Nucleoside Phosphonic Acids. II. The Synthesis of 5′-Deoxythymidine 5′-Phosphonic Acid and Its Pyrophosphate Derivatives*

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ABSTRACT: The chemical synthesis of the thymidylic acid analogs, 5′-deoxythymidine 5′-phosphonic acid, 5′-deoxythymidine 5′-phosphonylphosphate, 5′-deoxythymidine 5′-phosphonylpyrophosphate, and several other derivatives, are described. Diallyl 5′-deoxythymidine 5′-phosphonate was prepared from 3′-*O*-acetyl-5′-deoxy-5′-iodothymidine by a Michaelis–Arbuzov-type reaction with triallyl phosphite. After removal of the acetyl group with dilute ammonium hydroxide, the allyl ester groups were removed by catalytic reductive hydrogenolysis yielding di-*n*-propyl 5′-deoxythymidine 5′-phosphonate, *n*-propyl 5′-deoxythymidine 5′-phosphonate, and 5′-deoxythymidine 5′-phosphonic acid. The di- and triphosphate derivatives of 5′-deoxythymidine 5′-phosphonic acid were prepared by treating it with 1,1′-carbonyldiimidazole to yield the 5′-deoxythymidine 5′-phosphonyl imidazolidate and then condensing this substance with inorganic phosphate or pyrophosphate. In preliminary attempts, 5′-deoxythymidine 5′-phosphonylpyrophosphate, the analog of thymidine 5′-triphosphate, was not incorporated into thymidylyldeoxyadenylyl (dAT) copolymer in the presence of radioactive deoxyadenosine 5′-triphosphate (DNA) polymerase of *Escherichia coli* and the standard polymerizing mixture. In addition, this substance, only minimally if at all, inhibited the incorporation of thymidine 5′-triphosphate into polymer in the presence of a complete polymerization mixture.

Nucleoside phosphonic acids[1] are potential analogs of the naturally occurring nucleoside phosphates (nucleotides). In these substances the phosphorus atom is directly bonded to C-5′ of the pentose rather than *via* the oxygen atom of a phosphomonoester bond as in the nucleotides (Figure 1). No nucleoside phosphonic acids have been found in nature, but the isolation of several naturally occurring aliphatic phosphonates has been reported (Horiguchi and Kandatsu, 1959; Kittredge and Hughes, 1964). Although the biological function of these phosphonic acid derivatives is not understood, the similarity of the phosphonic acid group to the biologically ubiquitous phosphate ester group suggests that these substances serve as analogs of certain phosphate esters. Unlike phosphates, however, phosphonates are stable to hydrolysis by phosphomonoesterases. Our interest in the phosphonates, particularly nucleoside phosphonic acids, arose from the possibility that enzymes which require pyrophosphate activation for coenzyme and polymer synthesis might utilize the pyrophosphate derivatives of the phosphonic acids for these synthetic reactions or, alternatively, phosphonates could act as specific inhibitors of these reactions (Simon and Myers, 1961). The possibility that the pyrophosphate of a nucleoside phosphonic acid can function in enzyme-catalyzed polymerization reactions has been strengthened by the observation that 5′-adenylylmethylene diphosphonate, a phosphonate analog of adenosine 5′-triphosphate, containing a methylene group in place of the terminal pyrophosphate oxygen, can serve as a substrate for the DNA-directed RNA polymerase of *Escherichia coli* (Simon *et al.*, 1965). The polymerization of this latter derivative would yield a polymer containing a normal phosphodiester bond, while the polymerization of a nucleoside phosphonic acid would yield a polymer which would be expected to resist hydrolysis by cellular nucleases of the phosphodiesterase II type. Indeed, if they were effective analogs, nucleoside phosphonic acids could be used to study a variety of biochemical reactions which involve nucleoside phosphates either alone or in combination with other substances such as coenzymes. With these uses in mind, we have initiated a systematic study of the chemistry and biochemistry of nucleoside phosphonic acids, their pyrophosphate derivatives and their polymerization products. The chemical synthesis of a ribonucleoside phosphonic acid, 5′-

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[1] In this and the previous publication in this series (Yengoyan and Rammler, 1966) the nucleoside phosphonic acids have been described in keeping with the traditional names used for nucleic acid derivatives. The more exact nomenclature used by the American Chemical Society's organic indexing department for the principle compounds reported here are 5′-deoxy-5′-phosphonothymidine for 5′-deoxythymidine 5′-phosphonic acid (I), 5′-deoxy-5′-phosphonothymidine anhydride with phosphoric acid for 5′-deoxythymidine 5′-phosphonylphosphate (II), and 5′-deoxy-5′-phosphonothymidine anhydride with pyrophosphoric acid for 5′-deoxythymidine 5′-phosphonylpyrophosphate (III).

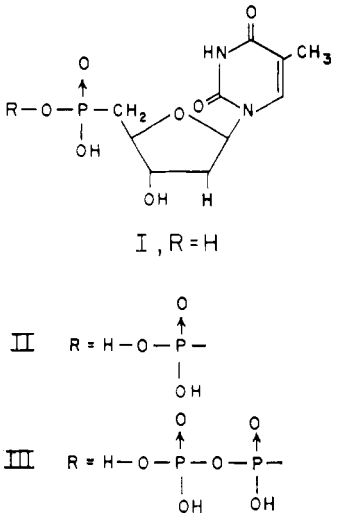


FIGURE 1: 5′-Deoxythymidine 5′-phosphonic acid and its pyrophosphate derivatives.

deoxyuridine 5′-phosphonic acid, and its pyrophosphate derivatives has been reported (Yengoyan and Rammler, 1966). In this communication, the syntheses of 5′-deoxythymidine 5′-phosphonic acid (I), 5′-deoxythymidine 5′-phosphonylphosphate (II), and 5′-deoxythymidine 5′-phosphonylpyrophosphate (III) (Figure 1) are described.

A general method for the preparation of aliphatic phosphonates has been described (Michaelis–Arbuzov reaction; see Kosolapoff (1950) for summarizing references). Using this procedure, the dialkyl esters of the

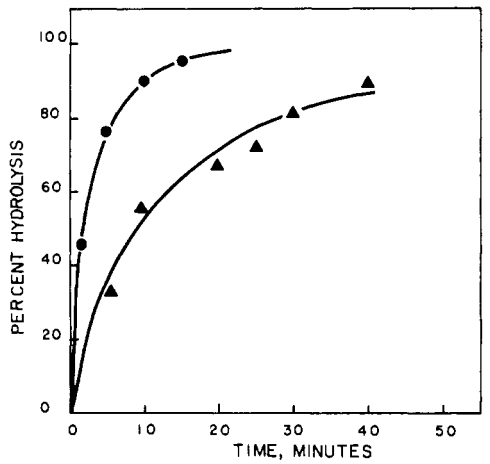


FIGURE 2: The alkaline hydrolysis of di-*n*-propyl 5′-deoxythymidine 5′-phosphonate (▲) and diallyl 5′-deoxyuridine 5′-phosphonate (●). The hydrolysis of the nucleoside phosphonates (0.12 mmole/ml) was carried out at 60° in sodium hydroxide (0.5 M). Samples were removed at the intervals indicated and were analyzed by paper chromatography in solvent A.

