The Phosphotriester Approach to Oligonucleotide Synthesis: Preparation of Oligo- and Poly-thymidylic Acids

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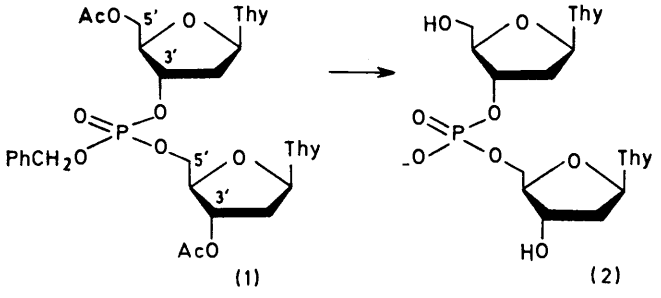
Fully-protected phosphotriester derivatives of $(Tp)_3T$, $(Tp)_7T$, $(Tp)_{15}T$, and $(Tp)_{31}T$ (18a; n = 2, 6, 14, and 30, respectively) were obtained in satisfactory yields by block synthesis. In all cases, stoicheiometric quantities of two starting materials (18b and c), each of which contained the same number of thymidine residues, were used. Phosphorylations were carried out in two steps with phenyl dihydrogen phosphate (16) and 2,4,6-tri-isopropylbenzenesulphonyl chloride (7b) in pyridine solution. Fully-protected TpT, $(Tp)_3T$, $(Tp)_7T$, and $(Tp)_{15}T$ (18a; n = 0, 2, 6, and 14, respectively) were converted into the corresponding unblocked oligothymidylic acids (21; n = 0, 2, 6, and 14, respectively) which were further purified by DEAE-cellulose or -Sephadex chromatography. In order to avoid terminal phosphoryl migration, the fully protected intermediates (18a) were converted into the corresponding tetrahydropyranyl derivatives (19) before they were submitted to alkaline hydrolysis to unblock the internucleotide phosphodiester linkages. The latter alkaline hydrolysis step led to *ca.* 3% cleavage per phosphotriester group and thus the phenyl protecting group or the present unblocking procedure is unsuitable for the preparation of oligonucleotides containing more than *ca.* 20 nucleotide residues.

THE first synthesis of a dinucleoside phosphate containing a natural 3′ ⟶ 5′ internucleotide linkage was carried out by Michelson and Todd [1] in 1955. These workers phosphorylated 3′-*O*-acetylthymidine with 5′-*O*-acetylthymidine 3′-(benzyl phosphorochloridate) and obtained the fully protected dinucleoside phosphate (1). Removal of the protecting groups gave (Scheme 1) thymidyl-(3′⟶5′)-thymidine (TpT) † (2). A related synthesis of the dinucleotide pTpT was reported in the same paper.[1] A noteworthy aspect of these preparations was that the internucleotide phosphodiester linkages were protected in the initially formed phosphorylation products and finally liberated in a deblocking step. Such procedures for the synthesis of oligonucleotides in which the internucleotide linkages are protected are now usually referred to as phosphotriester methods. Despite these promising initial results, the phosphotriester approach was not further investigated for 10 years.

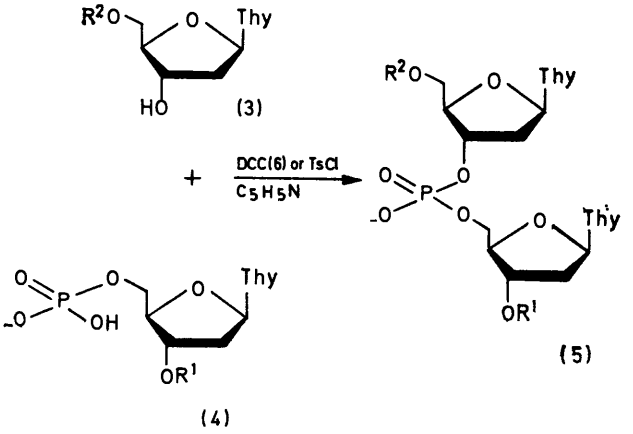
Soon after the publication of Michelson and Todd's results, Khorana and his co-workers reported [2] that



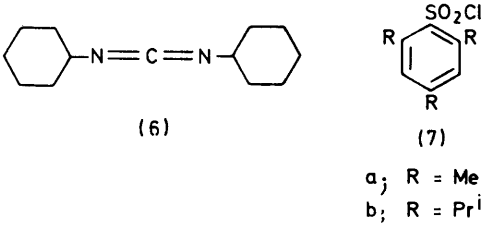
Thy = Thymin-1-yl

SCHEME 1

thymidine 5′-phosphate (4; R^1 = H) could be activated by toluene-*p*-sulphonyl chloride in anhydrous pyridine solution to phosphorylate 5′-*O*-tritylthymidine (3; R^2 = Ph_3C) and thereby give the protected TpT derivative (5; R^1 = H, R^2 = Ph_3C). Removal of the protecting group



SCHEME 2



gave TpT (2) in 45% isolated yield. The latter workers then reported [3] that *NN*′-dicyclohexylcarbodi-imide (DCC) (6) was a more satisfactory condensing agent than toluene-*p*-sulphonyl chloride and that the yield of TpT (2) could be further increased by protecting the 3′-hydroxy-group of the nucleotide component by acetylation as in (4; R^1 = Ac). A noteworthy aspect of these procedures for the synthesis of oligonucleotides, now usually referred to as phosphodiester methods, is that the internucleotide phosphodiester linkages are unprotected in the initially formed phosphorylation products [*e.g.* (5; R^1 = H or Ac, R^2 = Ph_3C)].

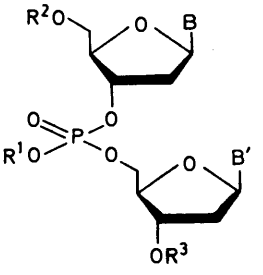
† A slight modification of the 1970 IUPAC–IUB nomenclature (*Biochemistry*, 1970, **9**, 1022) has been used in that thymidine has been abbreviated to T rather than to dT.

[1] A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 1955, 2632.
[2] H. G. Khorana, G. M. Tener, J. G. Moffatt, and E. H. Pol, *Chem. and Ind.*, 1956, 1523.
[3] H. G. Khorana, W. E. Razzell, P. T. Gilham, G. M. Tener, and E. H. Pol, *J. Amer. Chem. Soc.*, 1957, **79**, 1002.

In the following decade, Khorana and his co-workers [4] investigated the phosphodiester approach to oligonucleotide synthesis in great detail; these workers demonstrated [4] that this approach could be used successfully in the preparation of oligonucleotides of known sequence, containing all the main nucleoside building blocks, in both the deoxyribose and the ribose series. Khorana also introduced the use of 2,4,6-trimethyl- [5] and 2,4,6-triisopropyl- [6] benzenesulphonyl chlorides (7a and b, respectively) as alternative activating agents to DCC (6). The main disadvantage which toluene-*p*-sulphonyl and indeed 2,4,6-trimethylbenzenesulphonyl chlorides have as activating agents in oligonucleotide synthesis is their tendency to sulphonylate [6] the reaction component with a free hydroxy-group, especially if it has a 5'-hydroxy-group. Thus sulphonylation can compete with phosphorylation and lower yields of the desired products are obtained. Owing presumably to its much increased steric requirements, the tri-isopropyl derivative (7b) (TPS) is more specific in this respect and its use generally leads [6] to higher yields of oligonucleotides.

Phosphodiester methods have been used very successfully in the synthesis of low molecular weight oligodeoxyribonucleotides and high yields have often been obtained.[4] Such methods have also been used [4,7] in the stepwise and block synthesis of comparatively high molecular weight oligomers in the deoxy-series but substantial excesses of, respectively, the appropriate monomeric building block and the lower molecular weight components were then required for satisfactory yields to be obtained. Phosphodiester methods have, on the whole, been applied with less success in the ribose series [8] and our initial interest in the phosphotriester approach stemmed from difficulties [9] we encountered in the synthesis of oligoribonucleotides, from the ribonucleoside building blocks which we had prepared,[10] by phosphodiester methods. Two other groups of workers reported on the use of phosphotriester methods in oligodeoxyribonucleotide synthesis before our own studies along these lines had progressed far.

Letsinger and his co-workers [11] used the 2-cyanoethyl group to protect the internucleotide linkages [as in (8; $R^1 = CH_2{\cdot}CH_2{\cdot}CN$)] in a phosphotriester approach to oligodeoxyribonucleotide synthesis both on [11a] and without [11b] an insoluble polymer support. Eckstein and Rizk [12] then used the 2,2,2-trichloroethyl group [as in (8; $R^1 = CH_2{\cdot}CCl_3$)] for the same purpose. Clearly, in any phosphotriester approach the choice of the protecting group for the internucleotide linkages is a crucial decision upon which the whole success of the synthetic method will depend. This protecting group should (*a*) remain intact until a late stage or perhaps even until the end of the synthesis, (*b*) be stable under the conditions which are required to remove the protecting groups from the hydroxy-functions [R^2 and R^3 in (8)], and (*c*) finally be removable under conditions which do not lead to internucleotide cleavage or to degradation of the desired product in any other way.



(8)

After consideration of criteria (*a*)—(*c*), we thought that phenyl [13] and perhaps other aryl groups [10,14] [as in (8; R^1 = Ph, *etc.*)] would be suitable for the protection of the internucleotide linkages in the phosphotriester approach to oligonucleotide synthesis. At the outset we believed it likely that, with phenyl as the protecting group, criterion (*a*) would be fulfilled if strongly alkaline media could be avoided until the end of the synthesis, criterion (*b*) would be fulfilled if the protecting groups could be removed from the hydroxy-functions [*e.g.* R^2 and R^3 in (8)] again with the avoidance of strongly alkaline conditions, and criterion (*c*) would be fulfilled if the attack of hydroxide ion on phenyl-protected oligonucleotides [*e.g.* (8; R^1=Ph)] led exclusively to the expulsion of phenoxide ion with no resulting cleavage of the internucleotide linkages.

Our initial studies in the synthesis of low molecular weight oligodeoxyribo- [13] and oligoribo- [10,15-17] nucleotides by the phosphotriester approach were encouraging and suggested that phenyl was a suitable protecting group for the internucleotide linkages. However, we found that if one or both of the terminal hydroxy-func-

[4] For reviews of this work, see H. G. Khorana, *Pure Appl. Chem.*, 1968, **17**, 349: K. W. Agarwal, A. Yamazaki, P. J. Cashion, and H. G. Khorana, *Angew. Chem. Internat. Edn.*, 1972, **11**, 451.

[5] T. M. Jacob and H. G. Khorana, *J. Amer. Chem. Soc.*, 1964, **86**, 1630.

[6] R. Lohrmann and H. G. Khorana, *J. Amer. Chem. Soc.*, 1966, **88**, 829.

[7] For recent general reviews on oligonucleotide synthesis, see (*a*) R. I. Zhdanov and S. M. Zhenodarova, *Synthesis*, 1975, 222; (*b*) H. Kössel and H. Seliger, *Fortschr. Chem. org. Naturstoffe*, 1975, **32**, 298.

[8] (*a*) R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka, and H. G. Khorana, *J. Amer. Chem. Soc.*, 1966, **88**, 819; (*b*) A. Holý and J. Smrt, *Coll. Czech. Chem. Comm.*, 1966, **31**, 3800; (*c*) E. Ohtsuka, M. Ubasawa, S. Morioka, and M. Ikehara, *J. Amer. Chem. Soc.*, 1973, **95**. 4725.

[9] B. E. Griffin and C. B. Reese, *Tetrahedron*, 1969, **25**, 4057.

[10] C. B. Reese, *Colloques Internationaux du C.N.R.S.*, 1970, No. 182, 319.

[11] (*a*) R. L. Letsinger and V. Mahadevan, *J. Amer. Chem. Soc.*, 1965, **87**, 3526; *ibid.*, 1966, **88**, 5319; (*b*) R. L. Letsinger and K. K. Ogilvie, *J. Amer. Chem. Soc.*, 1967, **89**, 4801; 1969, **91**, 3350.

[12] F. Eckstein and I. Rizk, *Angew. Chem. Internat. Edn.*, 1967, **6**, 695, 949; *Chem. Ber.*, 1969, **102**, 2362.

[13] C. B. Reese and R. Saffhill, *Chem. Comm.*, 1968, 767.

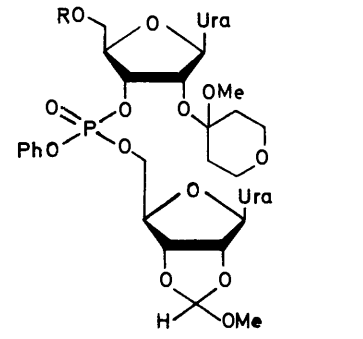
[14] J. C. M. Stewart, Ph.D. Thesis, Cambridge University, 1969, pp. 53 *et seq.*

[15] R. Saffhill, Ph.D. Thesis, Cambridge University, 1968, pp. 69 *et seq.*

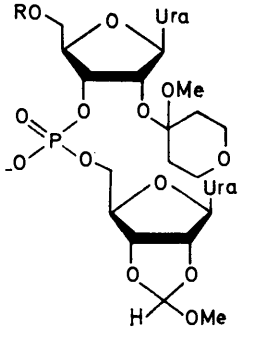
[16] G. R. Owen, Ph.D. Thesis, Cambridge University, 1971, pp. 37 *et. seq.*

[17] J. H. van Boom, P. M. J. Burgers, G. R. Owen, C. B. Reese, and R. Saffhill, *Chem. Comm.*, 1971, 869.

