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

# Studies on Polynucleotides. VI.[1] Experiments on the Chemical Polymerization of Mononucleotides. Oligonucleotides Derived from Thymidine-3′ Phosphate[2]

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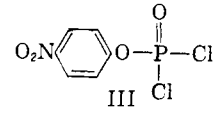
Thymidine-3′ phosphate has been synthesized in an over-all yield of 75% by the phosphorylation of 5′-*O*-tritylthymidine with *p*-nitrophenyl phosphorodichloridate followed by alkaline treatment of the product to remove the *p*-nitrophenyl group and then acidic treatment to remove the trityl group. Thymidine-3′ *p*-nitrophenyl phosphate, a useful assay substrate for certain phosphodiesterases, was prepared in 95% over-all yield by acidic treatment of the initial phosphorylation product, 5′-*O*-tritylthymidine-3′ *p*-nitrophenylphosphate. Thymidine-3′,5′-cyclic phosphate was demonstrated to be an intermediate in the alkaline hydrolysis of thymidine-3′ *p*-nitrophenyl phosphate. The polymerization of thymidine-3′ phosphate, after prior conversion to $P^1,P^2$-dithymidine-3′ pyrophosphate by reaction with an excess of dicyclohexylcarbodiimide in anhydrous pyridine, gave a complex mixture of products. Separation by chromatography on an ECTEOLA cellulose column followed by paper chromatography afforded a series of linear thymidine oligonucleotides bearing 3′-phosphate end groups and a series of cyclic oligonucleotides in which the linear compounds were cyclized by phosphorylation of the 5′-hydroxyl group at one end by the 3′-phosphomonoester group at the other end. Two other series of minor components also were obtained and the nature of these products is discussed. Results of the degradation of the linear thymidine oligonucleotides bearing 3′-phosphate end groups by venom phosphodiesterase are discussed.

Synthetic work in the polynucleotide field, which is in progress in this Laboratory, is being directed along two major lines.[3,4] The aim of the first of these is to develop routes to the specific synthesis of polynucleotides containing different nucleotide units in a predetermined sequence.[1,5] The second, on the other hand, aims at the polymerization of small units (*e.g.*, mononucleotides, preformed dinucleotides) so as to obtain somewhat more rapidly a range of simple polynucleotides. Some of our initial studies on the polymerization of thymidine-5′ phosphate were reported recently.[6] Before undertaking a more thorough investigation of the polymerization reaction with that nucleotide, it was considered desirable to explore a similar reaction with the isomeric class of deoxyribonucleotides and thymidine-3′ phosphate was chosen as an example. The present communication records a new and improved synthesis of this nucleotide and the study of the products formed by its polymerization according to the previously described procedure.[6] The polymerization experiments have made available moderate amounts of thymidine oligonucleotides terminated in 3′-phosphomonoester groups.

Thymidine-3′ phosphate[7] (I) was first prepared by Michelson and Todd[8] by the phosphorylation of 5′-*O*-tritylthymidine (II) with dibenzyl phosphorochloridate. While the yield of the nucleotide was not reported, phosphorylation was evidently not complete[9] since thymidine was found to be present after work-up. Repetition of their work showed that the phosphorylation was sluggish and the nucleotide isolated by a modified procedure was contaminated with a slower traveling material, probably thymidine-3′ pyrophosphate.[10] Pure nucleotide could only be obtained after a tedious work-up in yields of the neighborhood of 40%. An alternative method for the preparation of thymidine-3′ phosphate in quantity therefore was sought.

The crystalline *p*-nitrophenyl phosphorodichloridate (III) had been used earlier in this Laboratory in exploratory experiments on the synthesis of internucleotide bonds.[11] In the present work 5′-*O*-tritylthymidine (II) was treated with 2 mol. equiv. of the reagent in the presence of a limited quantity



III

of pyridine and after a simple work-up pure 5′-*O*-tritylthymidine-3′ *p*-nitrophenyl phosphate (IV) was obtained in virtually quantitative yield. Mild acidic treatment removed the trityl group and thymidine-3′ *p*-nitrophenyl phosphate (V) was obtained in an over-all yield of 94-97% based on II. The substance is a particularly useful assay substrate for one major class of phosphodiesterases.[12]

When thymidine-3′ *p*-nitrophenyl phosphate (V) was heated in alkali to remove the *p*-nitrophenyl group as had been done earlier in the preparation of guanosine-5′ phosphate,[13,14] the nucleotide obtained

(1) Paper V, P. T. Gilham and H. G. Khorana, THIS JOURNAL, **81**, 4647 (1959).

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

(3) G. M. Tener, P. T. Gilham, W. E. Razzell, A. F. Turner and H. G. Khorana, *Ann. N. Y. Acad. Sci.*, in press (1959).

(4) H. G. Khorana, *J. Cellular Comp. Physiol.*, in press (1959).

(5) P. T. Gilham and H. G. Khorana, THIS JOURNAL, **80**, 6212 (1958).

(6) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, *ibid.*, **80**, 6224 (1958).

(7) This nucleotide has been isolated recently by L. Cunningham [*ibid.*, **80**, 2546 (1958)] by the enzymic degradation of deoxyribonucleic acid.

(8) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 951 (1953).

(9) It may be noted that the reagent dibenzyl phosphorochloridate fails completely in the phosphorylation of 3′-hydroxyl groups in 5′-*O*-acetyldeoxyadenosine and deoxyguanosine; D. H. Hayes, A. M. Michelson and A. R. Todd, *ibid.*, 808 (1955).

