easily filterable. This crystallized when stirred in acetone; see Table VI.

9-(β-D-Xylopyranosyl)thioguanine (18).—A mixture of 2.00 g (6.64 mmol) of **15**, 70 ml of pyridine, and 13 ml of acetic anhydride was stirred at room temperature for 88 hr, then worked up to afford a tan foam (M), R_f 0.06 (major spot) and 0.20 (**16** and **17**, respectively) in solvent EC-100, when developed thrice. This foam was suitable for thiation. For analysis, the tan foam was stirred for several hours in ether, collected, and dried to afford 1.78 g (65%) of the triacetate **16**. This was recrystallized for analysis. The pure tetraacetate **17** was obtained from M which was partially freed of **16** by fractional precipitation from methylene chloride. The methylene chloride soluble material was then recrystallized from chloroform–carbon tetrachloride (1:1) to give **17**.

A 2.40-g portion (*ca.* 5.86 mmol) of the mixture of **16** and **17**, of purity equivalent to M, was heated with 10.0 g (45 mol) of phosphorus pentasulfide in 200 ml of pyridine[7] at reflux for 4.5 hr under a nitrogen atmosphere and worked up to afford 1.65 g (67%) of white, crystalline **19**. For analysis, see Table VI.

A solution of 1.20 g of **19**, 2.0 ml of 2-mercaptoethanol, and 0.20 g of sodium methoxide in 250 ml of methanol was heated at reflux for 3 hr under a nitrogen atmosphere. The solution was cooled to about 40°, treated with 20 g of IRC-50 (H^+) resin (prewashed with methanol), and stirred until pH 5–6 was attained (about 20 min). The mixture was filtered, treated with charcoal, filtered again, and evaporated. The residue was triturated with methanol–acetone (1:19) and the solid was collected to afford 0.68 g (73%) of **18**, homgeneous in solvent ME-30 with R_f 0.50. This was recrystallized once for analysis; see Table VI.

Registry No.—**2a**, 18520-77-9; **2b**, 18520-78-0; **3a**, 18520-79-1; **3b**, 18520-80-4; **4a**, 18520-81-5; **4b**, 18520-82-6; **5a**, 18520-83-7; **6a**, 18520-85-9; **6b**, 18520-84-8; **7a**, 18520-86-0; **7b**, 18520-87-1; **9b**, 18520-88-2; **10b**, 18520-89-3; **11**, 18520-90-6; **12**, 18520-91-7; **13**, 18520-92-8; **14**, 18520-93-9; **15**, 18520-94-0; **15** Na salt, 18520-95-1; **16**, 18520-96-2; **17**, 18598-35-1; **18**, 18598-36-2; **19**, 18530-32-0.

Acknowledgments.—The authors are indebted to Dr. Leon Goodman for advice and encouragement, to Drs. Goodman and Robert H. Iwamoto for stimulating discussions, to Dr. Peter Lim and staff for the spectral data, and to Mr. Osborne P. Crews, Jr., and staff for the preparation of many of the compounds in large quantities.

Oligonucleotide Syntheses on Insoluble Polymer Supports. II. Pentathymidine Tetraphosphate

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Received May 16, 1968

The linear oligonucleotide pentathymidine tetraphosphate (TpTpTpTpT) has been synthesized stepwise on an insoluble polymer support containing thymidine bound *via* 5′ ether linkage to a styrene–divinylbenzene copolymer containing methoxytrityl functional groups. Condensation of the bound thymidine with 3′-O-acetylthymidine 5′-phosphate activated by 2,4,6-triisopropylbenzenesulfonyl chloride or picryl chloride followed by 3′-O-deacetylation gave polymer-supported thymidylyl-(3′→5′)-thymidine in 70–80% conversions based on thymidine, corresponding to about 350–380 μmol of dinucleoside phosphate per gram of polymer. Repetition of the condensation and deacetylation steps gave the higher oligomers each in approximately 35–80% conversion based on the next lowest member. The over-all conversion into pentamer was about 10% based on initial polymer-bound thymidine.

The procedural advantages which accrue from stepwise synthesis of complex oligomeric substances on inert polymer supports have been discussed by Merrifield, particularly with respect to polypeptide synthesis;[1] more recently the application of this concept to oligonucleotides has been investigated in several other laboratories.[2–6] We have continued our work on oligonucleotides using an insoluble polymer bearing methoxytrityl chloride functional groups to which the nucleoside is attached by 5′-ether formation. The oligonucleotide chain is then extended by condensation of appropriately protected 5′-nucleotides with the free 3′-hydroxyl group of the polymer-bound nucleoside. Our system thus incorporates the characteristics of insolubility found in the polymers of Letsinger, *et al.*,[2] and the functionality of the soluble polymers described by Hayatsu and Khorana[3] and Cramer, *et al.*[4] The Letsinger system utilizes polymer-bound carbonyl chloride functional groups as nucleoside attachment sites through amide[2a,b] or ester[2c,d] formation. In other recent work Blackburn and coworkers have explored a system in which the polymer-bound oligonucleotide terminus is a nucleotide attached to an insoluble polymer by a phosphoramidate linkage.[6]

This paper will describe the synthesis of thymidine homooligonucleotides up to the pentanucleoside tetraphosphate stage. Several features different from our previous procedure[5] have led to increased per cent conversions. The effect of altering reaction variables will be discussed for each step of the synthesis outlined in Scheme I.[7]

(1) R. B. Merrifield, *Science*, **150**, 178 (1965).

(2) (a) R. L. Letsinger and V. Mahadevan, *J. Amer. Chem. Soc.*, **87**, 3526 (1965); (b) *ibid.*, **88**, 5319 (1966); (c) R. L. Letsinger, M. H. Caruthers, and D. M. Jerina, *Biochemistry*, **6**, 1379 (1967); (d) R. L. Letsinger, M. H. Carothers, P. S. Miller, and K. K. Ogilvie, *J. Amer. Chem. Soc.*, **89**, 7146 (1967).

