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

Studies on Polynucleotides. II.[1] The Synthesis and Characterization of Linear and Cyclic Thymidine Oligonucleotides[2]

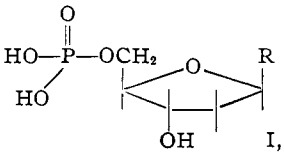
BY G. M. TENER, H. G. KHORANA, R. MARKHAM[3] AND E. H. POL

RECEIVED AUGUST 4, 1958

The reaction of thymidine-5′ phosphate with either *p*-toluenesulfonyl chloride or di*cyclo*hexylcarbodiimide in anhydrous pyridine at room temperature gives a number of polymeric products which can be separated satisfactorily on cellulose anion-exchange (DEAE and ECTEOLA cellulose) columns. The chromatographic pattern indicates that polynucleotides containing up to eleven units are present in the synthetic mixtures. The major peaks consist of two homologous series of compounds: (a) the *linear* oligonucleotides containing the repeating 5′→3′ internucleotidic linkage and bearing a 5′-phosphate group at one and a 3′-hydroxyl group at the other end of the chain; and (b), the *cyclic* oligonucleotides in which the linear compounds are cyclized by phosphorylation of the terminal 3′-hydroxyl group by the 5′-phosphate group at the other end. The extent of this intramolecular cyclization decreases with increase in chain length, such that only a trace of the cyclic pentanucleotide is formed. Members of both series up to the pentanucleotide have so far been isolated pure and characterized by chemical and enzymatic methods. A minor product formed during polymerization was identified as thymidine-3′,5′ cyclic phosphate (IV). Alternative satisfactory methods for the synthesis of this ester are described and some properties of this new type of cyclic phosphate are reported.

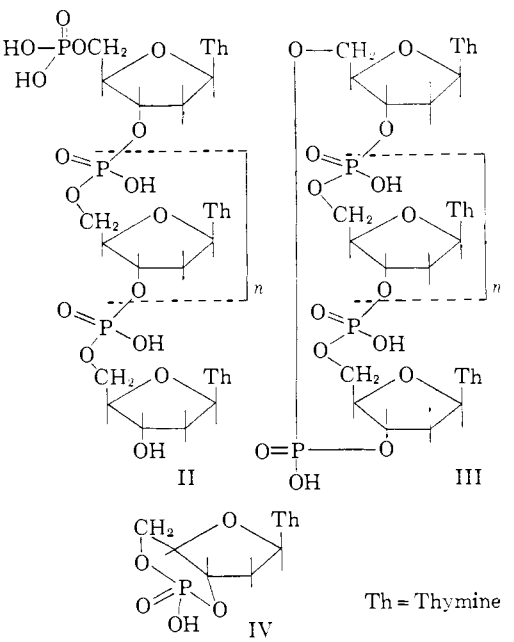
Studies in the protein field have benefited greatly from the availability of synthetic polypeptides consisting of only one type of amino acid residue. Similarly, ribo-polynucleotides, which have recently been prepared enzymatically,[4] are proving useful in current studies[5] of the nucleic acids. Chemical methods for the polymerization of mononucleotides would clearly be of similar value, especially as they might add to the range of size of such polymers and make the smaller-sized members more readily available in quantity. In the deoxyribopolynucleotide field, chemical methods have an added importance since polymers consisting of one type of deoxyribonucleotide residue have so far not been prepared. An alternative possibility for obtaining deoxyribo-oligonucleotides, namely, by the controlled enzymatic hydrolysis of high molecular weight deoxyribonucleic acids, leads to an exceedingly complex mixture of small fragments so that it is doubtful that oligonucleotides consisting of more than a few nucleotide residues can be isolated pure in any quantity.

The discovery of a chemical method for the synthesis of $C_5'\rightarrow C_3'$ internucleotide bonds in the deoxyribonucleotide series was reported recently from this Laboratory.[1,6,7] The method which involved the direct activation of the phosphomonoester group in mononucleotides offered an excellent opportunity for studying the polymerization of deoxyribonucleotides containing a free 3′-hydroxyl function (General formula I) and the nucleotide chosen first for studies of this type was thymidine-5′ phosphate (I, R = thymine). As already reported briefly,[6-8] a number of polymeric products



I, R = purine or pyrimidine

were indeed formed when this substance (I, R = thymine) was treated with either *p*-toluenesulfonyl chloride or di*cyclo*hexylcarbodiimide (DCC) in anhydrous pyridine at room temperature. The present communication contains a detailed report on the isolation and characterization of these products, which correspond to two homologous series; (a) *linear* oligonucleotides[9] of the type II



II III

IV

Th = Thymine

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

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

(3) On leave from the Agricultural Research Council Virus Research Unit, Cambridge, England, and wishes to thank the Wellcome Foundation for a travel grant.

(4) M. Grunberg-Manago, P. J. Ortiz and S. Ochoa, *Biochem. Biophys. Acta*, **20**, 269 (1956).

(5) R. C. Warner, *J. Biol. Chem.*, **229**, 711 (1957); A. Rich, *Nature*, **181**, 521 (1958); J. R. Fresco and P. Doty, THIS JOURNAL, **79**, 3928 (1957); L. A. Heppel, P. J. Ortiz and S. Ochoa, *J. Biol. Chem.*, **229**, 679 (1957); *Science*, **123**, 415 (1956); M. F. Singer, L. A. Heppel and R. J. Hilmoe, *Biochim. Biophys. Acta*, **26**, 447 (1957).

(6) H. G. Khorana, G. M. Tener, J. G. Moffatt and E. H. Pol, *Chemistry and Industry*, 1523 (1956).

(7) H. G. Khorana, W. E. Razzell, P. T. Gilham, G. M. Tener and E. H. Pol, THIS JOURNAL, **79**, 1002 (1957).

(8) H. G. Khorana, G. M. Tener, W. E. Razzell and R. Markham, *Federation Proc.*, **17**, 253 (1958).

