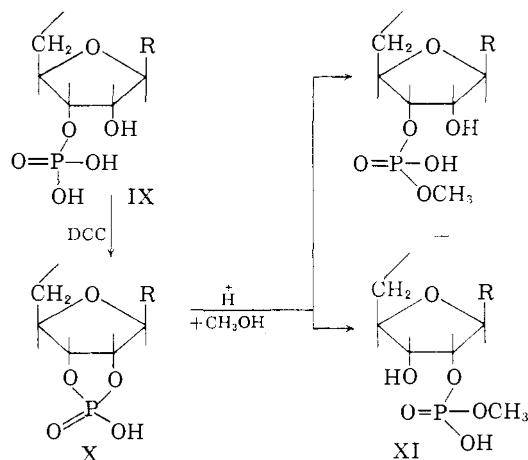
Scheme 1.—Venom diesterase degradation of the methyl ester of deoxycytidylyl-(5 → 3′)-thymidylic-(5′) acid (V).



Scheme 2.—Degradation of the methyl ester of adenylyl-(5′ → 3′)-adenylic-(5′) acid (VI).

Methylation of phosphomonoester end groups has been carried out with uniform success on all the typical oligonucleotides that are at present available in this Laboratory (Table I). These include deoxyribo-oligonucleotides bearing 5′- and 3′-phosphomonoester end groups and ribo-oligonucleotides bearing 5′-phosphomonoester groups. Very minor ultraviolet absorbing by-products have been noted on paper chromatograms in certain cases, but it is very probable that these arose from trace impurities in the starting materials. Retreatment of the isolated methyl esters of di- and trithymidylic acids and of di- and triadenylic acids under standard experimental conditions but with longer reaction periods the retreatment process was found to lead to no further reaction.

In principle, the only class of polynucleotides to which this technique will not apply directly are those illustrated by the partial structure IX. The phosphomonoester group bearing a vicinal hydroxyl group will react with DCC to form the 2′,3′-cyclic phosphate[8] X, preferentially.[10a] However, as was shown by Tener and Khorana[10b] for the simple ribonucleoside-2′,3′-cyclic phosphates, it would probably be possible to convert compounds of the type X to the acylic methyl esters XI by subsequent treatment with methyl alcohol under mild acidic catalysis.



The following general points may be made at the present time concerning the potentialities of the present technique in work on higher polynucleotides. Firstly, no interference is to be expected from any of the structural elements known to be present in the ribo- and deoxyribonucleic acids. The general lability of the ribonucleic acids may, however, require some caution. Secondly, solubility of the nucleic acids is not anticipated to be a serious problem. The formation of tri-*n*-butylammonium salts has a solubilizing effect and most encouraging are the observations of Geiduschek and Gray[11] concerning non-aqueous solvents (*e.g.*, ethyl alcohol) for deoxyribonucleic acid. Ribonucleic acids have also been extracted into 2-methyoxyethyl alcohol.[12] Furthermore, it is not even necessary to exclude water from the reaction mixtures and aqueous alcoholic solutions may be used advantageously.

The promise of the presence techniques resides especially in the use of C^{14}-methyl alcohol for labeling the end groups. Obviously the sensitivity will be increased by at least two orders of magnitude over ultraviolet spectrophotometry for end group analysis. The polynucleotides containing labeled end groups could be degraded partially by the use of appropriate nucleases and the fragments containing the label isolated and studied further. In this way

(10) (a) In the previous experiments with ribonucleoside-2′(3′) phosphates, methyl alcohol was not present in the medium of reaction. An experiment was therefore performed in which uridine-2′(3′) phosphate was treated in methyl alcohol with DCC in the presence of tri-*n*-butylamine; uridine-2′,3′ cyclic phosphate was the sole product. (b) G. M. Tener and H. G. Khorana, THIS JOURNAL, **77**, 5349 (1955).

(11) E. P. Geiduschek and I. Gray, *ibid.*, **78**, 879 (1956).

(12) K. S. Kirby, *Biochem. J.*, **64**, 405 (1956).

a beginning could be made on detailed analyses of polynucleotides in much the same way Sanger and co-workers[3] studied insulin fragments containing dinitrophenylamino end groups. A further possibility is that the use of a labeled end group will provide a method for determination of the molecular weight of polynucleotides by the principle already used by Craig and co-workers[13] in the polypeptide field.

Further work along these lines will be reported in due course.

