Synthesis of Some Nucleotides Derived from 3′-Deoxythymidine*

Alan F. Russell† and J. G. Moffatt

ABSTRACT: Phosphorylation of 3′-deoxy-3′-iodothymidine gives the corresponding 3′-deoxy-3′-iodothymidine 5′-phosphate in high yield. Activation of the phosphate group can be achieved by formation of the phosphoromorpholidate under anhydrous conditions, and subsequent condensation with tributylammonium pyrophosphate in anhydrous dimethyl sulfoxide gives 3′-deoxy-3′-iodothymidine 5′-triphosphate in modest yield. The latter reaction is complicated by simultaneous dehydrohalogenation giving the related 2′,3′-unsaturated nucleoside 5′-triphosphate and by extensive intramolecular displacement of iodide ion by phosphate giving a 3′,5′-cyclic phosphate with the 2-deoxy-β-D-*threo*-pentofuranosyl configuration. The same spectrum of products is obtained using 3′-deoxy-3′-iodothymidine 5′-phosphoroimidazolate prepared from the parent nucleoside and triimidazolephosphine oxide. The various products are characterized by enzymatic and spectroscopic techniques, and reduction of either the iodotriphosphate or the unsaturated triphosphate with hydrogen and palladium gives 3′-deoxythymidine 5′-triphosphate, the enzymatic properties of which are discussed in the accompanying paper. Phosphorylation of 1-(2-deoxy-β-D-*threo*-pentofuranosyl)thymine with diphenyl phosphorochloridate gives the crystalline 5′-diphenyl phosphate ester that can be converted with base into the same 3′,5′-cyclic phosphate obtained as a by-product during preparation of the triphosphates above. A pair of 3′,5′-cyclic phosphate triesters diastereoisomeric about their phosphorus atoms are intermediates in this cyclization reaction.

In recent years, much attention has been devoted to the study of compounds which inhibit the enzymatic synthesis of nucleic acids. Several types of nucleoside and nucleotide analogs impede nucleic acid synthesis and, at least in some cases, the inhibition is due to incorporation of a nucleoside which cannot support further chain elongation. Thus, the naturally occurring antibiotic cordycepin (Bentley *et al.*, 1951) which was subsequently shown to be 3′-deoxyadenosine (Kaczka *et al.*, 1964) has been shown to inhibit the synthesis of both DNA and RNA in Ehrlich ascites tumor cells (Klenow, 1963; Shigeura and Gordon, 1965). Under conditions of inhibition, an intracellular accumulation of 3′-deoxyadenosine 5′-mono-, di-, and triphosphates was observed (Klenow, 1963). More recent work (Shigeura and Boxer, 1964; Shigeura and Gordon, 1965) has demonstrated *in vitro* inhibition by 3′-deoxyadenosine 5′-triphosphate of RNA synthesis catalyzed by RNA polymerase and has related the observed inhibition to the incorporation of 3′-deoxyadenosine as the 3′ terminus of the growing RNA molecule. The absence of a 3′-hydroxyl group in this position obviates further chain elongation. 3′-Deoxy-3′-aminoadenosine (Shigeura *et al.*, 1966) and 3′-deoxyguanosine (Gitterman *et al.*, 1965) also appear to function in a similar way but have not, as yet, been so extensively studied.

Although not so obvious from a mechanistic point of view, 1-(β-D-arabinofuranosyl)cytosine also appears to inhibit nucleic acid synthesis (Cohen, 1966, and references therein) and evidence has been presented (Chu and Fischer, 1968) for incorporation of the analog into both terminal and internal positions of DNA and RNA. *In vitro* studies using ara-CTP and DNA polymerase (Momparler, 1969; M. R. Atkinson and A. Kornberg, unpublished observations), however, have shown that incorporation into DNA occurs only in the terminal position.

In a related study (Doering *et al.*, 1966; Toji and Cohen, 1969) 2′,3′-dideoxyadenosine has been shown to produce a lethal inhibition of DNA synthesis in an *Escherichia coli* auxotroph. Simultaneous addition of 2′-deoxyadenosine, but not of adenosine, will reverse this inhibition, but once DNA synthesis has been stopped, the effect is irreversible and is probably once again due to chain termination. 2′,3′-Dideoxycytidine and 2′,3′-dideoxyuridine, however, are nonlethal, perhaps due to these compounds not being substrates for cellular kinases.

Some years ago the synthesis of 3′-deoxythymidine 5′-phosphate (**1a**) and of the closely related 2′,3′-dideoxyuridine 5′-phosphate (**1b**) was described from this laboratory (Pfitzner and Moffatt, 1964). It was, thus, of interest to prepare isotopically labeled 3′-deoxythymidine 5′-triphosphate (**2**) and to examine in detail the effects of this compound upon reactions catalyzed by *E. coli* DNA polymerase (EC 2.7.7.7). In this paper, we report the synthesis of **2** and of some related compounds while in the accompanying paper (Atkinson *et al.*, 1969), a variety of enzymatic studies on (**2**) labeled with tritium in the sugar moiety are described.

Since a final product with a quite high specific activity was required for enzymatic studies, we preferred to introduce this label at as late a stage as possible in the synthesis. With this in mind, we undertook the synthesis of 3′-deoxy-3′-iodothymidine 5′-triphosphate (**7**), a compound in which the 3′-iodo function should readily be replaced by tritium by palladium-catalyzed reduction in tritium gas. Thus, 3′-deoxy-3′-iodothymidine (**3**) (Michelson and Todd, 1955), which is more conveniently prepared from 5′-*O*-tritylthymidine

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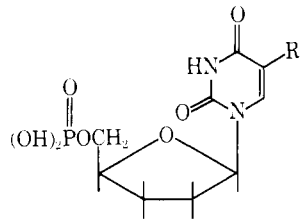
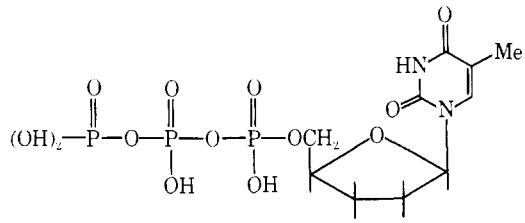
† Syntex Postdoctoral Fellow, 1968–1969.



