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Use of a Derivatized Merrifield Resin for the Polymer-Supported Synthesis of Oligodeoxyribonucleotides

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Commercial Merrifield resin was derivatized into a resin bearing acyl chloride groups. The derivatized resin can be linked to the 5′-hydroxyl group of thymidine *via* an acyl ester linkage. The polymer-supported thymidine gave thymidylylthymidine in a good yield on reaction with 3′-O-acetylthymidine 5′-phosphate in the presence of mesitylene sulfonyl chloride. When the polymer–supported thymidine was reacted with thymidine 5′-phosphate in the presence of dicyclohexylcarbodiimide, polymerization of thymidylic acid took place and polymer–supported oligothymidylic acid was obtained. The oligonucleotides were liberated from the resin by treatment with ammonia. It was found that this oligonucleotide mixture was composed exclusively of oligomers of the type, $Tp(Tp)_nT$. Oligomers up to the hexamer were purified and identified. This procedure would be convenient for the preparation of oligodeoxyribonucleotides of the general formula, $X(pY)_n$.

The utilization of the principle of supporter-aided synthesis of polymeric substances has furnished a striking success in the polypeptide synthesis, as exemplified by the recent achievement by Gutte and Merrifield of the total synthesis of ribonuclease.[2]

It is obviously attractive to adopt this principle for the synthesis of polynucleotides. Indeed, investigations on the polymer–supported synthesis of oligonucleotides have been made in a number of laboratories[3–7].

Hayatsu and Khorana[4] reported the use of a polymer support for the synthesis of thymidylyl (3′—5′) thymidylyl (3′—5′) thymidine. The polymer used there was the "soluble" type; it is soluble in the reaction solvent, pyridine, but not soluble in water, the solvent into which the reaction mixture may be poured and the resulting precipitate of polymer–supported oligonucleotide may be collected by filtration.

Whereas this soluble type polymer has an obvious advantage over the insoluble type polymer in that the reactions can be carried out at a rate comparable to that of the solution reactions, a difficulty exists in that the larger the size of the oligonucleotide on the sopporting polymer, the higher the solubility of the polymer in water, thereby making the recovery of the product less efficient.[8]

In contrast to the soluble type one, the insoluble type support is expected not to give such a solubility problem, although reactions on the solid polymer may not proceed so rapidly as those on the soluble support. Low efficiencies, in fact, have been observed in the solid phase synthesis of phosphodiester bonds.[3,5b,6,7] In spite of the discouraging previous works,

1) Location: *Hongo, Bunkyo-ku, Tokyo.*
2) B. Gutte and R.B. Merrifield, *J. Am. Chem. Soc.*, **91**, 501 (1969).
3) R.L. Letsinger and V. Mahadevan, *J. Am. Chem. Soc.*, **87**, 3526 (1965); **88**, 5319 (1966).
4) H. Hayatsu and H.G. Khorana, *J. Am. Chem. Soc.*, **88**, 3182 (1966); **89**, 3880 (1967).
5) *a*) F. Cramer, R. Helbig, H. Hettler, K.H. Scheit, and H. Seliger, *Angew. Chem.*, **78**, 640 (1966); *b*) F. Cramer and H. Köster, *Angew. Chem.*, **80**, 488 (1968).
6) L.R. Melby and D.R. Strobach, *J. Am. Chem. Soc.*, **89**, 450 (1967); *J. Org. Chem.*, **34**, 421 (1969); *J. Org. Chem.*, **34**, 427 (1969).
7) G.M. Blackburn, M.J. Brown, and M.R. Harris, *J. Chem. Soc.* (*C*), **1967**, 2483.
8) Such a case was observed in an attempt to synthesize a pentadeoxyribonucleotide on the polystylene support (H. Hayatsu and H.G. Khorana, unpublished experiment).

we considered it still attractive to utilize the "Merrifield resin", which is now commercially available, for the oligonucleotide synthesis and see how far and how efficiently an oligonucleotide chain can be elongated on this support, since it has been amply shown that long polypeptide chains can be very efficiently grown on this insoluble cross-linked polystyrene support.[2,9]

In this paper we wish to report the synthesis of oligothymidylic acids ($T(pT)_n$, $n=1$—5)[10] utilizing a derivatized Merrifield resin as the support.

Preparation of the Derivatized Resin

The commercial Merrifield resin (2% cross-linked polystylene beads with 1.36 mmole of $-CH_2Cl$ group/gram of resin, obtained from Cyclo Chemical Co.) was derivatized according to Chart 1 into a resin bearing an acyl chloride group. The procedures presented in the Scheme 1 are analogous to those conventionally used in the preparation of phenylacetyl chloride starting from benzyl chloride.[11] The reaction conditions employed in the resin derivatization, however, were made much stronger than those in solution reactions. Therefore,