(10) Michelson and Todd (ref. 8) noted the formation of a phosphorus-containing side product which was concluded to be dithymidine monophosphate. In our experiments, we also noted the presence of a spot on chromatograms corresponding to this product and, in addition, thymidine-3′ pyrophosphate.

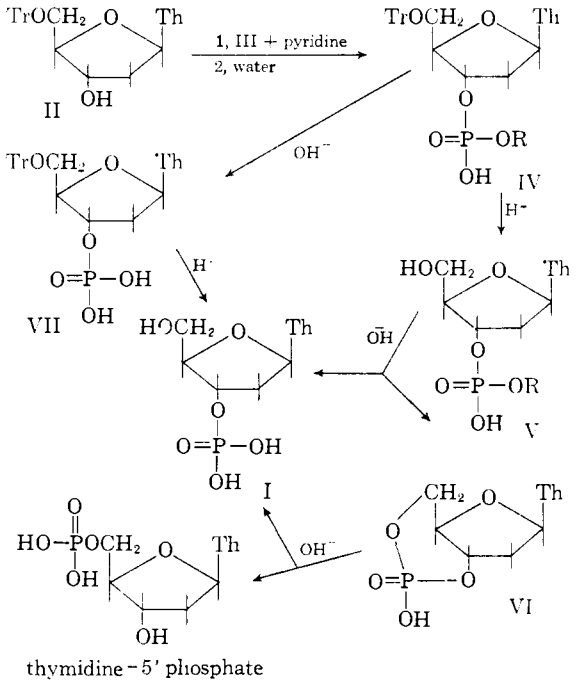
(11) G. M. Tener, H. G. Khorana and E. H. Pol, unpublished experiments referred to in ref. 5; see also W. E. Razzell and H. G. Khorana (*J. Biol. Chem.*, **234**, 2114 (1959) for preparation of thymidylyl-(5′ → 5′)-thymidine.

(12) A typical example is the spleen phosphodiesterase described by L. A. Heppel and R. J. Hilmoe (ref. 19); unpublished work of W. E. Razzell and H. G. Khorana.

(13) R. W. Chambers, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **79**, 3747 (1957).

(14) In the earlier work (ref. 13) alkaline removal of the *p*-nitrophenyl group from the 5′-phosphate ester was effected while the 2′- and 3′-hydroxyl groups were protected by an isopropylidene group.

proved in fact to be a mixture of thymidine-3′ and -5′ phosphates. Closer study of the alkaline hydrolysis showed that thymidine-3′,5′ cyclic phosphate[6] (VI) was formed as an intermediate.[14] Thymidine-5′ phosphate thus arose by the subsequent hydrolysis of the cyclic phosphate in either of the two ways.[6] For the preparation of thymidine-3′ phosphate, therefore, IV was first heated with alkali to give VII, which then was treated under mild acidic conditions to remove the trityl group. Pure nucleotide I was obtained in an over-all yield of 75%.

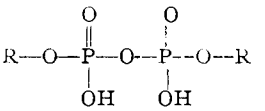



thymidine-5′ phosphate

Th = thymine; Tr = triphenylmethyl; R = *p*-nitrophenyl

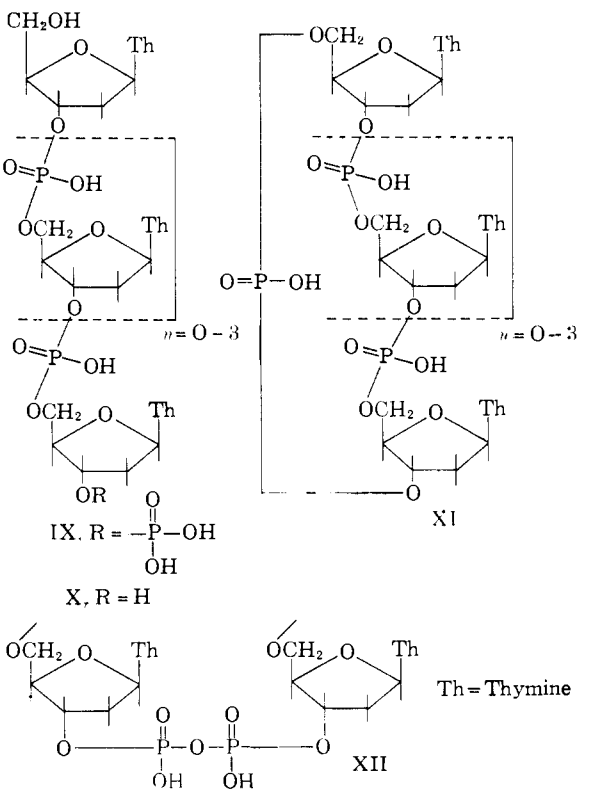
As described for the polymerization of thymidine-5′ phosphate,[6] it was intended to treat an anhydrous solution of thymidine-3′ phosphate in pyridine with dicyclohexylcarbodiimide. The pyridinium salt of the nucleotide crystallized, however, and was then relatively insoluble in the solvent. With the assumption[15] that the symmetrical dithymidine-3′ pyrophosphate (VIII) could serve equally well as the starting material in the polymerization reaction, the nucleotide (as its pyridine soluble tri-*n*-butylammonium salt) was converted to VIII by the procedure recently described.[16]

The pyrophosphate was formed in quantitative yield, as judged by paper chromatography, and its pyridinium salt[17] dissolved readily in pyridine, the