1a, R = Me
b, R = H



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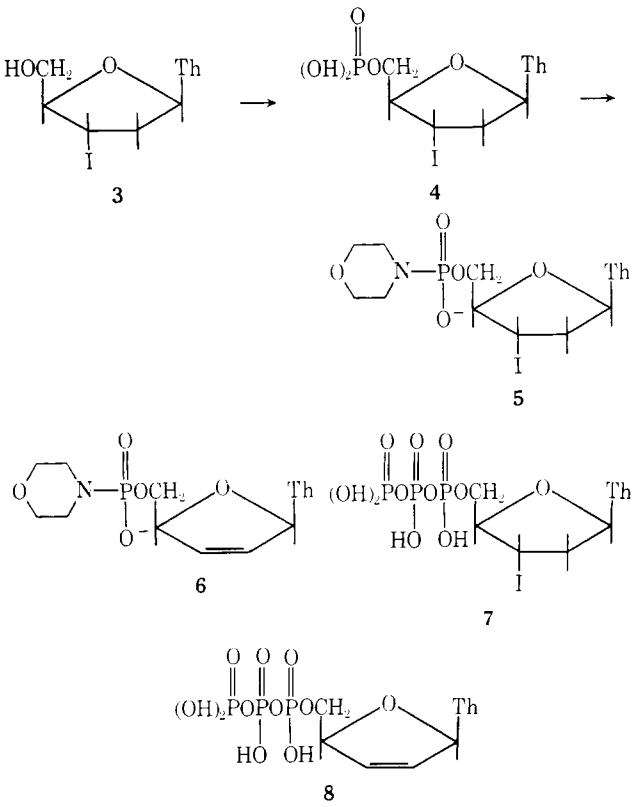
and methyltriphenoxyphosphonium iodide (Verheyden and Moffatt, 1964, 1969) followed by acidic hydrolysis, was phosphorylated using 2-cyanoethyl phosphate and dicyclohexylcarbodiimide[1] (Tener, 1961) giving 3′-deoxy-3′-iodothymidine 5′-phosphate (**4**) in 91% yield. Attempts to convert **4** into 3′-deoxy-3′-iodothymidine 5′-phosphoromorpholidate (**5**) by the usual reaction with morpholine and DCC[1] in refluxing aqueous *t*-butyl alcohol (Moffatt and Khorana, 1961) led to extensive decomposition probably due to either dehydrohalogenation or cyclonucleoside formation (see below) in the presence of morpholine. The desired morpholidate (**5**) could, however, be obtained in 66% yield by reaction of **4** with morpholine and DCC in anhydrous pyridine at room temperature for 20 hr. Extension of the reaction time led to a reduction in the yield of **5** due to formation of 2′,3′-didehydro-3′-deoxythymidine 5′-phosphoromorpholidate (**6**) presumably resulting from dehydrohalogenation of **5** by the strongly basic 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine formed as a by-product (Moffatt and Khorana, 1961). The unsaturated morpholidate (**6**) behaved as a monoanion during electrophoresis and was identified by its very rapid oxidation by permanganate on chromatograms and by its nuclear magnetic resonance spectrum which clearly showed the presence of a 2′,3′-unsaturated sugar. Thus, $C_{1'}H$ appeared as a broad singlet at 6.85 ppm rather than as the usual triplet, and the 2′ and 3′-vinyl protons appeared far downfield as broadened doublets at 6.40 and 5.90 ppm, respectively.

The reaction of the phosphoromorpholidate (**5**) with tributylammonium pyrophosphate in anhydrous Me_2SO (Moffatt, 1964) did not give the usual spectrum of products expected for such a reaction. After a 20-hr reaction time, paper chromatography indicated the formation of the desired triphosphate together with quite a large amount of unreacted **5**. Prolonged reaction, however, led to no further decrease in the amount of morpholidate present. Ion-exchange chromatography of the mixture after various periods of reaction showed roughly 10–20% of a neutral compound with an ultraviolet spectrum similar to thymidine, a large peak (30–50%) of a monoanionic material similar to **5**, a small amount of 3′-deoxy-3′-iodothymidine 5′-phosphate (5–10%), and two symmetrical peaks totalling 25–30% in the region of the expected 3′-deoxy-3′-iodothymidine 5′-triphosphate (**7**). The relative proportions of these two peaks, both of which showed a phosphorus:thymidine ratio of 3:1 and were homogeneous and distinct from one another by paper chromatography, appeared to vary with time. Thus, reactions carried out at room temperature for 2–4 days showed roughly equal amounts of the two triphosphate peaks while a similar reaction subsequently heated at 37° for a further 3 days showed only a single product corresponding to the first of those peaks. Upon hydrogenation using a palladium catalyst, both triphosphates were converted into a chromatographically common product which was identified as 3′-deoxythymidine 5′-triphosphate (**2**). Treatment of **2** from either source with purified snake venom phosphodiesterase gave a product indistinguishable from 3′-deoxythymidine 5′-phosphate (**1a**) while exhaustive incubation with *E. coli* alkaline phosphatase gave 3′-deoxythymidine (Michelson and Todd, 1955) which was identified by thin-layer and paper chromatography.

Incubation of the second triphosphate peak with venom phosphodiesterase gave a single product which was chromatographically identical with 3′-deoxy-3′-iodothymidine 5′-phosphate (**4**), thus providing evidence that it is the desired iodo triphosphate (**7**). This was confirmed by treatment of **7** with *E. coli* alkaline phosphatase which led to the direct crystallization of 3′-deoxy-3′-iodothymidine (**3**) from the incubation mixture. Similar incubation of the first triphosphate peak with venom diesterase gave a nucleotide that was chromatographically distinct from **4**, and complete dephosphorylation gave a product identical with an authentic sample of 2′,3′-didehydro-3′-deoxythymidine (**11**) (Horwitz *et al.*, 1966). The first triphosphate peak is thus shown to be 2′,3′-didehydro-3′-deoxythymidine 5′-triphosphate (**8**), its formation being presumably due to dehydrohalogenation of the initially formed iodo triphosphate (**7**) by excess tributylamine during prolonged reactions. Dehydrohalogenation probably did not occur during preparation of the anhydrous triethylammonium salt of **5** since the nuclear magnetic resonance spectrum of a similarly treated sample in Me_2SO showed the presence of no olefinic protons in the 5.5–6.5 ppm range and $C_{1'}H$ still appeared as a triplet at 6.18 ppm. The structure of **8** was confirmed by nuclear magnetic resonance spectroscopy in D_2O, the $C_{1'}$ proton appearing as a tight multiplet at 6.92 ppm showing small (1–3 Hz) vicinal, allylic, and homoallylic couplings to $C_{2'}H$, $C_{3'}H$, and $C_{4'}H$. The 2′ and 3′ protons appeared as multiplets at 6.50 and 5.91 ppm, the various assignments being verified by spin decoupling. All features of the spectrum of **8** were very similar to those of the spectrum of authentic 2′,3′-didehydro-3′-deoxythymidine (see Experimental Section) and leave no doubt as to the nature of the unsaturated nucleotide.