(3) (a) H. Hayatsu and H. G. Khorana, *ibid.*, **88**, 3182 (1966); (b) *ibid.*, **89**, 3880 (1967).

(4) F. Cramer, R. Helbig, H. Hettler, K. H. Scheit, and H. Seliger, *Angew. Chem. Intern. Ed. Engl.*, **5**, 601 (1966).

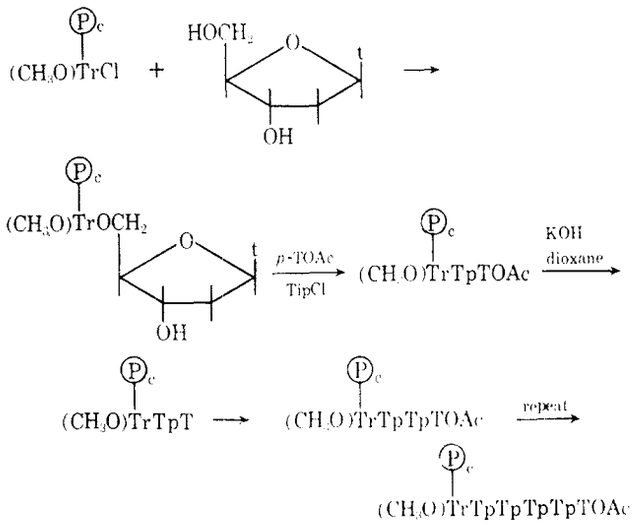
(5) L. R. Melby and D. R. Strobach, *J. Amer. Chem. Soc.*, **89**, 450 (1967).

(6) G. M. Blackburn, M. J. Brown, and M. R. Harris, *J. Chem. Soc., C*, 2438 (1967).

(7) The symbol —Ⓟc denotes the cross-linked polystyrene backbone and (CH_3O)TrT the pendant methoxytrityl group with thymidine (T) attached *via* 5′-ether linkage; TpT and TpTOAc, respectively, refer to thymidylyl-(3′→5′)-thymidine and its 3′-O-acetate. The higher oligomers are abbreviated in the conventional way.

SCHEME I

INSOLUBLE POLYMER-SUPPORTED OLIGONUCLEOTIDE SYNTHESIS[a]



[a] T = thymidine; t = thymine; TipCl = 2,4,6-triisopropylbenzenesulfonyl chloride. *p*-TOAc should read pTOAc.

Methoxytrityl Chloride Polymers and Condensation with Thymidine.—The synthesis of the cross-linked methoxytrityl chloride support polymer from the appropriate styrene–iodostyrene–divinylbenzene copolymer was described previously;[5] the same procedure was used to prepare polymers with 0.75, 1, 2, and 20% cross-linking by varying the amount of divinylbenzene in the monomer mixture. These variations in cross-linking imparted marked differences in the degree of solvent-induced swelling of the polymers but had little or no effect on the efficiency of conversion to supported thymidine polymers except at very high levels of cross-linking (Table I). However 0.75 or 1% cross-linked supports were preferred since they have higher thymidine loadings and allowed more facile acid-induced cleavage of products from the polymer.

Assay for Polymer-Supported Thymidine.—Previously we analyzed for polymer-bound thymidine by spectrophotometric determination of thymine released by exhaustive hydrolysis of the polymer in refluxing hydrochloric acid–acetic acid mixture.[5] We now find it more convenient to hydrolyze the polymer with 1% trifluoroacetic acid in benzene at room temperature. Under these conditions intact thymidine is released quantitatively in 24 hr. However, this reagent was not satisfactory for release of polymer-bound oligonucleotides (see below).[8]

Nucleotide Condensation with Polymer-Supported Thymidine.—In our previous paper we described the formation of polymer-bound dimer, thymidylyl-(3′→5′)-thymidine, in about 50% conversion, based on thymidine, using dicyclohexylcarbodiimide (DCC) as the condensing agent.[5] However, because of the long reaction times required and the low conversions obtained with DCC we turned to the arenesulfonyl chlorides which Khorana and coworkers had introduced as condensing agents for oligonucleotide synthesis.[9,10] We also investigated the use of picryl chloride.[11]

(8) Trifluoroacetic acid in chloroform or dioxane was used for product release in the soluble polymer work of Hayatsu and Khorana[3] and Cramer, *et al.*[4]

TABLE I

CONVERSION OF METHOXYTRITYL CHLORIDE POLYMER TO THYMIDINE DERIVATIVES[a]

Polymer degree of cross-linking, %[b]	Chloride polymer— % Cl found	Equiv μmol of Cl/g	Thymidine polymer, μmol of T/g— Theory[c]	Found	Convn, %
0.75	2.8	790	679	637	94
1.0	2.6	734	636	610	96
2.0	2.4	676	594	553	93
20.0[d]	1.9	535	482	382	79

[a] Reaction time, 48 hr; solvent, pyridine–benzene mixture. For details see ref 5. [b] Per cent by weight of divinylbenzene. [c] Calculated from per cent Cl found. These figures reflect the weight gain of the polymer resulting from substitution of Cl by thymidine. [d] This polymer was prepared using a monomer charge in which the iodostyrene content was increased to maintain potential functionality at about the same level as the less cross-linked polymers.

Table II summarizes synthesis conditions for a series of TpTOAc polymers. Maximum conversions (70–80%)[12] to TpTOAc were achieved using 2,4,6-triisopropylbenzenesulfonyl chloride in anhydrous pyridine as represented in expt 1, 6, and 7. The optimized conditions used therein form the basis for comparison of several reaction variables as follows.