Experimental

Paper chromatography was performed by the descending technique using mostly Whatman No. 1 or 3 MM paper. The solvent systems used routinely were isopropyl alcohol–ammonia–water (7:1:2) (solvent A) and *n*-butyl alcohol–acetic acid–water (5:2:3) (solvent B). Paper electrophoresis was carried out in an apparatus similar to that described by Markham and Smith.[14] The buffers used were 0.05 *M* sodium formate–formic acid (*p*H 3.5) and 0.05 *M* triethylammonium carbonate.[15] The venom phosphodiesterase used was one of the main fractions obtained upon chromatography on a diethylaminoethyl cellulose column, as described elsewhere.[5]

General Procedure for Methylation of Phosphomonoester End Groups.—The oligonucleotide salt (1–5 mg.) was passed through a pyridinium Dowex 50 ion exchange resin column (1.5 cm. × 1 cm.) and the effluent and washings evaporated in a vacuum at 25–35° bath temperature. To the residue was added 5 ml. of anhydrous pyridine and the solvent again evaporated in a vacuum. To the dry residue was now added 1.5 ml. of methyl alcohol and 0.05 ml. of triethylamine or tri-*n*-butylamine. Clear solutions were obtained with all the compounds studied. A pyridine solution[16] (0.25 ml. of 1 molar) of DCC was then added and the sealed reaction flask left at room temperature for 24–48 hr. The reaction was followed either by paper chromatography of an aliquot in solvent A or by electrophoresis at *p*H 7.5. In the former the product showed an increase, whereas in the latter it showed a decrease in mobility over the starting material. (In a few cases, there was found a small amount of starting material after 24 hr. and so a reaction time of 2 days was generally used.) At the end of the reaction, the total solution was directly applied to a 3-inch wide strip of Whatman 3 MM paper and the chromatogram developed in solvent A. Alternatively, water (1 ml.) was added and the mixture then evaporated partly to remove most of the methyl alcohol. Addition of a little more water and extraction with ether was followed by concentration of the aqueous layer under reduced pressure and chromatography of the concentrate as above. An extra band (R_f in solvent A, 0.35–0.45 in different runs) which was noted on observation under an ultraviolet lamp was found to correspond to N-methylpyridinium cation[16] (characteristic spectrum) and not nucleotidic material. The amount of this band increased when the reaction period was prolonged to several days. The methyl esters of oligonucleotides were eluted and treated as required. The compounds whose methyl esters have been prepared are listed in Table I, together with the R_f values of these products.

TABLE I

R_f'S OF THE MONOMETHYL ESTERS OF OLIGONUCLEOTIDES

Compound[a] Abbreviated name	Source, ref.	R_f of the methyl ester Solvent A	Solvent B
pT	9	0.48	0.31
pTpT	9	.28	.17
TpTp	17	.28	.10
pTpTpT	18	.13	.12
pTpTpTpT	18	.052	.07
pTp(d)A	9	.24	.16
pTp(d)C	19	.23	.17
pTpTp(d)C	19	.36[b]	.3[b]
pA		.39	.23
pApA	20	.15	.113
pApApA	20	.15[b]	.23[b]
pApApApA	20	.07[b]	.12[b]
pG[c]		.27	.15

[a] Instead of using the systematic nomenclature (ref. 9), abbreviations are used to designate the various oligonucleotides. In order to reduce confusion, which is evident in recent literature, we are using the system of abbreviations which has now been adopted by *J. Biol. Chem.*, thus the capital letters A, C T, are for the nucleosides, adenosine, cytidine and thymidine, respectively. Deoxynucleoside is indicated by adding the letter d in parentheses before the capital letter. The letter p to the left of the nucleoside initial indicates a 5'-phosphomonoester group; the letter p to the right, a 3'-phosphomonoester group. Thus pTpT is 5'-*O*-phosphoryl-thymidylyl-(3' → 5')-thymidine and TpTp is thymidylyl-(3' → 5')-thymidylic-(3') acid (ref. 9). [b] These values are with respect to the R_f of pA-methylester (1.0). [c] No oligonucleotides containing the base guanine have been available. Hence the technique has so far been tried on only guanosine-5' phosphate.