It might also be pointed out that prolonged incubation of both the 3′-deoxy triphosphate (**2**) and the 3′-iodo triphosphate (**7**) with crude *Crotalus adamanteus* venom leads to complete digestion to the corresponding nucleosides. On the other hand, the 2′,3′-unsaturated triphosphate (**8**) is degraded exclusively to the 5′-monophosphate. The observed resistance of the latter nucleotide toward the 5′-nucleotidase activity of crude venom is similar to that shown by 2′-*O*-methyluridine 5′-phosphate (Honjo *et al.*, 1964). Complete

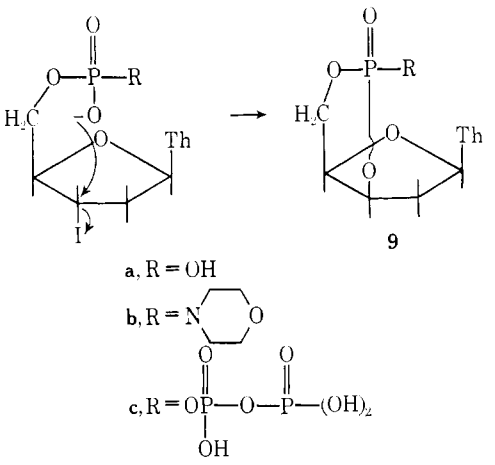
[1] Dicyclohexylcarbodiimide is hereafter abbreviated as DCC.



conversion of thymidine 5′-triphosphate into thymidine was not prevented in the presence of **8**.

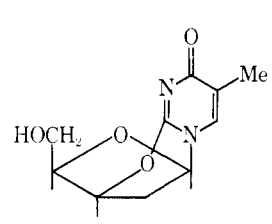
The striking incompleteness of the triphosphate-forming reaction became clear with the realization that the large amount of monoanionic material, thought at first to be unreacted **5**, was, in fact, distinguishable from **5** by paper chromatography, moving slower in solvent I. It was, however, clearly a monoanion, with an electrophoretic migration just greater than that of thymidine-5′-phosphoromorpholidate at pH 7.6. (As expected, **5** moved just slower than thymidine 5′-phosphoromorpholidate due to its increased mass). It was extremely stable to both acidic and alkaline hydrolysis and to the action of both crude snake venom and *E. coli* alkaline phosphatase. The most reasonable structure for this compound would appear to be 1-(2-deoxy-β-D-*threo*-pentofuranosyl)thymine 3′,5′-cyclic phosphate (**9a**) arising *via* intramolecular nucleophilic displacement of the 3′-iodo function by phosphate anion. Such a displacement could take place at the level of the iodomorpholidate **5**, the free nucleotide (**4**) arising by hydrolysis of **5** or, indeed, the triphosphate **7** giving the cyclic derivatives **9a–c**.

Mechanistically, the free 5′-phosphate (**4**) would appear to be the most likely precursor of **9a** since, unlike the morpholidate (**5**), it contains a more highly nucleophilic phosphate dianion. Indeed, a solution of the triethylammonium salt of **4** in anhydrous Me_2SO led to quite rapid formation of **9a** as shown by paper chromatography. Also, the major by-product arising during preparation of **5** in anhydrous pyridine appears to be **9a**. The conversion of **4** into **9a** is probably facilitated by the use of a dipolar aprotic solvent such as Me_2SO which is known to promote nucleophilic displacements (Parker, 1965). This view is supported by the apparent stability of **4** as the calcium salt or in aqueous solution where it is strongly solvated. Displacement of iodide can also, however, occur at the morpholidate level in Me_2SO. The neutral material eluted with the water wash during ion-exchange chromatography of the products from **5** and tributylammonium pyrophosphate was shown by analysis to contain 1 mole of bound phosphate/mole of thymine and its nuclear magnetic resonance spectrum showed the presence of a morpholine moiety by broad four-proton signals at 3.20 ppm (NCH_2) and 3.65 ppm (OCH_2). This neutral material thus appears to be the cyclic phosphoromorpholidate, **9b**. We have not been able to effect selective hydrolysis of the P–N bond in **9b** under a variety of acidic conditions. As yet, we have no evidence for the formation of the cyclic triphosphate (**9c**) from the iodo triphosphate (**7**) and it is likely that such a structure would quite rapidly hydrolyze to **9a**.



In an effort to more convincingly characterize the *threo* configuration of the cyclic phosphate, **9a**, we have undertaken an independent synthesis of this compound. Treatment of 3′-deoxy-3′-iodothymidine (**3**) with 1,5-diazabicyclo[3.4.0]-nonene-5 in acetonitrile led to the direct crystallization of O^2,3′-cyclothymidine (**10**) (Michelson and Todd, 1955) which was isolated in 81% yield. Chromatography of the mother liquors also gave 6% 2′,3′-didehydro-3′-deoxythymidine (**11**) (Horwitz *et al.*, 1966). The nuclear magnetic resonance spectrum of **11** is interesting since the $C_{1'}$ proton shows an extraordinarily large homoallylic coupling with $C_{4'}H$ ($J_{1',4'}$ = 3.64 Hz) in addition to the normal expected couplings to $C_{2'}H$ ($J_{1',2'}$ = 1.74 Hz) and $C_{3'}H$ ($J_{1',3'}$ = 1.53 Hz). This spectrum has been the subject of intensive analysis and computer simulation using the LAOCN3 program by Dr. M. L. Maddox and will be described elsewhere. A similar large homoallylic coupling has been observed in the unsaturated furan antibiotic furanomycin (Katagiri *et al.*, 1967) and also appears to be present in 2′,3′,5′-trideoxy-2′,3′-didehydroadenosine (McCarthy *et al.*, 1966). Alkaline hydrolysis of **10**, which is much more convenient than the previously reported acidic treatment, then gave crystalline 1-(2-deoxy-β-D-*threo*-pentofuranosyl)thymine (**12**) (Horwitz *et al.*, 1963) in high yield. Selective phosphorylation of the primary hydroxyl group of **12** with diphenyl phosphorochloridate gave the crystalline 5′-diphenyl phosphate ester (**13**) nearly quantitatively. By analogy with earlier work on diphenyl 1,2-*O*-isopropylidine-α-D-xylofuranose 5-phosphate (Moffatt and Khorana, 1957), **13** would be expected to undergo facile intramolecular transesterification under alkaline condi-