(9) In describing the simple oligonucleotides consisting of only one type of nucleoside and internucleotidic linkage it is felt unnecessary to give the systematic names according to the nomenclature proposed for polynucleotides.[1] Instead, the trivial names linear and cyclic thymidine oligonucleotides are used. The systematic name for the linear dinucleotide is thymidylyl-(5′→3′)-thymidylic-(5′) acid while

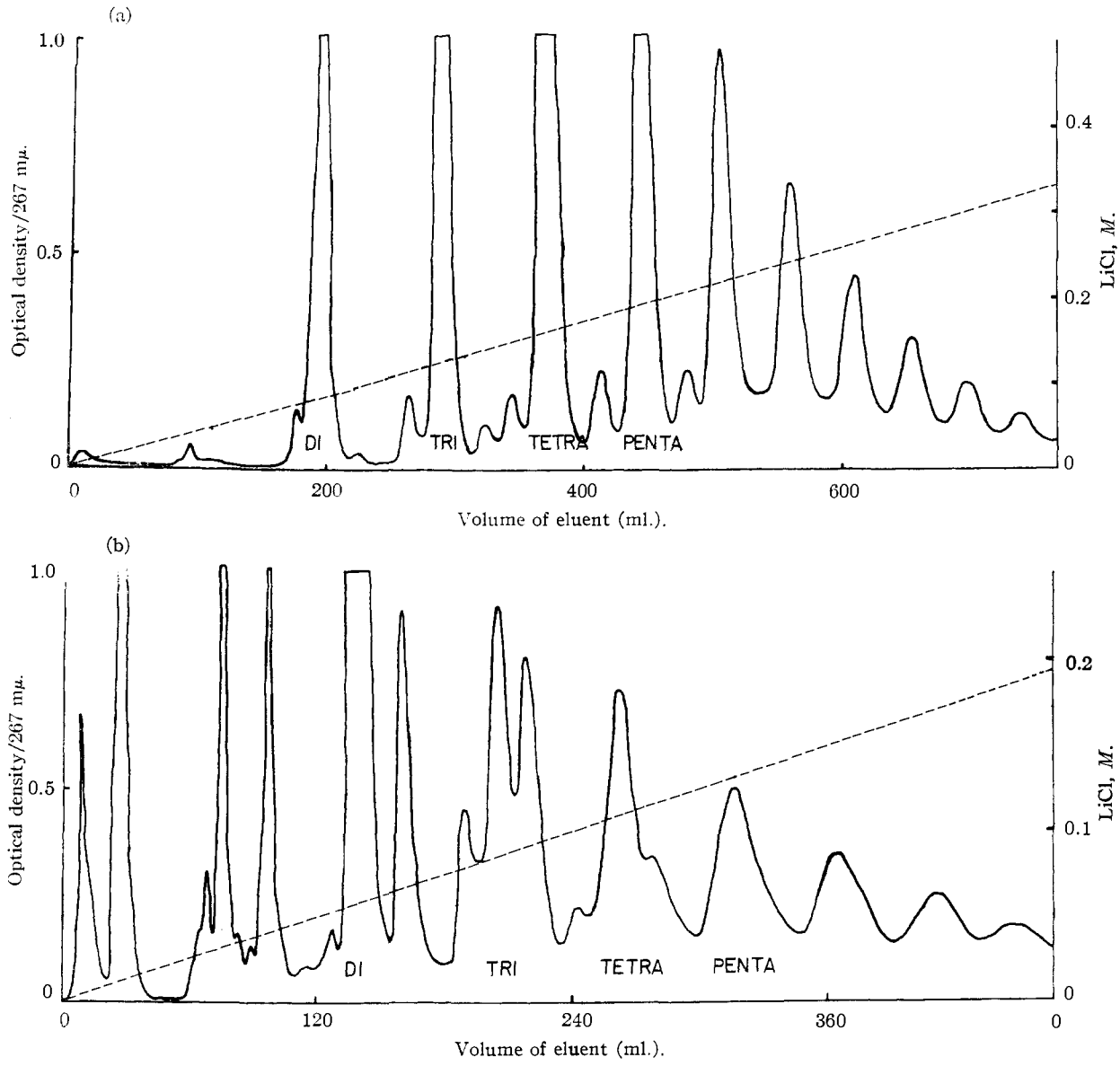


Fig. 1.—Chromatographic analysis of thymidine oligonucleotides: (a, top) products of reaction of thymidine-5′ phosphate with *p*-toluenesulfonyl chloride. The total mixture was dialyzed against distilled water and 220 optical density units (267 mμ) of the non-dialyzable material was chromatographed on a DEAE cellulose column (16.5 cm. × 0.9 cm.) using linear salt (lithium chloride) gradient as shown by the dotted line. Flow rate, about 1.2 ml./min. (b, bottom) total products of reaction of thymidine-5′ phosphate with DCC-chromatographed on an ECTEOLA cellulose column (8 cm. × 0.9 cm.) using linear salt gradient as shown by dotted line. Flow rate, 0.7 ml./min.

and (b) *cyclic* oligonucleotides[9] of the general structure III. In addition, a minor product obtained in these polymerization experiments was identified as thymidine-3′,5′ cyclic phosphate (IV).

Early experiments indicated that the products obtained by the reaction of thymidine-5′ phosphate with *p*-toluenesulfonyl chloride or DCC were polymeric in nature. In order to separate the products, paper chromatography was used initially with partial success. Anion-exchange resin columns which have been used previously for separation of mixtures of oligonucleotides[10] proved unsatisfactory in the present work. Compounds higher than the dinucleotide were held tenaciously on the resins and only a small portion of the total ultraviolet absorbing material applied was recovered. The anion exchangers which proved strikingly useful in the separation of present mixtures are the aminoalkyl-substituted celluloses which were first used by Peterson and Sober[11] and recently have been investigated by Bendich and co-workers[12] for fractionation of deoxyribonucleic acids. Both diethylaminoethyl (DEAE) cellu-

that for the cyclic dinucleotide is *cyclo*-[thymidylyl-(5′→3′)-thymidylyl-(5′→3′)]. Higher members would be named analogously.

(10) W. E. Cohn in "The Nucleic Acids," Vol. I, Academic Press, Inc., New York, N. Y., 1955, p. 211.

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

(12) A. Bendich, J. R. Fresco, H. S. Rosenkranz and S. M. Beiser, *ibid.*, **77**, 3671 (1955).