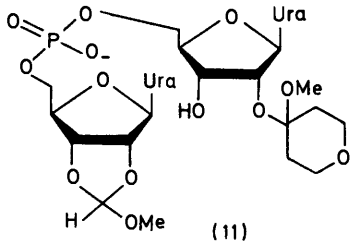
tions were to be protected by an acyl group or groups [as in (8; R^1 = Ph, R^2 and/or R^3 = RCO)], the latter must be removable under very mild basic conditions so that concomitant hydrolysis of the phosphotriester groups could be avoided [criterion (*b*)]. As indicated below, this is possible even in the synthesis of comparatively large oligonucleotides if aryloxyacetyl groups [18] are used to protect the hydroxy-functions. The phosphotriester groups are quite stable to the mildly acidic conditions required for the removal of methoxytetrahydropyranyl [19] protecting groups.



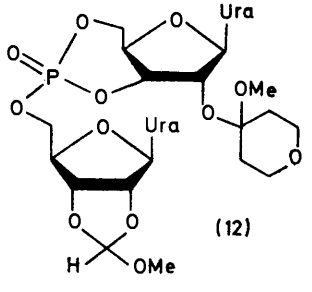
(9) a; R = Cl–C$_6$H$_4$–OCH$_2$CO
b; R = H
c; R =



(10) a; R = H
b; R =

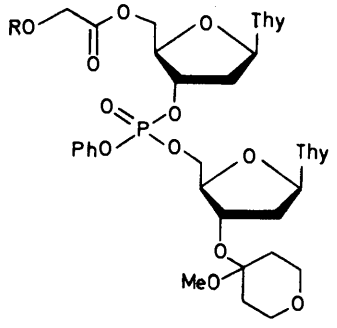


(11)

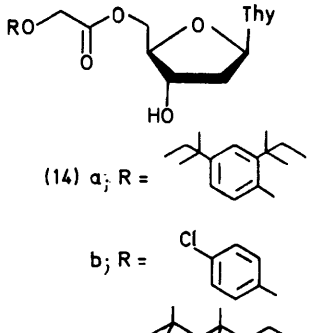


(12)

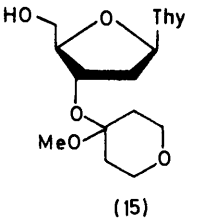
Ura = uracil-1-yl



(13) a; R =
b; R =



(14) a; R =
b; R =
c; R =
d; R =



(15)

In the course of our early studies on the synthesis of oligoribonucleotides by the phosphotriester approach we found [16,17] a complication in the unblocking of fully

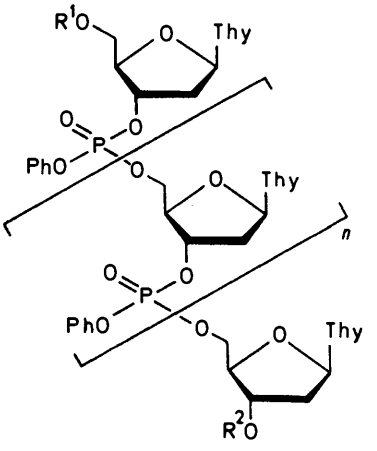
[18] C. B. Reese and J. C. M. Stewart, *Tetrahedron Letters*, 1968, 4273.

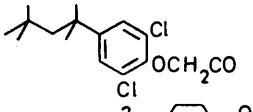
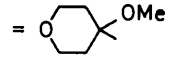
[19] C. B. Reese, R. Saffhill, and J. E. Sulston, *J. Amer. Chem. Soc.*, 1967, **89**, 3366; *Tetrahedron*, 1970, **26**, 1023.

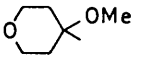
protected intermediates. Thus treatment of the fully protected dinucleoside phosphate (9a) with 0.1M-sodium hydroxide in aqueous dioxan gave a substantial quantity of the partially protected 5′ ⟶ 5′ dinucleoside phosphate (11) in addition to the desired product (10a). It seemed likely that the 3′,5′-cyclic phosphotriester (12) was an intermediate in the conversion of (9a) into (10a) and (11), and indeed we have recently concluded that the alkaline hydrolysis of (b) proceeds virtually exclusively [20] *via* the cyclic phosphotriester (12). Such neighbouring group participation and subsequent partial migration of the terminal internucleotide linkages is also observed [20] in the alkaline hydrolysis of phosphotriester intermediates with free 3′-hydroxy-functions in the ribose series and with both free 3′- and 5′-hydroxy-functions in the deoxyribose series. Neighbouring group participation analogous to the last process was observed [21] in the hydrolysis of a phosphodiester intermediate, thymidine 3′-*p*-nitrophenyl phosphate. Treatment of the latter compound with alkali gave [21] thymidine 5′-phosphate (11%) in addition to the expected 3′-phosphate (89%).

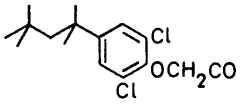
The formation of 3′,5′-cyclic phosphotriesters [such as (12)] can readily be prevented [16,17] in the unblocking of phosphotriester intermediates. For example, if (9b), which is obtained in high yield by the ammonolysis or mild alkaline hydrolysis of (9a), is tetrahydropyranylated [to give (9c)]before the internucleotide linkage is unblocked by alkaline hydrolysis under more drastic conditions, (10b) is obtained as virtually the sole product. Unprotected uridylyl-(3′ ⟶ 5′)-uridine, uncontaminated with its 5′ ⟶ 5′ isomer, may then be obtained [16,17] by the acidic hydrolysis of (10b). This tetrahydropyranylation procedure may also be used to prevent the formation of cyclic phosphotriester intermediates in the unblocking of oligonucleotides with terminal 3′-hydroxy-functions in the ribose series [20] and with both terminal 3′- and 5′-hydroxy-functions in the deoxyribose series.[20,22]

Although we were encouraged by our early studies on the phosphotriester approach in both the ribose and the deoxyribose series, we felt that it would not be possible to evaluate the real potential of this method before an attempt had been made to use it in the preparation of oligonucleotides of relatively high molecular weight. We therefore undertook a block synthesis of oligothymidylic acids by the phosphotriester approach. We decided to use phenyl groups to protect the internucleotide linkages although we realised that another aryl protecting group might eventually prove to be more suitable. Our first experiments in block synthesis [22] were carried out with the fully protected dinucleoside phosphate (13a) as the starting material. The dimer (13a) was designed in the hope that it would be possible by treatment with mild acid or mild alkali to unblock the 3′- or 5′-hydroxy-function specifically with virtually no phosphotriester hydrolysis. An aryloxyacetyl group with hydrocarbon side chains was selected to make the protected intermediate more lipophilic in the hope that purification by adsorption chromatography on silica gel would thereby be facilitated. The removal of the 3′-*O*-methoxytetrahydropyranyl group from (13a) was effected, under mild conditions of acidic hydrolysis, without any detectable side-reactions.[22] However, although it was found [22] that the 5′-*O*-2,4-di-t-pentylphenoxyacetyl



(18) a ; R^1 =  , R^2 = 

b ; R^1 = H , R^2 = 

c ; R^1 =  , R^2 = H

group could be removed from (13a) under relatively mild conditions of alkaline hydrolysis or ammonolysis, a small amount of concomitant phosphotriester hydrolysis occurred. It was anticipated that this lack of selectivity would prove a serious problem in the deacylation of large oligonucleotides containing a number of phosphotriester groups. It therefore seemed desirable to use a more base-labile but equally lipophilic aryloxyacetyl protecting group in the future.

The half-time of deacylation of 5′-*O*-2,4-di-t-pentylphenoxyacetylthymidine (14a) in M-ammonia at 20 °C was found (Table 1) to be *ca.* 100 min, as compared with *ca.* 15 min for *p*-chlorophenoxyacetylthymidine (14b). However, the latter compound is much less lipophilic, as indicated by its R_F value, than (14a). We then found that 5′-*O*-*p*-(1,1,3,3-tetramethylbutyl)phenoxyacetylthymidine (14c) was almost as lipophilic as (14a) but that it underwent deacylation over three times as quickly. Finally, we prepared 5′-*O*-2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetylthymidine (14d) as a crystalline compound in 70% yield (of isolated material) by the action of 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)-

[20] J. H. van Boom, P. M. J. Burgers, P. H. van Deursen, J. F. M. de Rooy, and C. B. Reese, *J.C.S. Chem. Comm.*, 1976, 167.

[21] A. F. Turner and H. G. Khorana, *J. Amer. Chem. Soc.*, 1959, **81**, 4651.

[22] N. J. Cusack, C. B. Reese, and J. H. van Boom, *Tetrahedron Letters*, 1973, 2209.

phenoxyacetyl chloride (see Experimental section) on thymidine; we found that while (14d) was almost as lipophilic as (14a), it underwent deacylation (Table 1)

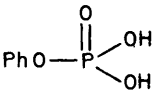
TABLE 1

Ammonolysis [a] of 5′-*O*-aryloxyacetylthymidines

Substrate	R_F [b]	$t_{\frac{1}{2}}$/min [c]
(14a)	0.42	100
(14b)	0.30	15
(14c)	0.38	30
(14d)	0.40	12

[a] In M-ammonia in dioxan–water (1 : 1 v/v) at 20 °C. [b] On Merck glass plates coated with silica gel 60 F_{254} in solvent system B [$CHCl_3$–MeOH (92 : 8 v/v)]. [c] Approximate values.

more than eight times as quickly. We therefore decided to repeat the block synthesis of oligothymidylic acids starting from the fully protected dinucleoside phosphate (13b), and now report our results in detail.



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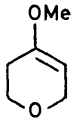


(17)

The fully-protected dinucleoside phosphate (13b) was prepared by treating (14d) (8.0 mmol) with a slight excess of phenyl dihydrogen phosphate [23] (16) in the presence of a 2.5-fold excess of TPS [6] (7b) in anhydrous pyridine solution at room temperature. After 19 h, 3′-*O*-methoxytetrahydropyranylthymidine (15) (8.0 mmol) and more TPS were added and the second step of the phosphorylation was allowed to proceed in concentrated pyridine solution for 24 h. The products were then worked up * and fractionated by short column chromatography [24] on silica gel to give the fully-protected dinucleoside phosphate (13b) (5.95 g, 71%) as a glass. 3′-*O*-Methoxytetrahydropyranylthymidine was obtained as a crystalline solid in 55% overall yield from thymidine by treating 5′-*O*-*p*-chlorophenoxyacetylthymidine (14b) with 5,6-dihydro-4-methoxy-2*H*-pyran [25] (17) in the presence of toluene-*p*-sulphonic acid and deacylating the product with methanolic dimethylamine. 5′-*O*-*p*-Chlorophenoxyacetylthymidine (14b) is even more easily accessible from thymidine than (14d).

The fully protected dinucleoside phosphate (13b) was divided into two equal portions of which one was treated with M-ammonia in aqueous dioxan (1 : 1 v/v) for 75 min at room temperature and the other with *ca.* 0.08M-hydrochloric acid in aqueous dioxan (3 : 4 v/v) for 135 min at room temperature. After work-up and short column chromatography, the partially protected dinucleoside phosphates [respectively, (18b; $n = 0$) and (18c; $n = 0$)] were isolated as solids in high yields (Table 2). The fully protected tetranucleoside triphosphate (18a; $n = 2$) was then obtained as a glass in 62% yield (of isolated material) from stoicheiometric quantities of the two partially protected dinucleoside phosphates (18b; $n = 0$) and (18c; $n = 0$), phenyl dihydrogen phosphate (16), and TPS (7b) by the procedure outlined above for the preparation of (13b); this material was then divided into two nearly equal portions of which one was treated with ammonia and the other with hydrochloric acid to give the partially protected tetramers [respectively, (18b; $n = 2$) and (18c; $n = 2$)], which were isolated as solids in good yields (Table 2).

The fully protected octanucleoside heptaphosphate (18a; $n = 6$) was then obtained in 54% yield from stoicheiometric quantities of (18b; $n = 2$) and (18c; $n = 2$) in the same way; this material was converted into each of the two partially protected octamers (18b and c; $n = 6$) in good yields (Table 2). It was clear from their t.l.c. properties that the fully and partially protected oligothymidylic acids (18a—c) were becoming more polar with increasing molecular weight. An important practical consequence of this was first encountered in the preparation of (18b; $n = 6$): the normal work-up procedure (see Experimental section) could not be used for the isolation of this product as it was only sparingly soluble in chloroform. However, it was possible to devise a reasonably satisfactory but more cumbersome alternative. It was found that this increase in polarity was also disadvantageous from the standpoint of the purification of the intermediates by short column chromatography. Indeed, we now feel that the protecting group for the internucleotide linkages should fulfil a fourth criterion [see above for criteria (*a*)—(*c*)] in that it should be lipophilic in order to facilitate dissolution of the protected oligonucleotide in chloroform.