tions with formation of the cyclic triester 1-(2′-deoxy-β-D-*threo*-pentofuranosyl)thymine 3′,5′-cyclic phenylphosphate (**14**). Treatment of **13** at room temperature with various amounts of triethylamine in dioxane led to slow transesterification, whereas dilute sodium hydroxide in dioxane gave some concomitant hydrolysis to ionic materials. Use of 1,5-diazabicyclo[3.4.0]nonene-5 in dioxane, however, led to complete conversion of **13** into two slightly more polar products in roughly equal amounts. Both of these have been isolated in crystalline form by preparative thin-layer chromatography and identified as the diastereoisomeric forms (around phosphorus) of the desired cyclic triester (**14a,b**). The nuclear magnetic resonance spectra of **14a** and **14b** are very similar, and for the moment we are not prepared to assign definitive stereochemistry to each isomer. Alkaline hydrolysis of **13**, or of either of the monophenyl esters (**14a,b**), gave a product identical in chromatographic behavior with **9a**. The nuclear magnetic resonance spectra of the compounds obtained from both sources were also identical. Hydrolysis of **9a** could not be achieved using large amounts of crude *C. adamanteus* venom or by treatment with barium hydroxide under conditions known to cleave ribonucleoside 3′,5′-cyclic phosphates (Smith *et al.*, 1961; Tener *et al.*, 1958). Treatment with 1 N sodium hydroxide at 100° for 4 days did hydrolyze roughly 80% of **9a** to a phosphate dianion. Enzymatic dephosphorylation of the latter readily gave a nucleoside which was chromatographically identical with 1-(2-deoxy-β-D-*threo*-pentofuranosyl)thymine (**12**) and clearly different from thymidine. The latter evidence would appear to provide clear proof for the inverted configuration of $C_{3'}$ of **9a**.



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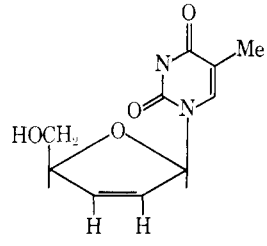

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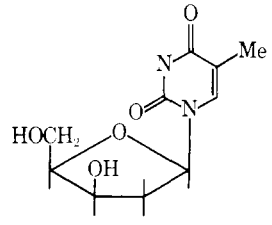

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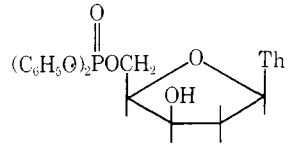

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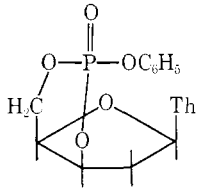
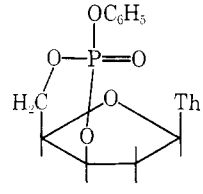
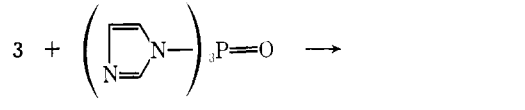


14a



14b

An alternative synthesis of the iodo triphosphate (**7**) has also been used to advantage. Thus, the reaction of 3′-deoxy-3′-iodothymidine (**3**) with triimidazolephosphine oxide (**15**) in tetrahydrofuran followed by immediate hydrolysis of the intermediate phosphorodiimidazolate (**16**) gave 3′-deoxy-3′-iodothymidine 5′-phosphoroimidazolate (**17**) which was isolated in 76% yield by ion-exchange chromatography. This elegant method (Cramer and Schaller, 1961; Scheit, 1968) for the direct conversion of a nucleoside into an activated nucleotide apparently avoids the side reaction leading to cyclic phosphate derivatives. While the preparation of the activated intermediate is somewhat simplified by this method, subsequent conversion into the triphosphate gives results similar to those using the morpholidate (**5**). Thus, in various reactions of **17** with tributylammonium pyrophosphate in Me_2SO, both the iodo triphosphate (**7**) and the unsaturated triphosphate (**8**) were obtained in a combined yield of only 25–30%. The relative proportions of **7** and **8** were quite variable and ranged from 26% and 5% in a 32-hr reaction to 13.5% and 12% in a 4-day reaction. In addition to **7** and **8**, the cyclic phosphate **9a** was a major product and a number of other uncharacterized mono- and diphosphates were also formed.



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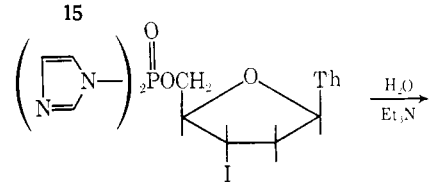

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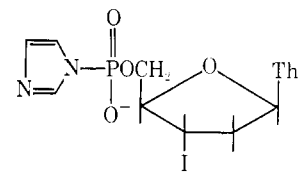

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Palladium-catalyzed hydrogenolysis of **7** on a preparative scale was done in the presence of triethylamine to neutralize the hydrogen iodide released and the resulting 3′-deoxythymidine 5′-triphosphate (**2**) was isolated by ion-exchange chromatography in 74% yield. The characterization of **2** has already been mentioned. Reduction of the unsaturated triphosphate (**8**) did not require the addition of triethylamine. The preparation of **2** labeled with tritium at $C_{3'}$ or at $C_{2'}$ and $C_{3'}$ has been achieved in collaboration with Dr. Walter Hafferl by reduction of **7** or **8**, respectively, with tritium gas. These procedures will be described in detail in a forthcoming paper.

Finally, it might be pointed out that synthesis of **2**, not containing an isotopic label, can probably be carried out in highest over-all yield by conversion of **1a** into 3′-deoxythymidine 5′-phosphoromorpholidate followed by reaction with tributylammonium pyrophosphate in the usual way (Moffatt, 1964). In the absence of the 3′-iodo function, the reduced yields observed in the present study will doubtless be avoided. A related synthesis of **2** from 3′-deoxythymidine using P^1-diphenyl P^2-morpholinopyrophosphorochloridate for activation has been mentioned (Ikehara and Ohtsuka, 1963) but no biological properties were reported.

