# Chemical Syntheses of Two Deoxyribopolynucleotide Fragments Containing the Natural Sequence of $T_4$-Lysozyme Gene e. V[1a]

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**Abstract:** Attaching an aromatic protecting group such as 2-phenylmercaptoethanol to the 5′-terminal phosphate group of deoxyribonucleotides strikingly increases their affinity for benzoylated DEAE-Cellulose and Sephadex. As a result, column chromatography with these adsorbents can easily fractionate condensation mixtures containing fragments thus protected, from the incoming nucleotides and undesired side products not bearing such an aromatic protecting group. The syntheses of nona- and dodecanucleotides containing the natural sequence of $T_4$-lysozyme gene e have been achieved in excellent purity.

Previously we introduced a new approach to oligonucleotide synthesis[1a] which was based upon the observation that attachment of an aromatic protecting group to the 5′-phosphate of a nucleotide greatly enhanced its binding affinity to benzoylated DEAE-Cellulose[2] or benzoylated DEAE-Sephadex.[3] Thus after condensation of an aromatic protected nucleotide with an incoming 3′-acetyl nucleoside 5′-phosphate component containing nonaromatic protecting groups, the aromatic group would be found only in the unreacted starting material and product. When such a reaction mixture was passed through a benzoylated DEAE-Sephadex or Cellulose column, only the components containing the aromatic protecting group were retained whereas all components lacking the aromatic protecting group and undesirable side products were eluted by washing with an aqueous buffer. The components retained on the column were eluted with higher molarity buffer containing ethyl alcohol. The desired product could then be isolated by gel filtration[4] or preparative tlc.[5] A similar approach has also been reported from various laboratories.[6]

In this paper, we wish to report (i) the synthesis of nonanucleotide **2** and dodecanucleotide **1** by the above mentioned approach without protecting the amino groups of the adenine and guanine moieties and (ii) the thin-layer chromatographic technique on silica gel $F_{254}$ plates with commonly available aqueous solvent systems for corroborating the purity of each oligonucleotide.[7] The unique sequence of these oligonucleotides **1** and **2** (Chart I) was established elegantly by Streisinger and coworkers[8] by their study of the frame-shift mutation in the strains eJJ44J42 and eJD10-eJD11 of phage $T_4$. These oligonucleotides were synthesized as templates to be used in the synthesis and sequence studies of $T_4$-lysozyme gene e by elongation from its 3′-hydroxyl end with DNA polymerase[9] in the presence of an appropriate complementary natural DNA strand.[10]

The present synthetic approach was developed by using 2-phenylmercaptoethanol, a novel phosphate-protecting group,[1,11] which is stable during all conditions commonly encountered in oligonucleotide synthesis and easily removed with 2 *N* sodium hydroxide at room temperature after oxidizing to the sulfoxide. Recently, it has also been noted that the corresponding sulfone[12] can be deblocked with very mild alkaline treatment.

The 5′-phosphate group of each deoxyribonucleoside (1 equiv) was protected by condensation with 2-phenylmercaptoethanol (PME) in the presence of triisopropylbenzenesulfonyl chloride (TPS) for 8 hr at room temperature. Additionally, there were reports in the literature[13] which inferred that the blocking of amino groups in the case of adenine and guanine bases might not be essential during oligonucleotide synthesis. We have verified this observation by performing the present synthesis without protecting (whenever convenient) the amino groups of these two bases. Initial protection of the 5′-phosphomonoester groups of deoxyadenosine and deoxyguanosine with 2-phenylmercaptoethanol and TPS was achieved in a mixture of anhydrous pyridine and hexamethylphosphoramide (HMPA). After the reaction was terminated with water, the aqueous solution was washed with ethyl acetate to remove excess 2-phenylmercaptoethanol then rendered anhydrous by evaporation in the presence of pyridine and the phosphate-protected mononucleotide was isolated by ether precipitation of this anhydrous pyridine solution. Final purification was achieved

(1) (a) Part IV: S. A. Narang, O. S. Bhanot, J. Goodchild, R. H. Wightman, and S. K. Dheer, *J. Amer. Chem. Soc.*, **94**, 6183 (1972); (b) NRCC No. 14286.