VIII, R = thymidine-3′

polymerization solvent. Treatment of an anhydrous solution with an excess of dicyclohexylcarbodiimide for four days at room temperature followed by Ecteola cellulose[6] and paper strip chromatography showed that a number of oligonucleotides were indeed formed. The results of chromatographic separation on a preparative scale using the linear gradient elution technique are shown in Fig. 1. As before,[6] the Ecteola cellulose column was effective in separating the mixture of products into groups mainly as a function of charge and, furthermore, partial resolution of the linear (IX) from the cyclic oligonucleotides (XI) (see below) was obtained. Subsequent purification of the materials recovered from the pooled fractions within a peak was achieved by prolonged paper chromatography in the isopropyl alcohol–ammonia–water system. In this way peaks were shown to consist of mixtures: peak G, for example, consisted mainly of linear trinucleotide (IX, $n$ = 1), with some cyclic trinucleotide (XI, $n$ = 1), and a minor component shown to




be the pyrophosphate (partial structure, XII) derived from thymidine-3′ phosphate and the linear dinucleotide (IX, $n$ = 0). The total yields of individual components thus isolated are presented in Table I and further characterization and discussion of these is presented according to the various series of homologous compounds isolated.

**Linear Oligonucleotides.**—The simplest member of this series, the linear dinucleotide (IX, $n$ = 0),

Uridine-5′ *p*-nitrophenyl phosphate has now been heated with alkali to learn if any participation from the 3′-hydroxyl group occurs during removal of the *p*-nitrophenyl group. No uridine-3′ phosphate could be detected in the uridine-5′ phosphate obtained. We are indebted to Dr. J. G. Moffatt for this experiment.

(15) As has been discussed elsewhere,[1,5,6] the first step in the phosphodiester bond synthesis using dicyclohexylcarbodiimide is considered to be the conversion of the mononucleotide to a meta (presumably tri- or higher) phosphate and the formation of this occurs, at least partly, *via* the symmetrical pyrophosphate VIII.

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

(17) For polymerization at room temperature it is necessary to remove the strong base, tri-*n*-butylamine; M. Smith, J. G. Moffatt and H. G. Khorana, *ibid.*, **80**, 6204 (1958).

TABLE I

YIELDS[a] OF INDIVIDUAL OLIGONUCLEOTIDES OBTAINED FROM THE POLYMERIZATION OF THYMIDINE-3′ PHOSPHATE

| Compound | Mono | Di | Tri | Tetra | Penta |
|---|---|---|---|---|---|
| Unidentified component | .. | 0.5 | 0.8 | 1.2 | 1.5 |
| Linear oligonucleotide (IX) | 0.1 | 0.3 | 3.5 | 3.4 | 4.0 |
| Cyclic oligonucleotide (XI) | 5.0 | 16.5 | 4.0 | 3.1 | 3.3 |
| Pyrophosphate polymer (XII) | .. | 0.3 | 1.1 | 1.0 | 1.3 |

[a] These are percentages of the total optical density (at 267 mμ) applied to the column.

was isolated in low yield from peak D and was identified by direct comparison with synthetic samples.[18] Characterization of the linear tri (IX, $n$ = 1), tetra (IX, $n$ = 2) and penta (IX, $n$ = 3) oligonucleotides which were the major components of peaks, G, I and K, respectively, was accomplished in several ways. Firstly, they behaved chromatographically and electrophoretically in exactly the same way as the isomeric thymidine oligonucleotides bearing 5′-phosphate end groups.[6] Because of their terminal phosphomonoester groups these compounds had greater electrophoretic mobility than their cyclic analogs at neutral $p$H. Secondly, these oligonucleotides were degraded rapidly to thymidine-3′ phosphate by the spleen phosphodiesterase preparation kindly furnished by Drs. Heppel and Hilmoe,[19] a degradation which is characteristic of oligonucleotides bearing a free 5′-hydroxyl group at one end and a 3′-phosphate group at the other end.[20] Finally, removal of the terminal phosphomonoester groups with prostate phosphomonoesterase[21] gave the homologous series of compounds (X, $n$ = 0–3) which were identical with the corresponding samples obtained previously by the use of the same enzyme from the oligonucleotides bearing 5′-phosphate end groups.[6]

**Cyclic Oligonucleotides.**—Intramolecular cyclization resulting from the esterification of the 5′-hydroxyl group by the 3′-phosphomonoester group at the other end of a polymer chain was, as in the earlier work,[6] a prominent reaction and the cyclic dinucleotide (XI, $n$ = 0), the major component of peak E, was by far the most abundant single product. The simplest member of this series, thymidine-3,5′ cyclic phosphate (VI) was present in peak C while the homologous cyclic tri- (XI, $n$ = 1), tetra-(XI, $n$ = 2) and penta- (XI, $n$ = 3)-nucleotides were the major constituents of peaks H, J and L, respectively. Characterization was effected by comparison (chromatographic and electrophoretic) with the same compounds prepared earlier.[6]

**Minor Products.**—Two other series of compounds which are present in relatively small amounts have been encountered in the polymerization mixtures.

(18) Synthesis of thymidylyl-(3′ → 5′)-thymidylic-(3′) acid has recently been reported (P. T. Gilham and H. G. Khorana, ref. 5). A much more satisfactory synthesis has been achieved more recently (G. M. Tener, in press).

(19) The preparation was a gift of these authors and was used according to their directions; L. A. Heppel and R. J. Hilmoe, "Methods in Enzymology," Vol. II, Academic Press Inc., New York, N. Y., 1955, p. 565.

(20) Hydrolysis by the spleen diesterase begins from the end of the chain bearing a 5′-hydroxyl group [W. E. Razzell and H. G. Khorana, THIS JOURNAL, **80**, 1770 (1958)]. Ribo-oligonucleotides bearing 5′-phosphate end groups have been found to be resistant [L. A. Heppel, P. J. Ortiz and S. Ochoa, *J. Biol. Chem.*, **229**, 679 (1957)].