Experimental Section

General Methods. Thin-layer chromatography was done on 0.25-mm layers of Merck silica gel GF and products were visualized by their ultraviolet absorption or by spraying with a 5% solution of ammonium molybdate in 10% sulfuric acid followed by brief heating at 150°. Preparative thin-layer chromatography was done on 20 × 100 cm glass plates coated with a 1.3-mm layer of Merck silica gel HF. Paper chromatography was done on Schleicher & Schuell No. 589 orange ribbon paper principally using the following systems: solvent I, isopropyl alcohol–concentrated NH_4OH–water (7:1:2); solvent II, isobutyric acid–1 M NH_4OH–0.1 M Na_4EDTA (100:60:1.6). Paper electrophoresis was done on the same paper impregnated with 0.05 M ammonium bicarbonate (pH 7.6) and exposed to a potential difference of 1500–2000 V for 30–45 min. Total phosphorus analyses were done according to King (1932). Ultraviolet spectra were recorded on a Cary Model 15 instrument and nuclear magnetic resonance spectra on a Varian HA-100 spectrometer. All evaporations were done at roughly 1 mm using a rotary evaporator with the condensing vessel cooled by circulating aqueous glycol at −15°. *E. coli* alkaline phosphatase, phosphodiesterase I from *C. adamanteus* (negligible 5′-nucleotidase activity), and crude *C. adamanteus* venom were obtained from the Worthington Biochemical Corp. and were made up to 100 μg, 500 μg, and 10 mg per ml, respectively, in 0.05 M Tris buffer (pH 8). Elemental analyses were obtained by Dr. A. Bernhardt, Mulheim, Germany, or by the Analytical Laboratory of the University of California, Berkeley.

3′-Deoxy-3′-iodothymidine 5′-Phosphate (**4**). An anhydrous solution of 3′-deoxy-3′-iodothymidine (352 mg, 1 mmole), cyanoethyl phosphate (from 700 mg, 2.2 mmoles, of the barium salt), and DCC (1.03 g, 5 mmoles) in pyridine (50 ml) was kept room temperature for 24 hr. After addition of water (5 ml), the at mixture was filtered and evaporated, and an aqueous solution of the residue was extracted with ether. The aqueous phase was made 0.5 M in lithium hydroxide and after 1 hr at 25° electrophoresis showed complete conversion into a dianion. The solution was adjusted to pH 8 with Dowex 50 (H^+) resin, filtered, and applied to a 3.5 × 35 cm column of DEAE-Sephadex (HCO_3^-). Elution with a linear gradient of triethylammonium bicarbonate (5 l., 0–0.2 M) gave a major peak containing 8750 OD_{267} units (91%) of **4** which was evaporated *in vacuo* and repeatedly coevaporated with methanol to remove residual salt. A portion of the chromatographically homogeneous product (R_F in solvents I and II, 1.40 and 1.55 relative to thymidine 5′-phosphate) was dissolved in ethanol and an excess of 1 M ethanolic calcium chloride was added. After thorough washing of the precipitate with ethanol and drying *in vacuo* at 25°, the calcium salt of **4** was obtained as the tetrahydrate: $\lambda_{max}^{H_2O}$ 267 mμ (ε 9700). The anhydrous salt was obtained after drying *in vacuo* at 100°.

Anal. Calcd for $C_{10}H_{12}N_2O_7PICa$: N, 5.96; I, 26.99; P:thymidine, 1.00. Found: N, 6.20; I, 27.06; P:thymidine, 1.00.

3′-Deoxy-3′-iodothymidine 5′-Phosphoromorpholidate (**5**). The triethylammonium salt of **4** (1 mmole) was passed through a column of Dowex 50 (H^+) resin, neutralized with excess pyridine, and evaporated to dryness. After several evaporations of solutions of the residue in anhydrous pyridine, the material was dissolved in pyridine (20 ml) together with morpholine (0.35 ml, 4 mmoles) and DCC (1.03 g, 5 mmoles) was added. After 20 hr at 25°, water (2 ml) was added and after 1 hr the solvent was evaporated and the residue was partitioned between water and ether with filtration. The aqueous phase was chromatographed on a 3.5 × 38 cm column of DEAE-Sephadex (HCO_3^-) using a linear gradient of triethylammonium bicarbonate (5 l., 0–0.25 M) giving one major and several minor ultraviolet-absorbing peaks. The major peak was pooled, evaporated to dryness, and repeatedly evaporated with methanol giving 6300 OD_{267} units (66%) of the chromatographically homogeneous triethylammonium salt of **5** with an R_F of 0.71 (1.12 relative to TMP-morpholidate in solvent I. A portion was converted into the calcium salt by treatment with ethanolic calcium chloride as with **4**, giving the monohydrate.

Anal. Calcd for $C_{28}H_{40}N_6O_{14}I_2P_2Ca \cdot H_2O$: N, 7.94; I, 23.98; P:thymidine, 1.00. Found: N, 7.79; I, 24.01; P:thymidine, 0.97.

A similar reaction to that described above was allowed to proceed for 72 hr with a second addition of morpholine (0.1 ml) and DCC (100 mg) after 48 hr. Chromatography as above gave five peaks, the first two of which were in the region expected for a monoanion. Peak I (18%) was evaporated to dryness and then repeatedly evaporated with methanol, giving the chromatographically homogeneous (R_F 1.00 relative to TMP-morpholidate in solvent I) triethylammonium salt of the unsaturated morpholidate **6**: nuclear magnetic resonance (Me_2SO-d_6) δ 1.65 ppm (s, 3, C_5Me), 2.9 (m, 8, NCH_2 from morpholine and Et_3N), 3.4 (m, 4, OCH_2), 3.8 (m, 2, $C_{5'}H_2$), 4.9 (m, 1, $C_{4'}H$), 5.90 (br d, 1, $J_{2',3'}$ = 6 Hz, $C_{3'}H$), 6.40 (br d, 1, $J_{2',3'}$ = 6 Hz, $C_{2'}H$), 6.85 (br s, 1, $C_{1'}H$), 7.67 (s, 1, C_6H).

Peak II (30%) was evaporated repeatedly with methanol, passed through a column of Dowex 50 (pyridinium) resin, evaporated to dryness, and coevaporated several times with pyridine and then with benzene giving the chromatographically homogeneous (R_F 1.12 relative to TMP-morpholidate in solvent I) pyridinium salt of the iodo morpholidate **5**: nuclear magnetic resonance (Me_2SO-d_6) δ 1.84 ppm (s, 3, C_5Me), 2.65 (br t, 1, $J_{1',2'}$ = 5 Hz, $C_{2'}H$), 3.1 (m, 4, NCH_2), 3.7–4.6 ($C_{3'}$, $C_{4'}$, $C_{5'}$, OCH_2), 6.20 (t, 1, $J_{1',2'}$ = 5 Hz, $C_{1'}H$).