TABLE 2

Yields of partially and fully protected oligothymidylic acids (phenyl esters)

Product	Yield (%)	Product	Yield (%)
(18a; $n = 0$) [a]	71 [b]	(18b; $n = 6$)	69 [g]
(18b; $n = 0$)	*ca.* 95 [c]	(18c; $n = 6$)	85 [g]
(18c; $n = 0$)	85 [c]	(18a; $n = 14$)	42 [h]
(18a; $n = 2$)	62 [d]	(18b; $n = 14$)	73 [j]
(18b; $n = 2$)	81 [e]	(18c; $n = 14$)	80 [j]
(18c; $n = 2$)	76 [e]	(18a; $n = 30$)	48 [k]
(18a; $n = 6$)	54 [f]		

[a] Or (13b). [b] Based on stoicheiometric quantities of (14d) and (15). [c] Based on (18a; $n = 0$). [d] Based on stoicheiometric quantities of (18b; $n = 0$) and (18c; $n = 0$). [e] Based on (18a; $n = 2$). [f] Based on stoicheiometric quantities of (18b; $n = 2$) and (18c; $n = 2$). [g] Based on (18a; $n = 6$). [h] Based on stoicheiometric quantities of (18b; $n = 6$) and (18c; $n = 6$). [j] Based on (18a; $n = 14$). [k] Based on stoicheiometric quantities of (18b; $n = 14$) and (18c; $n = 14$).

The fully protected hexadecamer (18a; $n = 14$) was prepared in 42% yield from stoicheiometric quantities of (18b; $n = 6$) and (18c; $n = 6$). This experiment was carried out with less than 0.1 mmol of each of the latter octamer blocks, each of which has a molecular weight of

* Care has to be taken to avoid acidic or basic conditions during work-up. Of the two work-up procedures described in the Experimental section, (*b*) is generally to be preferred.

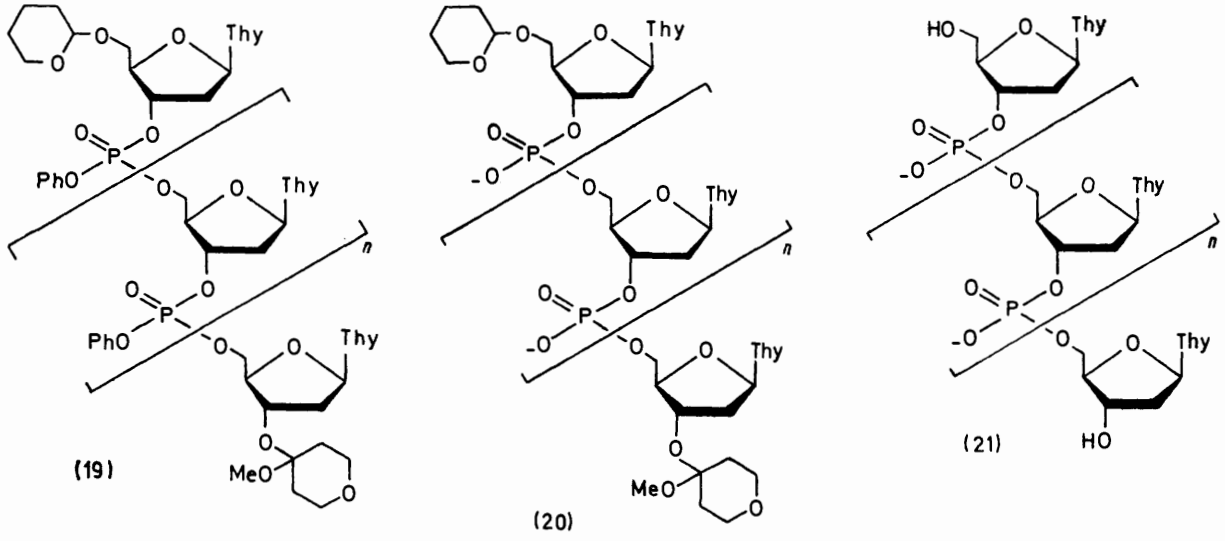
[23] G. R. Owen, C. B. Reese, C. J. Ransom, J. H. van Boom, and J. D. H. Herscheid, *Synthesis*, 1974, 704.

[24] B. J. Hunt and W. Rigby, *Chem. and Ind.*, 1967, 1868.

[25] R. Arentzen, C. B. Reese, and Y. T. Yan Kui, *Synthesis*, 1975, 509.

over 3×10^3 daltons. The material available was sufficient only for one condensation and it is possible that optimization of the reaction conditions would lead to a yield nearer to 50%. It should be emphasized that the phosphorylation procedure used has one significant limitation in that phenyl dihydrogen phosphate (16)–TPS(7b) is a bifunctional phosphorylating agent and its use can therefore lead to the formation of symmetrical products, derived from two molecules of (18b or c), in addition to the desired product. We found that symmetrical by-products, which usually account for *ca.* 10—15% of the fully protected nucleotide products obtained, could, in the cases of the dimer (18a; $n = 0$), tetramer (18a; $n = 2$), and octamer (18a; $n = 6$), be separated from the desired products by short column chromatography. However, purification of more polar, higher molecular weight products is much more difficult. Nevertheless, we were able to isolate the partially protected hexadecamers [respectively, (18a; $n = 14$) and (18c; $n = 14$)] in relatively pure states and in satisfactory yields (Table 2) from the ammonolysis and acidic hydrolysis products of (18a; $n = 14$). It is noteworthy that the yields of partially protected oligomers with free 5′-hydroxy-groups (18b) tend to decrease with increasing molecular weight (Table 2). This is not unexpected as 5′-deacylation of the fully protected oligonucleotides (18a) should become less selective as the number of phosphotriester groups is increased. Finally, we were able to link the partially protected hexadecamers (18b and c; $n = 14$) together to give (18a, $n = 30$) in *ca.* 48% yield. Although this material may be contaminated with symmetrical products, it is encouraging that it is possible to prepare a protected polynucleotide with a molecular weight of nearly 1.25×10^4 daltons and containing thirty-two nucleotide residues in approaching 50% yield from stoicheiometric quantities of two hexadecamer blocks.

The fully unblocked oligothymidylic acids (21) were obtained from the partially protected intermediates (18b) in three steps. As revealed above, it was necessary to protect the 5′-hydroxy-functions by tetrahydropyranylation * before unblocking the internucleotide linkages by alkaline hydrolysis. The partially protected dimer, tetramer, octamer, and hexadecamer (18b; $n = 0, 2, 6$, and 14, respectively) were rapidly tetrahydropyranylated by treatment with large excesses of 2,3-dihydro-4*H*-pyran in the presence of toluene-*p*-sulphonic acid in dioxan solution at room temperature. The products were worked-up and purified by short column chromatography to give the tetrahydropyranyl derivatives (19; $n = 0, 2, 6$, and 14, respectively) in good to high yields (see Experimental section). The latter products were then treated with alkali under a variety of conditions (see below) to hydrolyse the phosphotriester groups. The terminally protected oligothymidylic acids (20) thereby



obtained were kept in 0.01M-hydrochloric acid solution for 16 h at room temperature to remove the tetrahydropyranyl and methoxytetrahydropyranyl groups and thus give the completely unblocked oligonucleotides (21).

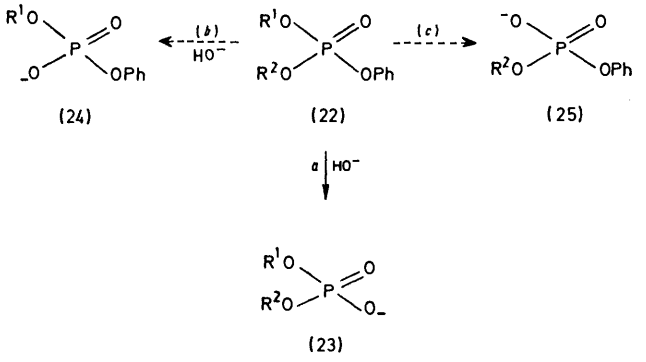
As suggested above, we selected the phenyl protecting group in the hope that it would fulfil criterion (*c*), that is that alkaline hydrolysis of the phosphotriester groups would proceed virtually exclusively by expulsion of phenoxide ion with no concomitant cleavage of the internucleotide linkages. Indeed we felt that because phenol is a stronger acid than a simple alcohol by five or six orders of magnitude, phenyl dialkyl phosphate esters (22) would, in general, undergo hydrolysis almost entirely by pathway (*a*) (Scheme 3) to give dialkyl phosphate esters (23). Unfortunately, this is not the case: alkaline hydrolysis of fully protected oligonucleotides corresponding to (22) [*e.g.* (19)] also proceeds by pathways (*b*) and (*c*) (Scheme 3) to give phenyl alkyl phosphate esters [(24) and (25)] in significant quantities.[22,26]

After the fully protected tetrahydropyranyl derivatives

* Other base-stable protecting groups, such as methoxytetrahydropyranyl, are also satisfactory for this purpose.

[26] J. H. van Boom, P. M. J. Burgers, P. H. van Deursen, R. Arentzen, and C. B. Reese, *Tetrahedron Letters*, 1974, 3785.

(19) had been submitted first to alkaline and then to acidic hydrolysis, the oligothymidylic acids (21) obtained were purified by anion-exchange chromatography on



SCHEME 3

DEAE-cellulose or DEAE-Sephadex. In the case of the TpT derivative (19; $n = 0$) (Table 3; experiment no. 1), alkaline hydrolysis was effected with aqueous potassium hydroxide and, after acidic hydrolysis, 98% of the u.v.-absorbing material eluted from the DEAE-cellulose column was the desired product, TpT (21; $n = 0$). However, when the same unblocking procedure was applied to the corresponding tetramer (19; $n = 2$) (experiment no. 2), the purified $(Tp)_3T$ (21; $n = 2$) isolated accounted for only 82% of the total nucleotide products. This loss of 18% of the material during unblocking indicates that, on the average, alkaline hydrolysis proceeds only 94% by pathway (*a*) and 6% by pathways (*b*) and (*c*) combined (Scheme 3) *per phosphotriester group*. Clearly, the extent of cleavage of the internucleotide linkages must be decreased if the synthesis of comparatively large oligonucleotides by this phosphotriester approach is to be feasible. Treatment of (21; $n = 2$) with 0.2M-sodium hydroxide in aqueous dioxan (experiment no. 3), the reagent which we used previously [22] for unblocking internucleotide linkages, leads to even more cleavage. More satisfactory results were obtained with 0.1M-potassium hydroxide in dimethyl sulphoxide–water (10 : 1 v/v) and 0.085M-tetraethylammonium hydroxide in acetonitrile–water (19 : 1 v/v) (experiment nos. 4 and 5, respectively). However, the use of the most suitable reagent which we have found so far, 0.085M-tetraethylammonium hydroxide in dimethyl sulphoxide–water (19 : 1 v/v) leads, in the unblocking of the tetramer (19; $n = 2$) (experiment no. 6), to only 6% of cleavage products. This last reagent was then used in the unblocking of the octamer and hexadecamer (19; $n = 6$ and 14, respectively) (experiment nos. 7 and 8) to give the fully unblocked oligothymidylic acids (21; $n = 6$ and 14) in 77.5 and 50% yields, respectively. In both cases, an average of *ca.* 3% internucleotide cleavage occurred for each phosphotriester group.

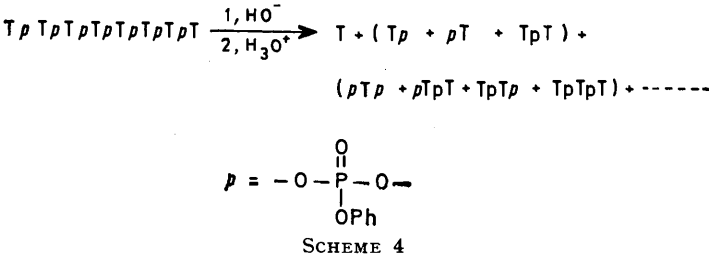
TABLE 3

Yields of fully unblocked oligothymidylic acids

Experiment no.	Substrate	Procedure [a]	Product	Yield [b] (%)
1	(19; $n = 0$)	A	(21; $n = 0$)	98
2	(19; $n = 2$)	A	(21; $n = 2$)	82
3	(19; $n = 2$)	B	(21; $n = 2$)	78
4	(19; $n = 2$)	C	(21; $n = 2$)	88
5	(19; $n = 2$)	D	(21; $n = 2$)	88.5
6	(19; $n = 2$)	E	(21; $n = 2$)	94
7	(19; $n = 6$)	E	(21; $n = 6$)	77.5
8	(19; $n = 14$)	E	(21; $n = 14$)	50

[a] In all experiments, the substrate was treated first with alkali and then with dilute hydrochloric acid (pH 2) at room temperature. The fully unblocked products were then fractionated by anion-exchange chromatography on DEAE-cellulose or DEAE-Sephadex. The procedures differed only in the alkaline reagents used which were as follows: A, aqueous M-KOH; B, 0.2M-NaOH in dioxan–water (1 : 1 v/v); C, 0.1M-KOH in Me_2SO–H_2O (9 : 1 v/v); D, 0.085M-$Et_4N^+HO^-$ in MeCN–H_2O (19 : 1 v/v); E, 0.085M-$Et_4N^+HO^-$ in Me_2SO–H_2O (19 : 1 v/v). [b] Estimated by dividing 100 × the number of absorbance units (measured at 267 nm) of oligothymidylic acid by the total number of absorbance units eluted from the DEAE-cellulose or -Sephadex anion-exhange column.