(21) Kind gift of Dr. R. Markham; R. Markham and J. D. Smith, *Biochem. J.*, **52**, 558 (1952).

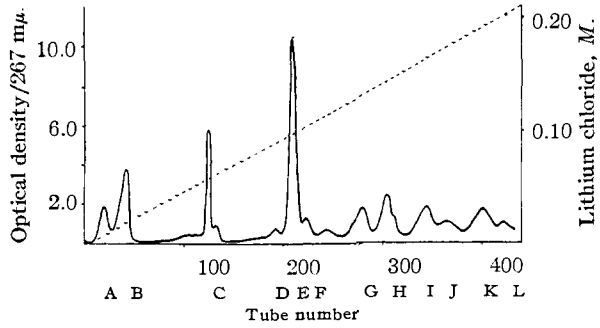



Fig. 1.—Preparative chromatography of thymidine oligonucleotides on an ECTEOLA cellulose column (35 cm. × 4 cm. diameter). Material applied was derived from 1.15 mmoles of thymidine-3′ phosphate and was eluted using a linear salt gradient as shown by the dotted line.

One of these series of compounds has the general structure XII, and contains the mononucleotide and linear oligonucleotides linked together in a pyrophosphate bond through their phosphomonoester groups. The identification of these components was accomplished by selective hydrolysis of the pyrophosphate linkage by mild acidic treatment. Thus a contaminant of peak D was $P^1,P^2$-dithymidine-3′ pyrophosphate (VIII). Brief hydrolysis of the minor component of peak G gave the linear dinucleotide (XI, $n$ = 0) and thymidine-3′ phosphate.

Acidic treatment of the contaminant present in peak I gave thymidine-3′ phosphate, and linear di- and trinucleotides. It would thus appear that the contaminant consisted of two pyrophosphates, a symmetrical one formed between two linear dinucleotide units and an unsymmetrical one, containing a mononucleotide and a trinucleotide. Similarly, a mixture of pyrophosphates must be present in the contaminant of peak K, which gave after acidic treatment oligonucleotides up to the tetranucleotide.

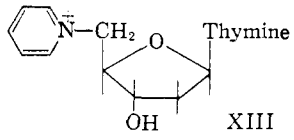
The presence of the above pyrophosphates can be explained readily if the first step in the polymerization reaction is accepted as the formation of one or more metaphosphate species. It should be added, however, that similar compounds were not detected in the analogous polymerization of thymidine-5′ phosphate and therefore the possibility must be raised that the mechanism of polymerization starting with the preformed dinucleotide pyrophosphate (VIII) may differ somewhat from that when the mononucleotide itself is used as the starting material. However, a strict comparison is made difficult at this stage by the fact that the polymerization reaction mixtures have not been homogeneous.

A second series of minor components was present in peaks E, H, J and L. It may be noted that the minor products with similar properties were also encountered in the previous work with thymidine-5′ phosphate (*e.g.*, peaks A and E of Fig. 2 in ref. 6) although their nature was not then discussed. Further work with those side products[22] and a parallel investigation of the constituents of peak B obtained in the present work have thrown light on the nature of these contaminants. Peak B could be separated by paper electrophoresis at $p$H 7.5 into two ultraviolet-absorbing components, one of which moved

(22) G. M. Tener, J. P. Vizsolyi and H. G. Khorana, unpublished work.

as a cation and the other as an anion. The latter had half the electrophoretic mobility of thymidine-3′ phosphate at *p*H 7.5 while at *p*H 4 it showed little net charge. The substance, which evidently contained a phosphomonoester group, could be dephosphorylated by incubation with prostate phosphomonoesterase and the product thus obtained was identical with the cationic component mentioned above. The ultraviolet absorption spectrum of the substance was characteristic and was similar to that of an equimolar mixture of thymidine and the N-methylpyridinium cation.

From these results it is tentatively concluded that the cation may have the structure XIII and that in the anion a phosphomonoester group is located at the 3′-position. If this is so then the contaminants in peaks E, J and L are probably homologous oligonucleotides in which the terminal nucleoside has the structure XIII.




XIII

**Degradation by Venom Phosphodiesterase.**—The exceptionally slow hydrolysis of deoxyribooligonucleotides bearing a 3′-phosphate end group by venom diesterase has been noted earlier.[23] Recent[24] detailed studies have elucidated the general mode of action of this enzyme and its action on the new substrates (IX, $n$ = 1 and 2) now has been investigated. As expected, degradation was extremely slow and using the conditions under which an equivalent amount of thymidylyl-(5′ → 3′)-thymidine was completely degraded in about 30 min., the linear trinucleotide (IX, $n$ = 1) was still only partially degraded in two days. The products formed were thymidine, thymidine-5′ phosphate, thymidine-3′,5′ diphosphate, dithymidine triphosphate (5′-*O*-phosphoryl-thymidylyl-(3′ → 5′)-thymidylic-(3′) acid) and the starting material, indicating a random mode of degradation. The results with the tetranucleotide were similar and no significant acceleration of the rate of degradation with increase in chain length was noted in this series.[25]

## Experimental

**General.**—Paper chromatography was performed by the descending technique using Whatman No. 1 or 3 MM paper. The solvent systems used were: isopropyl alcohol-ammonia-water (7:1:2, v./v.) (solvent A), *n*-butyl alcohol-acetic acid-water (5:2:3, v./v.) (solvent B) and (solvent C[26]) isobutyric acid-1 *M* ammonia (5:3, v./v.). Paper electrophoresis was carried out using Whatman 3 MM paper strips in an apparatus similar to that of Markham and Smith.[27] Phosphorus was determined by King's method.[28]