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(8) (a) E. Terzaghi, Y. Okada, G. Streisinger, J. Emrich, M. Inouye, and A. Tsugita, *Proc. Nat. Acad. Sci. U. S.*, **56**, 500 (1966); (b) Y. Okada, G. Streisinger, J. Owen, J. Newton, A. Tsingita, and M. Inouye, *Nature (London)*, **236**, 338 (1973); (c) T. H. Jukes, "Molecules and Evolution," Columbia University Press, New York, N. Y., 1966, p 145.

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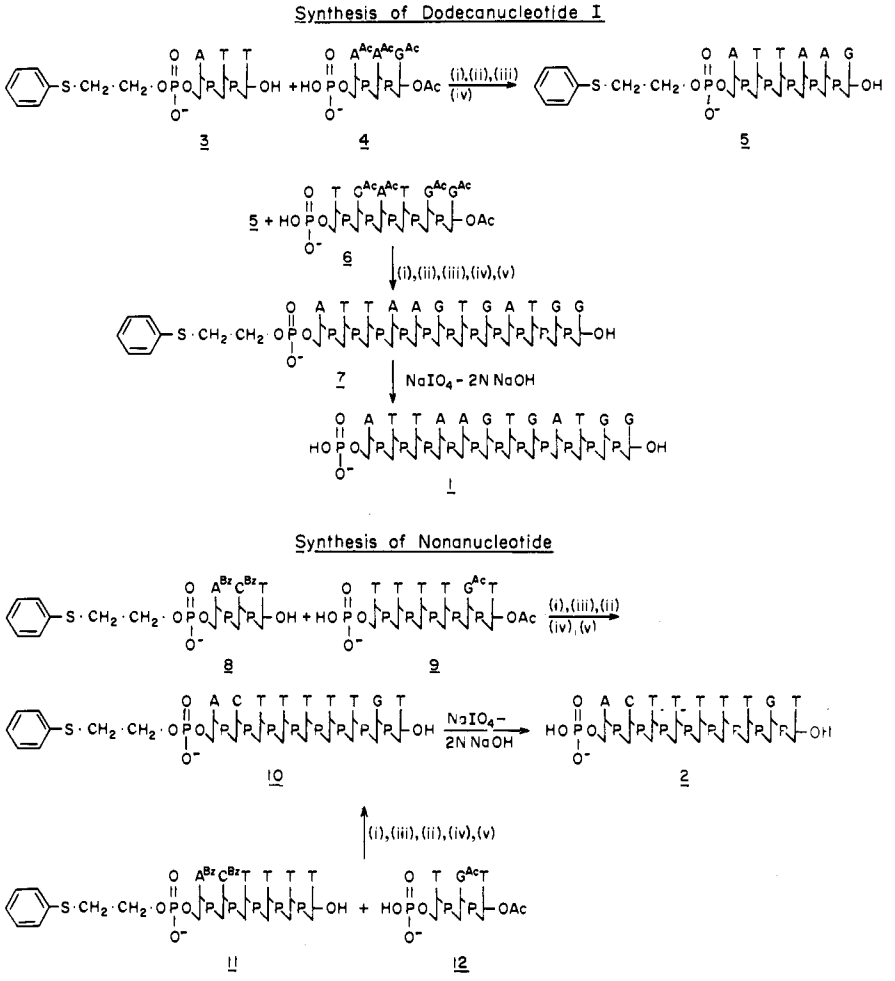
**Chart I.** (a) Base Sequence of a Specific Region of Wild-Type Lysozyme Gene e of $T_4$ DNA[7]

5′---ApCpApApApApGpTpCpCpApTpCpApCpTpTpApApTpGpCp---3′

3′---TpGpTpTpTpTpTpCpApGpGpGpTpApGpTpGpApApTpTpApCpGp---5′

2 1

**Chart II**




(i) mesitylenesulfonyl chloride
(ii) concentrated ammonium hydroxide 50°C/3hr
(iii) column chromatography on benzoated DEAE-cellulose column
(iv) gel-filtration on Sephadex G-75 (superfine)
(v) TLC on silica-gel plates

by gel filtration on Sephadex with isolated yields of 65–75%. However, protection of the amino group in cystosine was found to be essential,[13d] the benzoyl group[14] being found satisfactory.