The elution pattern obtained from the chromatography of the fully unblocked octamer (21; $n = 6$) on DEAE-cellulose is illustrated in Figure 1. The column was eluted with a linear gradient of triethylammonium hydrogen carbonate buffer (pH 7.5) and it appears that the cleavage products with charges of zero, −1, −2, −3, −4, −5, and to some extent −6 are separated from the desired $(Tp)_7T$ (21; $n = 6$) and the other possible products with a charge of −7. It can be seen from Scheme 4 that the unblocking of (19; $n = 6$) can lead to one product with zero charge (thymidine), three possible products with a charge of −1 (TpT and thymidine 3′- and 5′-phenyl phosphates), and four possible products with a charge of −2. There are also four possible products with charges of −3, −4, −5, −6, and −7. It is not clear why the elution pattern (Figure 1) shows only one peak in the monoanion region and at most only two peaks in the other regions. Clarification must await detailed analysis of all the cleavage products. The elution pattern obtained from the chromatography of the fully unblocked hexadecamer (21; $n = 14$) on DEAE-cellulose was less well resolved. Further purification of the combined later fractions on DEAE-Sephadex A-25 led to material



SCHEME 4

which we believe to be hexadecamer (21; $n = 14$) of high purity. Figure 2(b) shows that the latter material is eluted from DEAE–Sephadex A-25 as a relatively sharp peak. In comparison, Figure 2(a) illustrates the separation of an artificial mixture of TpT (21; $n = 0$), $(Tp)_3T$ (21; $n = 2$), and $(Tp)_7T$ (21; $n = 6$) on the same column. The hexadecamer (21; $n = 14$) requires a much higher concentration of triethylammonium hydrogen carbonate than the octamer (21; $n = 6$) for elution.

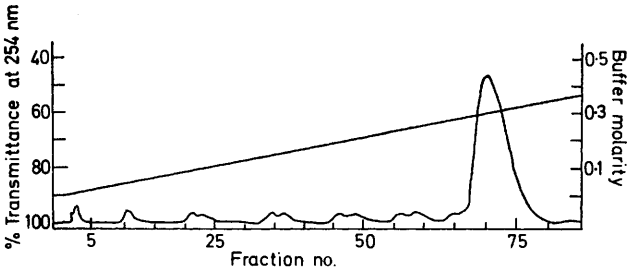


FIGURE 1 Elution pattern from chromatography of fully unblocked $(Tp)_7T$ (21; $n = 6$) on DEAE-cellulose with triethylammonium hydrogen carbonate buffer (pH 7.5; linear gradient, 0.001—0.6M; *ca.* 14.5 ml fractions)

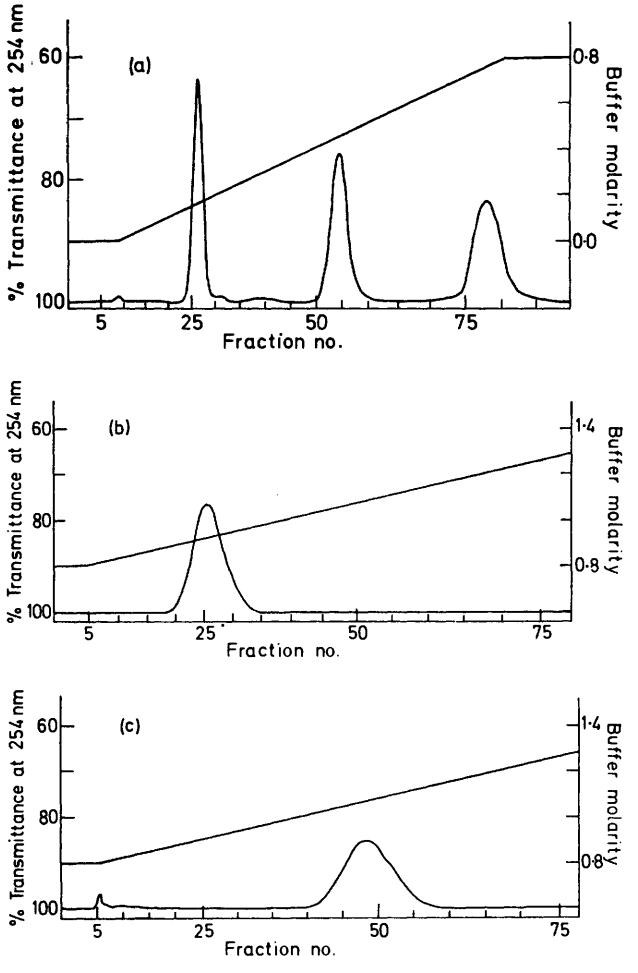


FIGURE 2 Elution patterns from chromatography on DEAE-Sephadex A-25 of (a) a mixture of *ca.* 45 O.D. units (at 267 nm) each of TpT, $(Tp)_3T$, and $(Tp)_7T$ (21; $n = 0$, 2, and 6, respectively) [linear gradient of triethylammonium hydrogen carbonate buffer (pH 7.5), 0.001—0.8M]; (b) $(Tp)_{15}T$ (21; $n = 14$) [linear gradient of triethylammonium hydrogen carbonate buffer (pH 7.5), 0.8—1.3M]; (c) high molecular weight products obtained from the unblocking of fully protected $(Tp)_{31}T$ (18: $n = 30$) [linear gradient of triethylammonium hydrogen carbonate (pH 7.5), 0.8—1.3M] (*ca.* 6.5 mol fractions in each case)

The extent of internucleotide cleavage observed in the unblocking of the hexadecamer (19; $n = 14$) (Table 3, experiment no. 8) emphasizes one of the limitations of the present phosphotriester approach to oligonucleotide synthesis. As was anticipated, considerable difficulty was encountered in the unblocking of fully protected $(Tp)_{31}T$ (18a; $n = 30$). In the first place it was not possible to remove the 5′-*O*-acyl group from (18a; $n = 30$) by the ammonolysis procedure without hydrolysis of the phosphotriester groups occurring to an appreciable extent. For this reason, the tetrahydropyranylation step was omitted before all the remaining protecting groups were removed by procedure E (Table 3). Although it is unlikely that, with an estimated 3% internucleotide cleavage occurring at each of thirty-one phosphotriester groups, a significant quantity of $(Tp)_{31}T$ (21; $n = 30$) survived the unblocking procedure, it was clear that an appreciable amount of polythymidylic acids of high molecular weight (possibly *ca.* $0.8—0.9 \times 10^4$ daltons) was obtained. The early fractions eluted from the Sephadex G-75 column, which presumably contained the polynucleotides of highest molecular weight,[27] were combined and rechromatographed on the same DEAE–Sephadex A-25 column as was used for the hexadecamer (21; $n = 14$) [Figure 2(b)] and for the separation of the mixture of dimer, tetramer, and octamer (21; $n = 0$, 2, and 6, respectively) [Figure 2(a)]. Figure 2(c) shows that the high molecular weight material was eluted from the DEAE–Sephadex column as a relatively sharp peak and that it required a significantly higher concentration of triethylammonium hydrogen carbonate than was required for the elution of the hexadecamer [Figure 2(b)]. This indicates that this high molecular weight portion of the products obtained by unblocking (19; $n = 30$) contains no material with a molecular weight of less than *ca.* 5×10^3 daltons. This conclusion is confirmed by the observation (see Figure 3) that this high molecular weight portion is completely eluted from a Sephadex G-75 column before the hexadecamer (21; $n = 14$).

In view of their method of preparation, there cannot be much doubt about the structures assigned to TpT, $(Tp)_3T$, $(Tp)_7T$, and $(Tp)_{15}T$ (21; $n = 0$, 2, 6, and 14, respectively). However, the purification procedures used become less effective with increasing molecular weight of the oligonucleotide. In the first place, as discussed above, it becomes more difficult to separate a fully protected oligonucleotide (18a; $n = 2y + 2$), by short column chromatography, from the symmetrical by-products derived from two molecules of either of its precursors (18b or c; $n = y$). Secondly, as indicated above, it becomes more difficult to separate the fully unblocked

[27] S. A. Narang and S. K. Dheer, *Biochemistry*, 1969, **8**, 3443.

oligonucleotides (21) from species with the same or with a slightly smaller number of negative charges by chromatography on DEAE-cellulose or DEAE-Sephadex. In any case, it is desirable to obtain as much confirmatory evidence as possible for all the structural assignments. The DEAE-cellulose elution patterns themselves provide supporting evidence especially for the structures of TpT, $(Tp)_3T$, and $(Tp)_7T$ (21; n = 0, 2, and 6, respectively).

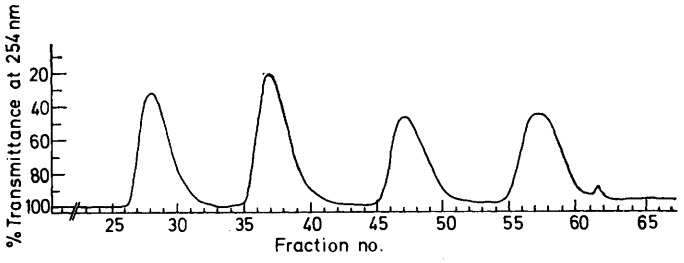


FIGURE 3 Elution pattern from chromatography of a mixture of *ca.* 45 O.D. units (at 267 nm) each of $(Tp)_3T$, $(Tp)_7T$, $(Tp)_{15}T$ (21; n = 2, 6, and 14, respectively) and the high molecular weight products obtained from the unblocking of fully protected $(Tp)_{31}T$ (18; n = 30) on Sephadex G-75 (ultrafine) with 0.1M-triethylammonium hydrogen carbonate buffer (pH 7.5) (*ca.* 3.8 ml fractions)

In addition to the actual concentrations of triethylammonium hydrogen carbonate required for the elution of the main components, the appearance of peaks corresponding to minor components with putative negative charges up to one less than the main components in the elution patterns obtained for TpT, $(Tp)_3T$, and $(Tp)_7T$ (Figure 1) provides valuable confirmatory evidence. Paper electrophoresis in 0.05M-sodium phosphate (pH 7.0) buffer (Table 4) revealed

TABLE 4

Chromatographic and electrophoretic properties of oligo- and poly-thymidylic acids

	R_F [a]	R_F [b]	R_F [c]	M [d]
T	0.74	0.79		0.37
pT	0.13	0.28		1.00
Tp	0.13	0.29		1.00
TpT	0.49	0.66	0.71	0.62
$(Tp)_3T$	0.11	0.32	0.13	0.96
$(Tp)_7T$		0.05	0.00	1.09
$(Tp)_{15}T$		0.00		1.14
$(Tp)_{31}T$ [e]		0.00		0.94

[a] Chromatography in system G on DC-Alufolien cellulose F_{254}. [b] Chromatography in system H on DC-Alufolien cellulose F_{254}. [c] Chromatography in aqueous 0.1M-sodium chloride on Merck Alufolien PEI cellulose; the R_F value of $(Tp)_7T$ in aqueous 0.4M-sodium chloride on PEI cellulose is 0.13. [d] Electrophoretic mobilities relative to pT (=1.00) in buffer system K. [e] Highest molecular weight material (see Experimental section) obtained in the deblocking of fully protected $(Tp)_{31}T$.

TpT, $(Tp)_3T$, $(Tp)_7T$, and $(Tp)_{15}T$ (21; n = 0, 2, 6, and 14, respectively) as homogeneous species with increasing mobilities. However, the high molecular weight (18; n = 30) had a lower electrophoretic mobility than material obtained by unblocking fully protected $(Tp)_{31}T$ (18; n = 30) had a lower electrophoretic mobility than $(Tp)_{15}T$ (21; n = 14). Cellulose and polyethyleneimine (PEI)-cellulose t.l.c. (Table 4) did not provide much confirmatory evidence for the structures of the synthetic oligothymidylic acids. However, high-pressure liquid chromatography (h.p.l.c.) * proved a valuable analytical technique; it provided better resolution than DEAE-Sephadex chromatography and was more rapid. The results obtained by h.p.l.c. suggested that the TpT and $(Tp)_3T$ (21; n = 0 and 2, respectively) were virtually pure and that the $(Tp)_7T$ (21; n = 6) was *ca.* 95% and the $(Tp)_{15}T$ (21; n = 14) *ca.* 90% pure.

When the synthetic oligothymidylic acids were incubated with either spleen or snake venom phosphodiesterase, TpT, $(Tp)_3T$, and $(Tp)_7T$ (21; n = 0, 2, and 6, respectively) were completely digested to thymidine 3′(or 5′)-phosphate and thymidine, and $(Tp)_{15}T$ (21; n = 14) and the high molecular weight material obtained from

TABLE 5

U.v. absorption spectra [a] of oligo- and poly-thymidylic acids

	$\lambda_{max.}$/nm	$\lambda_{min.}$/nm	$\varepsilon_{250}/\varepsilon_{260}$	$\varepsilon_{280}/\varepsilon_{260}$
TpT	266	236	0.66	0.68
$(Tp)_3T$	266	236	0.67	0.68
$(Tp)_7T$	266	236	0.68	0.66
$(Tp)_{15}T$	266	236	0.67	0.65
$(Tp)_{31}T$ [b]	266	238	0.70	0.66

[a] Of solutions in 0.01M-Tris hydrochloride buffer (pH 7.4), measured by Dr. A. F. Drake with a Cary 17 recording spectrophotometer. [b] Highest molecular weight material (see Experimental section) obtained in the deblocking of fully protected $(Tp)_{31}T$.

fully protected $(Tp)_{31}T$ (18; n = 30) were almost completely digested. Final confirmatory evidence for the structures of synthetic $(Tp)_3T$, $(Tp)_7T$, and $(Tp)_{15}T$ (21; n = 2, 6, and 14, respectively) was obtained † by incubating the latter oligonucleotides with [γ-^{32}P]-adenosine 5′-triphosphate in the presence of T_4-phosphokinase [28] and then fractionating the products by homochromatography on thin-layer DEAE-cellulose [29] or by gel electrophoresis on a 12% acrylamide slab.[30] One major labelled product was obtained from each substrate. The major products obtained from $(Tp)_7T$ and $(Tp)_{15}T$ (21; n = 6, or 14, respectively) were partially digested with venom exonuclease and the digests were submitted to homochromatography. In both cases, the expected number [31] of labelled digestion products was obtained.