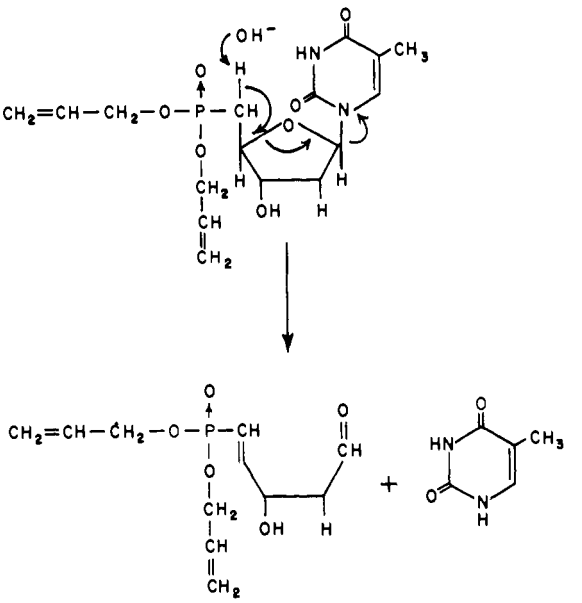
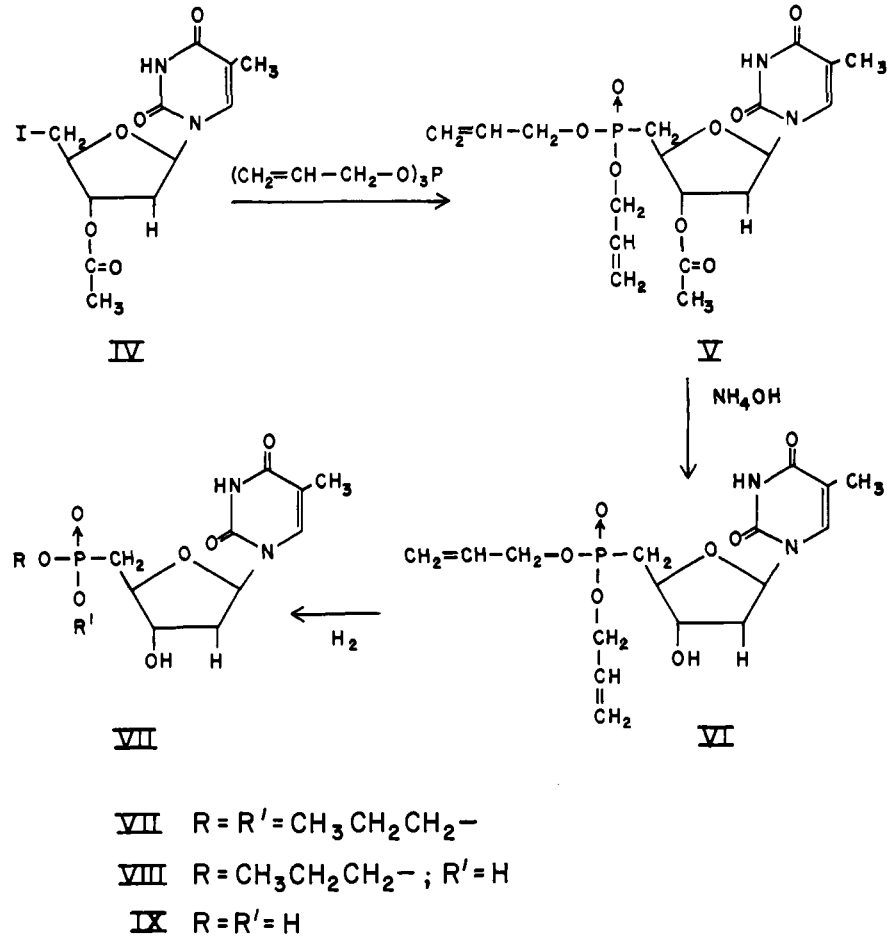


FIGURE 3: The alkaline hydrolysis of diallyl 5′-deoxythymidine 5′-phosphonate.

nucleoside phosphonic acids are readily prepared (Parikh *et al.*, 1957; Wolff and Burger, 1959; Bannister and Kagan, 1960). However, these derivatives have not been obtained as free acids. A major difficulty in the preparation of 5′-deoxynucleoside phosphonic acids from their aliphatic phosphonate esters (Bannister and Kagan, 1960) *via* base-catalyzed hydrolysis of the ester groups is the extreme alkaline lability of the 5′-deoxynucleoside 5′-phosphonate diester. For example, treatment of di-*n*-propyl 5′-deoxythymidine 5′-phosphonate with alkali yields thymine as the principal ultraviolet-absorbing product. The kinetics of the alkaline hydrolysis of diallyl 5′-deoxyuridine 5′-phosphonate and di-*n*-propyl 5′-deoxythymidine 5′-phosphonate are shown in Figure 2. At 60° (0.5 N, sodium hydroxide) the $t_{1/2}$ for the uridine derivative is about 2 min while that of the thymidine derivative is significantly longer. Analysis of the hydrolysis solution by paper chromatography demonstrated that the principal ultraviolet-absorbing component was uracil and thymine, respectively. Although there is no direct chemical evidence for the mechanism of this reaction, it is rationalized as indicated in Figure 3. A similar type of reaction has been proposed for the alkaline lability of 5′-sulphonium ribonucleosides (Baddiley *et al.*, 1962; Schlenk and Dainko, 1962) and the vitamin B_{12} coenzyme (Johnson and Shaw, 1961). In the hydrolysis of the thymidine and uridine phosphonates, the increase in the hydrolytic rate of the uridine derivatives is thought to result from an assist by the C-2′ hydroxyl in the concerted β elimination of the pyrimidine. Because of the extreme base lability of the nucleoside phosphonates, our attention was directed toward the preparation of these derivatives *via* the Michaelis–Arbuzov reaction with ester groups which could be

SCHEME I: The Synthesis of 5′-Deoxythymidine 5′-Phosphonic Acid.