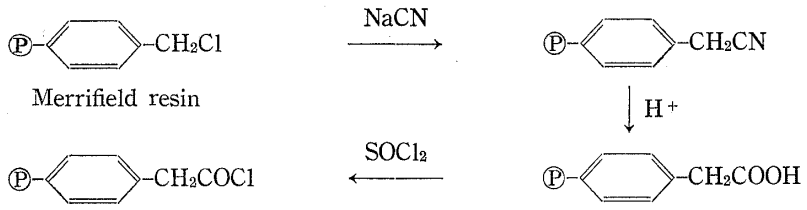


Chart 1

it is highly probable that these derivatization steps afforded necessary transformations of the resin. Satisfactory characterization of these derivatized resins was in fact obtained by infrared (IR) absorption and by elemental analysis. It should be noted that, in these resin reactions as well as in the following oligonucleotide synthesis on the resin, a solvent such as DMF, pyridine, or dioxane, which possesses a high capacity to swell up the resin, was usually contained in the reaction mixture. This was done in order to enhance the rate of reactions at the inner matrix of resin.[12]

Oligothymidylic Acid Synthesis on the Polymer Support

The total procedure with which the synthesis of oligothymidylic acid was carried out is shown in Chart 2. First, the resin bearing acyl chloride groups (Ⓟ–$C_6H_4CH_2COCl$) was reacted with thymidine in pyridine. After unreacted acyl chloride groups were alcoholized by treatment with methanol, the product, Ⓟ–$C_6H_4CH_2COO$–T–OH, was collected by filtration. A large excess of thymidine was used in this reaction hoping that the primary 5′-hydroxyl group (but not the secondary 3′-hydroxyl group) could be selectively bound to the resin. That this selective reaction indeed occurred was shown by the exclusive formation of 3′—5′ phosphodiester linkage in the synthesis of TpT starting from the Ⓟ–$C_6H_4CH_2COO$–T–OH (see below).

A gradual liberation of thymidine from this resin was observed on treatment of the resin with concentrated ammonia–dioxane (1:1, v/v). This fact demonstrated that the thymidine was bound to the polymer *via* acyl ester covalent linkage. The liberation of thymidine reached

9) R.B. Merrifield, *Scientific American*, March, 56 (1968).
10) Abbreviations used are: pT-OH for thymidine 5′-phosphate; pT-OAc for 3′-O-acetyl thymidine 5′-phosphate; TpT for thymidylyl (3′—5′) thymidine, TpTpT for thymidylyl (3′—5′) thymidylyl (3′—5′) thymidine *etc.*; DCC for dicyclohexylcarbodiimide; and P for cross-linked polystyrene skeleton.
11) *a*) R. Adams and A.E. Thal, in "Organic Syntheses", Collective Vol. 1, second Edition, Ed. by H. Gilman, and A.H. Blatt, John Wiley and Sons, Inc., New York, N.Y., 1944, pp. 107—109; *b*) Idem, *ibid.*, pp. 436—438.
12) R.B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).