DISCUSSION

The results indicate that the phosphotriester method is promising and suggest that it may become the method of choice for the chemical synthesis of oligo- and polydeoxyribonucleotides. Preliminary experiments [15-17] on the synthesis of oligoribonucleotides by the phosphotriester method have also been encouraging. The present

* These experiments were carried out by Dr. J. H. van Boom of the University of Leiden.

† These experiments were carried out by Dr. G. G. Brownlee of the M.R.C. Laboratory for Molecular Biology, Cambridge.

[28] C. C. Richardson, *Proc. Nat. Acad. Sci. U.S.A.*, 1965, **54**, 158.
[29] G. G. Brownlee and F. Sanger, *European J. Biochem.*, 1969, **11**, 395.
[30] F. Sanger and A. R. Coulson, *J. Mol. Biol.*, 1975, **94**, 441.
[31] E. Jay, R. Bambara, P. Padmanabhan, and R. Wu, *Nucleic Acids Res.*, 1974, **1**, 331.

study has clearly revealed a number of difficulties in our present approach which will have to be overcome if the promise of the phosphotriester method is to be fulfilled.

The most serious limitation of the present approach is the internucleotide cleavage (Table 3) which accompanies the unblocking of phenyl-protected internucleotide linkages. We have demonstrated that the extent of this cleavage is dependent on the alkaline reagent used for the hydrolysis of the phosphotriester groups and it is possible that the use of nucleophiles other than hydroxide ion will lead to more satisfactory results. Indeed, it has recently been suggested [32] that fluoride is more selective than hydroxide ion in this respect. Clearly, the possibility of using fluroride ion and other nucleophiles should be fully investigated. Another way in which the extent of internucleotide cleavage during unblocking may be decreased is by the use of aryl protecting groups derived from phenols which are more acidic than phenol itself. This approach has already been investigated: [14,17,26] thus it has been shown [26] that the extent of internucleotide cleavage can be decreased by a factor of *ca.* 2 by using *o*-chlorophenyl as the protecting group for the internucleotide linkages. An obvious advantage of aryl protecting groups is that their properties may readily be modified by the introduction of substituents. However, recent experiments suggest that if an aryl protecting group derived from a phenol more acidic than *o*-chlorophenol (pK_a 8.48) [33] is used, considerable difficulty may be encountered in the purification and handling of the phosphotriester intermediates. It therefore seems that, if aryl protecting groups are to be used successfully in the synthesis of polynucleotides of relatively high molecular weight, a nucleophilic species which will displace arenolate ion (derived from a phenol with $pK_a \geqslant 8.5$) from phosphotriester groups with not more than *ca.* 0.5% concomitant internucleotide cleavage is required. If these conditions cannot be met, it will be necessary to seek an alternative type of protecting group. In any case, in order to facilitate the purification of phosphotriester intermediates by short column chromatography (see above), it is desirable that the protecting group should be lipophilic. This criterion is easily fulfilled with aryl protecting groups by the introduction [34] of hydrocarbon *para*-substituents.

Finally, the ultimate success of the phosphotriester approach to oligonucleotide synthesis is likely to depend on the development of a more satisfactory phosphorylation procedure. The phosphorylating agent [phenyl dihydrogen phosphate (16)–TPS (7b)] is subject to two serious limitations. First, as it is bifunctional its use leads to the formation (see above) of appreciable quantities of symmetrical by-products which are sometimes difficult to remove and, in any case, their formation upsets the stoicheiometry of the reactions and inevitably leads to lower yields of the desired products. Secondly, the present procedure is rather unsuitable for the synthesis of guanine-containing oligonucleotides.[35] We are currently devoting much of our research effort in this field to a search for a phosphorylation procedure which is not subject to either of the latter two or indeed to any other serious limitations.

EXPERIMENTAL

U.v. absorption spectra were measured with a Cary 17 recording spectrophotometer. N.m.r. spectra (^1H) were measured at 60 MHz with a Perkin-Elmer R12B spectrometer and at 90 MHz with a Bruker HFX 90 spectrometer; tetramethylsilane was used as an internal standard. I.r. spectra were measured with a Perkin-Elmer 257 spectrometer.

Merck silica gel 60 F_{254} and DC-Alufolien cellulose F_{254} sheets were used for t.l.c. in the following solvent systems: A $CHCl_3$, B $CHCl_3$–MeOH (92 : 8 v/v), C $CHCl_3$–MeOH (90 : 10 v/v), D $CHCl_3$–MeOH (88 : 12 v/v), E $CHCl_3$–MeOH (85 : 15 v/v), F $CHCl_3$–MeOH (80 : 20 v/v), G aqueous M-NH_4OAc–EtOH (3 : 7 v/v), H aqueous M-NH_4OAc–EtOH (4 : 6 v/v), and J propan-2-ol–aqueous NH_3 (*d* 0.88)–water (7 : 1 : 2 v/v); t.l.c. was also carried out on Merck DC-Alufolien PEI–cellulose F in aqueous NaCl of various concentrations. Paper electrophoresis was carried out in a Savant tank on Whatman No. 1 paper in buffer system K [0.05M-sodium phosphate buffer (pH 7.0)]. Merck Kieselgel H and Reeve Angel silica gel CT were used for short column adsorption chromatography. Anion-exchange chromatography on DEAE-cellulose (Bio-Rad Cellex D, medium capacity) and DEAE–Sephadex A-25 was carried out with linear gradients of triethylammonium hydrogen carbonate buffer (pH 7.5).

Dioxan, acetonitrile, pyridine, and 2,6-lutidine were dried by heating, under reflux, with CaH_2 for 3—5 h; these solvents were then distilled at atmospheric pressure and stored over molecular sieves (no. 4A). Dimethylformamide and dimethyl sulphoxide were stirred at 20 °C for 16 h, then distilled under reduced pressure (at *ca.* 14 mmHg) and stored over molecular sieves (no. 4A). 2,3-Dihydro-4*H*-pyran was heated, under reflux, with CaH_2 for 1 h, then distilled and stored over molecular sieves (no. 4A). 2,4,6-Triisopropylbenzenesulphonyl chloride (TPS) and phenyl dihydrogen phosphate were recrystallized before use from light petroleum (b.p. 30—40 °C) and chloroform, respectively.

Enzymic digests were carried out with snake-venom phosphodiesterase in 0.1M-Tris, HCl, 0.01M-$MgCl_2$ at pH 8.9 and with spleen phosphodiesterase in 0.1M-NH_4OAc, 0.002M-EDTA, 0.05% Tween 80 at pH 7.0.

p-*Chlorophenoxyacetyl chloride*.—Freshly distilled thionyl chloride (50 ml, 0.69 mol), *p*-chlorophenoxyacetic acid (80.0 g, 0.47 mol), and dimethylformamide (*ca.* 0.5 ml) were swirled together, with the exclusion of moisture, several times during the course of 1 h. The reactants were then heated (oil-bath), under gentle reflux, for 2 h and the excess of thionyl chloride was removed under reduced pressure. Distillation of the residual oil under reduced pressure gave p-*chlorophenoxyacetyl chloride* (58 g, 65%), b.p. 80° at 0.05 mmHg, as a pale yellow liquid which solidified; $\nu_{max.}$ ($CHCl_3$) 1 810 cm^{-1}.

5′-O-p-*Chlorophenoxyacetylthymidine* (14b) (with Dr. J. H. van Boom).—A solution of *p*-chlorophenoxyacetyl

[32] K. K. Ogilvie, S. L. Beaucage, and D. W. Entwistle, *Tetrahedron Letters*, 1976, 1255.
[33] A. Albert and E. P. Serjeant, 'Ionization Constants of Acids and Bases,' Methuen, London, 1962, p. 130.
[34] R. E. Adamiak, R. Arentzen, C. B. Reese, and Y. T. Yan Kui, unpublished results.
[35] W. Markiewicz and C. B. Reese, unpublished results.

chloride (11.3 g, 55 mmol) in anhydrous acetonitrile (10 ml) was added dropwise, over 30 min, to a mixture of thymidine (12.1 g, 50.0 mmol), 2,6-lutidine (40 ml, 0.34 mmol), and anhydrous acetonitrile (75 ml) at 20 °C. After 24 h, the products were filtered and the residue washed, in order, with acetonitrile, chloroform, and ether. The filtrate and washings were concentrated and retained (see below). Recrystallization of the residue from ethanol–water (800 ml; 4 : 1 v/v gave 5′-O-p-*Chlorophenoxyacetylthymidine* (Found: C, 52.4; H, 4.7; N, 6.7. $C_{18}H_{19}ClN_2O_7$ requires C, 52.6; H, 4.7; N, 6.8%) as needles, m.p. 195—197°. A second crop of crystalline product was obtained from the mother liquors. Addition of chloroform to the concentrated filtrate and washings (see above) gave a solid precipitate which was recrystallized from ethanol–water (100 ml; 4 : 1 v/v) to give a third crop; total yield of pure crystalline material 14.4 g (70%); τ [$(CD_3)_2SO$] 2.55br (1 H, s), 2.6—3.2 (4 H, m), 3.79 (1 H, t, *J* 7.5 Hz), 5.14 (2 H, s), 5.6—5.9 (3 H, m), 6.04 (1 H, m), 7.88 (2 H, m), and 8.24 (3 H, s); $\lambda_{max.}$ (95% EtOH) 267 (ε 10 000), $\lambda_{min.}$ 241 nm (3 400); R_F 0.38 (C), 0.30 (B).

3′-O-4-*Methoxytetrahydropyran-4-ylthymidine* (15) (with Dr. N. J. Cusack).—5,6-Dihydro-4-methoxy-2*H*-pyran [25] (6.0 ml, *ca.* 49 mmol) was added to a stirred mixture of 5′-*O*-*p*-chlorophenoxyacetylthymidine (10.0 g, 24.3 mmol) and toluene-*p*-sulphonic acid monohydrate (*ca.* 0.001 g) in anhydrous dioxan (250 ml) at 20 °C. A homogeneous solution was obtained within 3 h. After 5 h the products were basified (to *ca.* pH 8) with a few drops of methanolic dimethylamine (10% v/v) and concentrated under reduced pressure; the residue was dissolved in methanol and the solution evaporated. After this process had been repeated once more, the resulting foam was dissolved in methanolic 10% dimethylamine (150 ml) and kept at 20 °C. After 45 min, when t.l.c. (system B) revealed that deacylation was complete, the products were concentrated under reduced pressure; the residue was dissolved in ethanol and the solution evaporated. After this process had been repeated twice more, the residue was dissolved in chloroform and the solution evaporated; the residue was then redissolved in chloroform–methanol (96 : 4 v/v; *ca.* 50 ml) and applied to a column of silica gel. The column was eluted, under slight pressure (*ca.* 8 cmHg), with chloroform–ethanol (96 : 4). The appropriate fractions were combined and concentrated under reduced pressure to give a foam. Crystallization from hot ethyl acetate (*ca.* 20 ml) gave 3′-O-4-*methoxytetrahydropyran-4-ylthymidine* (Found: C, 53.6; H, 7.0; N, 8.0. $C_{16}H_{24}N_2O_7$ requires C, 53.9; H, 6.8; N, 7.9%) as prisms (6.9 g, 79%), m.p. 128—130°; τ [$(CD_3)_2SO$] 2.32 (1 H, m), 3.86 (1 H, t, *J* 7.5 Hz), 5.53 (1 H, m), 6.08 (1 H, m), 6.2—6.6 (6 H, m), 6.86 (3 H, s), 7.82 (2 H, m), 8.22 (3 H, s), and 8.27 (4 H, m); $\lambda_{max.}$ (95% EtOH) 267 (ε 9 900), $\lambda_{min.}$ 236 nm (2 100); R_F 0.35 (B).

2,6-*Dichloro*-4-(1,1,3,3-*tetramethylbutyl*)*phenoxyacetyl Chloride.*—Sulphuryl chloride (300 g, 2.2 mol) was added dropwise over 1 h to technical *p*-(1,1,3,3-tetramethylbutyl)-phenol (206.4 g, 1.0 mol). The temperature of the mixture, which was stirred when it became possible, rose slowly to *ca* 35 °C. When addition of sulphuryl chloride was complete, the reactants were heated to 100 °C. After 15 min, when t.l.c. (system A) revealed that all the starting material (R_F 0.31) had been converted into a product (R_F 0.56), the reactants were cooled (ice–water) and partitioned between dichloromethane (450 ml) and saturated aqueous sodium hydrogen carbonate (300 ml). The dried ($MgSO_4$) organic layer was evaporated under reduced pressure and the residue distilled to give 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)-phenol as a pale yellow syrup (245 g, 89%), b.p. 132—135° at 0.4 mmHg; τ (CCl_4) 2.89 (2 H, s), 8.34 (2 H, s), 8.69 (6 H, s), and 9.24 (9 H, s).