VII R = R′ = $CH_3CH_2CH_2-$

VIII R = $CH_3CH_2CH_2-$; R′ = H

IX R = R′ = H

removed by a nonhydrolytic method. This was accomplished by preparing their diallyl esters which can be removed by catalytic reductive hydrogenolysis. The starting material for the synthesis of 5′-deoxythymidine 5′-phosphonic acid was 5′-iodo-5′-deoxythymidine (Scheme I). This was prepared by treatment of thymidine with *p*-toluenesulfonyl chloride which yielded 5′-*p*-toluenesulfonylthymidine in 53% yields (Michelson and Todd, 1955). Treatment of this substance with sodium iodide provided 5′-deoxy-5′-iodothymidine in 80% yields (Michelson and Todd, 1955). 3′-*O*-Acetyl-5′-deoxy-5′-iodothymidine (IV) was prepared from 5′-deoxy-5′-iodothymidine by acetylation with acetic anhydride. After chromatography on silicic acid, IV was isolated in crystalline form (Michelson and Todd, 1955). Reaction of the crystalline anhydrous[2] 3′-*O*-acetyl-5′-deoxy-5′-iodothymidine with excess triallyl phosphite for 30 hr at 130° yielded a mixture of products containing diallyl 3′-*O*-acetyl 5′-deoxythymidine 5′-phosphonate (V). Compound V was purified by chromatography on silicic acid. Removal of the acetyl group on V was accomplished by hydrolysis with dilute ammonia and the product of this reaction was purified by chromatography on silicic acid. The principal contaminants found with V are triallyl phosphite, diallyl allylphosphonate, and triallyl phosphate. An alternate method of purification is the preferential adsorption of V onto charcoal. This was effective in removing essentially all of the nonultraviolet-absorbing contaminants; however, quantitative desorption of V could not be achieved. Removal of the allyl ester groups on VI was carried out by reductive catalytic hydrogenolysis; but, because some reduction of the allyl ester groups occurs without hydrogenolysis, a mixture of products is obtained from this reaction. These side products, di-*n*-propyl 5′-deoxythymidine 5′-phosphonate (VII) and mono-*n*-propyl-5′-deoxythymidine 5′-phosphonic acid (VIII) are readily separated from 5′-deoxythymidine 5′-phosphonic acid (IX) by ion-exchange chromatography. The 5′-deoxythymidine 5′-phosphonic acid fraction isolated in this manner is contaminated with a small amount (1–2%) of thymidine 5′-phosphate. Although the mechanism for the formation of this phosphate

[2] In order to avoid the formation of polymeric side products during the Michaelis–Arbuzov reaction, 3′-*O*-acetyl-5′-deoxy-5′-iodothymidine must be anhydrous.

ester is unknown, it can arise by nucleophilic displacement of the iodo group on IV by diallyl phosphate. This latter substance can be generated *in situ* by oxidation of triallyl phosphite with its subsequent partial hydrolysis to yield its monoanion (Anand *et al.*, 1952). Thymidine 5′-phosphate can be removed from IX by treating the mixture with alkaline phosphomonoesterase and separating the resulting thymidine from IX by chromatography on DEAE-Sephadex A-25. Compound IX was characterized by several different methods. It was obtained whether the acetyl group on V was removed before or after the catalytic hydrogenolysis step, demonstrating that IX contains a free 3′-hydroxyl group. Its ultraviolet spectrum was similar to thymidine and on alkaline hydrolysis thymine is released, indicating that no substitution has occurred on the pyrimidine base. Phosphorus analysis indicates the presence of one phosphorus atom per thymine. Paper chromatographic and electrophoretic analysis demonstrated that IX is homogeneous and its R_F values and electrophoretic mobilities are consistent for a phosphonic acid (Yengoyan and Rammler, 1966).

The titration curve of 5′-deoxythymidine 5′-phosphonic acid and thymidine 5′-phosphate is shown in Figure 4. There is clearly a difference between the apparent secondary acid dissociations of these substances. The reported $pK_{a'_2}$ of thymidine 5′-phosphate is 6.5 (Schwarz BioResearch Inc., 1966) and this value was also found in this titration; however, 5′-deoxythymidine 5′-phosphonic acid's apparent pK_{a_2} is approximately 0.5 pH unit higher than its phosphate analog. This value is consistent with the finding that aliphatic phosphonic acids are in general weaker acids than their corresponding phosphate analogs (Freedman and Doak, 1957) and lends support to structure IX. Despite the differences in the $pK_{a'_2}$ of thymidine 5′-phosphate and IX, there would be expected to be no significant difference in the net charge of either their esters or pyrophosphate derivatives at pH 7.

5′-Deoxythymidine 5′-phosphonylpyrophosphate, the analog of thymidine 5′-triphosphate, was prepared from the imidazolidate (Hoard and Ott, 1965) of IX. Treatment of this compound with inorganic pyrophosphate yielded 5′-deoxythymidine 5′-phosphonylpyrophosphate. This derivative contained three phosphorus atoms per thymine and was susceptible to hydrolysis by both alkaline phosphomonoesterase and snake venom phosphodiesterase yielding 5′-deoxythymidine 5′-phosphonic acid as the only ultimate ultraviolet-absorbing product.

In Figure 5, the rate of the acid-catalyzed hydrolysis of thymidine 5′-triphosphate is shown while in Figure 6 a comparative study of the hydrolysis of 5′-deoxythymidine 5′-phosphonylpyrophosphate is depicted. The products of the hydrolytic reaction were examined by paper chromatography. Although the rate of hydrolysis of both derivatives is similar, the pattern of the products formed is clearly different for each. With the hydrolysis of thymidine 5′-triphosphate, there is a coincidental increase of thymidine 5′-diphosphate