Syntheses of the various dinucleotides were carried out by condensing an anhydrous pyridine solution of (PME protected) deoxymononucleotides with acetylated deoxymononucleoside 5′-phosphate in the presence of mesitylenesulfonyl chloride (MS) at room temperature. It was observed that amounts of MS smaller than a 3 *M* excess resulted in lower yields of di- and trinucleotide and concomitant amounts of pyrophosphate; whereas greater than 5 *M* excess of MS produced extensive degradation. After 2–3 hr reaction time, the reaction mixture was decomposed with 25% aqueous pyridine and treated with concentrated ammonium hydroxide to remove the acetyl protecting group. After removal of excess ammonia and pyridine, the aqueous solution was applied to a benzoylated DEAE-Sephadex column and elution was carried out with a linear gradient of triethylammonium bicarbonate (TEAB) buffer, pH 7.1 (0.05–0.5 *M*), to remove mononucleotide along with its pyrophosphate. Subsequent washing with 0.5 *M* TEAB–50% ethyl alcohol eluted from the column the strongly retained nucleotide components containing the aromatic protecting group. These latter fractions were pooled, concentrated *in vacuo*, and further fractionated by a Sephadex G-25 (superfine) gel-filtration column. The di- and trinucleotides were obtained in 40–60% yields. It has been observed that the yields of trinucleotides containing only the purine bases as d-pApApG and d-pGpApG were generally quite low *ca.* 20–30%. These trinucleotides have also been found to be unstable as examined by tlc.

In the case of hexanucleotide synthesis (Chart II), tri-*n*-decylammonium salts of the two appropriate

(14) R. H. Ralph and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 2926 (1961).

**Table I.** Synthesis of Hexa-, Nona-, and Dodecanucleotides[a]

| Phosphate protected compd | Amount, mmol | 3-*O'*-Acetyl component | Amount, mmol | MS, mmol | Products | Yield, % |
|---|---|---|---|---|---|---|
| | | Synthesis of Hexanucleotides | | | | |
| (PME)pA-T-T | 0.025 | dpacA-acA-acG(Ac) | 0.030 | 0.30 | (PME)pA-T-T-A-A-G | 25 |
| (PME)pT-G-A | 0.04 | dpT-acG-acG(Ac) | 0.08 | 0.80 | (PME)pT-G-A-T-G-G | 22 |
| (PME)pT-T-T | 0.08 | dpT-acG-T(Ac) | 0.16 | 1.6 | (PME)pT-T-T-T-G-T | 30 |
| (PME)pbzA-bzC-T | 0.038 | dpT-T-T(Ac) | 0.154 | 1.54 | (PME)pbzA-bzC-T-T-T-T | 35 |
| | | Synthesis of Nonanucleotide | | | | |
| (PME)pbzA-bzC-T | 0.07 | dpT-T-T-T-acG-T(Ac) | 0.04 | 0.8 | (PME)pA-C-T-T-T-T-T-G-T | 10 |
| (PME)pbzA-bzC-T-T-T-T | 0.05 | dpT-acG-T(Ac) | 0.08 | 1.6 | (PME)pA-C-T-T-T-T-T-G-T | 17 |
| | | Synthesis of Dodecanucleotide | | | | |
| (PME)pA-T-T-A-A-G | 0.002 | dpT-acG-acA-T-acG-acG(Ac) | 0.0017 | 0.068 | (PME)pA-T-T-A-A-G-T-G-A-T-G-G | 10 |

[a] PME stands for 2-phenylmercaptoethanol and MS for mesitylenesulfonyl chloride.

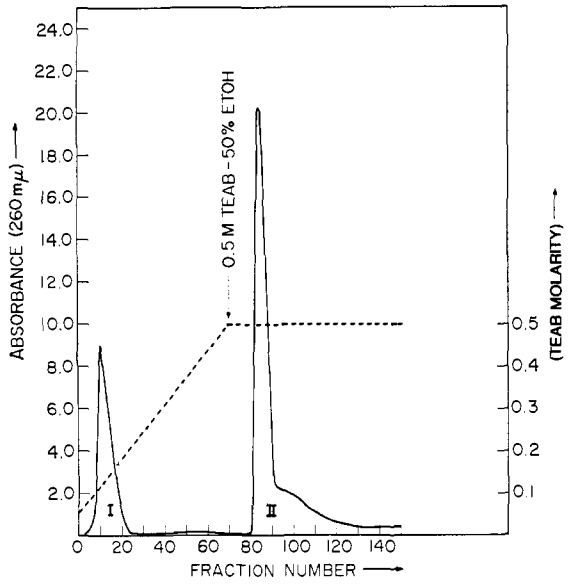



**Figure 1.** Column chromatography of the reaction mixture of hexanucleotide (PME-pApTpTpApApG) on a benzoylated DEAE-Cellulose column (3 × 10 cm). A linear gradient of 0.05—0.5 *M* triethylammonium bicarbonate buffer pH 7.1, 500 ml each, was applied and fractions of 15 ml were collected every 10 min. It was followed by elution with 0.5 *M* triethylammonium bicarbonate —50% ethyl alcohol, 1 l., and fractions of 10 ml were collected every 20 min. Peak II contained mainly a mixture of hexanucleotide and PME-pApTpT.