A. Degree of Polymer Cross-Linking.—At low levels of polymer cross-linking (0.75–2%) variation in the degree of cross-linking had little or no effect on per cent conversion of bound thymidine to the dimer TpTOAc despite large differences in solvent-induced polymer swellage (Table II, expt 1, 6, and 8). Absolute amounts of dimer formed differed because of variations in thymidine content of the polymers. However, on highly cross-linked polymer (20%), percentage conversion was reduced from 70–80% to about 60% (Table II, expt 11). This change probably reflects decreased accessibility of polymer-bound functional groups.

B. Nucleotide Salt Form and Mole Ratio Relative to Nucleoside.—Lohrmann and Khorana have shown that whereas DCC is an effective condensing agent only with pyridinium salts of nucleotides the arenesulfonyl chlorides are effective also with nucleotide salts of strongly basic amines.[10] We found bis(triethylammonium) 3′-O-acetylthymidine 5′-phosphate (pTOAc) to be preferable to the pyridinium salt (70–80% *vs.* 55% conversion, Table II, expt 9 and 10) and, furthermore, that the mono(triethylammonium) salt was less suitable than the bis salt (59% *vs.* 73% conversion, expt 1 and 3).

We usually used a three- to fivefold molar excess of nucleotide relative to polymer-bound nucleoside and observed no significant differences in conversion within these limits; no advantage was found in higher nucleotide ratios. At the nucleotide concentration used (~0.1 *M*), lower nucleotide/nucleoside ratios imposed solvent volumes so small that all of the solvent was

(9) R. Lohrmann and H. G. Khorana, *J. Amer. Chem. Soc.*, **88**, 829 (1967).
(10) T. M. Jacob and H. G. Khorana, *ibid.*, **86**, 1630 (1964).
(11) F. Cramer, R. Wittmann, K. Danek, and G. Weimann, *Angew. Chem.*, **75**, 92 (1963).
(12) As in our previous paper,[5] we distinguish between conversion and yield, the former being determined by the ratio moles of product/total moles of starting material and the latter by the ratio moles of product/moles of unrecovered starting material.

TABLE II

CONVERSION OF METHOXYTRITYLTHYMIDINE POLYMERS TO THYMIDYLYL-(3′→5)-THYMIDINE 3′-O-ACETATE DERIVATIVES

Expt no.	Precursor thymidine polymer: μmol of T/g	Precursor thymidine polymer: % cross-link	pTOAc,[a] mol/mol of T	Condensing agent,[b] mol/mol of pTOAc	Reaction time, hr	Product TpTOAc polymer: μmol of TpTOAc/g: Found	Product TpTOAc polymer: μmol of TpTOAc/g: Cor[c]	Convn, %
1	637	0.75	4.7	2	4	383	462	73
2	637	0.75	4.7	2	16	369	440	69
3	637	0.75	4.7	2	4	319	372	59
4	637	0.75	4.7	1	4	266	302	48
5	637	0.75	4.7	1	4	252	284	45
6	546	1.0	3.0	2	4	365	436	80
7	546	1.0	3.0	3	4	337	396	72
8	553	2.0	3.0	2	4	331	388	71
9	565	1.0	5.0	2	20	334	392	70
10	565	1.0	5.0	2	20	273	309	55
11	382	20.0	4.7	2	4	216	240	63
12	565	1.0	*d*		48	314	366	65
13	565	1.0	4.5	2	18	356	423	75

[a] The bis(triethylammonium) salt throughout excepting expt 3, and 5 for which the mono(triethylammonium) salt was used and expt 10 which utilized the pyridinium salt. The solvent was anhydrous pyridine. [b] 2,4,6-Triisopropylbenzenesulfonyl chloride in all cases excepting expt 13 for which picryl chloride was used. [c] The corrected figures take into account the polymer weight gain resulting from addition of the elements of pTOAc; see Experimental Section. [d] The phosphorylating solution from expt 1 was filtered under anhydrous conditions and added to the fresh batch of thymidine polymer.

imbibed by the polymer forming a nonfluid gel which could not be adequately mixed.

C. Condensing Agent/pTOAc Ratio.—Consistent with the conclusions of Khorana and coworkers we found a ratio of 2–3 mol of triisopropylbenzenesulfonyl chloride per mole of nucleotide to be optimum.

Such solutions retained phosphorylating capacity after having been used in an initial phosphorylating reaction. For example, when the solution from expt 1, Table II, was filtered onto a new batch of thymidine polymer, a further 65% conversion to dimer acetate, TpTOAc, was obtained (expt 12, Table II).

D. Reaction Time.—The mononucleotide and condensing agent in pyridine were allowed to react for 30 min prior to adding polymer. A subsequent reaction time of 4 hr was sufficient to achieve maximum yields.

E. Other Condensing Agents and Solvents.—Picryl chloride[11] proved to be an effective condensing agent although it gave a dark brown polymer; this color persisted as a contaminant in the isolated products but was readily separated by chromatography. Methanesulfonyl chloride proved to be unsatisfacory.

Anhydrous pyridine was used as solvent in all experiments of Table II. Other experiments showed that dichloromethane and dioxane were unsuitable solvents and that dimethylformamide diminished conversions particularly when present during the nucleotide activation period.

Acidic Release of Products from Oligonucleotide Polymer.—For polymer-supported oligonucleotide synthesis to be practical the product must be removed from the support under conditions sufficiently mild that the oligonucleotide is not degraded. With trityl-bound systems an acidic reagent must be used, and in our previous work the reagent of choice was 80% aqueous acetic acid saturated with benzene (HOAc–H_2O–C_6H_6, 16:4:5 v/v), the benzene assisting to swell the polymer; this remains the preferred reagent. With thymidine homooligonucleotides release times of about 18 hr allowed quantitative removal of the TpTOAc from all of the polymers investigated. In Table III are summarized the results of product cleavage with respect to reaction time and degree of cross-linking using several acidic reagents.