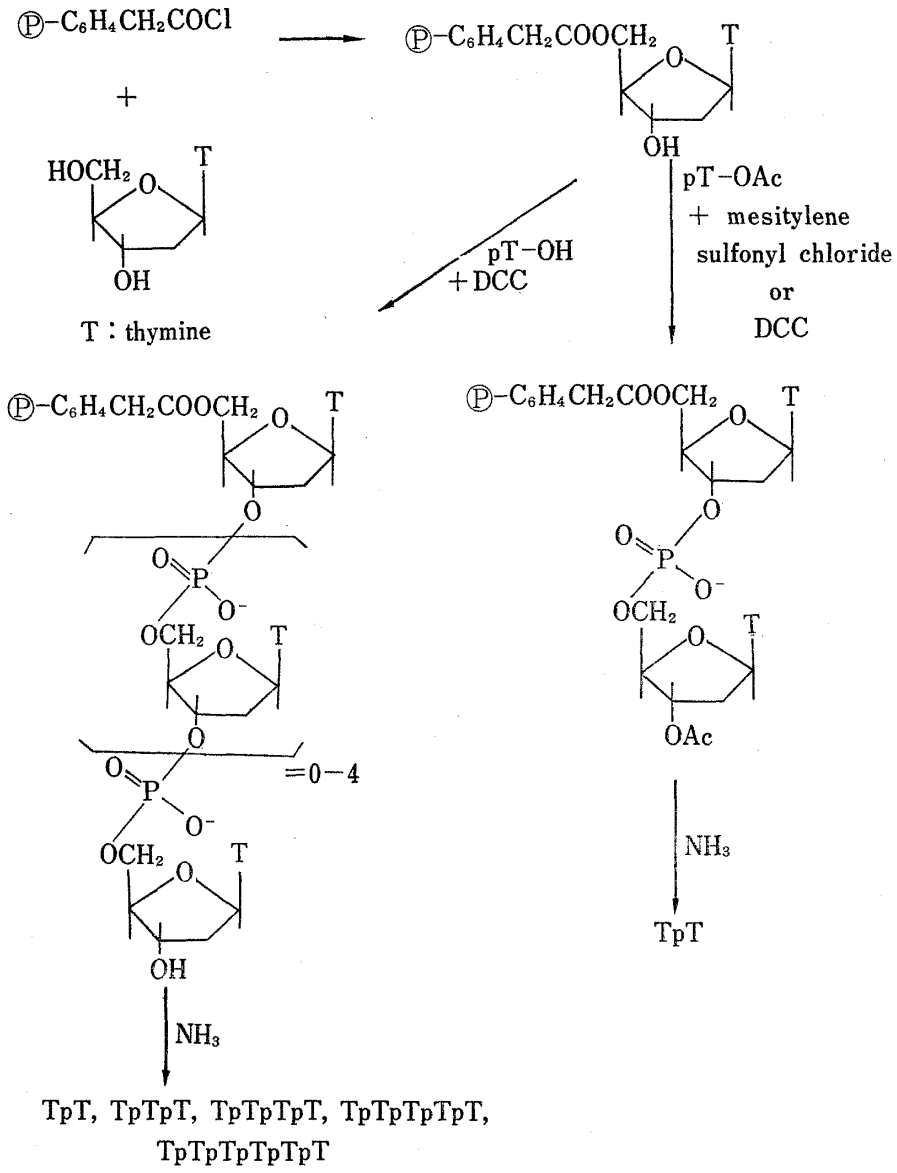


Chart 2

a plateau in about 72 hr. The amount of thymidine linked to the polymer was determined in this manner for several analogously prepared samples and it ranged 100—200 μmoles of thymidine/gram of resin.

The polymer-supported thymidine (Ⓟ-$C_6H_4CH_2COO$-T-OH) was next submitted to the internucleotide bond synthesis with pT-OAc using mesitylene sulfonyl chloride[13] or DCC[13] as the condensing agent (Chart 2). Thus, when Ⓟ-$C_6H_4CH_2COO$-T-OH (containing 10 μmoles of thymidine) was reacted with pyridinium pT-OAc (0.4 mmole) in the presence of mesitylene sulfonyl chloride (0.7 mmole) for 16 hr at room temperature using pyridine as the solvent, 94% of the thymidine residue was converted into TpT. When DCC was used as the condensing agent, and the reaction was allowed to proceed for 4 and half days, 85% conversion of the thymidine residue into TpT was obtained.

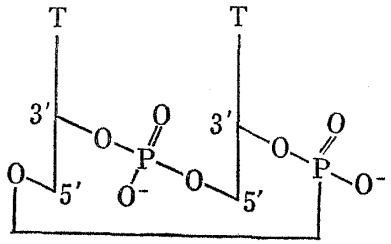
On enzymatic digestion with spleen phosphodiesterase of the TpT prepared as above, a complete hydrolysis into thymidylic acid and thymidine took place. This observation confirmed the exclusive presence of 3′—5′ internucleotide linkage in the TpT. This, in turn,

13) T.M. Jacob and H.G. Khorana, *J. Am. Chem. Soc.*, **86**, 1630 (1964).

indicated that the thymidine initially bound to the resin was linked to it by the 5′-O-acyl ester linkage.[14]

Being encouraged by the high efficiency of TpT formation on the polymer, P–$C_6H_4CH_2$–COO–T–OH was next reacted with pT-OH in the presence of DCC under conditions which will allow both the polymerization of pT-OH and the phosphodiester bond formation between P–$C_6H_4CH_2$COO–T–OH and thymine nucleotides (Chart 2).[15]

The polymerization experiment was designed with the following aims in mind. First, the use of polymer-supported thymidine as the initiator of the growing polynucleotide chain would produce only linear oligothymidylic acid of the type, $T(pT)_n$, being attached to the supporting resin. The series of cyclic molecules of the type I, which will be extensively formed during the polymerization,[16] would be present only in the solution phase, since the hydroxyl group at the 5′-end of the polynucleotide is protected by the linkage to the supporting polymer and therefore is unavailable to the cyclic compound formation. After collecting the resin by filtration, the linear polythymidylic acids could be liberated from the resin by treatment with ammonia and could easily be purified by a simple chromatographic procedure.



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Secondly, this experiment would indicate how far one can elongate the polynucleotide chain on the supporting solid resin. In principle, one could carry out the polymerization reactions repeatedly to the polymer-supported oligomers of the general formula, P–$C_6H_4CH_2$–COO–T–$(pT)_n$–pT–OH, and see the limitation, if there is any, in the size of the polynucleotide chain that can be grown on the resin.

The DCC-mediated polymerization of pT-OH was performed in the presence of P–C_6H_4–CH_2COO–T–OH under conditions analogous to those reported by Khorana and Vizsolyi.[16] After five days of the reaction in dry pyridine, the resin was treated with aqueous pyridine and then collected by filtration. Upon treatment with ammonia and subsequent paper chromatography, a homologous series of oligothymidylic acid with the general structure, $T(pT)_n$, was obtained. Oligomers up to the hexanucleotide were obtained pure in a single paper chromatographic separation. As expected, no cyclic compounds were detectable in the products liberated from the resin. Characterization of these oligothymidylic acids was done by the direct comparison with authentic samples which had been prepared by conventional polymerization of pT–OH and subsequent treatment of the resulting $pT(pT)_n$ compounds with phosphomonoesterase to remove the phosphomonoester groups at the 5′-end.[16]

The resin, P–$C_6H_4CH_2$COO–$(Tp)_n$–T–OH, which was the product obtained prior to the ammoniacal treatment, was submitted again to the DCC-mediated polymerization of pT-OH.