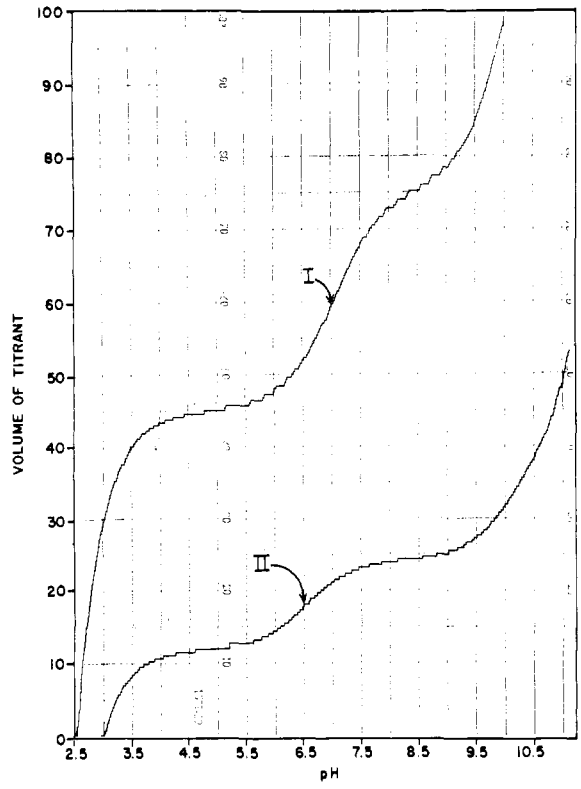


FIGURE 4: The titration curves of 5′-deoxythymidine 5′-phosphonic acid and thymidine 5′-phosphoric acid. Curve I is the titration curve of 5′-deoxythymidine 5′-phosphonic acid and curve II that of thymidine 5′-phosphoric acid. The titrant was sodium hydroxide. The apparent pK_{a2}'s are 7.0 and 6.5, respectively. The titration was carried out under nitrogen gas. The titrator was a Radiometer TTI apparatus (Radiometer, Copenhagen, Denmark).

formed with a subsequent increase in thymidine 5′-phosphate. This is consistent with a stepwise hydrolysis of the pyrophosphate bond occurring from the terminal or γ-phosphate group. However, the principal first product formed by acid hydrolysis of 5′-deoxythymidine 5′-phosphonylpyrophosphate (Figure 6) is not the nucleoside phosphonylphosphate, but rather 5′-deoxythymidine 5′-phosphonic acid, that is, the hydrolysis does not occur sequentially from the γ-phosphate group. From the titration data of both derivatives it is apparent that 5′-deoxythymidine 5′-phosphonic acid is a weaker acid than thymidine 5′-phosphate. Consistent with this view is the possibility that during acid hydrolysis of 5′-deoxythymidine 5′-phosphonylpyrophosphate protonation occurs not only on the terminal pyrophosphate bridge oxygen but also on the phosphonylpyrophosphate oxygen. Protonation of this latter bond would facilitate its hydrolysis resulting in the formation of 5′-deoxythymidine 5′-phosphonic acid and pyrophosphate.

In preliminary studies, 5′-deoxythymidine 5′-phosphonylpyrophosphate was tested for its ability to func-

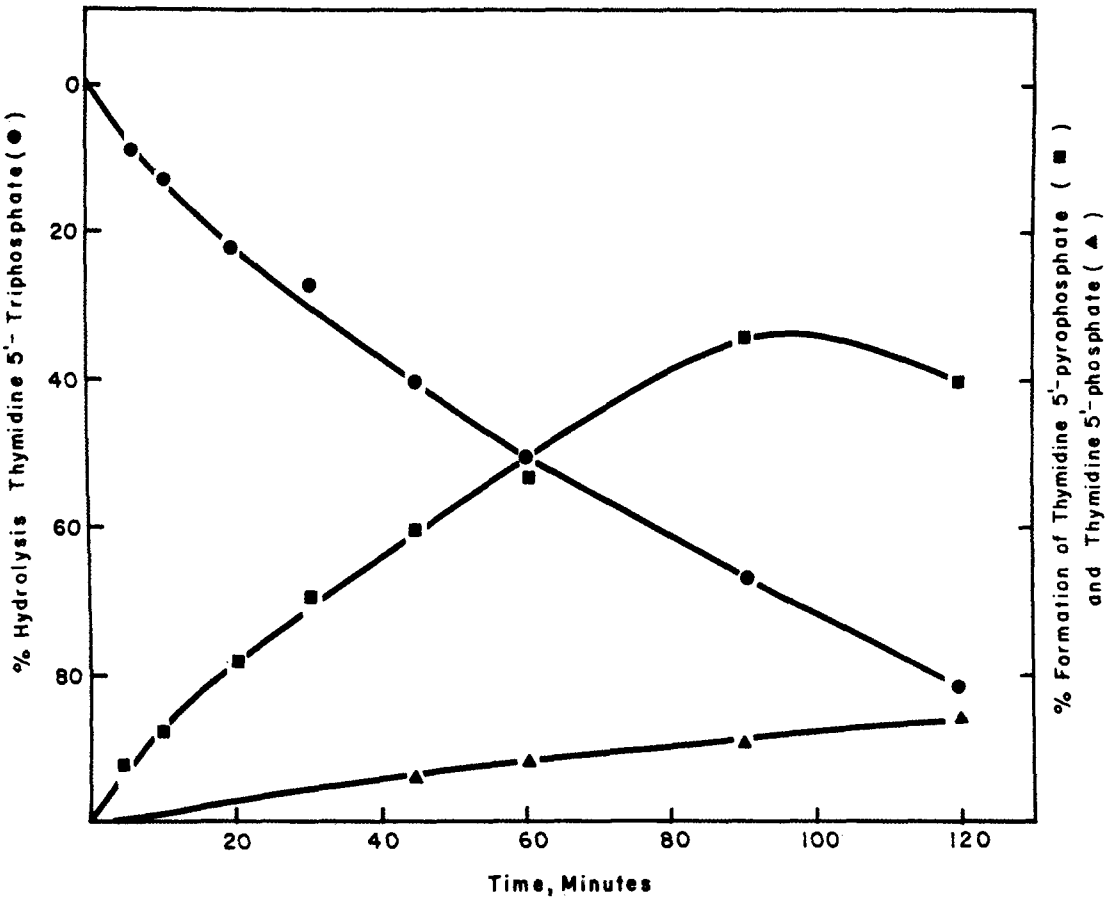


FIGURE 5: The acid hydrolysis of thymidine 5′-triphosphate. The nucleotide (2.0 μmoles/ml) in hydrochloric acid (0.5 N) was kept at 50° and aliquots were removed, neutralized with cold dilute ammonium hydroxide, and chromatographed in solvent A.

tion as a substrate for the DNA polymerase of *E. coli*. The polymerase activity was measured by the method of Richardson *et al.* (1964). In Table I are noted the results of an experiment in which 5′-deoxythymidine 5′-phosphonylpyrophosphate was examined for its ability to substitute for thymidine 5′-triphosphate[3] (TTP) in the synthesis of deoxyadenylylthymidylyl (dAT) copolymers. It has been previously demonstrated that the synthesis of dAT copolymer requires the presence of both dATP and TTP (Schachman *et al.*, 1960). Thus when [^{32}P]dAT was incubated with the polymerase in the absence of TTP, essentially no ^{32}P radioactivity could be detected in the acid-insoluble fraction. The addition of 5′-deoxythymidine 5′-phosphonylpyrophosphate to the reaction mixture in place of TTP failed to stimulate the incorporation of radioactivity into the acid-insoluble precipitate even when enzyme levels were raised ten times. Thus under standard polymerization conditions, it appears that 5′-deoxythymidine 5′-phosphonylpyrophosphate is not able to substitute for TTP in the polymerization reaction with DNA polymerase.