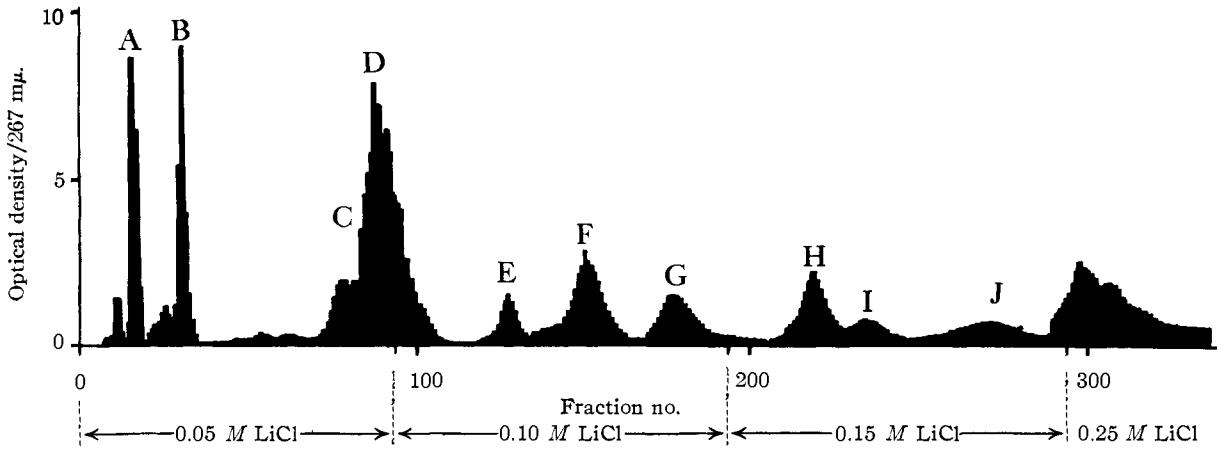


Fig. 2.—Preparative chromatography of thymidine oligonucleotide on an ECTEOLA cellulose column (23 cm. × 4 cm. diameter). Material applied was derived from 0.8 mmole of thymidine-5′ phosphate. Fraction size, approximately 20 ml., flow rate, approximately 3 ml. per minute. For identification of various peaks see Table I.

lose and "ECTEOLA"[11,13] cellulose have been used in the present work. Small scale exploratory columns were run overnight using a gradient elution technique and the results of two such separations are shown in Fig. 1a and 1b. In preparative work the total reaction mixture was separated using a stepwise elution procedure and each major peak was examined further by paper chromatography and paper electrophoresis. Sometimes the linear oligonucleotide emerged together with the corresponding cyclic member (Fig. 1a) and when this was the case, separation was achieved subsequently by chromatography on paper sheets. In the run reported here (Fig. 2), the linear and cyclic compounds larger than the dinucleotides were well resolved.

Linear Oligonucleotides

The linear di-, tri-, tetra- and pentanucleotides (II, $n = 0$–3) have been isolated pure and characterized. Their yields and the pattern of elution from the preparative column (Fig. 2) are shown in Table I. After the pentanucleotide had been eluted, the rest of the nucleotidic material was eluted together by 0.25 *M* lithium chloride solution. The optical density eluted this way corresponded to about 15% of the total applied to the column.

The linear dinucleotide (II, $n = 0$) was identical with the previously synthesized and well-characterized samples.[1] The general chemical and enzymatic methods used for the identification of the higher members were as noted: the homogeneity of each of the members was established by paper chromatography in three solvent systems (see Experimental) and by paper electrophoresis under acidic, neutral and alkaline conditions. The ratio of total phosphorus to thymidine (ultraviolet absorption measurement) was close to one for all the members. Purified snake venom diesterase degraded these compounds rapidly and quantitatively to thymidine-5′ phosphate, thus supporting the conclusion that the compounds were simple polymers of the mononucleotide.

(13) The procedure used for the preparation was a modification of one of the original procedures of Peterson and Sober.[11] The modified procedure was kindly communicated to us by Dr. A. Bendich.

The presence of the phosphomonoester end groups in the linear compounds was indicated by the increase in mobility on paper electrophoresis at *p*H 7.5 as compared with that at *p*H 3.5. Electrometric titration of the free tri- and tetranucleotides showed that the ratio of primary phosphoryl dissociations (alkali consumed for titration up to *p*H 4) to secondary phosphoryl dissociation (titration from *p*H 4 to *p*H 8) was, respectively, three and four. Determination of the ratio of the phosphorus (mono-esterified) released by prostatic phosphomonoesterase[14] to total phosphorus also showed the chain length[15] of the homologous members.

The series of compounds (V, $n = 0$–3) obtained after dephosphorylation of the oligonucleotides by phosphomonoesterase were degraded by snake venom diesterase[16] preparation to give mixtures of the mononucleotide (thymidine-5′ phosphate) and thymidine. Compounds of the type V also were degraded by spleen phosphodiesterase[16,17] to thymidine-3′ phosphate and thymidine. The ratios of the mononucleotide to nucleoside obtained in these degradations provided further check on the size of the oligonucleotides. Thus, the degradation of the dephosphorylated pentanucleotide (V, $n = 3$) by the snake venom diesterase gave 4 moles of

TABLE I

Product	(a) Linear oligonucleotides Peak	Yield, %[a]	(b) Cyclic oligonucleotides Peak	Yield, %[a]
Thymidine-5′ phosphate + thymidine-3′,5′ cyclic phosphate	B	3		
Dinucleotide	C	5	D	15–20
Trinucleotide	F	7	C	5–6
Tetranucleotide	H	6	I	3
Pentanucleotide	J	3	J	0.5

[a] These are the percentages of the total optical density eluted in the various peaks.

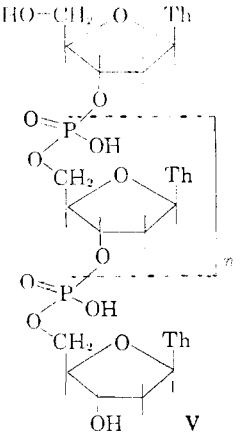
(14) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 558 (1952).

(15) *Cf.* R. L. Sinsheimer and J. F. Koerner, THIS JOURNAL, **74**, 283 (1952).

(16) W. E. Razzell and H. G. Khorana, *ibid.*, **80**, 1770 (1958).

(17) We are grateful to Dr. R. J. Hilmoe for a gift of the highly purified spleen diesterase.

thymidine-5′ phosphate for every mole of thymidine while degradation by the spleen diesterase gave 4 moles of thymidine-3′ phosphate for every mole of thymidine.



V

Cyclic Oligonucleotides

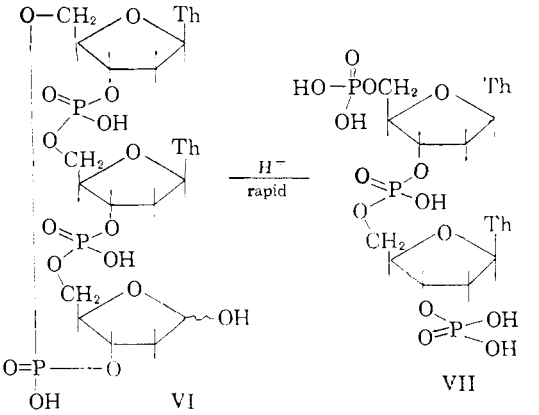
The yields and the order of elution of the cyclic di-, tri-, tetra- and penta-nucleotides (III, n = 0-3) are listed in Table I. It can be seen that the ratio of the individual cyclic oligonucleotides to the corresponding linear compounds decreases with increase in chain length. Thus, while at the dinucleotide level, the cyclic member (III, n = 0) is much more abundant than the linear dinucleotide, at the pentanucleotide stage, the cyclic compound forms only a small proportion of the total material.