trinucleotides in anhydrous pyridine were treated with MS for 2 hr. Any trace of insoluble material completely inhibited this condensation step so that the nucleotide solution was routinely filtered through a sintered glass funnel before treatment with MS. After the usual work-up, the aqueous solution was subjected to benzoylated DEAE-Cellulose chromatography and gel filtration on Sephadex G-75 (superfine). Typical profiles from these columns are illustrated in Figures 1 and 2. Reaction conditions and isolated yields are given in Table I.

The synthesis of nonanucleotide **2** was carried out by two different block condensation experiments. In the first case, the phosphate-protected trinucleotide (PME)pbzA-bzC-T[15] (**8**) was treated with dpT-T-T-T-acG-T(Ac) (**9**) in the presence of MS. After the usual work-up, the unreacted hexanucleotide was eluted by chromatography through a benzoylated DEAE-Cellulose column. The desired nonanucleotide (PME)pbzA-bzC-T-T-T-T-T-acG-T(Ac) retained on the column was eluted by washing with 0.5 *M* TEAB–50% ethyl alcohol; the appropriate fractions were pooled, concentrated *in vacuo*, treated with concentrated ammonium hydroxide, and further fractionated on Sephadex G-75 (superfine grade). This method offers an easier and better separation of the nonanucleotide but the yield of the product is quite low (*ca.* 10%). Alternatively, the synthesis of nonanucleotide was carried out by condensing hexanucleotide (PME)pbzA-bzC-T-T-T-T (**11**) with d-pT-acG-T(Ac) (**12**). After the usual work-up and isolation procedure, the final product was isolated in 15–20% yields. The reaction conditions and isolated yields are given in Table I.

The synthesis of dodecanucleotide (PME)-pA-T-T-A-A-G-T-G-A-T-G-G (**7**) was achieved by condensing phosphate-protected hexanucleotide (PME)-pA-T-T-A-A-G (**5**) with d-pT-acG-acA-T-acG-acG(Ac) (**6**) in the presence of MS (40-fold excess based on the acetylated component). After the usual work-up, the final product was isolated by the following sequence of chromatographic procedures: benzoylated DEAE-Cellulose column, gel filtration on Sephadex G-75 (superfine grade), and tlc on silica-gel plates in solvents A and B. The reaction conditions and yields are given in Table I.

The homogeneity of each oligonucleotide was checked by tlc on silica-gel $F_{254}$ plates in solvents A and B. These plates have the special property of quenching fluorescence from any coloring matter in the synthetic oligonucleotides thus making their identification on tlc much easier under ultraviolet light. The mobility of each synthesized oligonucleotide as (i) phosphate-protected oligonucleotide and (ii) unprotected oligonucleotide is given in Table II. The final characterizations of nona- **2** and dodecamer **1** were achieved by labeling the 5'-OH of the dephosphorylated product with $T_4$–polynucleotide kinase[16] and [$\gamma$-$^{32}$P]ATP. These labeled compounds were found to be homogeneous on a homochromatographic tlc plate[17] (DEAE-Cellulose: Cellulose, 1:9) in 2% partially hydrolyzed yeast RNA containing 7 *M* urea (homomixture V) at 60° developed by Wu.[18] Their characterization was achieved by digestion of the dephosphorylated compounds with snake venom or spleen phosphodiesterase enzymes

(15) The system of abbreviation is principally as has been suggested by IUPAC-IUB Commission, *Biochemistry*, **9**, 4022 (1970).

(16) C. C. Richardson, *Proc. Nat. Acad. Sci. U. S.*, **54**, 158 (1965).
(17) G. G. Brownless and F. Sanger, *Eur. J. Biochem.*, **11**, 395 (1969).
(18) We are grateful to Professor R. Wu for supplying us his experimental details.