It has been observed (Simon and Myer, 1961) that 5′-adenylylmethylene diphosphonate, an analog of adenosine 5′-pyrophosphate (ADP), can inhibit the polynucleotide phosphorylase catalyzed polymerization of ADP. It was of interest, therefore, to determine whether 5′-deoxythymidine 5′-phosphonylpyrophosphate could serve as an inhibitor of the dAT copolymer formation by DNA polymerase. The results of an experiment testing this possibility are noted in Table II. In these studies, the concentration of TTP in the standard polymerization mixture was reduced fivefold and the effect of increasing concentrations of 5′-deoxythymidine 5′-phosphonylpyrophosphate on the incorporation of TTP into dAT copolymer was observed. The results are presented in Table II and indicate that a small degree of inhibition is found only at high concentrations of the inhibitor. These results suggest that the binding site for TTP on the polymerase protein is unable to bind 5′-deoxythymidine 5′-phosphonylpyrophosphate efficiently. Although there is no direct evidence for this notion, it seems unlikely that the inability of DNA polymerase to utilize 5′-deoxythymi-

[3] Abbreviations used: TTP, thymidine 5′-triphosphate; dAT, deoxyadenylylthymidylyl copolymers; ADP, adenosine 5′-pyrophosphate; AMP, adenosine monophosphate.

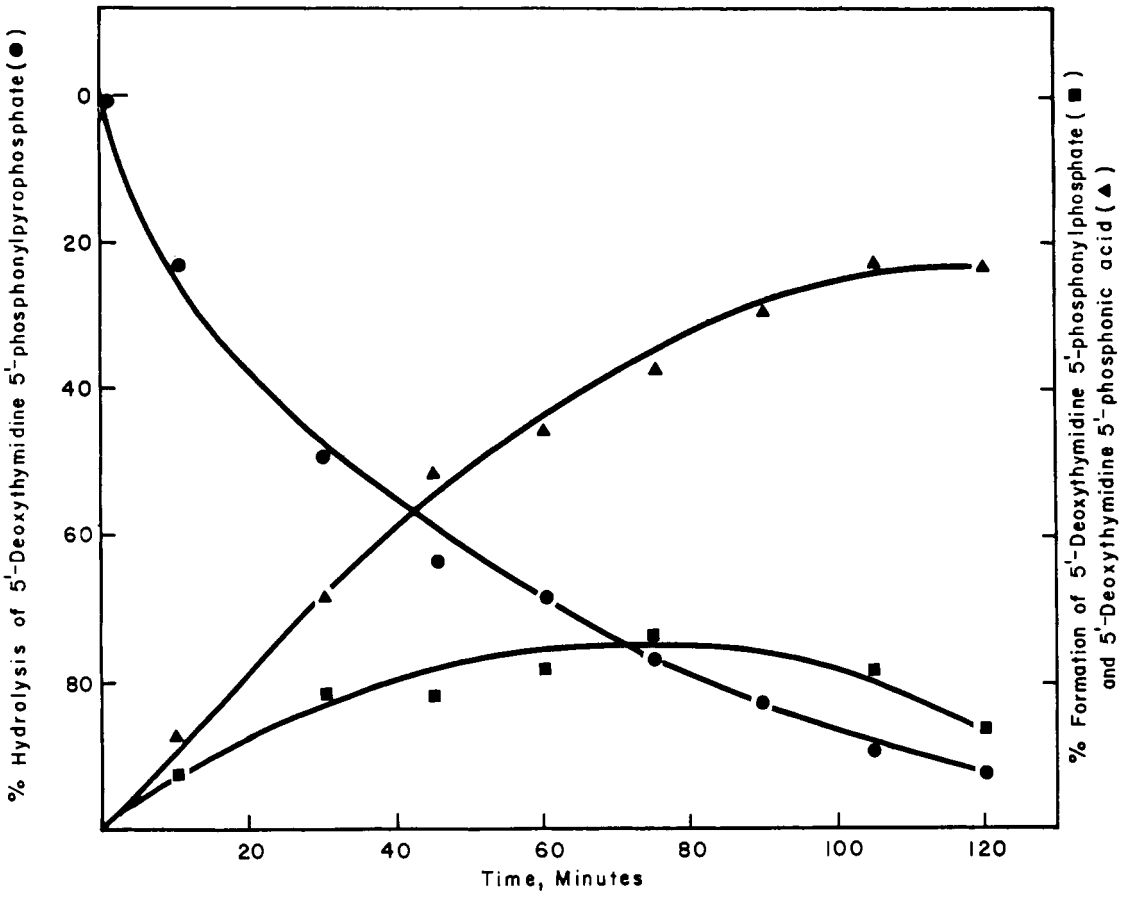


FIGURE 6: The acid hydrolysis of 5′-deoxythymidine 5′-phosphonic acid. The conditions used for its hydrolysis and analysis are similar to those described in Figure 5.

dine 5′-phosphonylpyrophosphate can be solely related to the phosphonylphosphate (pyrophosphate) bond, since the isosteric phosphonate, 5′-adenylmethylene diphosphonate, can substitute for ATP in the RNA polymerase reaction (Simon *et al.*, 1965). The absence of an oxygen atom at C-5′ of the deoxyribose will shorten the distance relationship between the reactive pyrophosphate linkage and the thymidine group. If the predominant element in the binding of the substrate to the enzyme is the deoxynucleoside moiety, this shortening could prevent the proper positioning of the phosphonylphosphate group for binding and reaction. In order to clarify these possible different effects, enzymatic studies using both 5′-deoxythymidine 5′-phosphonylpyrophosphate and the 5′-phosphonylpyrophosphate of homothymidine [1-(2,5-dideoxy-β-D-*erythro*-ribofuranosyl)thymine] should be undertaken.