Reaction of **5** *with Tributylammonium Pyrophosphate.* A. 48-HR REACTION. The triethylammonium salt of **5** (0.25 mmole) was carefully dried by repeated evaporation with anhydrous pyridine and residual pyridine was then removed by coevaporation with benzene. The residue was then dissolved in rigorously anhydrous Me_2SO together with tributylammonium pyrophosphate (1 mmole) which was prepared as previously described (Moffatt, 1964). After 48 hr, the mixture was diluted with water and directly applied to a 3.5 × 35 cm column of DEAE-Sephadex (HCO_3^-). After a water wash, the column was eluted with a linear gradient (4 l., 0–0.45 M) of triethylammonium bicarbonate giving four peaks. Peaks I (10%) and II (53%) were in the monoanion region (see later) while peaks III (12%) and IV (14%) were in the region of nucleoside triphosphates. Peak III (302 optical density units) was evaporated to dryness and repeatedly evaporated with methanol leaving a residue that was dissolved in methanol (1 ml) and treated with an excess of 1 M sodium iodide in acetone followed by acetone (10 ml). The resulting white precipitate was separated by centrifugation, washed three times with acetone, and dried *in vacuo* giving the chromato-

graphically homogeneous sodium salt of the unsaturated triphosphate **8** with almost quantitative recovery: R_F 0.06 (1.08 relative to TTP) in solvent I and R_F 0.36 (1.37 relative to TTP) in solvent II; nuclear magnetic resonance (D_2O) δ 1.86 ppm (s, 3, C_5Me), 4.17 (m, 2, $C_{5'}H_2$), 5.10 (m, 1, $C_{4'}H$), 5.91 (br d, 1, $J_{2',3'}$ = 6 Hz, $C_{3'}H$), 6.51 (q, 1, $J_{1',2'}$, = 1 Hz, $J_{2',3'}$ = 6 Hz, $C_{2'}H$), 6.92 (oct, 1, $J_{1',2'}$, $J_{1',3'}$, and $J_{1',4'}$ = 1–3 Hz, $C_{1'}H$), 7.58 (s, 1, C_6H). Treatment of 0.5 μmole of **8** with 20 μl of *E. coli* alkaline phosphatase for 16 hr led to complete conversion into **11** which was identified by thin-layer chromatography, while incubation with crude *C. adamanteus* venom gave a nucleotide, presumably 2',3'-didehydro-3'-deoxythymidine 5'-phosphate.

Anal. Required: total P:labile P:thymine = 3.00:2.00:1.00. Found: 3.12:2.01:1.00.

Peak IV (348 optical density units) was worked up in the same way giving the chromatographically homogeneous iodotriphosphate **7**: R_F 0.07 (1.25 relative to TTP) in solvent I and R_F 0.48 (1.82 relative to TTP) in solvent II; nuclear magnetic resonance (D_2O) δ 1.89 ppm (s, 3, C_5Me), 2.74 (br r, 2, $C_{2'}H_2$), 6.25 (t, 1, $J_{1',2a'}$, $J_{1',2b'}$ = 6 Hz, $C_{1'}H$), 7.74 (t, 1, J_{allylic} = 1 Hz, C_6H).

Incubation of 10 μmoles of **7** with 0.1 ml of *E. coli* alkaline phosphatase led to the separation of crystalline 3'-deoxy-3'-iodothymidine which was physically and chromatographically identical with an authentic sample, while treatment of 0.5 μmole of **7** with 50 μl of venom phosphodiesterase for 24 hr gave only **4**.

Anal. Required: total P:labile P:thymine = 3.00:2.00:1.00. Found: 3.04:2.00:1.00.

B. 8-DAY REACTION. A reaction using 0.5 mmole of **5** and 2 mmoles of tributylammonium pyrophosphate was conducted exactly as in A. After 5 days, at 25°, paper chromatography in solvent I still showed a large spot of monoanion similar to **5**. After a further 3 days at 37°, the mixture was chromatographed as in A. The water wash (880 optical density units at 267 mμ, 18%) was evaporated to dryness *in vacuo* (50°) and purified by preparative thin-layer chromatography using chloroform–methanol (85:15) giving 22 mg of a homogeneous syrup (**9b**) that was directly examined by nuclear magnetic resonance (pyridine-d_5): δ 2.07 ppm (d, 3, J_{allylic} = 1 Hz, C_5Me), 2.5 (m, 1, $C_{2a'}H$), 2.9 (m, 1, $C_{2b'}H$), 3.2 (m, 4, NCH_2), 3.6 (m, 4, O-CH_2), 6.65 (q, 1, $J_{1',2'}$ = 5 and 8 Hz, $C_{1'}H$), 8.70 (d, 1, J_{allylic} = 1 Hz, C_6H). Attempted hydrolysis of **9b** to **9a** led only to extensive decomposition.

Elution of the column with a linear gradient of triethylammonium bicarbonate (5 l. 0–0.45 M) gave five peaks, only one of which was in the region expected for a triphosphate. Peak I (9%) was impure and was discarded. Peak II (1330 optical density units, 28%, **9a**) was a monoanion by electrophoresis at pH 7.6 with a mobility of 1.25 relative to TMP morpholidate, R_F 0.49 in solvent I (0.85 relative to TMP morpholidate). The compound was resistant to venom diesterase, alkaline phosphatase, and hydrolysis with 1 M barium hydroxide solution at 100° or with 0.1 M hydrochloric acid at room temperature: nuclear magnetic resonance (D_2O) δ 1.87 ppm (d, 3, J_{allylic} = 1 Hz, C_5Me), 2.26 (br d, 1, J_{gem} = 16.5 Hz, $C_{2a'}H$), 2.76 (br m, 1, $C_{2b'}H$), 4.07 (m, 1, $C_{4'}H$), 7.84 (d, 1, J_{allylic} = 1 Hz, C_6H); $\lambda_{\max}^{H_2O}$ 267 mμ; P:thymidine 1.02:1.00.

Peaks III (3%) and IV (9%) were probably **4** and the corresponding diphosphate but were not examined further. Peak V (990 optical density units, 21%) was isolated as its sodium salt as in A giving the homogeneous unsaturated triphosphate (**8**) which was identical with that described above.

Alkaline Hydrolysis of **9a** *from Peak II.* A portion of peak II (10 μmoles) was evaporated to dryness, dissolved in 1 M sodium hydroxide, and heated at 100° for 4 days. Electrophoresis then revealed about 80% hydrolysis to a dianion with R_F 0.30 (1.55 relative to thymidine 5'-phosphate) in solvent I. The solution was then adjusted to pH 8 with Dowex 50 (H^+) resin and *E. coli* alkaline phosphatase (10 μg) was added. After overnight incubation at 37°, thin-layer chromatography revealed the presence of a single product with a mobility in several solvents (*e.g.*, chloroform–methanol, 85:15) identical with that of 1-(2-deoxy-β-D-*threo*-pentofuranosyl)thymine and distinctly different from thymidine.

