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]

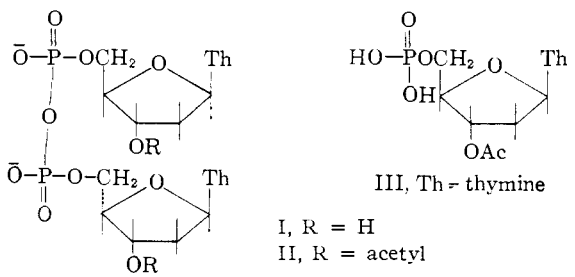
Nucleoside Polyphosphates. IX.[1] The Reversible Formation of Pyrophosphates from Monoesters of Phosphoric Acid by Reaction with Acetic Anhydride[2]

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.

P¹,P²-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.



III, Th = thymine

I, R = H
II, R = acetyl

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 P¹,P²-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.

TABLE II

PRODUCTS OF REACTION OF MONOETHYL PHOSPHATE WITH VARYING AMOUNTS OF ACETIC ANHYDRIDE IN ANHYDROUS PYRIDINE

Anhydrous pyridine solution of monoethylphosphate (*ca.* 0.2 molar) treated with acetic anhydride for three days at room temperature

Acetic anhydride, molar equiv.	Monoethyl phosphate, %	Diethyl pyrophosphate, %
0.5	21	79[a]
1.0	46	54
5.0	76	24

[a] The yield of pyrophosphate in this experiment is somewhat lower than that obtained under these conditions in other experiments, *e.g.*, Table I.

TABLE III

DEGRADATION OF SYMMETRICAL DIESTERS OF PYROPHOSPHORIC ACID BY REACTION WITH ACETIC ANHYDRIDE

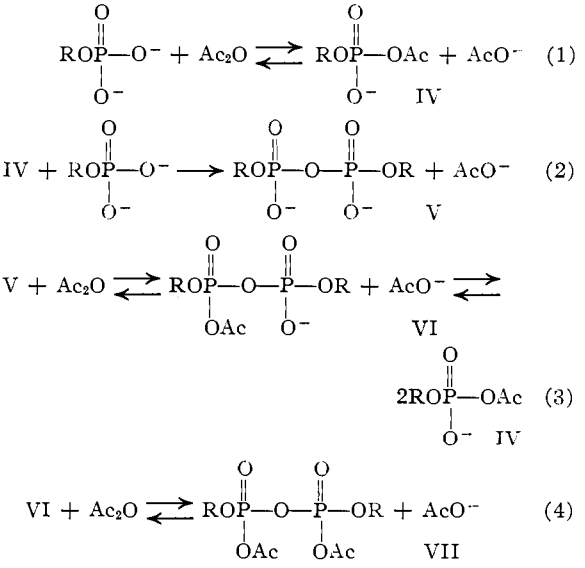
Anhydrous pyridine solution (*ca.* 0.2 molar) of P^1, P^2-diethyl pyrophosphate or di-(3′-*O*-acetylthymidine-5′) pyrophosphate treated with acetic anhydride at room temperature for three days

	Acetic anhydride, molar equiv.	Monoalkyl phosphate, %	Dialkyl pyrophosphate, %
P^1,P^2-Diethyl pyrophosphate	1	30	70
	5	44	56
	20	>95	<5
P^1,P^2-Di-(3′-*O*-acetylthymidine-5′) pyrophosphate	1	58	42
	5	88	12
	20	100	0

ester and the pyrophosphate using the same amounts of acetic anhydride are somewhat different. It is a good possibility that the exact positions of equilibria of the reactions (see below) vary with the phosphate esters used.

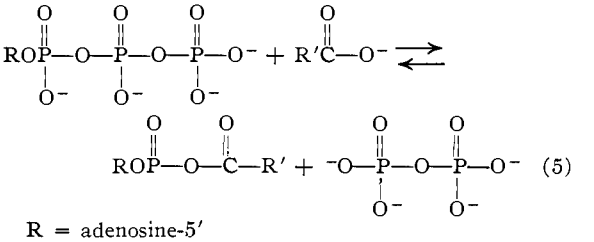
Discussion

The possible reactions and intermediates involved are shown in eq. 1-4.