**Preparation of Thymidine-3′ Phosphate (I) Using Dibenzyl Phosphorochloridate.**—Dibenzyl phosphorochloridate prepared fresh from dibenzyl phosphate (1.31 g., 5 mmoles) and N-chlorosuccinimide (0.66 g., 5 mmoles) was added to a frozen solution of 5′-*O*-tritylthymidine[1] (0.562 g., 1 mmole) in anhydrous pyridine (5 ml.). The mixture was allowed to warm sufficiently to obtain a homogeneous solution and then was kept at about −15° for 36 hr. Water (5 ml.) then was added and the mixture evaporated after 10 min. The yellow gum was dissolved in 25 ml. of 80% acetic acid and the solution heated in a boiling water-bath for 20 min. Acetic acid now was removed using re-evaporation with dioxane to assist complete removal. Water was added and after the removal of the insoluble triphenylcarbinol the aqueous solution was passed through a column of an excess of Amberlite IR-120 ($H^+$) resin. Some dibenzyl hydrogen phosphate which separated was removed and the total effluent was concentrated to about 50 ml. Dioxane (50 ml.) was added and the total solution hydrogenated in the presence of palladium oxide-barium sulfate catalyst.[29] When the hydrogen uptake ceased (2–3 hr.), the catalyst was removed and the solution concentrated, water added and extracted with ether. Chromatography indicated the presence of thymidine, thymidine-3′ phosphate, thymidine-3′ diphosphate and an unidentified component traveling at $R_f$ 0.45 (solvent A). The solution was concentrated to 5 ml., 0.5 ml. concentrated hydrochloric acid added and the mixture heated at 100° for 10 minutes. Water then was taken off at low temperature and pressure using re-evaporation with dioxane to assist complete removal and the resulting gum triturated several times with ether. Water was added, the solution neutralized with barium hydroxide to *p*H 7.5, and the resulting precipitate removed by centrifugation. The solid was washed with water and the combined aqueous liquors concentrated to small bulk and ethanol (3 vols.) added. The precipitate was washed with aqueous ethanol then acetone, dried at 100° (0.01 mm.), redissolved in water and reprecipitated with ethanol as above. Washing and drying in the same manner afforded the barium salt of thymidine-3′ phosphate which was chromatographically (solvents A and B) homogeneous.[30] The yield as estimated spectrophotometrically was 40%.

***p*-Nitrophenyl Phosphorodichloridate**[31] (By Dr. G. M. Tener).—Finely powdered anhydrous sodium *p*-nitrophenoxide[32] (41 g.) was added slowly to stirred precooled phosphorus oxychloride (250 ml.) in a flask equipped with a mechanical stirrer and reflux condenser. A vigorous reaction occurred, which was moderated by cooling with an ice-salt-bath, and the color of the added salt rapidly disappeared. When addition was complete (*ca.* 1 hr.), sodium chloride was removed by filtration, the excess of phosphorus oxychloride removed under reduced pressure and finally the residue was maintained at 0.01 mm. for 1 hr. The oil was distilled in a short-path apparatus and gave a light yellow oil, b.p. 128° (0.02 mm.), which crystallized to a product, m.p. 43.5–44.5°. Yields varied but were better than 40% provided a short-path apparatus[33] was used. Otherwise decomposition occurred, sometimes with explosive violence.

***p*-Nitrophenyl Thymidine-3′ Phosphate (V).**—A solution of 5′-*O*-tritylthymidine[1] (1.12 g., 1.98 mmoles) in anhydrous dioxane (5 ml.) was added dropwise with exclusion of moisture to a magnetically stirred solution of *p*-nitrophenyl phosphorodichloridate (1.016 g., 3.92 mmoles) in dioxane (5 ml.) and dry pyridine (0.63 ml., 7.84 mmoles). When the addition was complete (1 hr.), the mixture was kept at room temperature for a further two hr. A solution of pyridine (0.65 ml.) in water (3.0 ml.) then was added rapidly with stirring and the total solution evaporated to a gum in a vacuum. The residue was taken up in a mixture of chloroform and water and the chloroform layer was separated after careful agitation. The organic layer was re-extracted with 1 *M* pyridine hydrochloride solution (*p*H 5.5). Paper chromatography in solvent A showed that the chloroform layer contained *p*-nitrophenyl 5′-*O*-tritylthymidine-3′ phosphate (IV) and that the first water extract contained *p*-nitrophenyl phosphate and some $P^1$,$P^2$-di-(*p*-nitro-

(23) M. Privat de Garilhe, L. Cunningham, U. Laurila and M. Laskowski, *J. Biol. Chem.*, **224**, 751 (1957).

(24) W. E. Razzell and H. G. Khorana, THIS JOURNAL, **80**, 1770 (1958); *J. Biol. Chem.*, **234**, 2114.

(25) The acceleration in rate noted by J. F. Koerner and R. L. Sinsheimer (*ibid.*, **228**, 1049 (1957)) may obtain in longer polynucleotide chains.

(26) H. A. Krebs and R. Hems, *Biochim. et Biophys. Acta*, **12**, 172 (1953).

(27) R. Markham aod J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

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

(29) R. Kuhn and H. J. Haas, *Angew. Chem.*, **67**, 785 (1955).

(30) This material later was shown to contain an alkali-labile impurity.

(31) G. R. Cebrian, *Anales real soc. espan. fis. y quim. (Madrid)*, **47B**, 841 (1951), *C. A.*, **46**, 11140 (1952).