Cyclic Dinucleotide (III, n = 0).—The ratio of phosphorus to thymidine, as estimated spectrophotometrically, was found to be 1:1 for this substance. It was degraded by venom diesterase to thymidine-5′ phosphate, although much more slowly than the linear dinucleotide. These results indicated that the compound was probably a polymer of the mononucleotide. In electrophoretic mobility at pH 3.5 the substance was indistinguishable from the linear dinucleotide or P^1P^2-dithymidine-5′ pyrophosphate. It also resembled the latter in not having a secondary phosphoryl dissociation and in its mobility on paper chromatograms developed in the isopropylalcohol–ammonia–water solvent. However it was readily shown to be different from the pyrophosphate by a study of the acidic hydrolysis. While the pyrophosphate was hydrolyzed completely in about 2 hr. at 100° in 0.1 N hydrochloric acid to give thymidine-5′ phosphate, the cyclic dinucleotide was largely unaffected under these conditions. It required heating in 1 N hydrochloric acid at 100° for 3 hr. for complete hydrolysis and the products were thymidine-3′,5′ diphosphate, thymine and some inorganic phosphate. The acid stability and the formation of thymidine-3′,5′ diphosphate showed the presence of a 5′→3′-phosphodiester linkage in the compound.

The evidence presented above and the position of emergence of the substance from the column (Fig. 2) showed that in total charge and size it corresponded to a dinucleotide. The absence of a phosphomonoester group indicated it to be cyclic. Direct proof of structure was obtained by synthesis in over 80% yield from the linear dinucleotide by treatment of the latter at high dilution with DCC in pyridine.

Cyclic Tri-, Tetra- and Penta-nucleotides.—These compounds also were concluded to be cyclic by the absence of the phosphonomonoester end groups and by their complete degradation to thymidine-5′ phosphate on digestion with venom diesterase. That they represented a homologous series was clear from their R_f values on paper chromatograms and the fact that their relative electrophoretic mobilities were constant in acidic, neutral and alkaline buffers. Their order of elutions from the column (Fig. 2) was further evidence in support of this conclusion and enabled correlation with the linear series. Paper electrophoretic mobility of each cyclic homolog was identical with that of the corresponding linear compound at pH 3.5. Confirmation of structure of the cyclic trinucleotide (II, n = 1) also was obtained by its synthesis from the linear trinucleotide by treatment of the latter in high dilution with DCC.

Acidic Hydrolysis of Cyclic Oligonucleotides.—The first step in the acidic hydrolysis of the symmetrical cyclic oligonucleotides is the cleavage of one of the glycosyl bonds. As exemplified by the cyclic trinucleotide, the product would be VI, which



VI VII

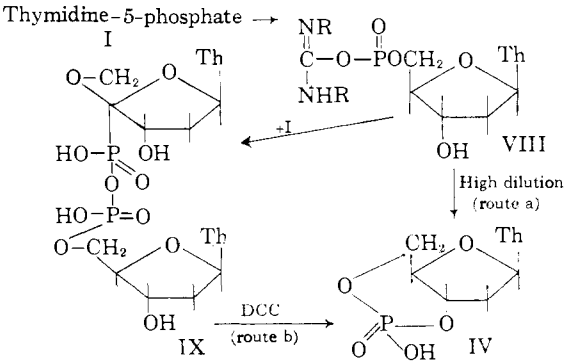
would suffer rapid degradation at points linking the phosphoryl groups to the reducing deoxyribose fragment to give dithymidine triphosphate (VII). Further degradation of VII would occur by an analogous manner to give thymidine diphosphate. This mode of degradation of the cyclic compounds makes it possible to correlate them with the linear series. The technique employed was to hydrolyze partially the cyclic oligonucleotide so as to isolate the largest linear product of degradation, namely, the product containing all the phosphorus of the original molecule but one less thymidine unit. Compounds of the type VII then were treated with prostate phosphomonoesterase to remove the phosphomonoester end groups and the resulting compounds V were identified by comparison with the same series of products obtained from the linear oligonucleotides II by phosphomonoesterase treatment. In the case of VII, the product after dephosphorylation was identical with a synthetic sample of dithymidine monophosphate.[1] The cyclic tetranucleotide gave as expected trithymidine

diphosphate as the major product and, similarly, the cyclic pentanucleotide gave tetrathymidine triphosphate.

Thymidine-3′,5′ Cyclic Phosphate.—A minor product obtained during the polymerization experiments, which emerges from the column with thymidine-5′ phosphate (peak B, Fig. 2) has been identified as thymidine-3′,5′ cyclic phosphate (IV). This substance may in fact be regarded as the smallest member of the cyclic series of compounds described above. The following evidence is consistent with the assigned structure. Its mobility on paper chromatograms was very similar to those of a large number of cyclic phosphates (for example, ribonucleoside-2′,3′ cyclic phosphates) studied previously.[18] Its electrophoretic mobility at an acid *p*H was equal to, but at neutral *p*H was lower than, that of thymidine-5′ phosphate. This finding showed the absence of a secondary phosphoryl dissociation. The substance, which was quite stable to alkali, hydrolyzed on heating at 100° in 1 *N* sodium hydroxide (50% hydrolysis in 2 hours) to give a mixture of thymidine-3′ phosphate (*ca.* 80%) and thymidine-5′ phosphate(*ca.* 20%). Hydrolysis by a snake venom diesterase fraction, although slow, gave again a mixture of the two isomeric thymidine monophosphates.[19,20] These results show that the 3′-hydroxyl group of thymidine is involved in esterification.