Anhydrous benzene (100 ml) was added to a stirred solution of the above material (245 g, 0.89 mol) and potassium hydroxide (85%; 50 g, 0.76 mol) in absolute ethanol (1 250 ml) and the resulting solution concentrated to *ca.* 500 ml. Ethyl chloroacetate (120 g, 0.98 mol) was then added and the reactants were heated, under reflux. After 2 h the products were cooled and filtered, and the residue was washed with ethanol (100 ml). The combined filtrate and washings were treated with aqueous 5M-sodium hydroxide (240 ml, 1.20 mol) at 20 °C. After 15 min, the hydrolysate was neutralised (to pH 8) with 5M-hydrochloric acid and concentrated under reduced pressure to remove most of the ethanol. More 5M-hydrochloric acid (750 ml) was added and the mixture extracted with ether (1 000 ml). The ether layer was washed with water (500 ml), dried ($MgSO_4$), and evaporated to give crude 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)-phenoxyacetic acid [320 g; containing 15—20% (by n.m.r.) of unchanged phenol] as an oil.

The above crude acid (160 g, *ca.* 0.48 mol), redistilled thionyl chloride (56 ml, 0.77 mol), and dimethylformamide (*ca.* 1 ml) were heated together, under reflux, for 3 h. Distillation gave crude 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)-phenoxyacetyl chloride [91.5 g; 52%, based on *p*-(1,1,3,3-tetramethylbutyl)phenol] as a pale orange syrup, b.p. 176—178° at 0.25 mmHg. Crystallization of this material from light petroleum (b.p. 30—40°) gave colourless needles (60 g, in 3 crops), m.p. 73.5—74.5° (Found: C, 54.7; H, 5.9. $C_{16}H_{21}Cl_3O_2$ requires C, 54.6; H, 6.0%); τ (CCl_4) 2.78 (2 H, s), 5.12 (2 H, s), 8.31 (2 H, s), 8.66 (6 H, s), and 9.23 (9 H, s); $\nu_{max.}$ (CCl_4) 1 827 cm^{-1}.

5′-O-2,6-*Dichloro*-4-(1,1,3,3-*tetramethylbutyl*)*phenoxyacetylthymidine* (14d).—A solution * of 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetyl chloride (7.74 g, 22 mmol) in anhydrous acetonitrile (15 ml) was added dropwise over 10 min to a stirred mixture of thymidine (4.845 g, 20 mmol), 2,6-lutidine (16 ml, 136 mmol), and acetonitrile (30 ml), with exclusion of moisture, at 20 °C. After 2 h, the products were concentrated under reduced pressure; the residue was dissolved in chloroform and the solution washed first with saturated aqueous sodium hydrogen carbonate and then with water. The dried ($MgSO_4$) chloroform layer was concentrated to an oil which was redissolved in ethanol, and the solution was evaporated. After this process had been repeated several times, the material was purified by short column chromatography on silica gel, eluted with chloroform–methanol (95.5 : 4.5 v/v). Crystallization of the resulting glass (8.8 g) from di-isopropyl ether containing a small amount of diethyl ether gave 5′-O-2,6-*dichloro*-4-(1,1,3,3-*tetramethylbutyl*)*phenoxyacetylthymidine* (Found: C, 56.0; H, 6.1; N, 4.9. $C_{26}H_{34}Cl_2N_2O_7$ requires C, 56.0; H, 6.15; N, 5.0%) as crystals (7.80 g, 70%), m.p. 99—100°; τ[$(CD_3)_2SO$] 2.54br (3 H, s), 3.78 (1 H, t, *J* 7.6 Hz), 5.30br (2 H, s), 5.66 (3 H, m), 6.03 (1 H, m), 7.80 (2 H, m), 8.29br (5 H, s), 8.69 (6 H, s), and 9.29 (9 H, s); $\lambda_{max.}$ (95% EtOH) 267 (ε 10 700), $\lambda_{min.}$ 240 nm (3 600); R_F 0.40 (B).

Deacylation of 5′-O-Aryloxyacetyl Derivatives of Thymidine.—Aqueous ammonia (2M; 0.5 ml, 1.0 mmol) was added to

* Unless this solution is kept warm, the acid chloride tends to crystallize in the dropping funnel.

a stirred solution of 5′-*O*-aryloxyacetylthymidine (*ca.* 0.01 g, 0.02 mmol) in dioxan (0.5 ml) at 20 °C. The approximate rates of deacylation were determined by t.l.c. (system B) on silica gel. The half-times ($t_{\frac{1}{2}}$) of deacylation of 5′-O-*p*-chlorophenoxyacetyl-, 5′-*O*-2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetyl-, 5′-O-*p*-(1,1,3,3-tetramethylbutyl)phenoxyacetyl-, and 2,4-di-t-pentylphenoxyacetylthymidines (14b, d, c, and a, respectively) were *ca.* 15, 12, 30, and 100 min, respectively. The R_F values (system B) of the four substrates were 0.30, 0.40, 0.38, and 0.42, respectively.

General Phosphorylation Procedure: Preparation of Fully Protected Oligonucleotides.—A concentrated anhydrous solution of the protected nucleoside or oligonucleotide building block with a free 3′-hydroxy-group (1.0 mol. equiv.), phenyl dihydrogen phosphate [23] (1.01 mol. equiv.), and TPS [6] (2.5 mol. equiv.) in pyridine is kept at room temperature. *Stringent precautions must be taken to exclude moisture.* After 8—24 h, when t.l.c. (system C) indicates that phosphorylation is complete, a solution of the protected nucleoside or oligonucleotide building block with a free 5′-hydroxy-group (1.0 mol. equiv.) and TPS (0.5 mol. equiv.) in anhydrous pyridine is added. The reactants are then concentrated to give a mobile syrup which is kept *again under stringently anhydrous conditions* at room temperature. After 24—72 h (see below), the products are worked up by one of the two following procedures.

(*a*) (with Dr. N. J. Cusack) Water (0.036 g per mmol of TPS used) is added to the products, which are then stirred for 1 h at room temperature. Powdered potassium hydrogen carbonate (0.30 g per mmol of TPS used) is added and the mixture stirred until effervescence ceases. Aqueous M-potassium hydrogen carbonate is then added dropwise until precipitation occurs or until the volume of the solution is doubled. The solution is then evaporated under reduced pressure (bath temperature 30—35 °C) to remove most of the pyridine and the concentrated products are partitioned between chloroform (or dichloromethane) and aqueous M-potassium hydrogen carbonate. If an emulsion is formed, the mixture is centrifuged (10 min at 1 500 rev. min^{-1} should be sufficient). Potassium 2,4,6-tri-isopropylbenzenesulphonate separates at the interface. The organic layer is stirred overnight with anhydrous Na_2SO_4 (containing some $KHCO_3$) and filtered; the filtrate is concentrated under reduced pressure and redissolved in ethanol. The resulting solution is evaporated and this process repeated a further two times. The residue is purified by short column chromatography.[24]

(*b*) The products are dissolved in pyridine, and a twofold excess (with respect to the total quantity of TPS used) of M-potassium phosphate buffer (pH 6.5—7.5) is added. The mixture is then concentrated under reduced pressure (bath temperature below 40 °C) and the oily residue partitioned between chloroform and water. If an emulsion is formed, the mixture is centrifuged at 1 500 rev. min^{-1}. The aqueous layer is extracted twice with chloroform and the combined organic layers are dried ($MgSO_4$) and evaporated. The residue is dissolved in toluene–chloroform, the solution evaporated, the residue dissolved in ethanol–chloroform, and the solution again evaporated. Finally the residue is dissolved in chloroform and the solution evaporated to give a foam, solid, or gum which is then purified by short column chromatography.[24]

After work-up by procedure (*a*) or (*b*) the material may be obtained as a solid by dropwise addition of its solution in chloroform or chloroform–ethanol (*ca.* 2 ml g^{-1}) to light petroleum (b.p. 30—40 °C; *ca.* 100 ml g^{-1}). The precipitated solid is then collected by centrifugation and dried.

General Procedure for Terminal 5′-Deacylation of Fully Protected Oligonucleotides.—Aqueous *ca.* 2M-ammonia is freshly prepared by diluting concentrated aqueous ammonia (*d* 0.88) to 10 times its original volume with water. The substrate is dissolved in a suitable volume * of dioxan (up to 50 ml g^{-1}) at room temperature and an equal volume of aqueous 2M-ammonia is added to the stirred solution. Deacylation is generally *ca.* 95% complete after 60—90 min, but the optimum reaction time for a specific substrate should be determined by t.l.c. on silica gel. Longer reaction times may lead to appreciable solvolysis of the phosphotriester functions and should therefore be avoided. When deacylation is nearly complete, aqueous M-sodium dihydrogen phosphate (1.1—1.15 mol. equiv. with respect to the amount of ammonia used) is added, and the product was concentrated under reduced pressure (bath temperature $<40°$) and then extracted with chloroform. The layers are separated, the aqueous layer is twice extracted with chloroform, and the organic layers are combined, dried ($MgSO_4$), and evaporated under reduced pressure. The gum, foam, or solid obtained is fractionated by short column chromatography.[24] The appropriate fractions are combined and evaporated and the product is isolated as a solid by precipitation from its solution in chloroform or chloroform–ethanol with light petroleum (b.p. 30—40 °C) as described above.

General Procedure for Terminal 3′-Deacetalation of Fully Protected Oligonucleotides.—The substrate is dissolved in a suitable volume of dioxan (up to 50 ml g^{-1}), and 0.2M-hydrochloric acid (*ca.* three quarters of the volume of dioxan used) is added to the stirred solution at room temperature. After *ca.* 2 h, M-potassium phosphate buffer (pH 7—7.5; 5.5—5.75 mol. equiv. with respect to the hydrochloric acid used) is added and the products are worked up as in the general procedure for 5′-deacylation. The crude products are again purified by short column chromatography [24] and isolated as solids by precipitation.

Preparation of Fully Protected TpT *Phenyl Ester* (13b).—5′-*O*-2,6-Dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetylthymidine (4.46 g, 8.0 mmol), phenyl dihydrogen phosphate [23] (1.407 g, 8.08 mmol), and TPS [6] (6.057 g, 20.0 mmol) were dissolved in anhydrous pyridine (50 ml) and the solution was concentrated (to *ca.* 20 ml). After 19 h, a solution of 3′-*O*-methoxytetrahydropyranylthymidine (2.851 g, 8.0 mmol) and TPS (1.211 g, 4.0 mmol) in pyridine was added and the resulting solution concentrated to give a mobile syrup (*ca.* 15 ml) which contained some crystalline TPS. The reaction was monitored by t.l.c. (system C) and worked up by procedure (*a*) to give a glass which was fractionated in two equal portions by short column chromatography [24] [$CHCl_3$–MeOH (97 : 3 v/v)] on Kieselgel H (250 g); yield of glass (this material was not precipitated) 5.95 g (71%).

Preparation of 3′- and 5′-*Partially Protected* TpT *Phenyl Esters* [(18c; $n = 0$) *and* (18b; $n = 0$), *respectively*].—The fully protected TpT phenyl ester (5.95 g, *ca.* 5.6 mmol) was dissolved in dioxan (225 ml) and the solution divided into two portions of equal weight.

(a) 5′-*Deacylation.* To one portion was added aqueous

* As the solubilities of various substrates in dioxan–aqueous ammonia differ, it is advisable to carry out a small scale deacylation in the first instance. This also permits the optimum reaction time to be determined.

2M-ammonia (115 ml) and the solution was stirred at room temperature. After 75 min, M-sodium dihydrogen phosphate (250 ml) was added; the products were worked up as above and fractionated by short column chromatography [24] [$CHCl_3$–MeOH (95 : 5 v/v)] on Kieselgel H (250 g). The pure product was isolated as a solid (2.126 g, *ca.* 2.9 mmol) by precipitation.

(b) 3′-*Deacetalation*. To the other portion was added 0.2M-hydrochloric acid (80 ml) and the resulting solution was stirred at room temperature. After 135 min, M-potassium phosphate buffer (pH 7; 100 ml) was added and the products were worked up as above and fractionated by short column chromatography [24] [$CHCl_3$–MeOH (95 : 5 v/v)] on Kieselgel H (250 g). The product was isolated as a solid (2.259 g, *ca.* 2.4 mmol) by precipitation.

The combined yield of pure 5′-deacylated and 3′-deacetalated products was 66%, based on monomeric starting materials.

Preparation of Fully Protected $(Tp)_3T$ *Triphenyl Ester* (18a; $n = 2$).—The 3′-deacetalated dimer (18c; $n = 0$) (2.157 g, 2.3 mmol), phenyl dihydrogen phosphate [23] (0.404 g, 2.323 mmol), and TPS [6] (1.741 g, 5.75 mmol) were kept together in concentrated pyridine solution at room temperature. After 15 h, a solution of the 5′-deacylated dimer (18b; $n = 0$) (1.694 g, 2.3 mmol) and TPS (0.348 g, 1.15 mmol) in pyridine was added and the mixture was concentrated to a mobile syrup and set aside at room temperature. Both phosphorylation steps were monitored by t.l.c. (system C). After 24 h, the products were worked up by procedure (*a*) and fractionated by short column chromatography [24] [$CHCl_3$–MeOH (95 : 5 v/v)] on Kieselgel H (250 g); yield of glass 2.6 g (62%).