Table I

	% of total optical density units (267 mμ) First polymerization	Second polymerization	*Rf*[a]
Thymidine	37	21.5	0.66
TpT	21.5	19	0.57
TpTpT	20	18	0.48
TpTpTpT	14	15	0.40
TpTpTpTpT	4	9.5	0.33
TpTpTpTpTpT	3.5	9.5	0.28
Higher oligomers	—	7.5	0—0.24

a) Paper chromatographic solvent used was: isobutyric acid–1M ammonium hydroxide (5:3, v/v).

The products were analysed in an identical manner as in the first polymerization. The results are summarized in Table I. It can be seen that the repeated reaction did bring about more utilization of the remaining initial thymidine residue, as well as the increased formation of higher members of oligothymidylic acids. It is to be stressed that, since no contaminating cyclic nucleotides were present in the oligothymidylic acids liberated from the resin, single paper chromatographic fractionation was enough to purify each member of the oligomers. Thus, the present synthesis is a procedure convenient for the preparation of oligothymidylic acid of the type, $T(pT)_n$ (n=1—5). In principle this polymerization method is applicable to the preparation of polynucleotides of the type $X(pY)_n$.

Even after the second polymerization, a considerable amount of unreacted thymidine residue remained. This was surprising when one reflected the high yield obtained in TpT formation from P–$C_6H_4CH_2COO$–T–OH and pT-OAc. The following explanations can be made for this phenomenon. (1) As the polymerization reaction proceeded, the concentration of pT-OH must have decreased quickly because of the self condensation of pT-OH molecules to give polythymidylic acids and cyclic compounds of the type I. This unavailability of pT-OH would inhibit the formation of TpT from the thymidine residue on the polymer support. Phosphodiester bond formation between the thymidine residue and the oligothymidylic acids of the type, $pT(pT)_n$, would be a slow reaction. It has been known that the internucleotide bond formation between 5′-O-tritylthymidine and pTpT-OAc is much slower than that between 5′-O-tritylthymidine and pT-OAc.[17] (2) Furthermore, it should be pointed out that the reaction conditions of the polymerization were significantly different from those of TpT formation. In the polymerization, the reaction was carried out with a very high initial concentration of pT-OH. Soon after the start of the reaction, the total solution became very thick. It is possible that under such conditions the molecules of thymine nucleotides were immobilized and the reaction on the insoluble polymer support was considerably slowed down.

Since Khorana and Vizsolyi[16] have shown that oligomers up to at least dodecanucleotide are formed in the DCC-mediated polymerization of thymidylic acid, we had tended to expect more efficient formation of higher oligomers in this polymer-supported reaction than we actually obtained. This inefficiency could have been due either to the inherent sluggishness of the phosphodiester bond formation in the solid phase, or to some other factors that give a limitation to the size of the oligonucleotide chain which can be put on to the inner matrix of the solid resin. Although further research is required to critically evaluate these points, it appears likely that the use of insoluble polystylene support is suited only for the synthesis of oligonucleotides with relatively short chain lengths.

In the present studies, the linkage employed for the binding of the 5′-end component of the oligonucleotide growing on the support is an alkali-labile acyl ester linkage. This choice was made because the acyl chloride group can be easily introduced in the Merrifield resin. In the stepwise synthesis of oligodeoxyribonucleotides in which the chain is grown in the direction from 5′ to 3′ end, however, alkali–stable rather than alkali–labile groups are employed for the protection of the 5′-end groups as well as the amino groups of the heterocyclic rings.[18] Therefore, although the use of an alkali–stable group such as the trityl[5b,6] or the phosphoroamidate[7] on a polymer support has been shown by other workers to be not

14) We could not exclude a possibility, although it appeared unlikely, that some thymidine residues were bound to the resin through its 3′-hydroxyl group and thymidylyl (5′—5) thymidine derived from them were extremely resistant to the ammonia treatment and therefore escaped our detection.

15) Gilham (P.T. Gilham, *J. Am. Chem. Soc.*, **86**, 4982 (1964)) have described the DCC-mediated fixation of oligothymidylic acids to cellulose. Although that experiment bears a similarity to the present one, it was aimed at the study of the use of such cellulose derivatives in column chromatography.

16) H.G. Khorana and J.P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961).

17) H. Kössel, M.W. Moon, and H.G. Khorana, *J. Am. Chem. Soc.*, **89**, 2148 (1967).

18) H.G. Khorana, T.M. Jacob, M.W. Moon, S.A. Narang, and E. Ohtsuka, *J. Am. Chem. Soc.*, **87**, 2954 (1965) and accompanying papers.

completely satisfactory, it would still be of importance to continue efforts to get a high yield in internucleotide bond synthesis starting from a nucleoside (or a nucleotide) that is linked to the supporting polymer through such an alkali-stable covalent bond.[19]

Conclusion

The present experiments have demonstrated the feasibility of the Merrifield resin in the synthesis of oligonucleotides with chain lengths up to about six. More work is necessary, however, to show the general applicability of this type of resin for the stepwise synthesis of oligodeoxyribonucleotides bearing defined nucleotide sequences.