3'-Deoxy-3'-iodothymidine 5'-Phosphoroimidazolate (**17**). A solution of 3'-deoxy-3'-iodothymidine (352 mg, 1 mmole) triimidazolephosphine oxide (Cramer and Schaller, 1961) (1 g, 4 mmoles), and 1,1'-carbonyldiimidazole (100 mg) in anhydrous tetrahydrofuran (20 ml) was kept at room temperature for 12 hr. Triethylamine (2 ml) and then water (5 ml) were added and after 30 min the solution was diluted with water and applied to a 3.5 × 40 cm column of DEAE-Sephadex (HCO_3^-). After a thorough water wash, the column was eluted with a linear gradient (4 l., 0–0.2 M) of triethylammonium bicarbonate. Several small peaks were followed by a single large peak containing 7275 optical density units (76%) of chromatographically homogeneous **17** with R_F 0.73 (1.24 relative to thymidine 5'-phosphoromorpholidate) in solvent I. This was evaporated to dryness and freed from residual bicarbonate by several evaporations with methanol. It behaved identically with **5** on paper electrophoresis at pH 7.6 but was unstable upon storage as both its triethylammonium and calcium salts. Accordingly, it was used directly in the next step.

3'-Deoxy-3'-iodothymidine 5'-Triphosphate (**7**), *via the Imidazolate* (**17**). A solution containing **17** (0.25 mmole of the triethylammonium salt) and tributylammonium pyrophosphate (1 mmole) in rigorously anhydrous Me_2SO (4 ml) was prepared in the same way as with phosphoromorpholidates (Moffatt, 1964). After 4 days at 25°, the mixture was diluted with water and applied to a 3.5 × 47 cm column of DEAE-Sephadex (HCO_3^-). After a thorough water wash, the column was eluted with a linear gradient (4 l., 0–0.5 M) of triethylammonium bicarbonate. Seven ultraviolet-absorbing peaks were obtained as follows: peak I (26%) contained the 3',5'-cyclic phosphate (**9a**) identical with that above; peaks II (3%), III (10%), IV (6%), and V (10%) were mixtures of iodo and unsaturated mono- and diphosphates and were not examined further; peak VI (200 optical density units, 8%) was the 2',3'-unsaturated triphosphate (**8**) while peak VII (412 optical density units, 17) was the 3'-iodo triphosphate (**7**). Peaks VI and VII were evaporated to dryness, carefully freed from residual bicarbonate, and finally precipitated as the sodium salts as above. The isolated products were chromatographically homogeneous and identical with those obtained from the morpholidate (**5**).

3'-Deoxythymidine 5'-triphosphate (**2**). A. *Via* THE IODO TRIPHOSPHATE (**7**). The sodium salt of **7** (25 mg, 305 optical density units) was dissolved in water (5 ml) containing trieth-

ylamine (0.1 ml) and vigorously stirred in an atmosphere of hydrogen at room temperature for 3 hr in the presence of a 10% palladium-on-carbon catalyst (25 mg). Paper chromatography then indicated complete conversion into the slower moving **2**. The catalyst was removed by centrifugation and washed with 1% ammonium hydroxide, and the combined supernatants were directly applied to a 2 × 30 cm column of DEAE-Sephadex (HCO_3^-) which was then well washed with water. Elution with a linear gradient of triethylammonium bicarbonate (1 l., 0–0.45 M) gave a major peak containing 225 optical density units (74%) of **2** which was evaporated to dryness, carefully freed from residual bicarbonate by repeated evaporation with methanol, and precipitated as the sodium salt (13 mg) as described above. The product was chromatographically homogeneous with R_F 0.12 (1.12 relative to thymidine 5′-triphosphate) in solvent I and R_F 0.36 (1.35 relative to thymidine 5′-triphosphate) in solvent II.

Anal. Required: total P:labile P:thymidine = 3.00:2.00:1.00. Found: 2.93:2.03:1.00.

B. *Via* THE UNSATURATED TRIPHOSPHATE (**8**). A solution of the sodium salt of **8** (40 mg) in water (3 ml) was vigorously stirred in a hydrogen atmosphere at room temperature for 24 hr in the presence of 50 mg of a 5% palladium-on-barium sulfate catalyst (Kuhn and Haas, 1955). The catalyst was removed by centrifugation leaving a clear solution of **2** which was chromatographically homogeneous and indistinguishable from the starting material **8** and the product from A. Completion of the reduction was confirmed by incubation of the crude product (2 μmoles) with *E. coli* alkaline phosphatase (20 μl) which led to quantitative conversion into 3′-deoxythymidine with no observable **11**. Complete reduction was also demonstrated by the complete absence of vinylic protons at 5.91 and 6.51 ppm, typical of **8** in the nuclear magnetic resonance spectrum of the crude product. The product was used directly in other studies (Atkinson *et al.*, 1969).

Anal. Required: total P:labile P:thymine = 3.00:2.00:1.00. Found: 2.95:1.89:1.00.

O²,3′-Cyclothymidine (**10**). A solution of 3′-deoxy-3′-iodothymidine (1.76 g, 5 mmoles) and 1,5-diazabicyclo[4.3.0]-nonene-5 (1.25 g, 10 mmoles) in acetonitrile (60 ml) was heated under reflux for 1 hr. Upon cooling *O²,3′*-cyclothymidine (0.80 g, 71%), mp 235–237° (lit. mp 230°, Michelson and Todd, 1955), crystallized from solution: $\lambda_{max}^{H_2O}$ 252 mμ (ε 8600); nuclear magnetic resonance (Me_2SO-d_6) δ 1.77 ppm (d, 3, $J_{allylic}$ = 1 Hz, C_5Me), 3.50 (d, 2, $J_{4',5'}$ = 7 Hz, $C_{5'}H_2$), 4.22 (hex, 1, $J_{4',5'}$ = 7 Hz, $J_{3',4'}$ = 2.5 Hz, $C_{4'}H$), 5.05 (m, 1, $C_{5'}OH$), 5.25 (br d, 1, $J_{3',4'}$ = 2.5 Hz, $C_{3'}H$), 5.83 (d, 1, $J_{1',2'}$ = 3 Hz, $C_{1'}H$), 7.57 (d, 1, $J_{allylic}$ = 1 Hz, C_6H). Purification of the mother liquors by preparative thin-layer chromatography using chloroform–methanol (9:1) gave a further 110 mg (total yield 81%) of **10** and also 65 mg (6%) of 2′,3′-didehydro-3′-deoxythymidine, mp 163–164° from ethanol–benzene (lit. mp 165–166°, Horwitz *et al.*, 1966). A detailed description of the nuclear magnetic resonance spectrum of this compound will be presented elsewhere by Dr. M. L. Maddox.

