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Studies on Polynucleotides Containing Hybrid Sequences. Synthesis of Oligonucleotides Containing Thymidine, Adenosine, and a Single Deoxyribonucleotidyl-(3′–5′)-ribonucleotide Linkage†

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ABSTRACT: The polymerization of thymidine 5′-phosphate in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride and N^6,$O^{2'}$,$O^{3'}$-triacetyladenosine 5′-phosphate yields two series of oligonucleotides, pdT-dT_n-dT and pdT-dT_n-A, as the major products. The two sets of products may be readily separated by chromatography on a column of dihydroxyboryl-substituted cellulose, and the individual members of each series can be subsequently resolved by anion-exchange chromatography on polystyrene resins using aqueous ethanol solutions containing linear gradients of salt concentration as the eluting solvents. The series, pdT-dT_n-A, and the dephosphorylated series, dT-dT_n-A, are shown to serve both as acceptor molecules for single addition reactions catalyzed by polynucleotide phosphorylase in the presence of 2′-*O*-(α-methoxyethyl)adenosine 5′-diphosphate, and also as acceptors for polymerization reactions involving adenosine 5′-diphosphate and polynucleotide phosphorylase. The products, pdT-dT_n-A_m-A and dT-dT_n-A_m-A, are suitable substrates for the study of the mode of action of nucleases on polynucleotides containing hybrid sequences.

The recent discoveries of the occurrence, in biological systems, of nucleic acids containing covalently linked ribo- and deoxyribopolynucleotide chains have generated an interest in their chemical, biochemical, and physical properties as well as an interest in the relationship that these properties might have to the biological function of such hybrid molecules. For example, the involvement of RNA primers in a number of DNA-synthesizing systems raises questions concerning both the nature of the enzymatic processing of the hybrid product formed and the physical and chemical properties of the hybrid that might be responsible for directing the processing. Apart from their obvious biological importance covalent hybrid polynucleotides are of some biochemical interest in that, in many *in vitro* enzyme systems, they are capable of serving in place of normal ribo- or deoxyribopolynucleotides, and this substitution can provide distinct diagnostic advantages in the study of the system. Canellakis and Canellakis (1963) have pointed out that DNA molecules containing a ribonucleotide at their 3′ terminals can serve as primers for a variety of nucleotide-polymerizing enzymes. The use of unusual primers of this type not only provides some indication of the primer requirements of the particular enzyme system but also facilitates the identification and characterization of the reaction products.

Up to the present time, studies on hybrid oligonucleotides have been limited to the synthesis and the investigation of the properties of some small hybrid molecules containing a single type of nucleotide base. For example, Kondo *et al.* (1972) have prepared adenosine–deoxyadenosine dinucleoside phosphates for a comparative study of their conformations in solution, while Moon *et al.* (1966) have synthesized five trinucleoside diphosphates containing adenosine and deoxyadenosine moieties for the study of their ability to bind lysyl-tRNA to ribosomes. Certain covalent hybrid molecules have been prepared by the action of polynucleotide phosphorylase. This enzyme, in the presence of the appropriate nucleoside diphosphates, has been shown to (i) catalyze the addition of

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one or two deoxyadenosine residues to the terminus of an adenosine oligonucleotide (Kaufmann and Littauer, 1969; Bon *et al.*, 1970), and (ii) effect the synthesis of large adenosine–deoxyadenosine copolymers (Chou and Singer, 1971). The present work concerns the development of methods for the synthesis of hybrid oligonucleotides of specific sequence and chain length in order to provide suitable materials for the study of the chemical and biochemical properties of molecules containing the hybrid internucleotide linkage. In particular, the hybrid molecules described in this paper are to be used initially in studies of their behavior toward those enzymes that normally degrade RNA and DNA.

Synthesis of Thymidine Oligonucleotides Containing a Terminal Adenosine. For the synthesis of compounds of the type, pdT-dT$_n$-A, as starting materials for the preparation of hybrid oligonucleotides it was decided that chemical methods should be investigated in order that large quantities of these species could be produced for subsequent enzymatic addition reactions. Homooligonucleotides containing a different terminal nucleotide have been synthesized before (Khorana and Vizsolyi, 1961; Schott *et al.*, 1973) and the usual technique has involved the polymerization of a nucleotide with either dicyclohexylcarbodiimide or a substituted benzenesulfonyl chloride in the presence of a second nucleotide whose hydroxyl functions have been blocked with acetyl groups.