(32) J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **79**, 3741 (1957).

(33) A modified Hickman flask was satisfactory [K. C. D. Hickman, *Chem. Revs.*, **34**, 51 (1944)].

phenyl) pyrophosphate[34] while the second water extract usually contained in addition a trace of the nucleotide derivative. The chloroform solution was taken to dryness and the resulting pale yellow gum was dissolved in 25 ml. of 80% acetic acid and heated on a boiling water-bath for 20 min. The solvent next was removed under reduced pressure, the residue diluted with water and the flask set aside for 18 hr. at 4°. The crystalline precipitate of triphenylcarbinol was removed and the aqueous solution was lyophilized. The solid was redissolved in water and the solution relyophilized. The product then was converted to the free acid by dissolving it in water and passing it through an Amberlite IR-120 ($H^+$) resin column. A small amount of *p*-nitrophenol which was present was removed at this stage by repeated ether extraction of the acidic solution. The aqueous solution then was neutralized with ammonium hydroxide. Lyophilization gave the ammonium salt as a dry powder (0.860 g., 94% yield). Calcd. for $C_{16}H_{17}N_3O_{10}P{\cdot}NH_4$: C, 41.7; H, 4.60; N, 12.2; P, 6.7. Found: C, 41.6; H, 5.1; N, 12.0; P, 6.4; $\epsilon_{max}/P$ = 15,400.[35] The compound travelled as a single spot in solvents A, B and *n*-BuOH–$H_2O$ (86:14, v./v.) and was homogeneous when examined electrophoretically under neutral and acid conditions; spectral ratios (in neutral solution), 250/260, 0.62; 260/270, 0.818; 280/260, 0.905; $\lambda_{max}$ 270.5 mμ.

**Alkaline Hydrolysis of *p*-Nitrophenyl Thymidine-3′ Phosphate.**—An approximately 5% solution of the above diester in 1 *N* sodium hydroxide was heated at 100° in a polyethylene tube. Aliquots were removed at intervals and were chromatographed in solvent A after treatment with an excess of pyridinium Dowex-50 ion exchange resin. It was found (a) that hydrolysis to a slow traveling spot identical in $R_f$ with thymidine-3′ phosphate was complete in 2 hr. and (b) that in the aliquots corresponding to 0.25, 0.5 and 1 hr. another spot with $R_f$ 0.52 was present. Larger amounts of the nucleotide and the material with $R_f$ 0.52 were collected by performing the alkaline hydrolysis for 3 and 0.25 hr., respectively, and subsequently chromatographing on a Whatman 3 MM paper strip. The following experiments were performed: (a) The nucleotide (*ca.* 1 μmole) was incubated with crude *Crotalus adamanteus* venom in 0.05 *M* tris-hydroxymethylaminomethane buffer (*p*H 8.5). Partial hydrolysis to thymidine occurred under the conditions that authentic thymidine-3′ phosphate was completely resistant. The extent of degradation was estimated to be about 11% by spectrophotometric measurement.

(b) The intermediate spot with $R_f$ 0.52 had $\lambda_{max}$ at 264.5 mμ and was found by paper chromatography (solvents A and B) and paper electrophoresis (*p*H 3.5 and 7.5) to be identical with thymidine-3′,5′-cyclic phosphate (VI).

**Thymidine-3′ Phosphate (I) by Using *p*-Nitrophenyl Phosphorodichloridate.**—5′-*O*-Tritylthymidine (0.57 g., 1.015 mmoles) was phosphorylated with *p*-nitrophenyl phosphorodichloridate as described above and the chloroform solution of *p*-nitrophenyl 5′-*O*-tritylthymidine-3′ phosphate was evaporated and the residual gum was dissolved in 2 *N* lithium hydroxide (10 ml.) and the orange solution heated for 4 hr. at 100° in a polyethylene tube under nitrogen. Amberlite IR-120 ($H^+$) resin then was added until the *p*H of the solution was about 10. The resin was separated on glass wool and set aside while the aqueous solution was concentrated to a sirup. This was redissolved in 50% aqueous dioxane (20 ml.), the above resin added back together with more IR-120 ($H^+$) resin sufficient to render the solution completely acidic then the reaction mixture left at room temperature. Paper chromatography showed that detritylation was complete after 72 hr. The resin was removed and washed thoroughly with aqueous dioxane. The total acidic solution of the nucleotide was neutralized to *p*H 3.5 with lithium hydroxide and concentrated to small bulk. More water was added and the suspension of triphenylcarbinol and *p*-nitrophenol extracted with ether. The solution then was passed through a free sulfonic acid resin column to remove lithium ions and the total acidic effluent neutralized with barium hydroxide to *p*H 7.5. A small amount of precipitate which formed was removed by centrifugation and barium thymidine-3′ phosphate was precipitated after concentration of the clear solution with two volumes of ethyl alcohol and washed as described above. The yield (0.389 g. of the trihydrate[36]) was 75% based on 5′-*O*-tritylthymidine. The material was electrophoretically and chromatographically homogeneous and had the characteristics: $\lambda_{max}$ (in neutral solution) 267 mμ; $\epsilon_{max}/P$ 9500; 250/260, 0.635; 250/270, 0.588; 280/260, 0.708.[7]