Several alternative methods for the synthesis of thymidine-3′,5′ cyclic phosphate have been discovered and these provide additional support for its structure. P^1P^2-Dithymidine-5′ pyrophosphate (IX) on being heated in anhydrous pyridine undergoes partial conversion to thymidine-3′,5′ cyclic phosphate. The reaction is another example of *intramolecular phosphorylation*[18] which has been observed in several cases during the cleavage of esters of pyrophosphoric acid (nucleotide coenzymes, etc.) containing suitably placed hydroxyl groups. Preparatively, a better yield of the thymidine-3′,5′ cyclic phosphate is obtained by heating bis-(tri-*n*-butylammonium)-dithymidine pyrophosphate (IX) in the presence of DCC (route b, Scheme I). The reaction is reminiscent of the conversion of bis-4-hydroxybutyl pyrophosphate to butane-1,4-diol cyclic phosphate on reaction with the carbodiimide.[18] Thymidine-3′,5′ cyclic phosphate also is formed, although as a minor product, when thymidine-3′ *p*-nitrophenyl phosphate is heated with alkali.[21,22] This reaction is also analogous to the previously described synthesis of cyclic phosphates by transesterification[18] of diesters of phosphoric acid. The best method for the synthesis of the cyclic phosphate IV is the reaction of thymidine-5′ phosphate with DCC in a very dilute pyridine solution at room temperature (route a, Scheme I).

SCHEME I



The 3′,5′-cyclic phosphate ring in IV causes a hypsochromic shift of the ultraviolet absorption maximum (λ_{max}, 264.5 mμ) and great labilization of the glycosyl bond to acidic treatment. The cyclic phosphate was hydrolyzed completely in 0.1 *N* hydrochloric acid at 100° in under five minutes. Thymidine-5′ phosphate is largely unaffected under these conditions for three hours.

Concurrently with the present work, Cook, Lipkin and Markham[23] have investigated the structures of some of the products formed during the heating in alkaline solution of the barium salt of adenosine-5′ triphosphate. One of the products formed has been shown by these authors to be adenosine-3′,5′ cyclic phosphate. This product is identical with the substance isolated by Sutherland and Rall[24] from tissue particles and found by them to stimulate the formation of glycogen phosphorylase.

A variety of cyclic phosphates have been described in recent years.[18] The usual method employed for the preparation of such esters has been the activation of the phosphomonoester group followed by an intramolecular cyclization with a suitably placed hydroxyl group. The ease of the cyclization reaction is very much a function of the stereochemistry of the phosphoryl group and the pertinent hydroxyl group, and it is easy to see why the nucleoside-3′,5′ cyclic phosphates were not encountered earlier in studies of reactions involving nucleoside-5′ phosphates and DCC. Thus, under identical conditions, while ribonucleoside-2′(3′) phosphates form on reaction with DCC the nucleoside-2′,3′ cyclic phosphates,[25] nucleoside-5′ phosphates give the dinucleoside-5′ pyrophosphates[26] (I→VIII→IX, scheme I). The preferred *bimolecular* reaction between the adduct VIII and

(18) H. G. Khorana, G. M. Tener, R. S. Wright and J. G. Moffatt, THIS JOURNAL, **79**, 430 (1957).

(19) This finding might imply that two distinct diesterases are present in the snake venom fraction used.

(20) Similarly, enzymatic hydrolysis of uridine-3′,5′ cyclic phosphate, gives as a product uridine-3′ phosphate which can be characterized by its reaction with DCC to form first uridine-2′,3′ cyclic phosphate and then the N-phosphoryl ureas (unpublished work of Dr. M. Smith in this Laboratory).

(21) It may be noted that the reaction involving tri-*n*-butylammonium salt occurs only on heating. At room temperature, the trialkylammonium salts of dinucleoside-5′, pyrophosphates are stable over prolonged periods (M. Smith and H. G. Khorana, THIS JOURNAL, **80**, 1141 (1958). The preparation of a nucleoside 3′,5′-cyclic phosphate (adenosine-3′,5′ cyclic phosphate) by heating of a trialkylammonium salt of nucleoside-5′ phosphate and DCC in pyridine was first carried out by Lipkin, Markham and Cook (unpublished results). This synthesis proceeds likewise *via* dinucleoside-5′ pyrophosphate (IX, route b, Scheme I).

(22) A. F. Turner and H. G. Khorana, unpublished results.

(23) W. H. Cook, D. Lipkin and R. Markham, THIS JOURNAL, **79**, 3607 (1957); D. Lipkin, W. H. Cook and R. Markham, Abstracts of the Am. Chem. Soc. 133rd National meeting, p. 4D. 1958.

(24) T. W. Rall and E. W. Sutherland, *J. Biol. Chem.*, **232**, 1065 (1958); E. W. Sutherland and T. W. Rall, *ibid.*, **232**, 1077 (1958).

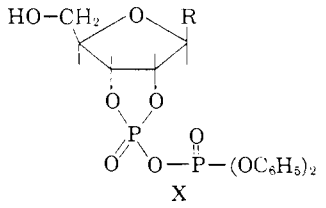
(25) C. A. Dekker and H. G. Khorana, THIS JOURNAL, **76**, 3422 (1954).

(26) H. G. Khorana, *ibid.*, **76**, 3417 (1954).

another nucleotide anion,[27] which leads to the formation of the pyrophosphate IX can however be suppressed by using high dilution reaction conditions and the adduct VIII then probably undergoes the sterically unfavored *intramolecular* reaction to form nucleoside-3′,5′ cyclic phosphate (IV) directly (route a, Scheme I).

General Remarks

The present work constitutes the first report on the successful chemical polymerization of a mononucleotide, in which the *phosphomonoester grouping* is activated *directly*. The mechanism of the reaction is complex and certain observations on it have been recorded previously. The method used by Michelson[28] in the recently reported synthesis of ribo-oligonucleotides containing random 2′→5′ and 3′→5′ internucleotide bonds involves the prior formation of ribonucleoside-2′,3′ cyclic phosphates and, presumably, their subsequent activation by formation of the mixed anhydride X. The latter is akin to the conventional fully protected mixed anhydrides which have been used as phosphorylating agents previously.[29]



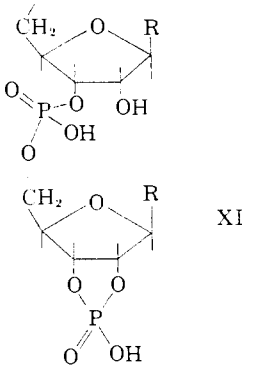
X

Of the two reagents used, DCC and *p*-toluenesulfonyl chloride, the former is preferred. Although by-products were formed (peaks A and E, Fig. 2) which remain to be identified, the polymeric mixture was cleaner than that obtained by using *p*-toluenesulfonyl chloride.

Cellulose anion exchangers of ECTEOLA type, which have proved useful in the present work, are promising for the separation of higher polynucleotides. A thorough study of these anion exchangers using the simpler synthetic polymers of mononucleotides is desirable.