TABLE III

ACIDIC CLEAVAGE OF THYMIDYLYL-(3′→5′)-THYMIDINE 3′-O-ACETATE FROM METHOXYTRITYL SUPPORT POLYMERS

Expt no.	Acidic reagent[a]	Polymer source[b]	Polymer cross-link, %	Reaction time, hr	TpTOAc liberated: Polymer,[c] μmol/g	TpTOAc liberated: % of total
1	A	1	0.75	18	383	100
2	A	1	0.75	0.25	373	97
3	A	3	0.75	18	319	100
4	B	3	0.75	18	270	84
5	C	3	0.75	18	136	43
5	A	6	1.0	18	365	100
6	A	6	1.0	0.25	276	76
8	A	13	1.0	18	356	100
9	A	13	1.0	86	356	100

[a] Reagent A is HOAc–H_2O–C_6H_6 (16:4:5 v/v); B trifluoroacetic acid–$CHCl_3$ (1:99 v/v); and C trifluoroacetic acid–C_6H_6 (1:99 v/v). For detailed work-up see Experimental Section. [b] The numbers refer to the first column of Table II. [c] Each value represents an average of at least two analyses reproducible to within ±2% of the value shown.

In previous sections of this paper it has been shown that in various condensation reactions with polymer-bound reactants the per cent conversions are relatively insensitive to the degree of polymer cross-linking. However, the latter property markedly affects the rate of the acidic cleavage reaction; thus oligonucleotide was nearly quantitatively released from 0.75% cross-linked polymer in 15 min while in the same period of time only about 75% of the product was released from 1% cross-linked polymer (Table III, expt 2 and 7).

In our previous paper[5] we remarked on the increased acid lability of the polymer–oligonucleotide trityl ether bond relative to the equivalent bond in the nucleoside polymer. Although that work was carried out with DCC condensing agent, the same increased lability is manifested in sulfonyl chloride condensed derivatives. For example, in expt 2, Table III, a reaction time of 15 min released 373 μmol of TpTOAc/g of polymer. This was the only product isolated (although with longer release times a small amount of unreacted thymidine was also released, *e.g.*, 30 μmol of T/g for expt 1). On the other hand, the thymidine

TABLE IV

DEACETYLATION OF POLYMER-SUPPORTED THYMIDYLYL-(3′→5′)-THYMIDINE 3′-O-ACETATE WITH 0.2 *M* KOH IN METHANOL–DIOXANE[a]

Expt no.	TpTOAc polymer source[b]	Polymer cross-linking, %	Hydrolysis time, hr	TpTOAc, μmol/g of polymer	Product TpT, μmol/g[c]
1	1[d]	0.75	0.25	385	295
2	9[d]	1.0	0.25	327	351
3	9[d]	1.0	24.0	327	343
4	6	1.0	0.5	365	297
5	13	1.0	0.25		356
6	8[d]	2.0	0.5	319	318
7	8	2.0	0.5	331	296
8	11	20.0	0.5	216	184

[a] For general conditions see Experimental Section. [b] Refers to the first column in Table II. [c] Homogeneous by paper chromatography in solvents A and C. [d] New preparations, prepared similarly to corresponding preparations of Table II.

polymer afforded only 44 μmol of T/g under identical conditions, less than 12% of that expected on the basis of the TpTOAc released.

The results in Table III also focus a peculiar characteristic of the trifluoroacetic acid–benzene reagent. This reagent was effective for quantiative assay of thymidine polymers, and yet under essentially identical conditions it released less than half of the TpTOAc from the derivative polymer. Thus the behavior of trifluoroacetic acid–benzene was qualitatively the converse of that of the acetic acid–water–benzene reagent.

Deacetylation of Polymer-Bound Thymidylyl-(3′→5′)-thymidine 3′-O-Acetate.—Prior to oligonucleotide chain extension to higher oligomers it is necessary to deacetylate the polymer-bound 3′-O-acetate. This hydrolysis was accomplished quantitatively by treating the polymer with 0.2 *M* potassium hydroxide in methanol–dioxane (1:9 v/v) for 15–30 min at room temperature.[13] Several other alkaline reagents were examined but none was as effective as the above combination. For analytical purposes with small amounts of polymer and large volumes of reagent, a hydrolysis time of 15 min was shown to be adequate, but for preparative purposes 30 min was allowed. Results are summarized in Table IV. In these experiments alkaline hydrolysis of the polymer was followed by an extensive washing procedure and isolation of dry polymer from which products were released by acetic acid treatment. Recoveries of the dimer TpT were erratic but several of the experiments clearly show a 10–25% loss of polymer dimer. These results suggest that the normally acid-labile trityl ether bond, when incorporated in the polymeric structure, is somehow further labilized so that cleavage occurs even in basic media.[14] Several different washing procedures were employed subsequent to the deacetylation step, but no correlation could be made between the extent of product loss and composition of the wash solvents (see below).

Conversion of Polymer-Supported Thymidylyl-(3′→5′)-thymidine into Thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine 3′-O-Acetate.—Since the nature of the activated pTOAc intermediate is unknown, the form of the phosphate group in the TpTOAc polymer immediately after condensation is uncertain. However, it is no doubt converted into the pyridinium or triethylammonium salt during the wash cycle (moist pyridine or dilute aqueous triethylamine in pyridine). Subsequent to deacetylation with potassium hydroxide the TpT exists as the potassium salt (KTpT), and in this form the polymer swelled markedly less in organic solvents than the onium salt forms. Assuming that swelling might affect subsequent reactions we examined the effect of converting the KTpT polymer into the pyridinium or triethylammonium salts by washing with the corresponding acetates in pyridine. Although this procedure restored the swelling characterisics of the polymer, it did not improve percentage conversion to trimer acetate. For example, the Et_3NH^+ form of a 1% cross-linked dimer polymer containing 297 μmol of TpT/g gave a product containing 211 μmol of TpTpTOAc/g while the K^+ form of a 2% cross-linked polymer gave an identical result corresponding to 78% conversion after correction for polymer weight gain.