Concluding Remarks

The utility of synthetic mononucleotide derivatives and oligonucleotides for chemical, physiochemical, and biological studies has been adequately demonstrated (Khorana, 1961; Michelson, 1963) and has assisted considerably in the understanding of a variety of diverse biological problems (Söll *et al.*, 1966). In general, synthetic phosphonic acid derivatives of biologically important substances have not been readily available and, in particular, the lack of nucleoside phosphonic acids has precluded biological studies with these analogs of nucleic acid precursors. The preparation of 5′-deoxythymidine 5′-phosphonic acid and 5′-deoxyuridine 5′-phosphonic acid (Yengoyan and Rammler, 1966) has made these substances available for a variety of biological studies. Thus, in the deoxyribonucleotide series, phosphonate oligonucleotides prepared from 5′-deoxythymidine 5′-phosphonic acid (D. H. Rammler and P. C. Bax, to be published) are now available and their chemistry and biochemistry is being actively investigated.

Experimental Section

Materials and Methods. Triallylphosphite and 1,1′-carbonyldiimidazole were products of Aldrich Chemical Co., Milwaukee, Wis. *E. coli* alkaline phosphomonoesterase and *Crotalus adamanteus* diesterase were obtained from Worthington Biochemicals, Freehold, N. J. Silicic acid was obtained from Brinkman Instruments, Westbury, N. Y. Paper chromatography and

TABLE I: Effect of 5′-Deoxythymidine 5′-Phosphonylpyrophosphate on the DNA Polymerase of *E. coli*.[a,b]

Additions	Omissions	[^{32}P]AMP Inc (cpm)
None	None	4173
None	TTP	19
None	Enzyme	8
TTP*	TTP	19
TTP* + 1.6 units of enzyme	TTP	35

[a] When indicated TTP was replaced by TTP* (5′-deoxythymidine 5′-phosphonylpyrophosphate) (10 mμmoles). Unless otherwise noted, all assays were done with 0.16 unit of enzyme. The specific activity of [α-^{32}P]dAT was 5.3 × 10^6 cpm/μmole. The incubation mixture (0.3 ml) contained 20 μmoles of potassium phosphate buffer (pH 7.4), 2 μmoles of $MgCl_2$, 0.3 μmole of 2-mercaptoethanol, 6 mμmoles of dAT copolymer, 10 mμmoles of TTP, 10 mμmoles of [α-^{32}P]dATP, and enzyme. After incubation for 30 min at 37°, the acid-insoluble fraction was isolated by centrifugation (Lehman *et al.*, 1958). [b] A kind gift from Professor A. Kornberg, Stanford University, Calif.

TABLE II: Effect of 5′-Deoxythymidine 5′-Phosphonylpyrophosphate on the Utilization of TTP by *E. coli* DNA Polymerase.[a]

Additions	Omissions	[^{32}P]dAMP Inc (cpm)	mμmoles of TTP*: mμmole of TTP
None	None	4599	0
None	Enzyme	16	0
TTP* (20 mμmoles)	None	4366	10
TTP* (36 mμmoles)	None	4149	18
TTP* (54 mμmoles)	None	4078	27

[a] The reaction mixture was the same as described in Methods (Table I) except that the concentration of TTP was reduced to 2 mμmoles. When indicated TTP* (5′-deoxythymidine 5′-phosphonylpyrophosphate) was added. All assays were done with 0.30 unit of enzyme. The specific activity of [^{32}P]dATP was 4.3 × 10^6 cpm/μmole. Incubation was carried out for 30 min at 37°.

electrophoresis were carried out using S & S No. 589 orange ribbon paper, a product of Schleicher & Schuell, N. H. The paper chromatographic solvents were: solvent A, isopropyl alcohol–ammonium hydroxide–water (7:1:2, v/v); solvent B, *n*-propyl alcohol–water–ammonium hydroxide (6:1:3, v/v); and solvent C, isobutyric acid–ammonia hydroxide–water (57:4:39, v/v). Paper electrophoresis was carried out using the following buffer systems: ammonium bicarbonate (0.05 M, pH 7.5) and sodium acetate buffer (0.05 M, pH 4.0). Phosphorus analysis was performed using the methods of Ames and Dubin (1960) and of Fiske and Subbarow (1925) as indicated in the text. All phosphorus values are related to thymidine 5′-monophosphate. The R_F values and electrophoretic mobilities of 5′-deoxythymidine 5′-phosphonic acid and its derivatives are contained in Table III. The enzymatic hydrolysis with venom diesterase and alkaline phosphomonoesterase was carried out at 37° in Tris·HCl buffer (0.1 ml, pH 8.0, 0.1 M) containing the nucleoside phosphonic acid derivative (2 μmoles) and enzyme (0.01 mg). The reaction solutions were incubated at 37° for 5 hr and then analyzed by paper chromatography in solvent B.

5′-Deoxy-5′-O-p-toluenesulfonylthymidine. This substance was prepared essentially as described by Michelson and Todd (1955), mp 172° dec, lit. mp 172° dec.

5′-Deoxy-5′-iodothymidine. This substance was prepared as described by Michelson and Todd (1955), mp 166° dec, lit. mp 168° dec.

3′-O-Acetyl-5′-deoxy-5′-iodothymidine. To an ice-cold solution of 5′-deoxy-5′-iodothymidine (356 mg, 1 mmole) in dry pyridine (5 ml) was added acetic anhydride (2 ml). After 18 hr at room temperature, a small amount of ice was added and after 30 min the total mixture was extracted with chloroform. The chloroform solution was reextracted with water and was then dried over anhydrous sodium sulfate. The dried chloroform solution was evaporated under vacuum and the residual oil was chromatographed on a silicic acid column developed with 5% methanol in chloroform. The principal ultraviolet-absorbing band was isolated and crystallized from methanol–ether–petroleum ether (bp 30–60°) in 80% yield (0.322 g, mp 129–131°). This substance, prepared by an alternate procedure (Michelson and Todd, 1955), has a melting point of 131°.

Diallyl 5′-Deoxythymidine 5′-Phosphonate. A solution of triallyl phosphite (15 ml) containing anhydrous 3′-*O*-acetyl-5′-deoxy-5′-iodothymidine (1.51 g, 4.0 mmoles) was heated to 130°. Dry nitrogen gas saturated with triallyl phosphite at 130° was bubbled through the solution to entrain any allyl iodide formed in the reaction. After 30 hr at 130°, about 90% of the starting material had reacted. The reaction mixture was cooled and the excess triallyl phosphite was removed by evaporation under high vacuum. The remaining oil (10.1 g) containing as side products diallyl allylphosphonate and triallyl phosphate was chromatographed on silicic acid (400 g). The column was de-

TABLE III: R_F Values of the Nucleoside Phosphonates and Derivatives.