Alkaline Degradation of the Methyl Ester of Adenylyl-(5' → 3')-adenylic-(5') Acid.—The methyl ester as eluted from a paper strip (*ca.* 20 optical density units at 260 mμ) was kept at 37° in 1 *N* sodium hydroxide for 18 hr. and the solution then passed through a 1.5 cm. × 1 cm. ammonium Dowex-50 column. The column was washed with 1 *N* ammonia solution and the total effluent was concentrated and applied to a 2" wide Whatman 3 MM strip. Chromatography in solvent A showed a band corresponding to adenosine and a slow traveling band of equal intensity. The slow traveling band which was found to be homogeneous electrophoretically gave after incubation with prostatic phosphomonoesterase[21] in ammonium acetate (*p*H 4.5) buffer, methyl adenosine-5' phosphate. The latter was compared with an authentic sample[8] by paper chromatography and paper electrophoresis.

Venom Diesterase Degradation of Methyl Esters.—In a typical experiment, about 0.5 μmole of the methyl ester of deoxy-cytidylyl-(5' → 3')-thymidylic-(5') acid was incubated in 0.2 ml. of solution with a purified venom diesterase preparation,[5] which had been standardized against thymidylyl-(5' → 3')-thymidine. Under the conditions that the latter required approximately 0.5 hr. for complete hydrolysis, the above methyl ester was completely hydrolyzed to cytidylic-(5') acid and methyl thymidylic-(5') acid in under 5 minutes. The products at this stage were further identified by paper electrophoresis against known samples and by the characteristic spectrum of cytidylic-(5') acid. Incubation for a further 0.5 hr. caused some 10% hydrolysis of the methyl ester of thymidylic-(5') acid.

Hydrolysis of 3'-*O*-Phosphorylthymidylyl-(5'→3')-thymidine Methyl Ester with Spleen Diesterase.—A solution of 1.5 μmoles of the ester in 0.1 ml. of water was incubated at 37° in 0.1 ml. of 1 *M* ammonium acetate (*p*H 6.5) buffer with 0.1 ml. of a spleen diesterase preparation.[22] Aliquots were removed and directly applied to a chromatogram which was developed in solvent A. Hydrolysis to form thymidine-3' phosphate and thymidine-3' methyl phosphate (R_f 0.55) was complete within 5 min. The subsequent hydrolysis of thymidine-3' methyl phosphate to

(13) A. R. Battersby and L. C. Craig, THIS JOURNAL, **73**, 1887 (1951); E. J. Harfenist and L. C. Craig, *ibid.*, **74**, 3087 (1952).

(14) R. Markham and J. D. Smith, *Biochem. J.*, **62**, 552 (1952).

(15) J. Porath, *Nature*, **175**, 478 (1955).

(16) In view of the formation, although slow, of N-methylpyridinium cation, pyridine, which is not required in the reaction, has been omitted in recent experiments. Instead, solid DCC has been used.

(17) G. M. Tener, unpublished work.

(18) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, THIS JOURNAL, **80**, 6212 (1958).

(19) Unpublished work of G. M. Tener, J. P. Vizsolyi and H. G. Khorana.

(20) These ribo-oligonucleotides were prepared by degradation of polyadenylic acid with a liver nuclei enzyme; L. A. Heppel, P. J. Ortiz and S. Ochoa, *Science*, **123**, 415 (1956); *J. Biol. Chem.*, **229**, 679 (1957). We are grateful to Dr. W. E. Razzell for a preparation of purified triadenylic acid.

(21) Kind gift of Dr. R. Markham, Cambridge, England.

(22) The spleen diesterase was a preparation kindly given by Dr. W. E. Razzell. It had been prepared according to an unpublished procedure supplied by Dr. R. J. Hilmoe. The diesterase preparation had an activity of 52 μmoles/hr./ml. as tested against thymidylyl-(3'–5')-thymidine.

form thymidine-3′ phosphate was slow, requiring about 12 hr. for completion. When the methyl ester of adenylyl-(5′ → 3′)-adenylic-(5′) acid was incubated with the diesterase under the above conditions, no hydrolysis was detected in 2.5 hr.[23]

(23) It has previously been determined by L. A. Heppel and R. J. Hilmoe ("Methods in Enzymology," Vol. II, 1955, p. 565) that dinucleotides terminated in 5′-phosphomonoester groups are resistant to this enzyme.

Acknowledgment.—The author is grateful to Mr. J. P. Vizsolyi for technical assistance in some of the experiments reported here.