Table II. $R_f$ Values[a] of Deoxyoligonucleotide on Silica-Gel Tlc Plates

| Compd | Tlc in solvent systems A | B | C |
|---|---|---|---|
| **Dinucleotides** | | | |
| PME-pApT | 3.1 | 1.7 | |
| d-pApT | | 0.9 | 0.8 |
| PME-pApA | 2.9 | 2.5 | |
| d-pApA | | 1.7 | 0.7 |
| PME-pApC | 4.1 | 2.0 | |
| d-pApC | | 1.5 | 0.8 |
| PME-pTpT | 4.0 | 1.4 | |
| pTpT | | 0.7 | 0.7 |
| PME-pTpG | 3.6 | 1.4 | |
| d-pTpG | | 0.8 | 0.6 |
| **Trinucleotides** | | | |
| PME-pApTpT | 2.7 | 1.5 | 1.4 |
| d-pApTpT | | 0.8 | 0.7 |
| PME-pApApG | 2.0 | 2.1 | 1.3 |
| d-pApApG | | 1.0 | 0.5 |
| PME-pApCpT | 3.0 | 1.8 | 1.5 |
| d-pApCpT | | 0.9 | 0.8 |
| PME-pTpTpT | 3.1 | 1.5 | 1.7 |
| pTpTpT | | 0.5 | 0.6 |
| PME-pTpGpT | 2.8 | 1.1 | 1.4 |
| d-pTpGpT | | 0.7 | 0.5 |
| PME-pTpGpA | 2.9 | 1.4 | 1.3 |
| d-pTpGpA | | 0.6 | 0.45 |
| PME-pTpGpG | 2.8 | 1.3 | 1.2 |
| d-pTpGpG | | 0.5 | 0.4 |
| **Hexanucleotides** | | | |
| PME-pApTpTpApApG | | 1.1 | 0.9 |
| d-pApTpTpApApG | | 0.4 | 0.35 |
| PME-pTpGpApTpGpG | | 0.9 | 0.7 |
| d-pTpGpApTpGpG | | 0.2 | 0.3 |
| PME-pTpTpTpTpGpT | | 1.2 | 1.1 |
| d-pTpTpTpTpGpT | | 0.3 | 0.35 |
| PME-pApCpTpTpTpT | | 1.0 | 0.9 |
| d-pApCpTpTpTpT | | 0.3 | 0.3 |
| **Nonanucleotide** | | | |
| PME-pApCpTpTpTpTpTpGpT | | 0.75 | 0.45 |
| d-pApCpTpTpTpTpTpGpT | | 0.30 | 0.20 |
| **Dodecanucleotide** | | | |
| PME-pApTpTpApApGpTpGpApTpGpG | | 0.65 | 0.34 |
| d-pApTpTpApApGpTpGpApTpGpG | | 0.20 | 0.10 |

[a] All $R_f$ values are with respect to pT.

followed by tlc on Avicel-Cellulose plates[5] in solvent system B. These results appear in Table III.

The enzymatic work using these synthetic templates and their sequence analysis by Sanger and Wu techniques will be published elsewhere.

## Experimental Section

**General Materials.** All deoxymononucleoside 5′-phosphates (Calbiochem.), mesitylenesulfonyl chloride (MS) (Aldrich), triisopropylbenzenesulfonyl chloride (TPS) (Aldrich), silica-gel $F_{254}$ tlc plates (Brinkman Inc.), $T_4$-induced polynucleotide kinase (Miles Laboratories Inc.), and [$\gamma$-$^{32}$P]ATP (New England Nuclear) were obtained commercially. 2-Phenylmercaptoethanol was prepared according to the published procedure.[19] Benzoylated DEAE-Sephadex was prepared according to the method described earlier[3] and benzoylated DEAE-Cellulose was purchased from Schwarz/Mann.

**Solvent Systems for Silica Gel $F_{254}$ Tlc.** Silica-gel plates (0.25-mm thickness) were developed with the following solvent systems by the ascending technique: A, isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2 v/v); B, isobutyric acid–1 *M* ammonium hydroxide–0.1 *M* EDTA (100:60:1.6); and C, *n*-propyl alcohol–concentrated ammonium hydroxide–water (55:10:35).

(19) H. H. Ford-Moore, R. A. Peters, and R. H. Wakelin, *J. Chem. Soc.*, 1754 (1949).