Although only the oligonucleotides up to the pentanucleotides have been characterized in the present work, the elution patterns (Fig. 1) indicate that homologs containing up to eleven units are present in the polymeric mixtures. Since, as mentioned above, intramolecular cyclization becomes negligible beyond the pentanucleotide stage, the higher homologs will probably consist esclusively of the linear series.

It seems important to point out that the name "cyclic oligonucleotides" in the ribonucleotide series previously has been used to designate certain fragments obtained by the degradation of ribonucleic acids by pancreatic ribonuclease.[14] These compounds which bear a five-membered cyclic phosphate group at one terminus of the linear chain are of the type XI. In our view, in future, the term "cyclic oligonucleotide" should be restricted to the class of compounds described in the present work, in which a "macro" ring is formed by "end to end" cyclization of the linear polynucleotide chain. These compounds are then analogous to the cyclic polypeptides in which the end groups of a linear peptide chain are involved in an amide bond to form a ring.



XI

Finally, the present work can be extended in a number of directions; for example, to the polymerization of different deoxyribonucleotides, singly and as mixtures, of performed di- and trinucleotides, and to the synthesis of polyribonucleotides.[30] These extensions will be reported in subsequent papers.

Experimental

Polymerization of Thymidine-5′ Phosphate: Experiment I, Using *p*-Toluene-sulfonyl Chloride.—(a) Pyridinium thymidine-5′ phosphate (0.5 mmole), was rendered anhydrous by repeated evaporations of its anhydrous pyridine solution under reduced pressure and at room temperature using a trap immersed in a trichloroethylene–Dry Ice-bath as the condenser. At the end of each operation the flask was opened to air dried by passage through a silica gel tube. In the fifth repetition of this operation the pyridine solution was partly evaporated to give about 3 ml. of the anhydrous solution. To this solution was added 150 mg. (0.79 mmole) of *p*-toluenesulfonyl chloride and the flask left sealed at 0° for 18 hr. Water (2 ml.) then was added and, after 0.5 hr., the solution evaporated under reduced pressure. The residual sirup was taken up in water and pyridine removed by adjusting the *p*H of the solution to around 8.5 with sodium hydroxide and evaporating *in vacuo*. The procedure was repeated until no further drop in the *p*H of the solution occurred on evaporation. The solution was made up to 10 ml. with water and analyzed by paper chromatography and cellulose anion-exchange columns as described below (Fig. 1a).

(b) In another experiment, only 50 mg. (0.263 mmole) of *p*-toluenesulfonyl chloride was first added to an anhydrous solution of pyridinium thymidine-5′ phosphate (0.5 mmole). After 18 hr. at 0°, a small aliquot was removed, diluted with water and examined by paper chromatography. P^1P^2-Dithymidine-5′ pyrophosphate was the sole product and its formation was quantitative. Its identification was accomplished by paper electrophoresis and rate of acidic hydrolysis (complete to thymidine-5′ phosphate in about 15 minutes at 100° in 1 *N* hydrochloric acid or in about 2-2.5 hr. at 100° in 0.1 *N* hydrochloric acid). To the bulk of the anhydrous reaction mixture then was added 103 mg. (0.54 mmole) of *p*-toluenesulfonyl chloride and the mixture left for another 24 hr. at 0°. Analysis of the reaction products then showed that the latter were very similar to those obtained above in (a).

Dialysis of the Polymeric Mixture.—In one run, the total mixture of products was freed from pyridine as described

(27) For a detailed discussion of the mechanisms of the carbodiimide reactions see M. Smith, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **80**, 6212 (1958).

(28) A. M. Michelson, *Nature*, **181**, 303 (1958).

(29) R. H. Hall, A. R. Todd and R. F. Webb, *J. Chem. Soc.*, 3291 (1957).

(30) The formation of ribo-oligonucleotides containing presumably random $C_3'\rightarrow C_5'$ and $C_3'\rightarrow C_3'$ internucleotidic linkages already has been reported.[27]

above and its aqueous solution dialyzed against distilled water (600-ml. portions). After three changes of water, the rate of dialysis of ultraviolet absorbing material was negligible and 47% of the total optical density was still in the cellophane bag. Paper chromatography showed the non-dialyzed portion to consist of cyclic dinucleotide (a part retained) and higher oligonucleotides. Figure 1a shows the elution pattern of this mixture upon chromatography on a DEAE cellulose column.

Polymerization of Thymidine-5′ Phosphate Using Dicyclohexylcarbodiimide (Experiment II).—Earlier experiments were carried out using 0.25 mmole of pyridinium thymidine-5′ phosphate. Analysis and comparison of the products with those obtained using toluenesulfonyl chloride as the reagent showed that the individual thymidine oligonucleotides were easier to purify when DCC was employed. The following large scale experiment then was carried out: pyridinium thymidine-5′ phosphate (4 mmoles) was rendered anhydrous by six evaporations in the presence of anhydrous pyridine (25 ml. each time) as described above. The gum then was dissolved in 30 ml. of dry pyridine (stored over calcium hydride) and DCC (4.20 g., 20.3 mmoles) then added and the sealed reaction flask was shaken rapidly. The mixture which was clear at first became turbid within five minutes and deposited semi-crystalline material. The reaction mixture was kept for five days at room temperature with occasional agitation and then diluted with 25 ml. of water. After 3 hr. at room temperature, the mixture was extracted six times with ether (100 ml. each time) and the aqueous layer then concentrated at 30° *in vacuo* to a sirup. This was dissolved in 50 ml. of water, the solution adjusted to *p*H 8 with lithium hydroxide and then evaporated. This process was repeated to ensure complete removal of pyridine. The final aqueous solution was filtered from a small amount of di*cyclo*hexylurea and made up to 100 ml. The total optical density at 267 mμ at acid *p*H was 36,000 units. Cellulose anion-exchange chromatography of portions of this solution is described below (Fig. 1b and Fig. 2)

Separation of Thymidine Oligonucleotides on Cellulose Anion Exchangers.—DEAE-cellulose (Type 20, 0.65 meq./g.)[31] was washed with 0.1 *N* sodium hydroxide and then 0.1 *N* hydrochloric acid (2 l. for 50 g. of the cellulose). This cycle was repeated twice and the exchanger, finally in the chloride form, was washed free of acid with distilled water. Fines were removed by decantation after allowing a stirred suspension of about 50 g. of the material in 8 l. of water to settle for 30 minutes. This process was repeated. The settled material was packed into a column as a thin slurry in water, first by allowing to settle under gravity and then by applying 1–2 lb./sq. inch air pressure. A column, 8 cm. long × 0.9 cm. diameter, was routinely used for a primary analysis of various reaction mixtures, which were applied as aqueous solutions of lithium or sodium salts (see above Experiments I and II) and contained about 150 optical density units at 267 mμ. After a water wash, elution was carried out using a linear gradient technique[32] with 500 ml. of water in the mixing vessel and an equal volume of 0.4 *M* lithium chloride containing 0.04 *M* lithium acetate (*p*H 5) buffer. An approximate flow rate of 1 ml./min. was maintained and the elution was followed on a recording spectrophotometer with the cell compartment modified to hold a flow cell. The results of such an analysis on the non-dialyzable portion of the mixture of products obtained in Experiment I (see above) are shown in Fig. 1a.