Support for reaction 1 comes from the elegant work of Avison[3] who showed that mixed anhydrides of the type IV can be prepared rapidly in high yield in aqueous pyridine. Under anhydrous conditions, reaction 1 is presumably a rapid and reversible one. The finding that monoesters of phosphoric acid may be converted to the corresponding symmetrical pyrophosphates in high yield by treatment with 0.5 equivalent of acetic anhydride is new and deserves close examination. Firstly, the pyrophosphate formation must involve the reaction shown in eq. 2, namely, a displacement of the acetate ion by a phosphomonoester anion. The slow rate of the pyrophosphate formation (Table I) is concluded to be due to the slowness of reaction 2. That this reaction is practically irreversible was shown by a separate experiment in which *di*thymidine-5 pyrophosphate (I) was kept for several days in aqueous pyridine with and without an excess of acetate anions. Reversal of reaction 2 would lead to the formation of IV which would rapidly hydrolyze in aqueous pyridine at room temperature (*cf.* Avison[3] and Berg[4]). The net effect would be one of catalysis of pyrophosphate hydrolysis by acetate ions. No such effect was actually found.

The synthesis of pyrophosphates according to equation 2 parallels the recent results of enzymatic studies on acyl phosphates of the type IV which have been reported from several laboratories. A number of mixed anhydrides of adenosine-5′ phosphate and other acids (carboxylic acids, α-amino acids, sulfuric acid) have been discovered which are formed by the pyrophosphorolysis of adenosin-5′ triphosphate[5] (eq. 5). The equilibrium



R = adenosine-5′

of the reaction appears to lie heavily to the left as far as has been ascertained.[6]

It must be emphasized that the formation of pyrophosphates by reaction 2 is due to the thermodynamic stability of the product. Kinetically, nucleophilic attack is preferred by far at the carbonyl carbon of the mixed anhydrides of the type IV.[7] This is demonstrated by the formation of hydroxamates,[8] anilides and peptides from aminoacyl phosphate esters.[9] It was also shown to be true, in the present work, for attack by phosphate anions (see below) and alcohols. Thus, when an excess of

(3) A. W. D. Avison, *J. Chem. Soc.*, 732 (1955).

(4) P. Berg, *J. Biol. Chem.*, **222**, 1015 (1956).

(5) For a recent review see A. Kornberg, *Advances in Enzymology*, **18**, 191 (1957).

(6) *Cf.* P. W. Robbins and F. Lipmann [*J. Biol. Chem.*, **233**, 686 (1958)] for a fuller discussion of the energetics of this reaction. See also P. Berg [*ibid.*, **222**, 991 (1956)] who first showed the correspondence in ATP formation and acetyl adenylate disappearance. The correspondence between ATP formation and α-aminoacyl adenylates disappearance has also been demonstrated in a number of other laboratories recently.

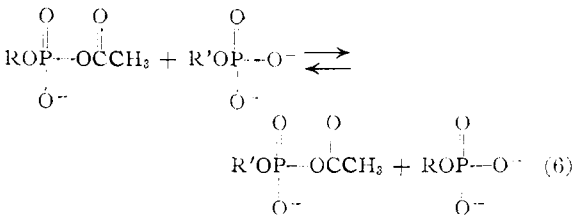
(7) This is so at least under neutral conditions when IV will be ionized. Under acidic conditions the situation could be different; see, *e.g.*, R. Bentley (THIS JOURNAL, **71**, 2765 (1949)) for mechanisms of hydrolysis of acetyl phosphate.

(8) F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **153**, 571 (1944).

(9) H. Chantranne, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **26**, 297 (1948); *Nature*, **164**, 576 (1949); Avison (ref. 3); Th. Wieland and F. Jaenicke, *Ann.*, **613**, 95 (1958).

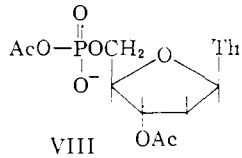
methyl alcohol was added to thymidine-5′ phosphate dissolved in acetic anhydride–pyridine mixture, none of the methyl ester of the nucleotide was formed.