The paper chromatographic mobility of the trimers from those polymers merit some special attention. TpTpT, deacetylated with potassium hydroxide while bound to polymer or derived from polymer-bound KTpT and deacetylated with ammonia *after* release from the polymer, moved as a single band with mobility very similar to reference pT in the ammoniacal solvent system A.[15] On the other hand, when trimer derived from KTpT was ion exchanged with triethylammonium acetate while bound to the polymer, and was released and deacetylated with ammonia, it moved as a multiple band with a major component of low mobility as above and a diffuse minor component with $R_f \sim 0.25$. Both forms were shown to be authentic TpTpT by specific enzymic hydrolysis. We ascribe the mobility differences to different salt forms of TpTpT of which three are possible. When potassium salt polymer was agitated for 4 hr with 1 *M* triethylammonium acetate in pyridine, 78% of the isolated TpTpT retained low mobility while 22% had been converted into the more mobile species. Further equilibration of the polymer with 1 *M* tetraethylammonium acetate in moist pyridine for 20 hr converted virtually all the trimer into the high-mobility form.

Conversion of Polymer-Bound Trimers into Higher Oligomers.—Repetition of the deacetylation and nucleotide condensation reactions gave the tetramer and pentamer derivatives (Tables V and VI). Conversions to these higher members were in the range 35–65% based on next lowest oligomer. Table V shows results only for the highest oligomer isolated at each stage but in each case it was accompanied by the lower oligomers. Complete assay at each step is illustrated in Table VI which shows satisfactory materials balance.[16]

Although the chromatographic mobility of TpTpT was significantly affected by its salt form, such effects

(13) N-Acyl groups apparently survive these conditions intact.

(14) In experiments with dimethoxytrityl analogs of the polymers described here we encountered severe loss of dimer as a result of extensive washing of the TpTOAc derivative with anhydrous pyridine.

(15) See Experimental Section; the R_f of reference sodium pT was 0.15 ± 0.01.

(16) Nucleoside is ignored in the materials balance since the acidic release times were insufficient to liberate all the thymidine.

TABLE V

PENTATHYMIDINE TETRAPHOSPHATE SYNTHESES

Polymer stage	Product[a]	% convn[b]	Product[a]	% convn[b]	Product[a]	% convn[b]
T	637[c,d]		565[d,e]		546[e]	
TpT	295	53	356	75	332	71
TpTpT	161	58	185	56	236	79
TpTpTpT	74	47	112	63	114	51
TpTpTpTpT	40	55			41	37

[a] μmoles per gram of polymer, uncorrected for weight gain. [b] After correction for weight gain. [c] 0.75% cross-linked. [d] Et_3NH^+-exchanged intermediates. [e] 1% cross-linked. [f] K salt forms throughout.

TABLE VI

TETRATHYMIDINE TRIPHOSPHATE SYNTHESIS
2% CROSS-LINKED POLYMER

Stage	Products, μmol/g of polymer T	TpT	TpTpT	TpTpTpT	Highest oligomer Amt, μmol/g cor	% convn
T	553					
TpTOAc	47	319			372	67
TpT	95	318			371	67
TpTpTOAc	38	109	212		232	73
TpTpT	35	108	216		236	74
TpTpTpTOAc		65	153	70	72	34

were virtually insignificant with the tetramer TpTpTpT and pentamer TpTpTpTpT.

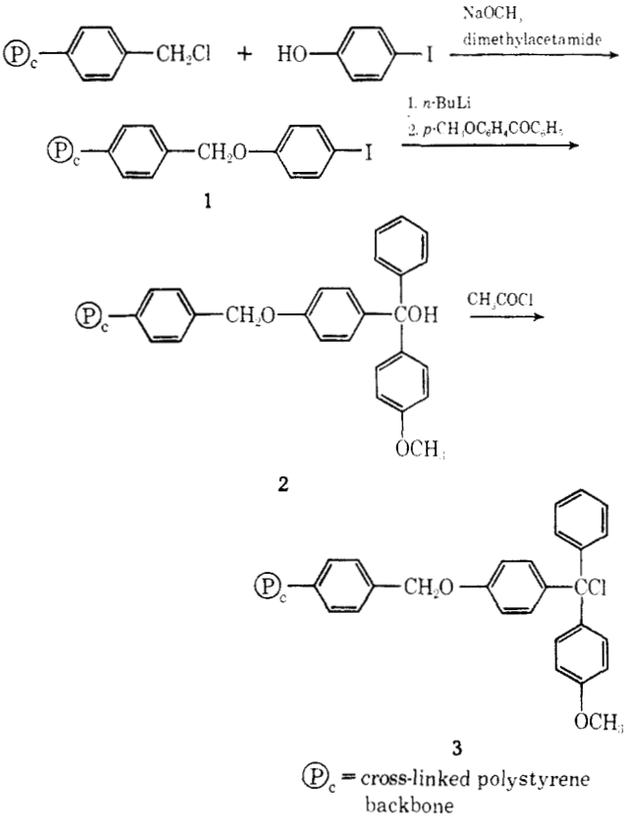
Other Support Polymers.—With the exception of the aminophenoxymethylpolystyrene of Blackburn, *et al.*, all other oligonucleotide support polymers previously described[2-5] have, as nucleoside attachment sites, functional groups bound to phenyl rings which are directly attached to the polyalkylene backbone of the polymer. We reasoned that the contiguity of the functional group and backbone might sterically hinder phosphorylation of the 3′-hydroxyl group of a polymer-supported nucleoside and that a more favorable environment might be provided by separating the trityl group from the polymer backbone by an extended bridge of several atoms. The preparation of one such "extended" polymer is outline in Scheme II and its use in synthesis of the pentanucleoside tetraphosphate TpTpTpTpT will be discussed below.