VANCOUVER 8, B.C., CANADA

[CONTRIBUTION FROM THE DIVISION OF CHEMISTRY, BRITISH COLUMBIA RESEARCH COUNCIL]

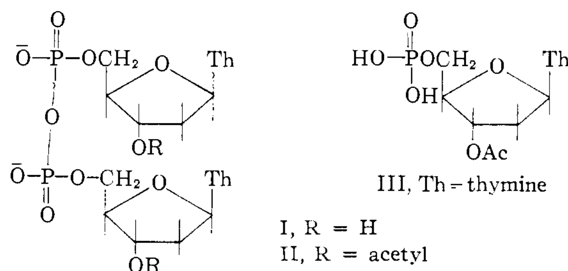
Nucleoside Polyphosphates. IX.[1] The Reversible Formation of Pyrophosphates from Monoesters of Phosphoric Acid by Reaction with Acetic Anhydride[2]

BY H. G. KHORANA AND J. P. VIZSOLYI

RECEIVED MARCH 13, 1959

The reaction of monoesters of phosphoric acid and of the corresponding pyrophosphates (symmetrical diesters of pyrophosphoric acid) with varying amounts of acetic anhydride in anhydrous pyridine has been studied. With half a molar equivalent of acetic anhydride (or acetyl chloride) symmetrical diesters of pyrophosphoric acid are formed in good yield from the monoalkyl esters of phosphoric acid. With increasing amounts of acetic anhydride the amount of the pyrophosphate decreases. In fact, diesters of pyrophosphoric acid may be degraded by treatment with an excess of acetic anhydride. Both synthetic and the degradative reactions, presumably, involve acyl phosphates as the intermediates. The new method of pyrophosphate formation has no significance for the specific formation of unsymmetrical pyrophosphates, due to exchange reactions.

P[1],P[2]-Dithymidine-5′ pyrophosphate (I) was treated with an excess of acetic anhydride–pyridine mixture with a view to preparing the corresponding acetyl compound (II, R-acetyl). The product isolated in quantitative yield was 3′-*O*-acetylthymidine-5′ phosphate (III). This cleavage of the pyrophosphate linkage prompted a closer examination of the reaction of phosphate esters with acetic anhydride in anhydrous pyridine. The results are reported in this communication.



Results

Two phosphomonoesters, monoethyl phosphate and 3′-*O*-acetylthymidine-5′ phosphate (III), and the corresponding pyrophosphates have been used in the present work.

Initial experiments showed that the treatment of pyridinium monoethyl phosphate in anhydrous pyridine with one-half mol. equiv. of acetic anhydride or acetyl chloride resulted in the formation of the corresponding pyrophosphate. In Table I are recorded the results of rate study of this reaction. Pyrophosphate formation is, thus, a relatively slow process. It should be noted that the conditions of the work-up (aqueous pyridine at room temperature) were such that no acyl phosphates would survive. In a subsequent experiment on a 2-mmole scale, diethyl pyrophosphate was isolated as the crystalline cyclohexylammonium salt in 75% yield after treating monoethyl phosphate with one-half mol. equiv. of acetic anhydride for three days. Similarly, the reaction of 3′-*O*-acetylthymidine-5′ phosphate in anhydrous pyridine gave the corresponding pyrophosphate II in 55% yield. The lower yield in this case could be due to difficulty in excluding traces of moisture on the small scale (0.5 mmole) used.

TABLE I

THE FORMATION OF SYMMETRICAL DIETHYL PYROPHOSPHATE FROM MONOETHYL PHOSPHATE ON REACTION WITH ACETIC ANHYDRIDE OR ACETYL CHLORIDE

Anhydrous pyridine solution of monoethyl phosphate (0.2 molar) and acetic anhydride or acetyl chloride (0.1 molar) at room temperature

	Time	Monoethyl phosphate, %	Diethyl pyrophosphate, %
Acetic anhydride	5 min.	100	0
	16 hr.	55	45
	72 hr.	10	90
Acetyl chloride	5 min.	100	0
	18 hr.	62	36
	72 hr.	26	74

Table II shows the results of a study of the reaction of pyridinium monoethyl phosphate with varying amounts of acetic anhydride, a reaction period of three days being used. Thus, the amount of pyrophosphate formed decreases with increasing amounts of acetic anhydride.

Table III shows the results of treatment of P[1],P[2]-diethyl pyrophosphate and di-(3′-*O*-acetylthymidine-5′) pyrophosphate (II) with varying amounts of acetic anhydride for three days in pyridine at room temperature. The results with the two pyrophosphates are in qualitative agreement with one another and show (see also above, Table II) that increasing amounts of acetic anhydride cause increasing breakdown of the pyrophosphate linkage. However, the actual proportions of the mono-

(1) Paper VIII, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **80**, 3756 (1958).

(2) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and the National Research Council of Canada, Ottawa.