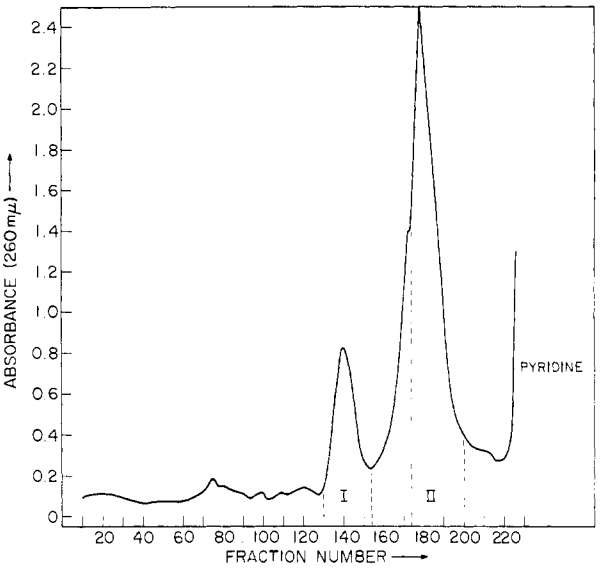



**Figure 2.** Gel filtration of peak II from the benzoylated DEAE-Cellulose column chromatography (Figure 1) on a Sephadex G-75 (superfine) K50-100 column for the isolation of PME-pApTpTpApApG. Peak I contained the desired hexanucleotide.

Photography and ultraviolet scanning of the tlc plates were carried out as described previously.[18]

**Protection of 5′-Phosphomonoester Group of Deoxyadenosine and Deoxyguanosine with 2-Phenylmercaptoethanol.** A mixture of the pyridinium salt of the deoxymononucleoside 5′-phosphate (10 mmol) and 2-phenylmercaptoethanol (50 mmol) in anhydrous pyridine (50 ml) and HMPA (100 ml) was treated with TPS (30 mmol) at room temperature for 8 hr. The reaction mixture was then decomposed with aqueous pyridine (50%, 100 ml) and further kept at room temperature overnight. The resulting solution was concentrated *in vacuo* to remove pyridine and then mixed with 0.2 *M* triethylammonium bicarbonate pH 7.1 buffer (500 ml). It was next extracted with ethyl acetate (5 × 200 ml) to remove excess 2-phenylmercaptoethanol and condensing reagent. Repeated evaporation *in vacuo* of this aqueous layer with excess pyridine poduced an anhydrous solution from which the desired ester was isolated in 65–70% yield by ether precipitation and subsequent gel filtration on Sephadex G-25 (superfine) column.

**General Method for the Synthesis of Di- and Trinucleotides.** An anhydrous pyridine solution (10 ml) containing the tri-*n*-hexylammonium salts of PME mono- or dinucleotide (1.0 mmol) and 3′-*O*,*N*-diacetylated deoxymononucleoside 5′-phosphate (1.5 mmol) was treated with MS (7.5 mmol) for 3 hr at room temperature.[20] The reaction mixture was then decomposed with aqueous pyridine (25%, 10 ml) and kept for 1 hr at room temperature before being treated with an equal volume of concentrated ammonium hydroxide (28–30%) for 3 hr at 50° in a sealed flask. After complete removal of ammonia by evaporation *in vacuo*, the aqueous solution (free from pyridine) was applied to a benzoylated DEAE-Sephadex column (3 × 30 cm) at 4°. The column was first washed with a linear gradient of triethylammonium bicarbonate buffer (pH 7.1) from 0.05–0.5 *M* (2 l. each) and then exhaustively with 0.5 *M* triethylammonium bicarbonate containing 50% ethyl alcohol. This latter eluent was concentrated *in vacuo* in the presence of pyridine to about 10 ml and then applied to a Sephadex G-25 (superfine) column K50-100 through a sample applicator. The desired compound was isolated by eluting the column with 0.1 *M* triethylammonium bicarbonate (pH 7.1) at 4°. The results are summarized in Table I.