Later work with a second lot of DEAE cellulose supplied by Brown Co. and stated to have 0.85 meq./g. anion exchange capacity gave unsatisfactory separations.

Very satisfactory separations were obtained by using ECTEOLA cellulose prepared according to the directions supplied by Dr. Bendich (*cf.* Peterson and Sober).[11] The results obtained using this material for the separation of products of Experiment II (see above) are shown in Fig. 1b. These separations were even better on the large scale column described below in which elution was carried out stepwise.

Large Scale Separation and Isolation of Thymidine Oligonucleotides.—A column of ECTEOLA cellulose (23 cm. long, 4.0 cm. diameter) was prepared as described above for DEAE cellulose and a 20-ml. portion (corresponding to 0.8 mmole of thymidine-5′ phosphate used) of the total solution obtained in Experiment II (see above) was applied on the top. After a water wash (50 ml.) elution was begun by passing eluents containing increasing amounts of lithium chloride (see Fig. 2). Twenty-five ml. fractions were collected with a flow rate of about 3 ml./min. Tubes corresponding to each peak were pooled. (For the sake of purity, tubes containing shoulders or tails at either end of the main peak were not included.) Each set of pooled fractions was evaporated first at 10 mm. pressure and finally at 0.01 mm. pressure to a solid white residue. Five milliliters of methyl alcohol was added to dissolve the lithium chloride. With the dinucleotides clear solutions were obtained at this stage, while the higher oligonucleotides remained partly undissolved. Subsequent addition of five volumes of acetone precipitated the polynucleotide. The precipitate was collected by centrifugation, redissolved (resuspended) in five milliliters of methyl alcohol and again precipitated by addition of an excess of acetone. The precipitate was dried *in vacuo*, and, where necessary, separated further into cyclic and linear oligonucleotides by paper chromatography on Whatman 3 MM sheets using Solvent A. The approximate yields of the various products are listed in Table I.

Formation of Thymidine Cyclic Dinucleotide (III, n = 0) from Linear Dinucleotide (II, n = 0).—Pyridinium thymidylyl-(5′→3′)-thymidylic-(5′) acid,[1] prepared from 7 mg. of the crystalline sodium salt, was taken up in pyridine and the solution evaporated. The residue then was redissolved in 30 ml. of anhydrous pyridine and 100 mg. of DCC added. After four days at room temperature water (25 ml.) was added and the solution extracted with ether. The aqueous solution was concentrated to 2 ml. and examined by paper chromatography. The major product formed in over 80% yield was identified as the cyclic dinucleotide (III, *n* = 0).

Synthesis of Thymidine-3′,5′ Cyclic Phosphate (IV).—An anhydrous pyridine solution (25 ml.) of thymidine-5′ phosphate (0.25 mmole) was added dropwise to a vigorously stirred solution of DCC (0.6 g.) in 50 ml. of dry pyridine over a period of 6 hr. at room temperature. The reaction mixture was kept overnight and then diluted with 10 ml. of water. The total mixture was evaporated *in vacuo* and the residual gum taken up in water, brought to *p*H 7.5 with sodium hydroxide and the aqueous solution re-evaporated *in vacuo*. Paper chromatography in Solvent A showed the major product (R_f, 0.45) to be thymidine-3′,5′ cyclic phosphate and, in addition, minor spots with R_f's 0.56, 0.30 and 0.15 and less were present. The yield of the cyclic phosphate was determined to be 45%. This product was isolated by chromatography on paper sheets.

Formation of Thymidine-3′,5′ Cyclic Phosphate from P^1-P^2-Dithymidine-5′ Pyrophosphate. (a).—To a mixture of pyridinium thymidine-5′ phosphate (0.04 mmole) and tri-*n*-butylamine (0.04 ml., 0.170 mmole) and pyridine (1 ml.) was added 110 mg. (0.52 mmole) of DCC and the total reaction mixture kept at room temperature. Paper chromatography after 23 hr. showed the quantitative formation of P^1 P^2-dithymidine-5′ pyrophosphate. A portion of the mixture was kept for seven days at room temperature and even after this period the pyrophosphate was the only product of reaction. The major portion (0.75 ml.) of the reaction mixture after the initial 23 hr. reaction period was heated at 100° after the addition of a further amount (100 mg.) of DCC. After a few hours some 50% of the pyrophosphate had disappeared and a new major spot corresponding to thymidine-3′,5′ cyclic phosphate had appeared, together with a weak spot of thymidine, and faint spots with low R_f values. The major product was identical with the material prepared above.

(b) In a separate experiment P^1P^2-dithymidine-5′ pyrophosphate was prepared as above and freed from the excess of the carbodiimide reagent by addition of water to the mixture and repeated extraction with ether. The aqueous solution was evaporated and the pyrophosphate taken up in pyridine. When this solution was heated at 100° for 4 hr. partial conversion to thymidine-3′,5′ cyclic phosphate occurred.

Properties of Thymidine-3′,5′ Cyclic Phosphate.—The substance was identical with the faster-travelling component of peak B (Fig. 2). The R_f values in three solvent systems are recorded in Table II. On paper electrophoresis, the product had the same mobility as thymidine-5′ phosphate at *p*H 3.5, while at *p*H 7.5, it travelled slower.

The ultraviolet absorption spectrum showed λ_{max} at 264.5 mμ both in acid and alkali. Except for the hypsochromic shift, the spectral characteristics in acid and alkali were very similar to those of thymidine.

(31) Brown Co., Berlin, New Hampshire.

(32) C. W. Paar, *Biochem. J.*, **56**, xxvii (1954).

The rate of alkaline hydrolysis was followed by paper chromatography in Solvent A. The substance was hydrolyzed to the extent of 50% in 2 hr. at 100° in 1 *N* sodium hydroxide. The product of hydrolysis corresponded to about 80% thymidine-3′ phosphate and about 20% thymidine-5′ phosphate, as judged by the extent of dephosphorylation by the specific 5′-nucleotidase in snake venom.