Acyl phosphates of the type IV cannot be used in specific synthesis of unsymmetrical pyrophosphates by treating them with different phosphate ester anions. Kinetic preference for attack at the carbonyl carbon, as discussed before, would lead to the exchange reactions, shown in equation 6, and ultimately to random pyrophosphate formation. There



(6)

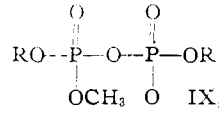
is the additional possibility of symmetrical pyrophosphate formation due to reaction 3. In one experiment, the formation of thymidine-5′ pyrophosphate was attempted by prior formation of the acetyl compound VIII[10] and subsequent addition of an anhydrous pyridine solution of tri-*n*-butyl-



VIII

ammonium orthophosphate.[11] On working up after a reaction period of three days, none of the nucleoside pyrophosphate was found; instead a considerable amount of inorganic pyrophosphate and smaller amounts of higher polyphosphates had formed. These results indicate that, as expected, the initial reaction of the orthophosphate anions with VIII led exclusively to the formation of acetyl phosphate which was attacked preferentially by orthophosphate anions or by itself to form inorganic pyrophosphate and higher polyphosphates. Adaptations of techniques using acetic anhydride can however be visualized for preparation of nucleoside polyphosphates in much the same way as the carbodiimide reagents[12] have been used. The approach is clearly inferior to the specific methods for unsymmetrical pyrophosphates which have recently been developed in this laboratory.[1,13]

Reaction 3 is postulated for the cleavage of the pyrophosphate bond by treatment with an excess of acetic anhydride and involves a disproportionation reaction of two anhydrides to form the mixed anhydride IV; the latter will hydrolyze rapidly during work-up to give monoalkyl phosphate. As shown by the results of Table II and III, reaction 3 is an equilibrium reaction, a large excess of acetic anhydride being required to complete the formation of the mixed anhydride IV. An alternative mechanism for cleavage of the pyrophosphates is represented by reaction 4. In this case the fully-substituted pyrophosphate VIII would have to hydrolyze on treatment with water to first form acyl phosphates IV which will hydrolyze further to give monoalkyl phosphates. Evidence against this mechanism was obtained by treating P^1,P^2-di-(3′-*O*-acetylthymidine-5′) pyrophosphate (II) with an excess of acetic anhydride and pyridine and subsequently adding an excess of methyl alcohol. No monomethyl ester of thymidine-5′ phosphate was obtained.[14] It is interesting, however, that a small amount of monomethyl ester of di-thymidine-5′ pyrophosphate (IX) was present. Most of the nucleotide was recovered as thymidine-5′ phosphate after alkaline treatment of the reaction products.



IX, R = thymidine-5′

Finally, it is interesting to compare the behavior of different anhydrides toward esters of phosphoric acid. In studies on the synthesis of diesters of phosphoric acid from monoalkyl esters, it has been previously shown[15] that the reaction of a monoester of phosphoric acid with a half molar equivalent of a powerful anhydride such as *p*-toluenesulfonyl chloride leads to the formation of the corresponding diester of pyrophosphoric acid. The latter, on further reaction with the anhydride, is apparently converted to polyphosphates or metaphosphates which are powerful phosphorylating agents for hydroxyl functions. The present work has shown that while acetic anhydride also shares the property of converting monoalkyl esters of phosphoric acid to the corresponding pyrophosphates, subsequent reaction with this anhydride leads only to degradation of the pyrophosphate linkage.

Experimental

Analytical Methods.—Paper chromatography was performed by the descending technique. The solvent systems used routinely were isopropyl alcohol–ammonia–water (7:1:2, v./v.) (solvent A) and *n*-butyl alcohol–acetic acid–water (5:2:3, v./v.) (solvent B). Other solvents used for specific purposes are mentioned in text.

Paper electrophoresis was carried out using 2″ or 3″ wide strips in an apparatus similar to that of Markham and Smith.[16] Phosphorus analyses were carried out by King's method.[17]

Barium Monoethyl Phosphate.—Commercially available sirupy ethylphosphoric acid was purified as follows: Twenty grams of the sirup was dissolved in 200 ml. of water and the solution heated on a water-bath at 100° for one hour to hydrolyze pyrophosphates. The cooled solution was neutralized to *p*H 8 with saturated barium hydroxide. The precipitate of barium phosphate was removed by centrifugation after one hour and the clear supernatant concentrated to about 50 ml. under reduced pressure, when barium ethyl phosphate largely crystallized. An equal volume of ethyl alcohol was added to complete crystallization and the total material was collected by centrifugation. It was washed successively with 50% ethyl alcohol, ethyl alcohol and ether.