**General Method for the Synthesis of Hexa-, Nona-, and Dodecanucleotide.** A pyridine solution of the tri-*n*-decylammonium salts of phosphate-protected trinucleotide and the 3′-*O*,*N*-diacetylated deoxytrinucleotide (1.5–2.0 *M* equiv) was rendered anhydrous by repeated evaporation with anhydrous pyridine. The pyridine solution was then filtered through a sintered glass funnel (fine grade) to remove any trace of insoluble material and the filtrate further concentrated, and finally this clear solution (*ca.* 5 ml) was treated

(20) S. A. Narang, T. M. Jacob, and H. G. Khorana, *J. Amer. Chem. Soc.*, **89**, 2158 (1967).

**Table III.** Spleen Phosphodiesterase Digestion of Deoxyoligonucleotides

| Compd | Nucleotides:nucleoside | Molar ratio of products | |
|---|---|---|---|
| | | Found | Theor |
| d-ApTpTpApApG | d-Ap:Tp:d-G | 3.1:1.9:0.9 | 3:2:1 |
| d-TpGpApTpGpT | Tp:d-Gp:d-Ap:T | 2:1.8:1.1:1 | 2:2:1:1 |
| d-TpTpTpTpGpT | Tp:d-Gp:T | 3.8:0.8:1 | 4:1:1 |
| d-ApCpTpTpTpT | d-Ap:d-Cp:Tp:T | 1:0.9:2.9:0.9 | 1:1:3:1 |
| d-ApCpTpTpTpTpTpGpT | d-Ap:d-pC:Tp:pG:T | 0.9:1:4.8:0.9:1 | 1:1:5:1:1 |
| d-ApTpTpApApGpTpGpApTpGpG | d-Ap:Tp:d-Gp:d-G | 3.9:3.7:3.1:0.9 | 4:4:3:1 |

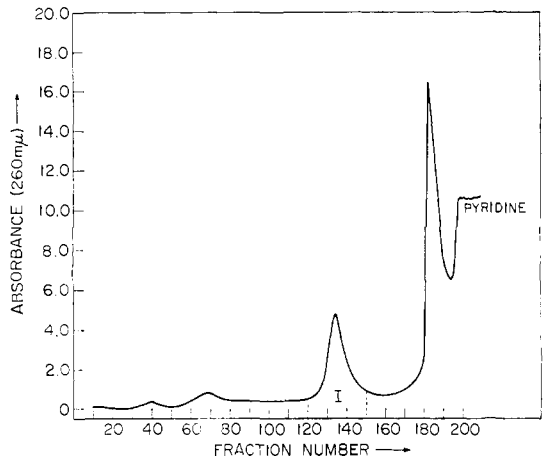



**Figure 3.** Gel filtration of the second peak (eluted with 0.5 *M* triethylammonium bicarbonate buffer 50% ethyl alcohol) from the benzoylated DEAE-Cellulose column chromatography on a Sephadex G-75 (superfine) K25-100 for the isolation of nonanucleotide PME-pApCpTpTpTpTpTpGpT. Peak I contained the desired nonanucleotide.

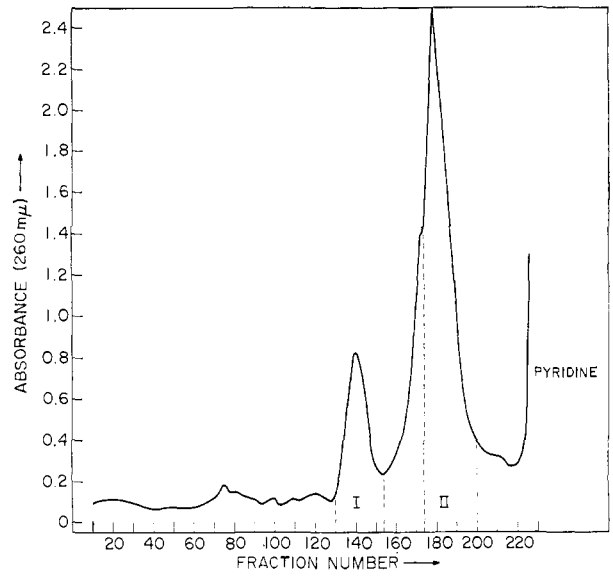



**Figure 4.** Gel filtration of the second peak (eluted with 0.5 *M*) triethylammonium bicarbonate–50% ethyl alcohol) from the benzoylated DEAE-Cellulose column chromatography on a Sephadex G-75 (superfine) K25-100 for the isolation of dodecanucleotide PME-pApTpTpApApGpTpGpApTpGpG. Peak I contained the desired dodecanucleotide.

with MS (10 *M* equiv for hexa, 20 for nona, and 40 for dodeca) for 2 hr at room temperature. The reaction mixture was decomposed with aqueous pyridine (25%, 5 ml) and kept for 1 hr at room temperature and then treated with an equal volume of concentrated ammonium hydroxide (28–30%) for 3 hr at 50°. After removal of excess ammonium hydroxide and pyridine by evaporation *in vacuo*, the reaction mixture was subjected to initial chromatography on benzoylated DEAE-Cellulose at 4° (*vide supra*). A typical elution pattern is shown in Figure 1 and the elution pattern of the second peak after subsequent gel filtration on Sephadex G-75 (superfine) is presented in Figure 2.