Experimental

Materials and Methods——The Merrifield resin was a commercial sample obtained from Cyclo Chemical Co., Los Angeles, Cal. U.S.A. (lot No. A1502). It was a 2% cross-linked copolymer of polystyrene and divinyl benzene bearing 1.36 mmole of $-CH_2Cl$ groups per gram. *Anal.* Calcd. for $(C_6H_5CHCH_2)_{0.83}\cdot(C_6H_4\text{-}(CHCH_2)_2)_{0.02}\cdot(C_6H_4CHCH_2(CH_2Cl))_{0.15}$: C, 87.9; H, 7.4; N, 0.0. Found: C, 87.5; H, 7.4; N, 0.7.

DMF was distilled before use. Anhydrous pyridine was prepared according to the method of Lohrmann and Khorana.[20] Toluene and ether were distilled and kept dry over sodium linings. Reagent grade dioxane was used without further purification. Mesitylene sulfonyl chloride was a product of Aldrich Chem. Co., Milwaukee, Wis., U.S.A., and recrystallized from pentane before use. DCC was purchased from Nakarai Chem. Ltd., Kyoto, Japan, and used without further purification.

All evaporations were carried out under reduced pressure at room temperature. All resin-reactions were performed under mechanical stirring. Paper chromatography was carried out ascendingly on Toyo Roshi No. 53 paper. For quantitative determination, the nucleotidic material on the paper chromatogram was eluted with water and submitted to spectrophotometric analysis using Beckmann DU spectrophotometer. Blank absorption due to the paper was always taken into account by submitting a blank area to the identical treatment and subtracting the resulting absorbance value from that obtained with the nucleotidic compounds.

Capacity of Various Solvents to Swell up the Merrifield Resin——In a graduated centrifuge tube, 0.3 ml of dry Merrifield resin was placed, and 2 ml of a solvent was added to it. After gently shaking for about 1 min, the mixture was allowed to stand at room temperature for 1 hr. The mixture was then centrifuged and the volume of the swelled-up resin was determined. The results were as follows. Solvent/Swelling capacity (swelled-up volume/original volume): pyridine/3.0; dioxane/2.5; dioxane–water (3:1, v/v)/1.7; toluene/2.8; ethanol/1; ethanol–water (2:1, v/v)/1; DMF/2.0; acetic acid/1. Thionyl chloride apparently has a high swelling capacity although those values were not determined.

Cross-linked Polystyrene bearing $-CH_2CN$ Groups (P-$C_6H_4CH_2CN$)[21]——The Merrifi eldresin (5.0 g) was suspended in a mixture of DMF (10 ml) and water (2 ml). To this was added a solution of sodium cyanide (2 g) in DMF (20 ml)-water (4 ml), and the mixture heated at 115—120° (bath temperature) under mechanical stirring for 19.5 hr.[22] The brown colored mixture was filtered hot, and the resin was washed with water (10 ml×6), dioxane–water (1:3, v/v, ×5), dioxane–water (1:1, v/v, ×5), dioxane–water (3:1, v/v, ×4), dioxane (×3), dioxane–ethanol (2:1, v/v, ×4), dioxane–ethanol (1:2, v/v, ×3), ethanol (×3), and ether (×3), successively. The resin was finally dried *in vacuo* over phosphorus pentoxide. Yield, 4.95 g. IR

19) Harpold and Calvin (M.A. Harpold and M. Calvin, *Nature*, **219**, 486 (1968)) have recently reported that they observed a phosphodiester bond formation when the Merrifield resin itself and adenosine 5′-phosphate were simply treated in pyridine. According to them, this is a benzyl ester-type covalent-bond formation between 5′-phosphate group and the benzyl chloride group on the resin, and the nucleotide can be recovered upon treatment with aqueous acetic acid. When we treated pyridinium thymidine 5′-phosphate with the Merrifield resin in dry pyridine at room temperature for 24 hours, however, we could not obtain such a covalent linkage. True, that thymidine 5′-phosphate could be recovered from the resulting polymer by a brief treatment with 80% acetic acid, but it could also be completely recovered by treatment with aqueous 1% sodium chloride solution at room temperature for 20 min. The nucleotide could not be liberated from the resin by treatment with water-ethanol (3:1, v/v). These results apparently indicated that the thymidine 5′-phosphate had been bound to the resin through a linkage of ionic character. It is possible that pyridine had reacted with benzyl chloride groups of the resin to form quaternary alkylpyridinium cations.

20) R. Lohrmann and H.G. Khorana, *J. Am. Chem. Soc.*, **86**, 4188 (1964).

21) After completion of the present work, we became aware that this resin-derivative has been prepared by Ayres and Mann (J.J. Ayres and C.K. Mann, *J. Polymer Science, Polymer Letters*, **3**, 505 (1965)).