1-(2-Deoxy-β-D-threo-pentofuranosyl)thymine (**12**). A solution of *O²,3′*-cyclothymidine (448 mg, 2 mmoles) in 0.1 N sodium hydroxide (10 ml) was heated at 100° for 1 hr. The cooled solution was then passed through a 1 × 10 cm column of Dowex 50 (H^+) resin and evaporated to dryness. Crystallization from ethyl acetate–methanol gave 397 mg (82%) of **12**, mp 173.5–174.5° (lit. (Horwitz *et al.*, 1963) mp 170–171°).

1-(2′-Deoxy-β-D-threo-pentofuranosyl)thymine 5′-Diphenyl Phosphate (**13**). Diphenyl phosphorochloridate (300 mg, 1.1 mmoles) was added to a suspension of **12** (242 mg, 1 mmole) in anhydrous pyridine (5 ml). After 16 hr, water (0.2 ml) was added and the solvent was evaporated *in vacuo* giving a residue that was dissolved in ethyl acetate, washed with water, dried (Na_2SO_4), and evaporated leaving 456 mg of a crystalline residue. Recrystallization from ethanol gave 403 mg (85%) of **13**, mp 148–149°; $\lambda_{max}^{dioxane}$ 263 mμ (ε 9300); $[\alpha]_D^{25}$ +5.3° (*c* 0.14, dioxane); nuclear magnetic resonance ($CDCl_3$) δ 1.83 ppm (br s, 3, C_5Me), 2.1 (m, 1, $C_{2a'}H$), 2.5 (m, 1, $C_{2b'}H$), 3.98 (m, 1, $C_{4'}H$), 4.23 (m, 1, $C_{3'}H$ giving q, 1, J = 2 Hz, 5 Hz with D_2O), 4.35–4.7 (m, 2, $C_{5'}H_2$), 6.15 (q, 1, $J_{1',2'}$ = 2 Hz, 8 Hz, $C_{1'}H$), 7.2 (m, 10, OC_6H_5), 7.64 (d, 1, $J_{allylic}$ = 1 Hz, C_6H).

Anal. Calcd for $C_{22}H_{23}N_2O_8P$: C, 55.70; H, 4.89; N, 5.91. Found: C, 55.60; H, 4.76; N, 5.96.

1-(2′-Deoxy-β-D-threo-pentofuranosyl)thymine 3′,5′-Cyclic Phenyl Phosphate (**14a,b**). A solution of **13** (237 mg, 0.5 mmole) and 1,5-diazabicyclo[4.3.0]nonene-5 (0.1 ml) in dioxane (10 ml) was stored at room temperature for 30 min at which point thin-layer chromatography, using chloroform–methanol (9:1), showed complete conversion into two more polar products which, after evaporation, were separated by preparative thin-layer chromatography using three developments with the same solvent. Crystallization of the faster band (100 mg) from ethanol gave 76 mg (40%) of a pure isomer, mp 247–249°; λ_{max}^{MeOH} 266 mμ (ε 9200); $[\alpha]_D^{25}$ −24.8° (*c* 0.10, MeOH); nuclear magnetic resonance (Me_2SO-d_6) δ 1.74 ppm (d, 3, $J_{allylic}$ = 1 Hz, C_5Me), 2.25 (m, 1, $C_{2a'}H$), 2.85 (m, 1, $C_{2b'}H$), 4.20 (m, 1, $C_{4'}H$), 4.75 (m, 2, $C_{5'}H_2$, overlapping quartets), 6.24 (q, 1, $J_{1',2'}$ = 2 Hz, 8 Hz, $C_{1'}H$), 7.46 (d, 1, $J_{allylic}$ = 1 Hz, C_6H), 7.3 (m, 5, OC_6H_5).

Anal. Calcd for $C_{16}H_{17}N_2O_7P$: C, 50.53; H, 4.51; N, 7.37. Found: C, 50.75; H, 4.84; N, 7.31.

Crystallization of the slower band from ethanol gave 57 mg (21%) of the pure isomer, mp 203–205°; λ_{max}^{MeOH} 265 mμ (ε 9700); $[\alpha]_D^{25}$ −11.0° (*c* 0.13, MeOH); nuclear magnetic resonance (Me_2SO-d_6) 1.56 ppm (br s, 3, C_5Me), 2.25 (m, 1, $C_{2a'}H$), 2.85 (m, 1, $C_{2b'}H$), 4.27 (m, 1, $C_{4'}H$), 4.80 (m, 2, $C_{5'}H_2$), 5.35 (m, 1, $C_{3'}H$), 6.22 (q, 1, $J_{1',2'}$ = 3 Hz, 8 Hz, $C_{1'}H$), 7.50 (d, 1, $J_{allylic}$ = 1 Hz, C_6H), 7.3 (m, 5, OC_6H_5).

Anal. Calcd for $C_{16}H_{17}N_2O_7P$: C, 50.53; H, 4.51; N, 7.37. Found: C, 50.60; H, 4.60; N, 7.27.

Hydrolysis of **13**. To a solution of **13** (237 mg, 0.5 mmole) in dimethylformamide (5 ml) was added 1 M aqueous sodium hydroxide solution (4 ml) and the mixture was stored at room temperature. After 6 days, electrophoresis showed almost complete conversion into a monoanion. The mixture was diluted to a total volume of 200 ml with water and directly applied to a column (40 × 2.5 cm) of DEAE-Sephadex (HCO_3^-) which was then washed with water. Elution with a linear gradient of triethylammonium bicarbonate (2 l., 0–0.1 M) gave a major peak containing 4312 optical density units (89.4% yield) of **9a** which was evaporated *in vacuo* and repeatedly coevaporated with methanol. The final residue was passed through a column of Dowex 50 (H^+) resin and the effluent adjusted to pH 5.5 with sodium hydroxide. The solution was evaporated to dryness and the dried residue was dissolved in methanol (2 ml). Addition of ether (10 ml)

precipitated the sodium salt of **9a** (127 mg) which was chromatographically and electrophoretically homogeneous and identical with the compound isolated from reactions of either **5** or **17** with pyrophosphate. The nuclear magnetic resonance spectra of the samples were also identical.

Treatment of either **14a** or **14b** with sodium hydroxide under similar conditions also gave a single product chromatographically identical with **9a** obtained as above.

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