Reaction of chloromethylated 1% or 2% divinylbenzene–styrene copolymer[17] containing 1.7–2.0 mmol of Cl/g with sodium 4-iodophenoxide in dimethylacetamide proceeded in essentially quantitative conversion to give the iodo polymer **1**. When a limited amount of the phenoxide was used, remaining chloromethyl groups were capped by reaction with excess sodium methoxide. In this way, polymers with a wide range (up to 1380 μmol/g) of iodine content were conveniently prepared. Conversion of **1** into **3** was carried out essentially as described for the *p*-iodostyrene–styrene copolymer[5] to obtain polymers containing 300–1100 μmol of Cl/g. Condensation of extended polymer of type **3** containing 400–500 μmol of Cl/g with thymidine in pyridine–benzene[3] gave thymidine polymers in 90–94% conversion, but polymers of higher chloride content (>1 mmol/g) gave lower conversions (85%) into thymidine polymer in-

(17) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963).

SCHEME II

SYNTHESIS OF EXTENDED SUPPORT POLYMER



1 2 3

Ⓟc = cross-linked polystyrene backbone

dicating inaccessibility of an increasing proportion of chloride groups.

Optimum nucleoside loading for the formation of polymer-supported TpTOAc was determined by reaction of 3′-O-acetylthymidine 5′-phosphate with thymidine polymers having 150–700 μmol of T/g. Polymers containing 400–700 μmol of T/g gave TpTOAc in amounts of 200 ± 15 μmol/g; thus the percentage conversion decreased as the initial nucleoside content of the polymer increased. On the other hand, polymers containing thymidine in the range of about 150–400 μmol of T/g gave fairly constant (60 ± 5%) conversions into TpTOAc. These data are for 2% cross-linked polymers which gave slightly higher conversions than did the 1% cross-linked polymer support. These observations imply that at loadings up to about 400 μmol of T/g about 40% of the supported thymidine is inaccessible to phosphorylation, but any thymidine in excess of 400 μmol/g is totally inaccessible.

Stepwise synthesis of thymidine oligomers up to the pentamer, TpTpTpTpT, was carried out to compare the results with those obtained using copolymer supports. Accordingly, an "extended" polymer containing 410 μmol of T/g was phosphorylated under the optimum conditions described above, to give a product containing 216 μmol of TpTOAc/g (59% conversion). Successive steps of deacetylation and phosphorylation gave polymers containing 108 μmol of TpTpTOAc/g, 41 μmol of TpTpTpTOAc/g and 20 μmol of TpTpTpTOAc/g which represent 52, 39, and 49% conversions, respectively, based on the next lower member in the series. The over-all conversion into the pentanucleoside tetraphosphate based on initially bound

thymidine was 6%.[18] These data show that use of the copolymer support[5] gives somewhat higher over-all conversions for the synthesis of thymidine oligomers than does the "extended" support and emphasize that the method of introduction of the functional group into the polymer support may have significant effects in solid phase oligonucleotide synthesis.

Product Characterization.—The thymidine oligonucleotides were completely hydrolyzed by spleen and venom phosphodiesterase. The nucleotide/nucleoside ratios were determined and shown to be satisfactory. Paper chromatographic mobilities of the oligomers are listed in Table VII.

Summary.—The efficiency of any polymer-supported synthesis scheme must be evaluated in terms of several characteristics, *e.g.*, high yields and conversions into products, absolute amounts of products carried by the polymer, rapidity of synthesis, ease of handling, polymer and product recoveries, and availability of intermediates. Compared to other systems the scheme described here meets reasonable requirements in all aspects including yields, but at rather low conversions in steps leading to the higher oligomers. Moreover, the conditions established are suitable for the polymer-supported synthesis of deoxycytidine and purine-containing oligonucleotides which are the subject of the succeeding paper in this series.

Experimental Section

General Methods and Materials.—Paper chromatography was carried out by the descending technique using Whatman No. 40 paper. Solvent systems used A, 2-propanol-concentrated ammonium hydroxide–water (7:1:2, v/v); B, ethanol–1 *M* ammonium acetate (pH 7.5) (7:3); and C, 1-butanol–acetic acid–water (5:2:3).

Ultraviolet spectra were determined with a Cary Model 15 recording spectrophotometer. The expression OD_{267} unit is defined as that amount of substance in 1 ml of solution which gives an optical density of 1.00 through a 1-cm path length at the indicated wavelength. The following molar extinction coefficients (267 mμ) were used: TpT, 18,500;[19] TpTpT, 25,400;[20] TpTpTpT, 34,000;[20] and TpTpTpTpT, 42,500.[20]

Pyridinium 3′-O-acetylthymidine 5′-phosphate (pTOAc) (0.1 *M* in anhydrous pyridine), prepared as previously described,[5] was converted into the mono- or bis(triethylammonium) salt by addition of 1 or 2 equiv of anhydrous triethylamine which had been distilled from potassium hydroxide. 2,4,6-Triisopropylbenzenesulfonyl chloride[21] was recrystallized from hexane. Methoxytrityl chloride support polymer was prepared and condensed with thymidine as previously described.[5]

Small-scale reactions (3-ml volume or less) were carried out in screw-cap vials. Larger scale reactions (5 ml or larger) were carried out in reactors made by fusing a Teflon fluorocarbon resin stopcock to the outlet of a coarse-frit cylindrical funnel and a screw-cap top to the input. The outlet tube below the stopcock was equipped with a one-hole rubber stopper for insertion into a filter flask for removal of liquid components. The screw-cap tops were obtained from commercially available screw-cap erlenmeyer flasks and allow the use of polyethylene-lined caps which provide liquid and air-tight seals and permit easy access to the reactor contents. Moisture-sensitive reactants were mixed and transferred in a drybox.