22) For the conversion of benzyl chloride into benzyl nitrile, the former compound is heated under reflux with sodium cyanide in water for 4 hr.[11a]

(KBr): 2250 cm^{-1} (m) (CN). *Anal.* Calcd. for $(C_6H_5CHCH_2)_{0.83}\cdot(C_6H_4(CHCH_2)_2)_{0.02}\cdot(C_6H_4CHCH_2(CH_2CN))_{0.15}$: C, 90.7; H, 7.4; N, 1.9. Found: C, 89.9; H, 7.5; N, 2.4. The absence of the $-CH_2Cl$ groups in this preparation was evidenced as follows. Ten milligrams of the resin were suspended in a mixture of DMF (0.2 ml) and 20% aqueous sodium hydroxide (0.2 ml), and the mixture was heated for 10 min at 120°. After cooling, the supernatant solution was made acidic by addition of 1N nitric acid. Upon addition of an aqueous silver nitrate solution to it, no precipitation took place. In contrast, the starting material, the Merrifield resin, gave a massive precipitation of white silver chloride on similar treatment.

Cross-linked Polystyrene bearing $-CH_2COOH$ Groups (P-$C_6H_4CH_2COOH$)——P-$C_6H_4CH_2CN$ (4.85 g) was suspended in a mixture of concentrated sulfuric acid (15 ml), acetic acid (15 ml) and water (15 ml), and was heated under stirring at 115—120° (bath temperature) for 10 hr.[23] The slightly yellow resin was collected by filtration, washed with water (×6), dioxane–water (1:3, v/v; ×4), dioxane–water (1:1, v/v; ×4), dioxane–water (3:1, v/v; ×3), dioxane (×3), dioxane–ethanol (3:1, v/v; ×3), dioxane–ethanol (1:1, v/v; ×3), dioxane–ethanol (1:3, v/v; ×3), ethanol (×3), and ether (×3), successively, and finally dried *in vacuo* over phosphorus pentoxide. Yield, 4.99 g. IR (KBr): 1705 cm^{-1} (CO) (the starting material, P-$C_6H_4CH_2CN$, does not give any absorption band between 1620 cm^{-1} and 1780 cm^{-1}), and no absorption at 2250 cm^{-1} (CN) was detectable. *Anal.* Calcd. for $(C_6H_5CHCH_2)_{0.83}\cdot(C_6H_4(CHCH_2)_2)_{0.02}\cdot(C_6H_4CHCH_2COOH)_{0.15}$: C, 88.4; H, 7.45; O, 4.3; N, 0.0. Found: C, 87.5; H, 7.6; O, 4.4; N, 0.8. The resin showed a property of a cation exchanger. Thus, upon addition of the resin into a mixture of dioxane and 2M aqueous sodium chloride (1:1, v/v), the aqueous phase became acidic.

Polymer bearing $-CH_2COCl$ Groups (P-$C_6H_4CH_2COCl$)——P-$C_6H_4CH_2COOH$ (4.56 g) was mixed with toluene (20 ml) and thionyl chloride (20 ml), and the mixture was heated under exclusion of moisture ($CaCl_2$-tube) with mechanical stirring. After 24 hr of refluxing, when the initially strong evolution of gaseous hydrogen chloride subsided, the mixture was filtered. A great care was taken not to introduce moisture during this filtration and subsequent washing steps: only dried air was allowed to contact with the resin. The resin washing was carried out with anhydrous toluene (×5), and ether (×3), successively. The dried resin weighed 5 g, and was kept in a tightly stoppered container which was placed in a dessicator. IR (KBr): 1735 cm^{-1} (CO).

Polymer-supported Thymidine (P-$C_6H_4CH_2COO$-T-OH)——A mixture of P-$C_6H_4CH_2COCl$ (2.0 g) and thymidine (3.06 g; 12.4 mmoles) in anhydrous pyridine (15 ml) was stirred in a tightly stoppered flask. After allowing the reaction to proceed at room temperature for 22 hr, methanol (1 ml) was added and the stirring continued further for 20 hr. The resin was then collected by filtration, and washed with pyridine (×4), pyridine–water (3:1, v/v, ×3), pyridine–water (1:1, v/v, ×3), pyridine–water (1:3, v/v, ×3), water (×3), water–ethanol (1:1, v/v, ×2), ethanol (×3), and ether (×3), successively. The resin was then dried *in vacuo* over phosphorus pentoxide. Yield, 1.7 g. Determination of the thymidine content of this product by treatment with dioxane–concentrated ammonia (1:1, v/v) for 94 hr gave a value of 210 μmoles thymidine/gram of the polymer. Four other similarly prepared samples of P-$C_6H_4CH_2COO$-T-OH showed the following thymidine content: 100 μmoles, 120 μmoles, 110 μmoles and 120 μmoles of thymidine/gram of the polymer.