(10) Acetyl chloride was used in this experiment since the equilibrium of reaction 1 then would be expected to be rather irreversibly to the right and at least the exchange reactions due to reversal of this step would be avoided.

(11) M. Smith and H. G. Khorana, THIS JOURNAL, **80**, 1141 (1958).

(12) H. G. Khorana, *ibid.*, **76**, 3517 (1954).

(13) R. W. Chambers and H. G. Khorana, *ibid.*, **80**, 3749 (1958).

(14) There was detected a bare trace of a thymidine-containing product traveling close to the solvent front on paper chromatograms. This could be dimethyl thymidine-5′ phosphate.

(15) (a) H. G. Khorana, G. M. Tener, J. G. Moffatt and E. H. Pol, *Chemistry & Industry*, 1523 (1956); (b) P. T. Gilham and H. G. Khorana, THIS JOURNAL, **80**, 6212 (1958); (c) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, *ibid.*, **80**, 6224 (1958).

(16) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 522 (1952).

(17) E. J. King, *ibid.*, **26**, 292 (1932).

The yield of air-dried material was 21 g. Paper chromatography showed a trace impurity of P^1,P^2-diethyl pyrophosphate.[18] The material was further purified by recrystallization. The total product was dissolved in 400 ml. of water at room temperature, the solution was filtered from a little suspension and 50 ml. of ethyl alcohol was added cautiously under agitation. The slightly turbid solution deposited crystals which were collected after three hours at room temperature. This crop which was pure was collected and washed as described above. The yield was 11 g. Phosphorus analysis showed it to be a trihydrate.

Reaction of Monoethyl Phosphate with Acetic Anhydride or Acetyl Chloride: General Method.—Pyridinium ethyl phosphate was prepared from the barium salt by treatment with pyridinium Dowex-50 cation exchanger, and the aqueous solution evaporated to a gum. The residue was dissolved in anhydrous pyridine and the solution re-evaporated. This process was repeated three times and a standard solution of the phosphate ester in anhydrous pyridine was then prepared. Appropriate portions containing 1 mmole of the substance were again evaporated twice after additions of anhydrous pyridine. To anhydrous pyridine solutions (*ca.* 5 ml.) were then added appropriate amounts of acetic anhydride or acetyl chloride and the sealed reaction flask kept at room temperature. Where the amount of acetic anhydride to be added was below 0.1 ml., a freshly prepared 10% solution of acetic anhydride in pyridine was used. At the end of appropriate reaction period, the reaction mixture was poured into 20 ml. of water and the aqueous pyridine solution left at room temperature usually for about 30 minutes to hydrolyze acetyl monoethyl phosphate. For analysis, one-fourth of the total reaction product was applied on a 6'' wide strip of Whatman 3 MM paper and monoethyl phosphate and symmetrical diethyl pyrophosphate were separated by chromatography in solvent A. Bands corresponding to the phosphate esters were located by spraying a thin strip cut out from one edge of the chromatogram. The bands were then eluted and finally made up to 10 or 25 ml. Phosphorus analysis was performed on suitable aliquots. The results obtained are recorded in the tables.

Preparation of P^1,P^2-Diethyl Pyrophosphate by Reaction of Monoethyl Phosphate with Acetic Anhydride.—Pyridinium monoethyl phosphate (2 mmoles) was rendered completely anhydrous as described above and was taken up in 20 ml. of anhydrous pyridine and the solution again evaporated to about 5 ml. Acetic anhydride (0.11 ml., 1.1 mmoles) was added and the sealed reaction flask kept at room temperature for three days. The reaction mixture was then poured into 20 ml. of water and the solution concentrated under reduced pressure after 0.5 hour to about 2 ml. The total concentrate was applied on two sheets of Whatman 3 MM paper sheets and chromatographed using solvent A. The major product, P^1,P^2-diethyl pyrophosphate was eluted with water and the total solution concentrated to a few ml. and passed through a Dowex-50 (H^+) resin column (6 × 1 cm.) in the cold at 2°. The total acidic effluent was neutralized with cyclohexylamine to *p*H 5 and evaporated under reduced pressure to a sirup which was dissolved in methyl alcohol and re-evaporated. Addition of methyl alcohol (2 ml.) to the residue followed by acetone (20 ml.) gave a crystalline crop (400 mg.) which was recrystallized twice from methyl alcohol–acetone in the same way. The final yield of pure material (m.p. 209–211°) was around 325 mg., more material being present in the mother liquors. The theoretical yield for pure bis-cyclohexylammonium P^1,P^2-diethyl pyrophosphate is 432 mg. Paper chromatography in solvent A showed that the only phosphorus-containing material in the purified sample and mother liquors was the pyrophosphate.