(18) These values are for the same polymer carried continuously from T to TpTpTpTpT. Highest percentage conversions observed with similarly prepared polymers for the isolated reactions T → TpT, TpT → TpTpT, etc., were 66, 64, 40, and 49%, respectively, or an over-all 8% conversion into pentamer.

(19) P. T. Gilham and H. G. Khorana, *J. Amer. Chem. Soc.*, **80**, 6212 (1958).

(20) T. M. Jacob and H. G. Khorana, *ibid.*, **87**, 368 (1965).

(21) Aldrich Chemical Co., Milwaukee, Wis.

TABLE VII

PAPER CHROMATOGRAPHY OF THYMIDINE OLIGONUCLEOTIDES

	Mobility (R_f)		
	Solvent A		
Compound	Onium salt	Potassium salt	Solvent C[a]
TpT	0.47	0.47	0.36
TpTpT	0.26	0.16	0.19
TpTpTpT	0.14	0.13	0.12
TpTpTpTpT	0.09	0.08	0.08

[a] No distinguishable difference conferred by salt form.

Iodo Polymer 1. Scheme II.—A mixture of 21.7 g of chloromethylpolystyrene[17] containing 1.96 mmol of Cl/g, 9.5 g (43.1 mmol) of 4-iodophenol, 2.75 g (51 mmol) of sodium methoxide, and 125 ml of freshly distilled dimethylacetamide was stirred at 85° for 18 hr with exclusion of moisture. After cooling the mixture, solids were collected by filtration, and the polymer was washed on the filter with a large volume of dimethylformamide (DMF), water, DMF, and methanol. The composition of wash solvents was changed gradually during all washing operations. After being dried at 100° *in vacuo*, the polymer weighed 28.4 g. *Anal.* Calcd: I, 18.3. Found: I, 17.0.

Methoxytrityl Alcohol Polymer 2. Scheme II.—Into a resin kettle, which had a 9-cm coarse fritted disk base and a stopcock beneath for removal of liquids, was placed 20 g of 1 and 400 ml of reagent grade benzene. The suspension was stirred under nitrogen during and after the addition of 100 ml of 1.6 *M* *n*-butyllithium in *n*-hexane.[22] After 24 hr, liquid reagents were removed and the lithio polymer was washed by suspension in 400 ml of benzene for 15 min. The wash liquid was removed and replaced with a solution of 25 g (0.12 mol) of 4-methoxybenzophenone in 400 ml of benzene. The reaction mixture was stirred for 24 hr under nitrogen. Excess reagents were removed, and the polymer was washed with benzene, 50% acetic acid, water, DMF, and methanol. The vacuum-dried product weighed 21.8 g. *Anal.* Found: I, 0.13. The polymer was converted into the chloride derivative with acetyl chloride in benzene as previously described.[5]

Thymidine Polymer Assay.—To 10–20 mg of thymidine polymer in a small vial was added 3 ml of 1% trifluoroacetic acid in benzene (v/v), the vial was sealed and agitated at room temperature for 18–24 hr. The mixture was filtered, the vial was rinsed, and the polymer was washed with five 1-ml portions of 80% aqueous acetic acid on the filter. The filtrate was evaporated to dryness on a rotary evaporator at a temperature not exceeding 25°. The residue was taken up in a precisely measured volume of water (50–100 ml) and the amount of thymidine determined spectrophotometrically.

Condensation of Polymer-Supported Thymidine with pTOAc.—In a typical experiment a solution of 600 μmol of $(Et_3NH^+)_2$-pTOAc in 6 ml of anhydrous pyridine was treated with 360 mg (1200 μmol) of 2,4,6-triisopropylbenzenesulfonyl chloride, and the mixture was allowed to stand at room temperature for 30 min during which time triethylammonium chloride separated. This "activated" mixture was added to 400–500 mg of thymidine-containing polymer using about 2 ml of dry pyridine as rinse. The reactor was capped and agitated continuously at room temperature for the appropriate period of time (usually 4 hr). The mixture was filtered and the polymer washed by shaking for 1 min or so with each of five 8-ml portions of reagent grade pyridine then by mechanical agitation for 15 min with each of four 8-ml portions of pyridine and four 8-ml portions of aqueous pyridine (1:99 v/v) (total wash time about 2 hr). The polymer was then washed by agitation with several portions of ethanol and vacuum dried over phosphorus pentoxide for a minimum of 6 hr.

In those instances where solvent and/or condensing agents were varied, the calculated amount of pTOAc in pyridine was evaporated to dryness under anhydrous conditions and the appropriate solvent was added to the residue. Condensing agent was added and the reaction continued as described above.

Oligonucleotide Removal from Polymer.—A 10–20-mg portion of oligonucleotide polymer (*e.g.*, the TpTOAc derivative above) was continuously agitated with 3 ml of acetic acid–benzene reagent (HOAc–H_2O–C_6H_6, 16:4:5 v/v) for the appropriate

(22) Foote Mineral Co., Exton, Pa.

period of time, and the mixture was then filtered and washed as described under the thymidine assay procedure. The filtrate was evaporated to dryness, and the residue was treated for 1 hr with 1 ml of concentrated ammonium hydroxide. The hydrolysate was concentrated to a small volume and chromatographed on Whatman No. 40 filter paper. The uv-absorbing bands were cut out and eluted with water, and the solution was analyzed spectrophotometrically.