Release of Thymidine from P-$C_6H_4CH_2COO$-T-OH——P-$C_6H_4CH_2COO$-T-OH (23.0 mg) was treated with a mixture of dioxane and concentrated ammonia (1:1, v/v, 3 ml) in a stoppered test tube with mechanical stirring. At intervals, the mixture was centrifuged and an aliquot (100 μl) of the supernatant solution was taken up. The aliquot was paper chromatographed in the solvent, *n*-butanol–water (86:14, v/v). A single UV-absorbing spot was detectable. The *Rf* value of the spot was identical with that of thymidine. For 4 hr-aliquot, the absorbance at 267 mμ was determined before and after the chromatography. The optical density units before the paper chromatography were 3.6, and those recovered from the spot on the chromatogram were 3.0. From these observations, it was concluded that the thymidine content can be determined by directly measuring the absorbance at 267 mμ of the aliquot. For this purpose, the aliquot was evaporated to dryness and the residue dissolved in water (3 ml). Absorbance at 267 mμ of this solution was then determined.

In the calculation of the amount of thymidine released from the polymer, a correction was made to account the volume change due to the withdrawal of the aliquots. The following values were obtained. Time of ammonia treatment/thymidine released per gram of the resin/% of total thymidine releasable: 24 min/77 μmoles/36%; 31 min/107 μmoles/50%; 1 hr/125 μmoles/58%; 5 hr/151 μmoles/70.5%; 25 hr/183 μmoles/85.5%; 49 hr/198 μmoles/92.5%; 73 hr/211 μmoles/98%; 94 hr/214 μmoles/100%; 120 hr/216 μmoles/100%.

Synthesis of TpT on the Polymer Support——Method A. Using Mesitylene Sulfonyl Chloride as the Condensing Agent: To a mixture of P-$C_6H_4CH_2COO$-T-OH (50 mg, containing 10 μmoles of thymidine) and pyridinium 3′-O-acetylthymidine 5′-phosphate (180 mg, 0.4 mmole) was added anhydrous pyridine (1 ml). The mixture was then evaporated to dryness, and such evaporation of anhydrous pyridine was further repeated five times to render the mixture anhydrous. Finally, pyridine (2 ml) and mesitylene sulfonyl chloride (152 mg, 0.7 mmole) were added to the dry residue and the reaction mixture allowed to

23) The acid hydrolysis of benzyl nitrile to phenyl acetic acid is carried out by refluxing the nitrile in concentrated sulfuric acid–water (3:4, v/v) for 3 hr.[11b]

stand at room temperature for 16 hr with mechanical stirring. Up to this step, whenever the reaction flask had to be opened, it was done in a dry atmosphere. Next, pyridine (3 ml) and water (0.6 ml) were added to the reaction mixture under cooling, and the stirring was continued further for 3.5 hr. The resin was collected by centrifugation and washed with pyridine (3 ml×8), pyridine–water (1:1, v/v, ×6), water (×4), ethanol (×4), and ether (×3), successively. When dried *in vacuo*, the resin weighed 40 mg.

A portion (17.7 mg) of this sample was treated with dioxane–conc. ammonia (1:1, v/v) (2.5 ml) at room temperature under stirring. At intervals an aliquot (100 μl) was taken up and evaporated to dryness. The residue was dissolved in water (3 ml) and the absorbance at 267 mμ was determined. The results were; Time of ammonia treatment/optical density units per gram of the resin: 24 hr/3060; 49 hr/3180; 70 hr/3330. Approximately 4 optical density units of the 70 hr aliquot was submitted to the ascending paper chromatography using the solvent system, iso-propanol–concentrated ammonia–water (7:1:2, v/v). Only two UV-absorbing spots were detectable; one was TpT (*Rf*, 0.44), and another was thymidine (*Rf*, 0.69). The optical density units (at 267 mμ) of these spots were, 3.80 for TpT and 0.13 for thymidine. From these values, the conversion of thymidine into TpT was calculated to be 94%, and the recovery was 72% as based on the initial thymidine on the polymer. The molecular extinction coefficients employed in these calculations were, 18500 for TpT[24] and 9600 for thymidine.[25]

Method B. Using DCC as the Condensing Agent: A mixture of P–$C_6H_4CH_2COO$–T–OH (50 mg, containing 10 μmoles of thymidine) and pyridinium 3′-O-acetylthymidine 5′-phosphate (185 mg, 0.4 mmole) was azeotropically dried by repeated evaporation with anhydrous pyridine (six times). To the dry residue, DCC (190 mg, 0.9 mmole) and anhydrous pyridine (2 ml) were added, and the mixture allowed to stand at room temperature under mechanical stirring for four and half days. The mixture was then centrifuged and the supernatant solution removed. The resin was mixed with pyridine (3 ml) and water (0.8 ml), and allowed to stand further at room temperature for two days under stirring. The resin was collected by centrifugation and washed with warm ethanol (3 ml×5), pyridine (×5), pyridine–water (1:1, v/v, ×6), water (×5), warm ethanol (×5), and ether (×5), and finally dried over phosphorus pentoxide. Yield, 40 mg. The extent of conversion of thymidine into TpT was determined in an identical manner as in Method A, and was found to be 85%.