The above synthetic sample was identical (m.p., mixed m.p., and paper chromatography) with a sample which had been previously prepared and characterized in this Laboratory by Dr. J. G. Moffatt by the reaction of pyridinium monoethyl phosphate with 0.5 mol. equiv. of *p*-toluenesulfonyl chloride.[15]

Anal. Calcd. for $C_{16}H_{38}N_2O_7P_2$: C, 44.45; H, 8.85; N, 6.48. Found: C, 44.34; H, 8.91; N, 6.55.

(18) Higher yields of pure monoethyl phosphate will probably be obtained if the heating of the aqueous solution of the acidic commercial product is prolonged until paper chromatography in solvent A shows the complete breakdown of the pyrophosphates.

Preparation of P^1,P^2-Di-(3′-*O*-acetyl Thymidine-5′) Pyrophosphate.—The procedure used was similar to that described by Smith and Khorana.[11] Pyridinium 3′-*O*-acetylthymidine-5′ phosphate[19] (1 mmole) was dissolved in anhydrous pyridine (10 ml.) and purified triethylamine (0.28 ml., 2 mmoles) and, then, dicyclohexylcarbodiimide (1.125 g., 5.46 mmoles) added. After 16 hours at room temperature, paper electrophoresis at *p*H 7.5 showed complete conversion to the pyrophosphate. Water (20 ml.) was added to the reaction mixture and after two extractions with ether, the aqueous solution was concentrated in a vacuum. The concentrate was passed through a pyridinium Dowex-50 ion exchange column and the total effluent and washings lyophilized. The product traveled as a single spot electrophoretically at *p*H 7.5. On treatment with 0.1 *N* sodium hydroxide for 15 minutes at room temperature it was quantitatively converted to P^1,P^2-dithymidine-5′ pyrophosphate (I). Incubation with crude snake venom in tris-hydroxymethylamino methane buffer (*p*H 8.9) gave quantitatively 3′-*O*-acetylthymidine-5′- phosphate. The absence of thymidine in this experiment confirms that none of the 3′-*O*-acetyl groups had been removed during the preparation of the pyrophosphate.

Formation of P^1,P^2-Di-(3′-*O*-acetylthymidine-5′) Pyrophosphate from 3′-*O*-Acetylthymidine-5′ Phosphate and Acetic Anhydride.—Anhydrous pyridine solution (5 ml.) of 3′-*O*-acetylthymidine-5′ phosphate (0.5 mmole) was prepared as described above for ethyl phosphate and was treated with 0.275 mmole of acetic anhydride (0.138 ml. of a solution prepared by taking 1 ml. of the anhydride and diluting with pyridine to 5 ml.). The sealed reaction mixture was kept for three days and then poured into 10 ml. of cold water. A 2-ml. portion of the aqueous solution was evaporated under reduced pressure and redissolved in 1 ml. of water. The *p*H of the solution was raised to around 10 with sodium hydroxide and the solution was then made 0.1 *N* with respect to this base[20] and kept for about 20 minutes at room temperature to remove the acetyl group. The alkali was then removed by treatment with an excess of pyridinium Dowex-50 ion exchanger and the solution chromatographed in solvent A. The spots corresponding to thymidine-5′ phosphate and dithymidine-5′ pyrophosphate (I) were present. These were eluted and their optical densities determined. Triplicate analysis showed that conversion of the mononucleotide to the corresponding pyrophosphate was 55%.