Preparation of 3′-*Partially Protected* $(Tp)_3T$ *Triphenyl Esters* [(18b; $n = 2$) *and* (18c; $n = 2$), *respectively*].—The fully-protected $(Tp_3)T$ triphenyl ester (2.6 g, *ca.* 1.4 mmol) was dissolved in dioxan (60 ml) and the solution divided into two portions (53 and 47% by weight).

(a) 5′-*Deacylation*. To the larger portion was added aqueous 2M-ammonia (30 ml) and the solution was stirred at room temperature. After 120 min, M-sodium dihydrogen phosphate (250 ml) was added and the products were worked up and fractionated by short column chromatography [24] [$CHCl_3$–MeOH (93 : 7 v/v)] on Kieselgel H (100 g). The product was isolated as a solid (0.917 g, 0.61 mmol) by precipitation with light petroleum (b.p. 30—40° C) from its solution in $CHCl_3$–MeOH. Deacylation and deacetalation (see below) were monitored by t.l.c. (system E).

(b) 3′-*Deacetalation*. To the smaller portion were added 0.2M-hydrochloric acid (20 ml) and more dioxan (3.5 ml). The resulting solution was stirred at room temperature. After 165 min, M-potassium phosphate buffer (pH 7; 25 ml) was added; the products were worked up as above and fractionated by short column chromatography [24] [$CHCl_3$–MeOH (93 : 7 v/v)] on Kieselgel H (100 g). The product was isolated as a solid (0.869 g, 0.51 mmol).

The combined yield of 5′-deacylated and 3′-deacetalated products was 49%, based on the partially protected dimeric starting materials (18b and c; $n = 0$).

Preparation of Fully Protected $(Tp)_7T$ *Heptaphenyl Ester* (18a; $n = 6$).—The 3′-deacetalated tetramer (18c; $n = 2$) (0.849 g, 0.50 mmol), phenyl dihydrogen phosphate [23] (0.088 g, 0.505 mmol), and TPS [6] (0.379 g, 1.25 mmol) were kept together in concentrated pyridine solution at room temperature. After 18 h, a solution of the 5′-deacylated tetramer (18b; $n = 2$) (0.749 g, 0.50 mmol) and TPS (0.076 g, 0.25 mmol) in pyridine was added and the reactants were concentrated to give a mobile syrup which was set aside at room temperature. Both phosphorylation steps were monitored by t.l.c. (system E). After 24 h the products were worked up by procedure (*b*) and fractionated by short column chromatography [24] [$CHCl_3$–MeOH (94 : 6 v/v)] on Reeve Angel silica gel (250 g); yield of solid product 0.90 g (54%).

Preparation of 3′- *and* 5′-*Partially Protected* $(Tp)_7T$ *Heptaphenyl Esters* [(18b; $n = 6$) *and* (18c; $n = 6$) *respectively*].—The fully protected $(Tp)_7T$ heptaphenyl ester (0.90 g, *ca.* 0.27 mmol) was dissolved in dioxan (11 ml) and the solution divided into two parts (54 and 46% by weight).

(a) 5′-*Deacylation*. The larger part was stirred at room temperature while aqueous 2M-ammonia (8 ml) was added in portions to ensure that the reactants remained in solution throughout. After 4 h, M-sodium dihydrogen phosphate (20 ml) was added and the mixture concentrated under reduced pressure (bath temperature <40 °C). The residue was treated with water (10 ml), and the aqueous extract decanted from the insoluble gummy material and centrifuged. The supernatant was discarded and the residue and gummy material were re-extracted with water. The insoluble residue and gummy material were dissolved in tetrahydrofuran (10 ml) and the solution was evaporated under reduced pressure. After this process had been repeated several times, the residue was fractionated by short column chromatography [24] [$CHCl_3$–MeOH (91.5 : 8.5 v/v)] on Reeve Angel silica gel (50 g). The product was isolated as a solid (0.305 g, 0.10 mmol) by precipitation with light petroleum (b.p. 30—40 °C) from its solution in $CHCl_3$–MeOH. Deacylation and deacetalation (see below) of fully protected $(Tp)_7T$ heptaphenyl ester were monitored by t.l.c. (system E).

(b) 3′-*Deacetalation*. The smaller part was stirred at room temperature while 0.2M-hydrochloric acid (5 ml) was added in portions to ensure that the reactants remained in solution throughout. After 4 h, M-potassium phosphate buffer (pH 7; 12 ml) was added and the products were worked up by the procedure described above for 5′-deacylation of the fully protected octamer. After fractionation by short column chromatography [24] [$CHCl_3$–MeOH (91.5 : 8.5 v/v)] on Reeve Angel silica gel (50 g), the product was isolated as a solid (0.342 g, 0.106 mmol).

The combined yield of 5′-deacylated and 3′-deacetalated products was 41.5%, based on the partially protected tetramers (18b and c; $n = 2$).

Preparation of Fully Protected $(Tp)_{15}T$ *Pentadecaphenyl Ester* (18a; $n = 14$).—The 3′-deacetalated octamer (18c; $n = 6$) (0.306 g, 0.095 mmol), phenyl dihydrogen phosphate [23] (0.0167 g, 0.096 mmol), and TPS [6] (0.145 g, 0.48 mmol) were kept together in concentrated pyridine solution room at temperature. After 24 h, a solution of the 5′-deacylated octamer (18b; $n = 6$)(0.287 g, 0.095 mmol) and TPS (0.039 g, 0.13 mmol) in pyridine was added and the reactants were concentrated to give a mobile syrup which was set aside at room temperature. Both phosphorylation steps were monitored by t.l.c. (system F). After 72 h, the products were diluted with pyridine and M-potassium phosphate buffer (pH 7—7.5; 1.8 ml) was added. The resulting mixture was concentrated under reduced pressure (bath temperature <40 °C) and then worked up by the water–tetrahydrofuran procedure described in the deacylation of the fully protected octamer (18a; $n = 6$). The material obtained was fractionated by short column chromatography

[$CHCl_3$–MeOH (93—91.5 : 7—8.5 v/v)] on Reeve Angel silica gel (150 g). The product was isolated as a solid (0.254 g, 42%) by precipitation with light petroleum (b.p. 30—40 °C) from its solution in $CHCl_3$–MeOH (9 : 1 v/v).

Preparation of 3′- and 5′-Partially Protected $(Tp)_{15}T$ *Pentadecaphenyl Esters* [(18b; $n = 14$) *and* (18c; $n = 14$), *respectively*].—(a) *5′-Deacylation*. Aqueous 2M-ammonia (7.5 ml) was added dropwise to a stirred solution of the fully protected hexadecamer (0.140 g, 0.022 mmol) in dioxan (7.5 ml) at room temperature. After 70 min, M-sodium dihydrogen phosphate (17 ml) was added to the products, which were then worked up by the water–tetrahydrofuran procedure and fractionated by short column chromatography [24] [$CHCl_3$–MeOH (90.5 : 9.5 v/v)] on Reeve Angel silica gel (12 g) to give, after precipitation, the product (0.096 g, 72%) as an almost pure [t.l.c. (system E)] solid.

(b) *5′-Deacetalation*. 0.2M-Hydrochloric acid (5 ml) was added dropwise to a stirred solution of the fully protected hexadecamer (0.103 g, 0.016 mmol) in dioxan (7.5 ml) at room temperature. After 4 h, M-potassium phosphate buffer (pH 7; 12 ml) was added to the products, which were then worked up by the water–tetrahydrofuran procedure and fractionated by short column chromatography [24] [$CHCl_3$–MeOH (91 : 9 v/v)] on Reeve Angel silica gel (12 g) to give, after precipitation, the product (0.081 g, 80%) as a virtually homogeneous [t.l.c. (system E)] solid.

Preparation of Fully Protected $(Tp)_{31}T$ (18a; $n = 30$).—The 3′-deacetalated hexadecamer (18c; $n = 14$) (0.075 g, 0.012 mmol), phenyl dihydrogen phosphate [23] (0.002 1 g, 0.012 1 mmol), and TPS[6] (0.020 g, 0.066 mmol) were kept together in concentrated pyridine solution at room temperature. After 31 h, a solution of the 5′-deacylated hexadecamer (18b; $n = 14$) (0.073 g, 0.012 mmol) and TPS (0.020 g, 0.066 mmol) in pyridine was added and the reactants were concentrated to give a mobile syrup which was set aside at room temperature. Both phosphorylation steps were monitored by t.l.c. (system F). After 85 h, the products were diluted with pyridine and M-potassium phosphate buffer (pH 7—7.5; 1 ml) was added. The mixture was then worked up by the water–tetrahydrofuran procedure and fractionated by short column chromatography [24] [$CHCl_3$–MeOH (91.5 : 8.5 v/v)] on Reeve Angel silica gel (50 g). The product was isolated as a solid (0.072 6 g, 48.5%) by precipitation with light petroleum (b.p. 30—40 °C) from its solution in $CHCl_3$–MeOH (9 : 1 v/v). It was not possible to effect a complete separation of the products by short column chromatography. Thus the isolated material was not completely homogeneous [t.l.c. (system D); two developments].

Attempted 5′-Deacylation of Fully Protected $(Tp)_{31}T$.—Aqueous 2M-ammonia (5 ml) was added dropwise to a stirred suspension of the fully protected $(Tp)_{31}T$ (0.070 g, *ca.* 0.005 6 mmol) in dioxan (5 ml) at room temperature. After 65 min, M-sodium dihydrogen phosphate (11 ml) was added to the gummy mixture obtained and the products were worked up by the water–tetrahydrofuran procedure. As t.l.c. (system F) revealed much unchanged starting material, the residue was treated with tetrahydrofuran (3 ml) and aqueous 2M-ammonia (3 ml). The reactants were stirred for 3 h and then treated with M-sodium dihydrogen phosphate (10 ml). The material obtained after water–tetrahydrofuran work-up was dissolved in anhydrous pyridine and the solution evaporated under reduced pressure. The residue was dissolved in a small volume of pyridine and added to ether. The resulting precipitate was washed several times with ether and then dried; yield 0.043 g. The major component or components of this material had R_F 0 (system F).

Preparation of Fully Protected Intermediates by 5′-O-Tetrahydropyranylation of Deacylated Products.—(a) TpT. 2,3-Dihydro-4*H*-pyran (0.4 ml, *ca.* 4.4 mmol) was added to a stirred solution of 5′-deacylated dimer (18b; $n = 0$) (0.295 g, 0.40 mmol) and toluene-*p*-sulphonic acid monohydrate (0.010 g, 0.05 mmol) in anhydrous dioxan (4 ml) at room temperature. After 10 min, the products were neutralized with methanolic dimethylamine (10% v/v) and partitioned between dichloromethane (40 ml) and saturated aqueous sodium hydrogen carbonate (20 ml). The aqueous layer was extracted with dichloromethane (2 × 40 ml), and the organic extracts were combined, dried ($MgSO_4$), and concentrated under reduced pressure. The residual gum was dissolved in ethanol (10 ml) and the solution evaporated. After this procedure had been repeated once more, the residue was purified by short column chromatography [24] [$CHCl_3$–MeOH (96 : 4 v/v)] on Merck Kieselgel H (20 g) to give, after precipitation from light petroleum (b.p. 30—40 °C), the fully-protected dimer (19; $n = 0$) (0.308 g, 94%) as a homogeneous [t.l.c. (system C)] solid.

(b) $(Tp)_3T$. 2,3-Dihydro-4*H*-pyran (0.06 ml, *ca.* 0.66 mmol) was added to a stirred solution of 5′-deacylated tetramer (18b; $n = 2$) (0.090 g, 0.06 mmol) and toluene-*p*-sulphonic acid monohydrate (0.001 g, 0.005 mmol) in anhydrous dioxan (0.6 ml) at room temperature. After 10 min, the products were neutralized with methanolic ammonia (half-saturated at 0 °C) and worked up as above except that chloroform was substituted for dichloromethane. Purification by short column chromatography [24] [$CHCl_3$–MeOH (95 : 5 v/v)] on Merck Kieselgel H (12 g) gave, after precipitation from light petroleum (b.p. 30—40 °C), the fully protected tetramer (19; $n = 2$) (0.085 g, 90%) as a homogeneous [t.l.c. (system C)] solid.

(c) $(Tp)_7T$. 2,3-Dihydro-4*H*-pyran (0.01 ml, *ca.* 0.11 mmol) was added to a stirred solution of 5′-deacylated octamer (18b; $n = 6$) (0.021 g, *ca.* 0.007 mmol) and a trace of toluene-*p*-sulphonic acid monohydrate in anhydrous dioxan (0.1 ml) at room temperature. After 10 min, when t.l.c. (system E) revealed that the reaction was complete, the products were worked up as above and purified by short column chromatography [24] [$CHCl_3$–MeOH (94 : 6 v/v)] on Reeve Angel silica gel (12 g). After precipitation from light petroleum (b.p. 30—40 °C), the fully protected octamer (19; $n = 6$) (0.013 g, 62%) was obtained as a homogeneous [t.l.c. (system E)] solid.