The substance was hydrolyzed completely in 0.1 *N* hydrochloric acid at 100° within five minutes, whereas at room temperature it hydrolyzed to the extent of about 50% in 24 hr. The sole ultraviolet adsorbing product formed was found to be thymine.

The substance (30 optical density units at 267 mμ) was incubated in tris buffer (*p*H 8.5) with crude snake venom (*ca.* 5 mg). After incubation at 37° for 12 hr., the products were examined by paper chromatography in Solvent A. Approximately one-half of the starting material had disappeared and a spot with the same R_f as thymidylic acid (presumably, thymidine-3′ phosphate) and thymidine had appeared. The latter material obviously arose by the action of the 5′-nucleotidase on the initially formed thymidine-5′ phosphate. The ratio of thymidine-3′ phosphate to thymidine was about one.

General Analytical Methods.—Paper chromatography was carried out by the descending technique and using Whatman No. 1, 4 or 3 MM paper. The solvents which proved useful in this work 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 the new solvent system isopropyl alcohol–ammonia–acetic acid–water (4–1–2–2, v./v.) (solvent C), which is especially suited for the separation of the homologous series of oligonucleotides. The R_f's of the various compounds in these solvent systems are listed in Table II.

Paper electrophoresis was carried out on Whatman 3 MM strips using the buffers 0.05 *M* ammonium acetate (*p*H 4.5), 0.05 *M* potassium phosphate buffer (*p*H 7.5) and 0.05 *M* sodium borate buffer (*p*H 9.2).

Phosphorus analysis was carried out using the method of E. J. King.[33]

Enzyme Experiments.—Crude snake venom was purchased from Ross Allen's Reptile Institute, Florida. The Experiments using venom were carried out in 0.05 M tris buffer (*p*H 8.2–9.9). The snake venom diesterase used in some experiments was prepared using acetone fractionation procedure of Koerner and Sinsheimer.[34] We are grateful to Dr. W. E. Razzell for this preparation and for performing several of the enzymic experiments.

Prostate phosphomonoesterase was prepared as described by Markham and Smith.[14] It was virtually free from diesterase activity as tested against thymidylyl-(5′→3′)-thymidine.[1] The standard procedure used for removal of phosphomonoester end groups was based on the rate of dephosphorylation of linear thymidine dinucleotide. This rate was determined by adding one volume of a solution of the monoesterase (5–10 mg./ml.) in 1 *M* ammonium acetate buffer, *p*H 5.0, to three volumes of the dinucleotide (about 25 optical density units at 267 mμ) solution in water. Dephosphorylation of the oligonucleotides was followed by paper chromatography and was usually complete in a few hours at 37°.

TABLE II

R_f VALUES OF THYMIDINE OLIGONUCLEOTIDES AND RELATED COMPOUNDS

Compound	Solvent A	Solvent B	Solvent C
Thymidylic	0.15	0.36	0.69
Linear dithymidylic	.08	.21	.51
Linear trithymidylic	.045	.14	.41
Linear tetrathymidylic	.02	.09	.31
Linear pentathymidylic	.01	.06	.21
Thymidine	.67	.60	
Dithymidine monophosphate (V, $n = 0$)	.42		
Trithymidine diphosphate (V, $n = 1$)	.21		
Tetrathymidine triphosphate (V, $n = 2$)	.11		
Pentathymidine tetraphosphate (V, $n = 3$)	.045		
Thymidine-3′,5′ cyclic phosphate (IV)	.45	.31	.78
Cyclic dithymidylic	.28	.21	.56
Cyclic trithymidylic	.12	.16	.48
Cyclic tetrathymidylic	.051	.13	.32
Cyclic pentathymidylic	.026	.08	.22

The spleen diesterase was used according to the procedure of Heppel and Hilmoe,[35] the hydrolysis being followed by paper chromatography.

Acidic Hydrolysis of Cyclic Oligonucleotides.—The oligonucleotides were heated in 1 *N* hydrochloric acid at 100° for about 45 minutes, during which some 20–30% of the starting material had disappeared. The products then were separated on paper chromatograms, run in solvent A for 24–48 hours and the slowest band, which corresponded to dithymidine triphosphate or trithymidine tetraphosphate, etc., was eluted with water containing 0.5% Versene. The eluate was evaporated under reduced pressure and dephosphorylated with prostate monoesterase as described above except that incubation was usually prolonged to several hours. The resulting compounds V, dithymidine monophosphate, trithymidine diphosphate, etc., were compared on paper chromatograms with the corresponding samples obtained by monoesterase treatment of the linear oligonucleotide. The R_f values are listed in Table II.

(33) E. J. King, *Biochem. J.*, **26**, 292 (1932).

(34) J. F. Koerner and R. L. Sinsheimer, *J. Biol. Chem.*, **228**, 1039 (1957).

(35) L. A. Heppel and R. J. Hilmoe, in "Methods in Enzymology," Vol. II, Academic Press, Inc., New York, N. Y., 1955, p. 565.

VANCOUVER 8, CANADA

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN]

3′-Hydroxy-3,5-diiodo-L-thyronine[1]

BY RAYMOND W. DOSKOTCH AND HENRY A. LARDY

RECEIVED JULY 14, 1958

The synthesis of 3′-hydroxy-3,5-diiodo-L-thyronine is described. This compound has but little thyroid hormone activity.

Because the thyroid hormones and the adrenal medullary hormones augment one another,[2] and because thyroid hormones appear to interfere with catechol amine destruction,[3] the possibility exists that metabolic hydroxylation of the partially iodinated thyronine molecule forms a compound which is competitively antagonistic to catechol amine destruction. To test this hypothesis, the synthesis of

(1) Taken in part from the M.S. Thesis submitted by Raymond W. Doskotch, 1957. Presented at the 132nd National Meeting of the American Chemical Society, New York, 1957. Supported by grants from the U. S. Public Health Service and the Office of Naval Research.

(2) R. M. Morris, M. S. Witter and S. Weiss, *Proc. Soc. Exptl. Biol. Med.*, **21**, 149 (1924); J. Schaeffer and O. Thibault, *Compt. rend. soc. biol.*, **139**, 855 (1945); H. W. Swanson, *Endocrinol.*, **59**, 217 (1956).

(3) Z. M. Bacq, *J. Physiol. (London)*, **87**, 87P (1936); M. Zile, M.S. Thesis, University of Wisconsin, 1956.