Deacetylation of Polymer-Supported 3-O-Acetate Derivatives.—The hydrolyzing medium was prepared by dilution of 2 *M* potassium hydroxide in methanol with nine volumes of dioxane. In a typical hydrolysis reaction 8 ml of this solution was added to 300 mg of dry TpTOAc polymer, and the mixture was continuously agitated at room tempeature for 30 min. The mixture was filtered and the polymer washed with eight 8-ml portions of methanol–dioxane (1:9, v/v), 15 min for each portion, then with methanol, and vacuum dried as before.

In those instances where ion exchange was desired, the polymer was washed with four or five portions of methanol–dioxane mixture, 5 min each, then with eight portions of 10% triethylammonium acetate in pyridine (or 10% pyridinium acetate in pyridine) for 30 min each, then twice with dimethylformamide to remove a trace of unidentified flocculent white solid, and finally with several changes of reagent grade pyridine, then with methanol, and dried.

Higher Oligomers.—Further condensation to higher oligomers was conducted as described for the initial supported thymidine–pTOAc condensation.

Enzymic Hydrolyses.—Spleen phosphodiesterase solution was prepared by dissolving 10–15 units of lyophilized enzyme[23] in 2 ml of 0.2 *M* aqueous ammonium acetate (pH 5.7). To ten OD_{267} units of oligonucleotide in 10 μl of water was added 40 μl of enzyme solution and the mixture was incubated at 37° for 5–6 hr. The hydrolysis mixture was spotted on Whatman No. 40 paper and chromatographed.

Venom phosphodiesterase solution was prepared by dissolving 5–7 mg of lyophilized enzyme[23] in 1 ml of 0.1 *M* Tris HCl buffer (pH 8.9). Samples of 20 μl solution per ten OD units of oligonucleotide were used in the hydrolysis with subsequent treatment as above. Nucleotide/nucleoside mole ratios found were within ±8% of theory.

Calculation of Corrected Values of Polymer-Bound Products.—If it is assumed that the weight increase of the polymer during the reaction

$$\text{Ⓟ}_c\text{Tr(OCH}_3\text{)T(pT)}_n\text{OAc} \longrightarrow \text{Ⓟ}_c\text{Tr(OCH}_3\text{)T(pT)}_{n+1}\text{OAc}$$

is due only to the added protected nucleotide, then the polymer weight increase is kA, where k is the molecular weight of the protected nucleotide[24] times 10^{-6} and A is the number of μmoles of $T(pT)_{n+1}OAc$ oligomer formed. If B is the number of μmoles of $T(pT)_{n+1}OAc$ oligomer *found* per gram of product polymer, then for 1.000 g of starting polymer

$$B = \frac{A}{1.000 + kA} \quad \text{or} \quad A = \frac{B}{1.000 + kB}$$

When $n = 0$, $k = 447 \times 10^{-6}$; $k = 405 \times 10^{-6}$ for all other values of n. The value of A thus determined was used to calculate the percentage conversion for all reactions described herein.

Registry No.—Pentathymidine tetraphosphate, 17853-36-0.

Acknowledgments.—We wish to thank Dr. R. E. Benson for helpful discussions and Miss Eleanor Applegate for invaluable technical assistance.

(23) Worthington Biochemical Corp., Freehold, N. J.

(24) The weight increase included 1 mol of triethylamine.

Oligonucleotide Syntheses on Insoluble Polymer Supports. III. Fifteen Di(deoxyribonucleoside) Monophosphates and Several Trinucleoside Diphosphates

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Received May 16, 1968

The previously described insoluble styrene–divinylbenzene copolymer containing methoxytrityl functional groups has been condensed with the N-acylated deoxyribonucleosides N-benzoyldeoxyadenosine, N-anisoyldeoxycytidine, and N-acetyldeoxyguanosine to give the corresponding supported nucleosides in amounts corresponding to 325–360 μmol/g of polymer. Condensation of these products, and a similar thymidine-containing polymer, with the protected nucleotides N-3′-O-diacetyldeoxyadenosine 5′-phosphate, N-anisoyl-3′-O-acetyldeoxycytidine 5′-phosphate, N-3′-O-diacetyldeoxyguanosine 5′-phosphate, and 3′-O-acetylthymidine 5′-phosphate in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride gave 15 dinucleoside phosphates in isolated conversions of 10–60% based on polymer-bound nucleoside. Several dinucleoside monophosphate-containing polymers were 3′-O-deacetylated and further condensed to trinucleoside diphosphate derivatives from which were isolated deoxyadenylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine (dApTpT), deoxyguanylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine (dGTpT), deoxycytidylyl-(3′→5′)-deoxycytidylyl-(3′→5′)-thymidine (dCpCpT), deoxycytidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine (dCpTpT), and thymidylyl-(3′→5′)-deoxycytidylyl-(3′→5′)-thymidine (dTpCpT) in conversions of 10–75% based on dinucleoside phosphate. Specific enzymic hydrolysis showed the products to contain exclusively 3′→5′ phospho diester linkages.

A large proportion of previously reported work on polymer-supported oligonucleotide syntheses has dealt with thymidine-containing homooligonucleotides[1–5] while only limited studies on polymer-supported heterooligonucleotides have been described. These include the synthesis of thymidylyl-(3′→5′)-deoxyadenosine (dTpA), thymidylyl-(3′→5′)-deoxycytidine (dTpC), and thymidylyl-(3′→5′)-deoxyguanosine (dTpG)[6] on a soluble support as reported by Khorana and coworkers,[1] the deoxycytidine-containing products deoxycytidylyl-(3′→5′)-thymidine (dCpT), deoxycytid-

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(5) G. M. Blackburn, M. J. Brown, and M. R. Harris, *J. Chem. Soc., C*, 2438 (1967).

(6) Conventional oligonucleotide symbolism and abbreviations are used throughout this paper; see previously cited references. For simplicity, dinucleoside phosphates will sometimes be referred to as dimers and trinucleoside diphosphates as trimers.