Polymerization of Thymidine 5′-Phosphate. The Conventional Method——This experiment was done in order to prepare standard samples of oligothymidylic acids which were needed for the identification of the products in the polymer support synthesis of oligothymidylic acids. The procedure was essentially identical with that reported by Khorana and Vizsolyi.[16] A mixture of pyridinium thymidine 5′-phosphate (1.5 mmole) and pyridinium 3′-O-acetylthymidine 5′-phosphate (0.5 mmole) was azeotropically dried by evaporation with pyridine (5 ml×5). Anhydrous pyridine (2 ml) and DCC (4 mmoles) were added to the dried residue and the solution allowed to stand at room temperature for 4 and half days. After appropriate work-up,[16] the products were fractionated by DEAE cellulose column chromatography as described previously.[16] Satisfactory separation of oligothymidylic acids was achieved and the linear oligomers with the general formula, $pT(pT)_n$, were obtained. From the oligomers, the terminal phosphomonoester groups were removed by treatment with alkaline phosphatase from *E. coli*[20] using conditions previously described.[16] The resulting oligomers of the type, $T(pT)_n$, were characterized by treatment with spleen phosphodiesterase.[27] Satisfactory ratio of thymidylic acid to thymidine was obtained. Oligomers up to the hexamer were prepared and characterized in this manner. These were subsequently used as the standard samples in the experiments described below.

Polymerization of Thymidine 5′-Phosphate in the Presence of P-$C_6H_4CH_2COO$-T-OH.——A. First Polymerization: A mixture of P–$C_6H_4CH_2COO$–T–OH (350 mg, containing 70 μmoles of thymidine) and pyridinium thymidine 5′-phosphate (1 mmole) was azeotropically dried by repeated evaporation with anhydrous pyridine (1 ml×5). To the dry residue, pyridine (1 ml) and DCC (620 mg, 3 mmoles) were added, and the mixture allowed to stand at room temperature with mechanical stirring. After 5 days, pyridine (3 ml) and water (1 ml) were added and the stirring was continued further for 2 days. The resin was collected by centrifugation and washed with pyridine (3 ml×5), pyridine–water (1:1, v/v, ×5), water (×5), warm ethanol (×5), and ether (×3). After drying, a portion of the resin was treated with dioxane-concentrated ammonia (1:1, v/v) at room temperature for 44 hr. When the solution was submitted to paper chromatography (isobutyric acid–1M ammonium hydroxide (5:3, v/v)), six clearly separated spots were obtained. They were thymidine, TpT, TpTpT, TpTpTpT, TpTpTpTpT, and TpTpTpTpTpT. The relative amounts of these oligonucleotides as well as their *Rf* values are summarized in Table I. The identification of these oligothymidylic acids was done by direct comparison with the authentic samples which were co-chromatographed. These materials were found to be homogeneous when checked by two other paper chromatographic

24) P.T. Gilham and H.G. Khorana, *J. Am. Chem. Soc.*, **80**, 6312 (1958).
25) Schwarz BioResearch Inc. Catalog 1966.
26) A. Garen and C. Levinthal, *Biochim. Biophys. Acta*, **38**, 470 (1960).
27) W.E. Razzell and H.G. Khorana, *J. Biol. Chem.*, **136**, 1144 (1961).

systems: (1) *n*-butanol–acetic acid–water (5:2:3, v/v), and (2) iso-propanol–concentrated ammonia–water (7:1:2, v/v).

B. Second Polymerization: A portion of the resin (100 mg) obtained as above prior to the ammoniacal treatment was mixed with pyridinium thymidine 5′-phosphate (0.34 mmole) and the mixture azeotropically dried by evaporation with anhydrous pyridine (1 ml×6). To the dried mixture, DCC (280 mg; 1.36 mmole) and dry pyridine (0.3 ml) were added. The reaction was then allowed to proceed at room temperature for eight days. Pyridine (6 ml) and water (1 ml) were next added and the mixture further allowed to stand for a day. The resin was collected by centrifugation and washed with warm ethanol (3 ml×3), pyridine (×6), pyridine–water (1:1, v/v, ×6), water (×5), warm ethanol (×5), and ether (×3), successively. The resin was finally dried *in vacuo* over phosphorus pentoxide. Yield, 80 mg.

When the resin (30.0 mg) was treated with dioxane–concentrated ammonia (1:1, v/v, 4 ml) at room temperature for 71 hr, 109 optical density units (at 267 mμ) of nucleotidic material came out into the solution (96 optical density units at 25 hr-, and 102 optical density units at 48 hr-reaction period). On paper chromatography (iso-butyric acid–1M ammonium hydroxide (5:3, v/v)) of this material (about 12 optical density units), six distinctly separated spots were again obtained as had been the case with the first polymerization product. Homogeneity of these materials was confirmed as in the first polymerization experiment. The optical density units (267 mμ) of these oligonucleotides were determined. They were: thymidine, 2.32; TpT, 2.06; TpTpT, 1.92; TpTpTpT, 1.63; TpTpTpTpT, 1.01; TpTpTpTpTpT, 1.02. The optical density units obtained from the zone, *Rf* 0—0.24, was 0.82. The material contained in this zone was assumed to be oligomers with chain lengths longer than hexa. However, the nucleotidic materials were widely spread in this zone and no distinct UV-absorbing area was detectable.

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