**Polymerization of Thymidine-3′ Phosphate.**—Thymidine-3′ phosphate was first converted to $P^1,P^2$-dithymidine-3′ pyrophosphate as follows.[16] An aqueous solution of the barium salt (2.18 moles) was passed through Amberlite IR-120 ($H^+$) resin column and to the total effluent containing the free acid was added an excess of pyridine and the solution evaporated. The pyridinium salt was rendered anhydrous by successive evaporations with five portions (5 ml. each) of dry pyridine. The salt crystallized during these evaporations and was thereafter mostly insoluble in pyridine. To the solid, pyridine (10 ml.) and tri-*n*-butylamine (1.04 ml., 4.4 mmoles) were added and the resulting homogeneous solution was treated with dicyclohexylcarbodiimide (2.25 g., 11 mmoles). Paper-electrophoresis after 12 hr. showed that conversion to the pyrophosphate was essentially complete. The solvent was then removed, and the residue shaken with a mixture of water and ether. The aqueous layer was separated and extracted once again with ether. The aqueous solution of the tri-*n*-butylammonium salt was passed through a pyridinium Amberlite IR-120 resin column and the resulting solution of the pyridinium salt[17] evaporated under reduced pressure. It was rendered anhydrous by repeated addition and evaporation of dry pyridine and thus was obtained as a pyridine-soluble gum. Ten ml. of dry pyridine finally was added followed by dicyclohexylcarbodiimide (2.25 g., 11.0 mmoles). The sealed reaction mixture which deposited some insoluble material was shaken at room temperature for 4 days. Most of the solvent then was evaporated under reduced pressure and water and ether added. (Crystalline urea remained partly undissolved at this stage.) The aqueous layer was separated and re-extracted with ether. The aqueous solution was evaporated after adjusting its *p*H to 8 with lithium hydroxide. The residue was dissolved again in water and the solution adjusted to the above *p*H and evaporated. The process was repeated three times to ensure complete removal of pyridine. Spectrophotometric determination of an aliquot of the final aqueous solution of the polymeric mixture showed that a total of 17,500 optical density units (267 mμ) was present.

**Column Chromatography:** (a) **Analytical Run.**—An appropriate aliquot containing 105 optical density units (267 mμ) of the above solution was applied to a column (12 cm. height × 1 cm. diameter) of Ecteola[37] ($Cl^-$ form) cellulose and after a water wash, elution was begun by a linear gradient technique as described before.[6] The salt solution vessel contained 0.25 *M* lithium chloride + 0.025 *M* lithium acetate buffer of *p*H 5.0. The resulting elution pattern was essentially that shown in Fig. 1. (b) **Preparative Run.**—A portion (13.0 ml., 9,100 O.D. units) of the polymer solution was washed onto a column of Ecteola ($Cl^-$ form) cellulose[6,37] (35 cm. height × 4 cm. diameter) with water and eluted with a linear salt gradient delivered from water (4 liters) and lithium chloride solution (0.25 *M*) buffered with lithium acetate (0.025 *M*) at *p*H 5.0 (4 liters). The flow rate was 1.50 ml./minute and fractions of 15 ml. were collected. A plot of the results is reproduced in Fig. 1.

**Isolation of the Oligonucleotides.**—The bulked peaks were concentrated to a small volume by flash-evaporation and processed to remove salt by either of the following methods: (a) The solution was taken to dryness at low pressure and the crystalline residue dissolved in the minimum volume of methanol. Acetone was added in sufficient quantity to precipitate the nucleotide as a fine grayish-white powder and the solid removed by centrifugation. The precipitate was washed with a little methanol–acetone, then redissolved in methanol and reprecipitated as above. In this way peak H was dissolved in methanol (4 ml.) and

(34) A separate experiment showed that when *p*-nitrophenyl phosphorodichloridate was hydrolyzed in aqueous pyridine using a limited amouot of water, an appreciable amount of the symmetrical di-*p*-nitrophenylpyrophosphate was formed.

(35) The discrepancy between the observed and calculated (16,900) $\epsilon_{max}$ is greater in this case than for the 5′-isomer. Thymidine-5′ *p*-nitrophenyl phosphate has an $\epsilon_{max}$ of 16,250 and methyl *p*-nitrophenyl phosphate an $\epsilon$270 of 7700 (Dr. J. G. Moffatt, private communication).

(36) As ascertained by phosphorus analysis.

(37) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1956).

TABLE II

$R_f$ VALUES OF THYMIDINE OLIGONUCLEOTIDES AND OTHER DERIVATIVES

| Compound | Solvent A | Solvent B or C |
|---|---|---|
| Unidentified component of peak E | 0.030 | |
| Unidentified component of peak H | .0083 | |
| Unidentified component of peak J | .0034 | |
| Unidentified component of peak L | .0021 | |
| Linear dinucleotide (IX, $n$ = 0) | .077 | 0.45 C |
| Linear trinucleotide (IX, $n$ = 1) | .043 | .35 C |
| Linear tetranucleotide (IX, $n$ = 2) | .018 | .29 C |
| Linear pentanucleotide (IX, $n$ = 3) | .0099 | .21 C |
| Pyrophosphate component of peak D | .36 | |
| Pyrophosphate component of peak G | .18 | |
| Pyrophosphate components of peak I | .090 | |
| Pyrophosphate components of peak L | .043 | |
| Thymidine-3′ phosphate (I) | .18 | .36 B |
| Thymidine-3′ pyrophosphate | .11 | |
| 5-*O*-Tritylthymidine-3′ *p*-nitrophenyl phosphate (IV) | .81 | .78 B |
| 5′-*O*-Tritylthymidine-3′ phosphate (VII) | .61 | .72 B |
| *p*-Nitrophenyl phosphate | .45 | .48 B |
| Thymidine-3′ *p*-nitrophenyl phosphate (V) | .70 | .63 B |

ume of 10 ml. onto charcoal (430 mg.) and after washing, elution and evaporation, gave the nucleotide. The recovery of optical density by this process was 95%.