(d) $(Tp)_{15}T$. 2,3-Dihydro-4*H*-pyran (0.01 ml, *ca.* 0.11 mmol) was added to a stirred solution of 5′-deacylated hexadecamer (18b; $n = 14$) (0.027 g, *ca.* 0.004 4 mmol) and a trace of toluene-*p*-sulphonic acid monohydrate in anhydrous dioxan (0.4 ml) at room temperature. After 1 h, when t.l.c. (system E) revealed that starting material remained, more 2,3-dihydro-4*H*-pyran (0.01 ml) was added. After a further 30 min, the products were neutralized with methanolic ammonia, concentrated under reduced pressure, and purified * by short column chromatography [24] [$CHCl_3$–MeOH (92.5—93 : 7.5—8 v/v)] on Reeve Angel silica gel (12 g). After precipitation from light petroleum (b.p. 30—40 °C) the fully protected hexadecamer (19; $n = 14$) (0.020 g, 74%) was obtained as a homogeneous [t.l.c. (system E)] solid.

* Owing to the solubility properties of the material, partitioning between chloroform and aqueous sodium hydrogen carbonate was omitted.

Complete Deblocking of 5′-O-*Tetrahydropyranylated Fully Protected Oligonucleotides*.—(a) TpT. Fully protected dimer (19; $n = 0$) (0.032 8 g, 0.04 mmol) was stirred with aqueous M-potassium hydroxide (1.6 ml) at room temperature. A homogeneous solution was soon obtained and, after 2 h, when t.l.c. (system E) revealed that no starting material remained, the hydrolysate was applied to a column of Dowex AG 50 × 8 (200—400 mesh; NH_4^+ form; 10 ml) cation-exchange resin. The column was eluted with water and the appropriate fractions were pooled, concentrated under reduced pressure to small volume, and finally freeze-dried to give a solid. A solution of this material (0.015 g) in 0.01M-hydrochloric acid (3 ml) was set aside at room temperature. After 16 h, when t.l.c. (system G on Merck DC-Alufolien cellulose F_{254}) revealed that deblocking was complete, the products were neutralized with dilute aqueous ammonia and then extracted with dichloromethane (2 × 3 ml). The aqueous layer was concentrated (to *ca.* 1 ml under reduced pressure) and applied to a DEAE–cellulose column (15 cm × 2 cm), equilibrated with 0.001M-triethylammonium hydrogen carbonate buffer. The column was eluted with triethylammonium hydrogen carbonate buffer (linear gradient, 0.001—0.2M over 2 l). The appropriate fractions containing TpT [332 A_{267} units, 98% of u.v. absorbing material (at 267 nm) eluted from the column] were pooled, concentrated under reduced pressure to small volume, and then freeze-dried.

(b) $(Tp)_3T$. (i) Fully protected tetramer (19; $n = 2$) (0.023 7 g, 0.015 mmol) was dissolved in a freshly prepared 0.085M-solution of tetraethylammonium hydroxide in dimethyl sulphoxide–water (19 : 1 v/v; 3 ml) at room temperature. After 24 h, the products were neutralized with aqueous 10% acetic acid and concentrated under reduced pressure. The residue was dissolved in water and the solution evaporated. After this process had been repeated once more, the products were treated as above to give 465 A_{267} units [*ca.* 94% of u.v.-absorbing material (at 267 nm) eluted from the DEAE–cellulose column] of completely unblocked $(Tp)_3T$. This material was concentrated and freeze-dried as above. Earlier fractions eluted from the DEAE–cellulose column contained *ca.* 11 A_{267} units of material with a putative charge of −1 and *ca.* 17 A_{267} units of material with a putative charge of −2.

(ii) Fully protected tetramer (19; $n = 2$) (0.011 2 g, 0.007 mmol) was dissolved in 0.1M-potassium hydroxide in dimethyl sulphoxide–water (9 : 1 v/v; 1.5 ml) at room temperature. After 24 h, the products were treated as in (b) (i) above. Fractionation of the products on a DEAE–cellulose column gave *ca.* 8 A_{267} units with a putative charge of zero, *ca.* 23 A_{267} units with putative charges of −1 and −2, and 218 A_{267} units [*ca.* 88% of u.v.-absorbing material (at 267 nm) eluted from the column] of $(Tp)_3T$.

(iii) Fully protected tetramer (19; $n = 2$) (0.023 7 g, 0.015 mmol) was stirred with aqueous M-potassium hydroxide (0.6 ml) and the work-up and acidic hydrolysis were carried out as described above for the fully protected dimer. Fractionation of the products on a DEAE–cellulose column gave *ca.* 3 A_{267} units of material with a putative charge of −1, *ca.* 29 A_{267} units of material with a putative charge of −2, and 236 A_{267} units [*ca.* 82% of u.v.-absorbing material (at 267 nm) eluted from the column] of $(Tp)_3T$.

(iv) A solution of the fully protected tetramer (19; $n = 2$) (0.010 7 g, *ca.* 0.007 mmol) in a 0.2M-solution of sodium hydroxide in dioxan–water (1 : 1 v/v; 0.6 ml) was set aside at room temperature for 16 h. After the usual work-up and acidic hydrolysis, the products were fractionated on DEAE–cellulose to give *ca.* 9 A_{267} units of material with a putative charge of zero, *ca.* 19 A_{267} units with a putative charge of −1, *ca.* 22.5 A_{267} units with a putative charge of −2, and 179 A_{267} units [*ca.* 78% of u.v.-absorbing material (at 267 nm) eluted from the column] of $(Tp)_3T$.

(v) A solution of the fully protected tetramer (19; $n = 2$) (0.023 7 g, 0.015 mmol) in a 0.085M-solution of tetraethylammonium hydroxide in acetonitrile–water (19 : 1 v/v; 3 ml) was set aside at room temperature. Hydrolysis was complete after 16 h. After 40 h, the products were worked up and subjected to acidic hydrolysis by the usual procedure. Fractionation of the completely unblocked material on DEAE–cellulose gave *ca.* 7 A_{267} units of material with a putative charge of zero, *ca.* 19 A_{267} units with a putative charge of −1, *ca.* 29 A_{267} units with a putative charge of −2, and 429 A_{267} units [*ca.* 88.5% of the u.v.-absorbing material (at 267 nm) eluted from the column] of $(Tp)_3T$.

(c) $(Tp)_7T$. Fully protected octamer (19; $n = 6$) (0.015 3 g, *ca.* 0.005 mmol) was dissolved in a freshlyprepared 0.085M-solution of tetraethylammonium hydroxide in dimethyl sulphoxide–water (19 : 1 v/v; 2 ml) at room temperature. After 16 h, aqueous 1.7M-tetraethylammonium hydroxide (0.1 ml) was added and, after a further 7 h, the products were worked up [as in (b) (i) above], subjected to acidic hydrolysis, and then fractionated on a DEAE-cellulose column [linear gradient, 0.001—0.6M-triethylammonium hydrogen carbonate (pH 7.5); 2 l] to give 248 A_{267} units [*ca.* 77.5% of u.v.-absorbing material (at 267 nm) eluted from the column] of completely unblocked $(Tp)_7T$. This material was concentrated and freeze-dried as above. Earlier fractions eluted from the DEAE–cellulose column contained *ca.* 3 A_{267} units of material with a putative charge of zero and *ca.* 69 A_{267} units of material with putative charges from −1 to −6 (see Figure 1).

(d) $(Tp)_{15}T$. Fully protected hexadecamer (19; $n = 14$) (0.021 7 g, *ca.* 0.003 5 mmol) was dissolved in a freshly prepared 0.085M-solution of tetraethylammonium hydroxide in dimethyl sulphoxide–water (19 : 1 v/v; 3 ml) at room temperature. After 16 h, aqueous 1.7M-tetraethylammonium hydroxide (0.15 ml) was added and, after a further 7 h, the products were worked-up [as in (b) (i) above], subjected to acidic hydrolysis, and then fractionated on a DEAE–cellulose column [linear gradient, 0.001—0.5M-triethylammonium hydrogen carbonate (pH 7.5); 2 l]. The later fractions [containing 335 A_{267} units, *ca.* 72% of u.v.-absorbing material (at 267 nm) eluted from the column] were combined, concentrated, and re-chromatographed on a DEAE–Sephadex A-25 column [13 × 2 cm; prepared from 10 g of DEAE–Sephadex], equilibrated with 0.7M-triethylammonium hydrogen carbonate buffer (pH 7.5). The column was eluted with triethylammonium hydrogen carbonate buffer [linear gradient, 0.7—1.2M; 1 l] to give 235 A_{267} units [*ca.* 71% of u.v.-absorbing material (at 267 nm) but only *ca.* 50% of the total number of A_{267} units originally applied to the above DEAE–cellulose column] of completely unblocked $(Tp)_{15}T$. This material was concentrated and freeze-dried as above. Earlier fractions eluted from the DEAE–Sephadex column contained *ca.* 95 A_{267} units and the earlier (un-rechromatographed) fractions eluted from the DEAE–cellulose column contained *ca.* 133 A_{267} units of material.

Complete Deblocking of the Partially Protected $(Tp)_{31}T$.—Partially protected $(Tp)_{31}T$ (0.020 g), obtained (see above) by treating fully protected $(Tp)_{31}T$ (18a; $n = 30$) with

ammonia, was dissolved in a freshly prepared 0.085M-solution of tetraethylammonium hydroxide in dimethyl sulphoxide–water (19 : 1 v/v; 3 ml) at room temperature. After 16 h, aqueous 1.7M-tetraethylammonium hydroxide (0.15 ml) was added and, after a further 7 h, the products were worked up [as in (b) (i) above], subjected to acidic hydrolysis, and then applied to a Sephadex G-75 (ultrafine) column (75 × 2 cm), equilibrated with 0.1M-triethylammonium hydrogen carbonate buffer (pH 7.5.) A hydrostatic pressure (*ca.* 100 cm) was applied to the column, which was eluted with the latter buffer (flow-rate *ca.* 7.5 ml h^{-1}) and 140 fractions (each *ca.* 3.8 ml) were collected: (*a*) fractions 19—22 were combined and contained *ca.* 45 A_{267} units; (*b*) fractions 23—26 were combined and contained *ca.* 105 A_{267} units; (*c*) fractions 27—30 were combined and contained *ca.* 66 A_{267} units; (*d*) fractions 31—75 were combined and contained *ca.* 165 A_{267} units.

Combined fractions (*a*)—(*c*) were separately re-chromatographed on a DEAE–Sephadex A-25 column (13 × 2 cm), equilibrated with 0.8M-triethylammonium hydrogen carbonate buffer (pH 7.5). In each case, the column was eluted with triethylammonium hydrogen carbonate (linear gradient, 0.8—1.3M; 500 ml) and 80 fractions (each *ca.* 6.5 ml) were collected. In the re-chromatography of combined fractions (*a*)—(*c*), eluted material was found in fractions 41—58 (maximum concentration in fractions 48 and 49), fractions 31—60 (maximum concentration in fractions 45 and 46), and fractions 25—60 (maximum concentration in fractions 41 and 42), respectively.

Combined fractions (*b*) were then re-chromatographed on Sephadex G-75 (ultrafine) under the conditions described above: (i) fractions 24—27 were combined and contained *ca.* 40 A_{267} units; (ii) fractions 28—31 were combined and contained *ca.* 55 A_{267} units. Combined fractions (*b*) (i) and (*b*) (ii) were then separately re-chromatographed on DEAE–Sephadex under the conditions described above: the eluted material was found in fractions 39—58 (*ca.* 40 A_{267} units) and 34—58 (*ca.* 55 A_{267} units), respectively. Combined fractions (*a*) and (*b*) (i) were then themselves combined, concentrated, and freeze-dried as above. This material (*ca.* 85 A_{267} units) accounts for *ca.* 22% of the total u.v. absorbing material (at 267 nm) eluted from the original Sephadex G-75 column.

Enzymic Digestion of Oligo- and Poly-thymidylic Acids.—(a) *With venom phosphodiesterase.* A solution of substrate (0.2—0.3 mg) in 0.1M-Tris hydrochloride buffer (pH 8.9; 0.01M with respect to magnesium chloride; 0.08 ml) was treated with stock enzyme solution (1.0 mg enzyme per ml of same buffer; 0.02 ml) at 37 °C. All substrates were completely digested to thymidine [R_F (Merck DC-Alufolien cellulose F_{254}) 0.74 (G), 0.79 (H)] and thymidine 5′-phosphate [R_F 0.13 (G), 0.28 (H)] within 3—7 h.

(b) *With spleen phosphodiesterase.* A solution of substrate (0.2—0.3 mg) in 0.1M-ammonium acetate buffer (pH 7.0; 0.002M with respect to EDTA and containing 0.05% Tween 80; 0.08 ml) was treated with stock enzyme solution (1.0 mg per ml of same buffer; 0.02 ml) at 37 °C. TpT, $(Tp)_3T$, and $(Tp)_7T$ were completely digested to thymidine and thymidine 3′-phosphate [R_F 0.13 (G), 0.29 (H)] within 3—7 h. $(Tp)_{15}T$ and the highest molecular weight material obtained in the deblocking of fully protected $(Tp)_{31}T$ were almost completely digested under these conditions.

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