Degradation of P^1,P^2-Di-(3′-*O*-acetylthymidine-5′) Pyrophosphate by Treatment with Acetic Anhydride.—A series of experiments in which anhydrous pyridine solution of the pyrophosphate II (0.25 mmole) was treated with varying amounts of acetic anhydride was set up exactly as described above for monoethyl phosphate. After a reaction period of three days, the reaction mixtures were analyzed as described in the preceding experiment. The results are recorded in Table III along with those obtained on the treatment of P^1,P^2-diethyl pyrophosphate with acetic anhydride.

Attempted Formation of Thymidine-5′ Polyphosphates. Tri-*n*-butylammonium orthophosphate (600 mg., 5.18 mmole, of 85% orthophosphoric acid + 2.5 ml. of tri-*n*-butylamine in 10 ml. of pyridine) was rendered anhydrous by repeated evaporation of its pyridine solution and finally dissolved in 5 ml. of anhydrous pyridine. An anhydrous pyridine solution of 3′-*O*-acetyl thymidine-5′ phosphate (0.9 mmole) was similarly prepared and to it was added 0.08 ml. (1.12 mmoles) of acetyl chloride and after 5 minutes the two solutions were rapidly mixed with exclusion of moisture and the sealed reaction mixture kept for three days. Twenty milliliters of water was then added and one-tenth portions of the total solution were examined as follows: (a) A portion was evaporated under reduced pressure, and the concentrate diluted with 2 ml. of water. Sodium hydroxide was added to alkaline *p*H and more added to bring the concentration to 0.1 *N*. Tri-*n*-butylamine which separated was extracted with ether, and after 30 minutes at room temperature the aqueous alkaline solution was treated with an excess of pyridinium Dowex-50 ion exchange resin and the

(19) Ref. 15b. A very large excess of acetic anhydride–pyridine was routinely used in the experiments on acetylation of mononucleotides.

(20) It was separately established that dithymidine-5′ pyrophosphate is completely stable to 0.1 *N* sodium hydroxide at room temperature for at least 25 hours.

supernatant concentrated under reduced pressure. Paper chromatography in the solvent system isopropyl alcohol (75 ml.)–water (25 ml.)–trichloroacetic acid (5 g.)–ammonia[21] (0.25 ml.) showed the presence of a strong spot corresponding to inorganic pyrophosphate as well as two spots corresponding to higher polyphosphates. (b) A second portion was treated as above under (a) up to the point of alkaline hydrolysis of the acetyl group. After two evaporations of the aqueous solution to remove last traces of pyridine, dilute hydrochloric acid was added to *p*H 3. The ultraviolet-absorbing material was adsorbed onto Norite A by successive additions of small portions of the latter and the charcoal was collected by centrifugation and washed twice with 5-ml. portions of water. Four elutions were carried out in the centrifuge tube with 5-ml. portions of 50% ethyl alcohol containing 2% ammonia. The total eluate was concentrated under reduced pressure and examined in solvents A and B and isopropyl alcohol–1% aqueous ammonium sulfate (2:1) and 1 *M* ammonium acetate (*p*H 7.5)–ethyl alcohol (3:7.5, v./v.) and also by paper electrophoresis at acid and neutral *p*H. Thymidine-5′ phosphate was the only ultraviolet-absorbing material present.

Methanolysis of Reaction Mixtures Containing Phosphate Esters, Acetic Anhydride and Pyridine.—(a) Pyridinium thymidine-5′ phosphate (0.1 mmole) was treated with acetic anhydride (0.5 ml.) in pyridine (2 ml.). After 11 hr. at room temperature methyl alcohol (2 ml.) was added and the mixture kept overnight. Repeated evaporations under reduced pressure with water gave a gum which was treated with 0.1 *N* sodium hydroxide (2 ml.) for 30 minutes. An excess of pyridinium Dowex-50 ion exchange resin was then added and the supernatant solution chromatographed in solvent A. The only nucleotidic band present was that of thymidine-5′ phosphate.