The nucleotide then was applied to Whatman 3 MM paper and chromatographed in solvent A. The purified nucleotides finally were isolated by elution with water. In general it was possible to identify the components of a peak by their relative $R_f$'s in solvent A since they were always of the same general order. Thus in peaks G, I and K the slow, medium and fast moving components were always the linear, cyclic and pyrophosphate type oligonucleotides, respectively. Similarly peaks H, J and L each gave slow, medium and fast moving components which were the "unidentified component," linear and cyclic oligonucleotides, respectively. $R_f$'s of the compounds are given in Table II.

**Venom Diesterase Degradation of the Linear Tri-(IX, $n$ = 1) and Tetra-(IX, $n$ = 2) Oligonucleotides.**—The oligonucleotide (0.3–0.4 μmole) was treated with purified *Crotalus adamanteus* phosphodiesterase[38] (0.10 ml. of a solution of the enzyme in tris-hydroxymethylaminomethane buffer *p*H 8.5) in a final volume of 0.20 ml. and the mixture incubated at 37°. Samples were withdrawn at varying times and chromatographed on Whatman 1 paper in solvent A. After development, the spots were cut out and eluted with water and estimated spectrophotometrically. The yields of the various compounds, expressed as a percentage of the total optical density of the aliquots, are shown in Table III.

The "slowest moving" spots from the trinucleotide digests were eluted and the product subjected to electrophoresis (*p*H 7.5) to remove unreacted oligonucleotide. The area corresponding to thymidine-3′,5′ -diphosphate and dithymidine triphosphate was cut out and eluted with water

TABLE III

HYDROLYSIS OF OLIGONUCLEOTIDES BY VENOM DIESTERASE

| Time (hr.) | | 2.0 | 4.0 | 8.0 | 16.5 | 43.5 |
|---|---|---|---|---|---|---|
| Linear trinucleotide | Slowest moving spot,[a] % | | 80.0 | 81.0 | 70.5 | 55.0 |
| (IX, $n$ = 1) | Thymidine-5′ phosphate, % | | 8.5 | 12.9 | 19.8 | 29.0 |
| | Thymidine, % | | 11.9 | 6.2 | 10.3 | 16.0 |
| Linear tetranucleotide | Slowest moving spot,[b] % | 88.0 | 88.0 | 80.5 | 71.0 | 65.7 |
| (IX, $n$ = 2) | Thymidine-5′ phosphate, % | 10.7 | 12.0 | 17.7 | 24.2 | 19.3 |
| | Thymidine, % | 1.3 | 0.0 | 1.73 | 4.83 | 15.2 |

[a] This represents the sum of unchanged linear trinucleotide, dithymidine triphosphate and thymidine-3′,5′-diphosphate. [b] This represents the sum of unchanged linear tetranucleotide, trithymidine tetraphosphate, dithymidine triphosphate and thymidine-3′,5′ diphosphate.

nucleotide precipitated by the addition of acetone (12 ml.). Washing with methanol–acetone and reprecipitation in the same way gave the salt-free nucleotide. (The recovery of optical density by this process was 70–75%).

(b) The solution was stirred with sufficient charcoal to adsorb all of the optical density then the mixture filtered on a bed of Celite. Complete removal of the salt was effected using water, then the nucleotide eluted by washing with aqueous ethanolic concd. ammonium hydroxide (49:49:2 v./v.). Evaporation of solvent gave the nucleotide as a gum. In this way peak G was adsorbed in a volume of 10 ml. onto charcoal (430 mg.) and after washing, elution and evaporation, gave the nucleotide. The recovery of optical density by this process was 95%.

The nucleotide then was applied to Whatman 3 MM paper and chromatographed in solvent A. The purified nucleotides finally were isolated by elution with water. In general it was possible to identify the components of a peak by their relative $R_f$'s in solvent A since they were always of the same general order. Thus in peaks G, I and K the slow, medium and fast moving components were always the linear, cyclic and pyrophosphate type oligonucleotides, respectively. Similarly peaks H, J and L each gave slow, medium and fast moving components which were the "unidentified component," linear and cyclic oligonucleotides, respectively. $R_f$'s of the compounds are given in Table II.

**Venom Diesterase Degradation of the Linear Tri-(IX, $n$ = 1) and Tetra-(IX, $n$ = 2) Oligonucleotides.**—The oligonucleotide (0.3–0.4 μmole) was treated with purified *Crotalus adamanteus* phosphodiesterase[38] (0.10 ml. of a solution of the enzyme in tris-hydroxymethylaminomethane buffer *p*H 8.5) in a final volume of 0.20 ml. and the mixture incubated at 37°. Samples were withdrawn at varying times and chromatographed on Whatman 1 paper in solvent A. After development, the spots were cut out and eluted with water and estimated spectrophotometrically. The yields of the various compounds, expressed as a percentage of the total optical density of the aliquots, are shown in Table III.

The "slowest moving" spots from the trinucleotide digests were eluted and the product subjected to electrophoresis (*p*H 7.5) to remove unreacted oligonucleotide. The area corresponding to thymidine-3′,5′ -diphosphate and dithymidine triphosphate was cut out and eluted with water and the solution digested with prostate phosphomonoesterase.[27] Chromatography indicated the presence of thymidine and dithymidine phosphate. Similarly the "slowest moving" spots from the tetranucleotide digest was shown to consist of unchanged tetranucleotide and a material which could be dephosphorylated to thymidine, dithymidine phosphate and trithymidine diphosphate.

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(38) We are indebted to Dr. W. E. Razzell for this preparation.