Elution patterns in the synthesis of various nona- and dodecanucleotides are given in Figures 3 and 4, respectively. The final purification was achieved by preparative tlc on silica-gel plates and the results are documented in Table I.

**Deblocking of the 2-Phenylmercaptoethyl Group from the Phosphate-Protected Oligonucleotide.** An aqueous solution (2 ml) of protected oligonucleotide (0.01 mmol) was treated with freshly prepared 0.1 *M* sodium metaperiodate (approximately 5 *M* excess) for 1 hr at room temperature. After decomposition of excess periodate with ethylene glycol, the solution was concentrated to 1 ml and treated with 2 *N* sodium hydroxide (2 ml) for 90 min at room temperature. After neutralization with Dowex-50 (pyridimium salt) resin, the unprotected oligonucleotide was isolated by gel filtration on Sephadex G-25 (superfine) at 4°, in *ca.* 80–90%.

**Characterization of Oligonucleotides.** (a) **Tlc on Silica Gel $F_{254}$ Plates.** The homogeneity of each intermediate oligonucleotide (protected or unprotected) was checked by tlc on silica-gel $F_{254}$ plates in solvents A, B, and C. Not only do these plates quench any fluorescence in the reaction mixture but they permit detection of traces of impurities using $\leq 1.0$ $OD_{260\ nm}$ of product. The mobility of each compound with respect to pT is documented in Table II.

(b) **Homochromatography.** The dephosphorylated nona- **2** and dodecamer **1** were labeled at 5′-end with $^{32}P$ by polynucleotide kinase[16] and isolated by the Sephadex G-50 (superfine K10-100) gel-filtration[4] method. The desired peak was concentrated to 10 μl. A sample of 2 μl was applied to homochromatographic tlc plate DEAE-Cellulose:Cellulose (1:9)[17] (10 × 40 cm), the top of which was clipped to Whatman 3 MM paper. The plate was first developed in water after 0.5 μl of the standard dye mixture had been spotted 2 cm apart along the origin. Chromatography was then carried out in homomixer V developed by Wu and his coworkers[21] at 60° until the blue dye marker was 1 in. from the top of the plate. The X-ray chromatograph showed a single spot for nona- and dodecamers of $R_f$ value with respect to blue dye marker as 0.36 and 0.25, respectively.

(c) **Enzymatic Degradation.** (i) **Treatment with Bacterial Alkaline Phosphomonoesterase.** Unprotected oligonucleotide 1–2 $OD_{260\ nm}$ in 0.1 *M* ammonium bicarbonate solution pH 8.5 (50 μl) was treated with bacterial alkaline phosphomonoesterase (Worthington) (20 μl), (5 mg/5 ml of solution) at 70° for 30 min. The dephosphorylated product was isolated by tlc on silica-gel plates in solvents A or C and finally B.

(ii) **Spleen Phosphodiesterase Degradations.** The dephosphorylated oligonucleotide (0.5–1.0 absorbance unit at 260 nm in 0.25 ammonium acetate buffer pH 5.6 (50 μl)) was treated with spleen phosphodiesterase (Worthington) (20 μl) (1 mg/5 ml) for 3–5 hr at 37°. The solution was then concentrated to about 10 μl *in vacuo* and chromatographed on Avicel-Cellulose tlc plates in solvent B. The nucleotide material was extracted from the appropriate spot with 0.01 *N* hydrochloric acid. The absorbance of the solution was recorded on a Gilford spectrophotometer Model 2400 using the extract from an equivalent area of cellulose as a blank. The results of enzymatic degradations are given in Table III.

(21) E. Jay, R. Bambara, P. Padmanabhan, and R. Wu, *Nucleic Acids Res.*, **1**, 331 (1974).