	Chromatographic Solvent Systems		
	A	B	C
Diallyl 5′-deoxythymidine 5′phosphonate	0.83	—	0.93
Di-*n*-propyl 5′-deoxythymidine 5′-phosphonate	0.81	—	0.91
n-Propyl 5′-deoxythymidine 5′-phosphonate	0.68	—	0.85
5′-Deoxythymidine 5′-phosphonic acid	0.20	0.27	0.59
5′-Deoxythymidine 5′-phosphonylphosphate	0.13	0.15	0.25
5′-Deoxythymidine 5′-phosphonylpyrophosphate	0.07	0.10	0.15
Thymidine 5′-phosphate	0.19	0.25	0.41

	Electrophoretic Mobilities Relative to Thymidine 5′-Phosphate	
	pH 7.5	pH 4.0
5′-Deoxythymidine 5′-phosphonic acid	0.94	0.95
5′-Deoxythymidine 5′-phosphonylphosphate	1.24	1.62
5′-Deoxythymidine 5′-phosphonylpyrophosphate	1.39	2.20
n-Propyl 5′-deoxythymidine 5′-phosphonate	0.52	1.05

veloped with chloroform until all of the nonultraviolet-absorbing side products were eluted. Methanol was next passed through the column and 87% of the ultraviolet-absorbing material was eluted. Removal of the methanol by evaporation yielded an oil (1.1 g) which contained three ultraviolet-absorbing bands as evidenced by chromatography on preparative thin layer silicic acid plates (five plates, 1 m × 20 cm) developed with 7% methanol in chloroform. Good resolution of the ultraviolet-absorbing reaction products was obtained when the solvent was run about one-third of the plate distance initially, dried at room temperature, rechromatographed with the solvent front running two-thirds of the plate distance, dried, and finally chromatographed with the solvent front extending to the top of the plate. By chromatographing in this fashion four distinct ultraviolet bands were obtained. The band moving with an R_F just behind the starting material was removed and the phosphonate isolated by repeated extraction of the silicic acid with methanol. A light yellow oil (0.7 g) remained after removal of the solvent by evaporation. Examination of the oil by means of its nuclear magnetic resonance (nmr) spectrum in deuteriochloroform demonstrated the presence of the expected C-5′ methylene protons ($CH_2P(=O)$) centered at δ 2.28 as a multiplet, the methyl protons of the C-3′ *O*-acetyl group ($CH_3C(=O)$) at δ 2.15 as a singlet, and the methylene ester protons ($(=CH-CH_2O)_2P(=O)$) at δ 4.6 as a quartet. The nmr spectrum indicated the presence of a small amount of impurity which was also demonstrated by a high phosphorus:thymine ratio (ϵ:P 6500), λ_{max}^{MeOH} 267 mμ, λ_{min}^{MeOH} 232 mμ.

Diallyl 5′-Deoxythymidine 5′-Phosphonate. The oil (0.7 g) containing diallyl 5′-deoxythymidine 5′-phosphonate was dissolved in methanol (5 ml) and ammonium hydroxide was added (5 ml, 28%). After 1 hr at room temperature, the solvent was evaporated to dryness and the residue was chromatographed on silicic acid (50 g). The developing solvent was a 10% methanol–chloroform solution. The peak containing the ultraviolet-absorbing component yielded a clear oil (0.64 g, 1.6 mmoles) after evaporation of the solvent, λ_{max}^{MeOH} 268 mμ, λ_{min}^{MeOH} 234 mμ (ϵ:P 9300). Its nmr spectra showed the expected C-5′ methylene protons ($CH_2P-(=O)$) centered at δ 2.28 as a multiplet. The methyl protons of the acetyl group ($CH_3C(=O)$) were absent. The infrared spectra of this substance revealed the presence of the phosphonate ester linkage ($P(=O)OCH$) at 1070 cm^{-1} and the semipolar oxygen bond ($P(=O)$) at 1255 cm^{-1}.

5′-Deoxythymidine 5′-Phosphonic Acid. Diallyl 5′-deoxythymidine 5′-phosphonate (0.64 g, 1.6 mmoles) in methanol (10 ml) was added to a suspension of (25 mg) prereduced 5% palladium on barium sulfate (Kuhn and Hass, 1955) in 50% acetic acid (10 ml). After 3 hr of hydrogenation, the catalyst was removed by centrifugation, washed with water, and recentrifuged. The combined supernatants were evaporated to dryness and the residual oil was dissolved in water (15 ml) containing 1 drop of 9 M ammonium hydroxide. This solution was chromatographed on a column (2.5 × 25 cm) of DEAE-Sephadex (A-25) in the bicarbonate form. Elution was carried out using a linear gradient of triethylammonium bicarbonate at pH 7.5. The mixing vessel contained 500 ml of 0.05 M triethylammonium bicarbonate and the reservoir contained 500 ml of 0.2 M triethylammonium bicarbonate. The flow rate was 1 ml/min and 17-ml fractions were collected. Three principal ultraviolet-absorbing peaks were obtained. The initial neutral peak (13%) contained di-*n*-propyl 5′-deoxythymidine 5′-phosphonate. This material was characteristically labile to alkali (Yengoyan and Rammler, 1966). Using standard enzymatic assay conditions (see Methods), this material was completely stable to the action of the diesterase from *C. adamanteus* venom, λ_{max}^{MeOH} 268 mμ; λ_{min}^{MeOH} 233 mμ (ϵ:P 9100). It did not migrate on paper electrophoresis at pH 4 and 7.0. Peak II contained *n*-propyl 5′-deoxythymidine 5′-phosphonate (47%). This substance was homogeneous in solvents A and B and migrated as a singly charged species at pH 7.5 and 4.0. *n*-Propyl

5′-deoxythymidine 5′-phosphonate prepared from 5′-deoxythymidine 5′-phosphonic acid and *n*-propyl alcohol (Rammler and Khorana, 1962) was identical with the material obtained from peak II in every respect.

Peak III contained 5′-deoxythymidine 5′-phosphonic acid (39%). After removal of the eluent by evaporation under vacuum, the residual oil was dissolved in ammonium carbonate (1 ml, 0.1 M, pH 8.5) and *E. coli* alkaline phosphomonoesterase (0.2 mg) was added. After 12 hr at 37°, it was chromatographed on DEAE-Sephadex (A-25) as described above. Two ultraviolet-absorbing fractions were obtained. The first contained thymidine (2%) and the second 5′-deoxythymidine 5′-phosphonic acid. This substance was completely homogeneous as evidenced by paper chromatography in solvents A and B and by paper electrophoresis at pH 7.5 and 4.0. At pH 7.5, its mobility was 0.94 that of thymidine 5′-phosphate, as expected for a phosphonic acid derivative. Its ultraviolet spectrum was similar to thymidine 5′-phosphate with a $\lambda_{max}^{pH\ 7.5}$ 267 mμ and a $\lambda_{min}^{pH\ 7.5}$ 7.5 232 mμ. Phosphorus analysis indicated essentially 1 mole of phosphorus/mole of thymine. The ε:P is 9300.