(b) P^1,P^2-Di-(3′-*O*-acetylthymidine-5′) pyrophosphate (0.03 mmole) was treated in anhydrous pyridine (1 ml.) with 0.45 ml. of acetic anhydride for two days at room temperature. Methyl alcohol (2 ml.) was then added and after a further period of 2 hr. the reaction products treated and chromatographed as above under (a). A very strong band corresponding to thymidine-5′ phosphate, a weak band corresponding to dithymidine-5′ pyrophosphate, and another weak band (about 10%) corresponding approximately in mobility to methyl thymidine-5′ phosphate (R_f 0.52) were present. An extremely faint band close to the solvent front with ultraviolet spectrum of thymidine chromophore was also noticeable. A comparison of the band with R_f 0.52, after elution, with methyl thymidine-5′ phosphate showed it to have slightly lower mobility on paper chromatograms developed in solvents A and B as well as paper electrophoresis at *p*H 7.5. The substance on treatment with 0.5 *N* sodium hydroxide at 100° for 15 minutes gave thymidine-5′ phosphate and methylthymidine-5′ phosphate. A similar result was obtained on heating in 20% acetic acid for 15 minutes at 100°. Authentic methylthymidine-5′ phosphate was found to be completely stable during the above acidic and alkaline treatments. Incubation with a *Crotalus adamanteus* venum diesterase preparation[22] gave first thymine-5′ phosphate and methylthymidine-5′ phosphate and ultimately thymidine-5′ phosphate as the only ultraviolet-absorbing product. From these data the substance was concluded to be IX.

When, in the above reaction between the pyrophosphate II and acetic anhydride–pyridine, methyl alcohol was added after only 0.5 hr., the yield of IX was about the same, an appreciable amount (20–30%) of dithymidine-5′ pyrophosphate (I) was present and the remainder of the ultraviolet-absorbing material was thymidine-5′ phosphate.

(21) J. P. Ebel, *Bull. soc. chim. (France)*, 991 (1953).

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

VANCOUVER 8, B.C., CANADA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS]

Rearrangement of Isopelletierene Oxime[1]

BY ROBERT L. AUGUSTINE

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Isopelletierene oxime, on treatment with phosphorus pentachloride in phenetole, gave the amidine, 2-methyl-1,3-diazabicyclo[4:3:0]-2-nonene (IX); IX is hydrolyzed to N-(2-pipicolyl)-acetamide and 2-pipicolylamine. In view of the pelletierene–isopelletierene identity recently proposed, these results are used in a re-evaluation of the evidence presented in the determination of the structure of pelletierene.

Pelletierene, one of several alkaloids found in *Punica granatum* L., was first assigned the structure β-(2-piperidyl)-propionaldehyde (I) by Hess in 1917.[2] Since that time numerous attempts have been made at the synthesis of this compound, all of which have ended in failure as the amino-aldehyde underwent self-condensation with great facility.[3] Thus, some doubt was shed on the reliability of the structure assigned to pelletierene as it was difficult to see how a material which was so unstable as to prevent synthesis could be the same material described as a relatively stable, mobile liquid with well defined physical characteristics.[4] In 1954, Galinovsky and Hollinger reported that treatment of a commercial sample of pelletierene hydrobromide with benzoyl chloride gave the benzamide of isopelletierene, 2-acetonylpiperidine (V), another of the alkaloids found in *Punica granatum* L.[5] It was known, however, that commercial samples of pelletierene salts contained various amounts of isopelletierene, pseudopelletierene, and other materials, as well as pelletierene.[3] Thus, the results tended to be inconclusive.

Confirmation of the identity of pelletierene with isopelletierene was accomplished somewhat later by Wibaut and Hirschel[3] who compared a sample of synthetic isopelletierene hydrobromide with a pure sample of pelletierene hydrobromide obtained from natural sources. They found no depression on mixed melting point determination and identical infrared spectra and X-ray powder patterns.

In the light of this evidence it would seem that a re-evaluation of the work described by Hess in determining the structure of pelletierene would be of some interest. His structure proof was based primarily on the following reaction sequence.

(1) This work was supported by a grant from the University of Texas Research Institute.

(2) K. Hess and A. Eischel, *Ber.*, **50**, 1192 (1917).

(3) J. P. Wibaut and M. J. Hirschel, *Rec. trav. chim.*, **75**, 225 (1956), and references cited therein.

(4) K. Hess, *Ber.*, **50**, 368 (1917).

(5) F. Galinvosky and R. Hollinger, *Monatsh.*, **85**, 1012 (1954).