5′-Deoxythymidine 5′-Phosphonylphosphate. 5′-Deoxythymidine 5′-phosphonic acid (0.02 mmole) as its monotri-*n*-butylammonium salt was dissolved in dry dimethylformamide (1.2 ml). To this solution was added 1,1′-carbonyldiimidazole (32 mg) and the mixture was shaken until a solution was obtained. The tightly stoppered reaction mixture was kept in a desiccator at room temperature for 18 hr (Hoard and Ott, 1964). After this time, anhydrous tri-*n*-butylammonium phosphate (0.2 mmole) in dry dimethylformamide (1.1 ml) was added to the reaction mixture. After 18 hr, ice-cold water (10 ml) was added and the solution was kept cold for 1 hr. Ammonium hydroxide (0.3 ml, 28%) was then added and the solution was chromatographed on a DEAE-Sephadex (A-25) column as described previously (Yengoyan and Rammler, 1966) with the exception that the elution was carried out at 0°. Two ultraviolet-absorbing fractions were obtained from the column. The initial peak contained unreacted 5′-deoxythymidine 5′-phosphonic acid (55%) while the second peak eluted at a salt concentration of 0.17 M triethylammonium bicarbonate was identified as 5′-deoxythymidine 5′-phosphonylphosphate (40%). The product was homogeneous at pH 4.0 and 7.5 and it migrated as a dianion at the former pH and as a trianion at the latter pH. It was completely hydrolyzed in 1 N HCl at 100° for 7 min and yielded 1 mole of inorganic phosphate/mole of 5′-deoxythymidine 5′-phosphonate, $\lambda_{max}^{pH\ 7.5}$ 266 mμ, $\lambda_{min}^{pH\ 7.5}$ 231 mμ (ε:P 5200).

5′-Deoxythymidine 5′-Phosphonylpyrophosphate. The conditions for the formation of this derivative were the same as described above. The imidazolidate was prepared by the reaction of 1,1′-carbonyldiimidazole (70 mg) and the monotri-*n*-butylammonium salt of 5′-deoxythymidine 5′-phosphonic acid (0.04 mmole) in dry dimethylformamide (10 ml). After 8 hr at room temperature, an anhydrous solution of ditri-*n*-butylammonium pyrophosphate (0.2 mmole) in dimethylformamide was added. After 18 hr, the reaction mixture was worked up as described above. The DEAE-Sephadex (A-25) column was eluted with triethylammonium bicarbonate (pH 7.5, 200 ml, 0.06 M, in the mixing vessel; 200 ml, 0.55 M in the reservoir). Four ultraviolet-absorbing fractions were obtained after chromatography. The initial fraction came off at a salt concentration of 0.06 M and contained 5′-deoxythymidine 5′-phosphonic acid (35%). The second fraction was obtained at a salt concentration of 0.15 M and contained 5′-deoxythymidine 5′-phosphonylphosphate (10%). The third fraction eluted with 0.35 M triethylammonium bicarbonate and contained 5′-deoxythymidine 5′-phosphonylpyrophosphate (40%). The fourth fraction was eluted with 0.46 M triethylammonium bicarbonate and is tentatively assumed to be 5′-deoxythymidine 5′-phosphonyltriphosphate on the basis of its electrophoretic mobility. 5′-Deoxythymidine 5′-phosphonylpyrophosphate was shown to be homogeneous in solvents A and B and its R_F values were consistent for its structure. On paper electrophoresis at pH 4, this substance moved as a trianion and at pH 7.5 as a tetraanion. Enzymatic hydrolysis with alkaline phosphomonoesterase yielded 2 moles of inorganic phosphate (Fiske and Subbarow, 1925) per mole of 5′-deoxythymidine 5′-phosphonic acid. Treatment with 1 N HCl for 7 min at 100° completely hydrolyzed the pyrophosphate bonds, $\lambda_{max}^{pH\ 7.5}$ 265 mμ, $\lambda_{min}^{pH\ 7.5}$ 231 mμ (ε:P 3600).

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N^6-(Δ^2-Isopentenyl)adenosine. A Component of the Transfer Ribonucleic Acid of Yeast and of Mammalian Tissue, Methods of Isolation, and Characterization*

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ABSTRACT: A nucleoside has been isolated from yeast transfer ribonucleic acid (tRNA) which has been identified as 6-*N*-(3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine (I). tRNA (60 g) was hydrolyzed enzymatically to its constituent nucleosides and the hydrolysate was subjected to partition chromatography on a column. The separated nucleoside was isolated and crystallized (15 mg). The nucleoside was assigned structure I on the basis of elemental analysis, mass spectroscopy, and nuclear magnetic resonance (nmr) spectroscopy. Compound I was synthesized by condensing γ,γ-dimethylallylamine with 6-chloro-9-β-D-ribofuranosylpurine. The natural compound was identical with the synthetic nucleoside. In mild acid solution I loses the ribose residue and undergoes hydration of the allylic double bond to form 6-*N*-(3-hydroxy-3-methylbutylamino)purine which on further acid treatment yields 3H-7,7-dimethyl-7,8,9-trihydropyrimido-[2,1-*i*]purine. Mild permanganate oxidation of I yields 6-*N*-(2,3-dihydroxy-3-methylbutylamino)-9-β-D-ribofuranosylpurine. In concentrated ammonium hydroxide I is converted to adenosine. Compound I exhibits potent cytokinin activity; that is, it stimulates cell division and cell differentiation in plant systems.

Transfer ribonucleic acid contains many components which are structural modifications of the four major nucleosides. Methylation represents the most common form of modification known and the occurrence of 19 different methylated nucleosides in tRNA has now been reported. The function of the methylated components of RNA remains unknown but from physicochemical considerations the methyl groups could be instrumental in maintaining the secondary and tertiary structure of RNA molecules. Other types of modified nucleosides exist in which a larger and chemically more active group than a methyl is attached to the parent nucleoside, as for example, 2′-(3′)-*O*-ribosyladenosine (Hall, 1965), N^6-(aminoacyl)adenosine (Hall and Chheda, 1965), and N^4-acetylcytidine (Zachau *et al.*, 1966a). These nucleosides represent nucleic acid components that are capable of undergoing a variety of unique biochemical reactions.

We recently isolated from yeast tRNA another nucleoside containing a functional group attached

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