Thymidine 5′-phosphate was polymerized in the presence of N^6,$O^{2'}$,$O^{3'}$-triacetyladenosine 5′-phosphate and the reaction products were treated first with acetic anhydride–pyridine to remove any pyrophosphate bonds (Khorana *et al.*, 1962) and then with aqueous ammonia to remove the acetyl-blocking groups. The majority of the products of this procedure belong to two series of polymers, pdT-dT$_n$-dT and pdT-dT$_n$-A, and two techniques have been investigated for the separation of these oligomers. The oligonucleotide series containing the terminal adenosine can be readily separated from the rest of the products by passing the crude mixtures through a column of *N*-[*N*′-(*m*-dihydroxyborylphenyl)succinamyl]-aminoethylcellulose, DBAE-cellulose (Weith *et al.*, 1970). It had previously been shown that this cellulose derivative is capable of separating polynucleotide species that contain a 2′,3′-diol group at their 3′ terminals from those that do not (Rosenberg and Gilham, 1971; Rosenberg *et al.*, 1972). Polynucleotides that contain the 2′,3′-diol group form specific cyclic complexes with the cellulose-bound dihydroxyboryl groups at pH values of 8–9 and the bound polymers can be subsequently recovered by elution of the cellulose with buffers of lower pH values. The subsequent separation of the individual members of an oligonucleotide series can be achieved by anion-exchange chromatography on polystyrene resins using some new solvent systems. The compositions of these solvent systems are based on the observation that nucleosides, nucleotides, and polynucleotides can be fractionated on ion-exchange resins with aqueous ammonium chloride solutions containing certain percentages of ethanol (Ho and Gilham, 1973). The ethanol in the elution solvent serves to reduce the strong nonionic interactions that are normally superimposed on the ionic binding of nucleotides to polystyrene ion-exchange resins (Volkin and Cohn, 1953).

On chromatography at pH 8.7 on a column of DBAE-cellulose about half of the material from the polymerization reaction was bound. This product was recovered by elution at pH 5.5 and then fractionated by anion-exchange chromatography to yield the individual members of the series, pdT-dT$_n$-A (Figure 1). The yields in ODU_{260nm} based on the total

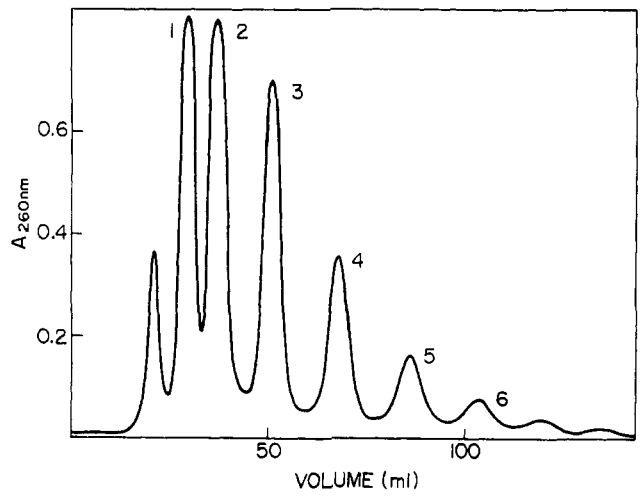

FIGURE 1: Elution pattern from the separation of the series, pdT-dT$_n$-A. The column of Dowex 1-X2 (−400 mesh) ion-exchange resin had dimensions of 100 × 0.4 cm and the elution was carried out at 12 ml/hr, under pressure, with 200 ml of 40% ethanol solution containing a linear gradient of 0.3–0.5 M ammonium chloride which had been brought to pH 8 with ammonia. Peaks 1, 2, 3, 4, 5, and 6 correspond to the series pdT-dT$_n$-A, where n = 1, 2, 3, 4, 5, and 6, respectively.

amount of hybrid oligonucleotides obtained ranged from 21% for pdT-dT-A to 3% for pdT-dT$_6$-A. The oligonucleotide fraction that was not bound to the column of DBAE-cellulose was shown to be free of hybrid molecules since the fraction could be passed through the column a second time with no loss of material. This product was then fractionated by anion-exchange chromatography and was subsequently shown to consist of the series, pdT-dT$_n$-dT, together with cyclic oligonucleotides corresponding to this series.

The crude polymerization mixture can also be fractionated in two steps in the reverse order to that described above. This alternative procedure is particularly useful when the preparation of a larger quantity of a certain hybrid oligonucleotide is desired. The procedure involves the separation of the mixture into isopliths by ion-exchange chromatography on DEAE-cellulose in the presence of 7 M urea (Tomlinson and Tener, 1963) followed by the separation of the components, pdT-dT$_n$-dT and pdT-dT$_n$-A, of each isoplith by DBAE-cellulose chromatography as described above. The yields obtained by this procedure were similar to those obtained in the first method.

The members of the series, pdT-dT$_n$-A, have been degraded with snake venom phosphodiesterase and the analysis of the products by ion-exchange chromatography yields the composition and chain length of each oligonucleotide (Table I). In addition, each member of the series was shown to be sensitive to the action of alkaline phosphatase and the products, dT-dT$_n$-A, are also listed in Table I together with their chromatographic properties. Some of the members of this dephosphorylated series have also been hydrolyzed with snake venom diesterase to confirm the assignment of compositions and chain lengths. All of the oligonucleotide species obtained from the fraction that was not bound to the DBAE-cellulose possessed ultraviolet spectra corresponding to the spectra of homopolymers of thymidine phosphate. In addition, two of the members of the series were shown to be pdT-dT and pdT-dT-dT by dephosphorylation to dT-dT and dT-dT-dT, respectively, and by subsequent degradation with snake venom diesterase to give thymidine 5′-phosphate and thymidine in the appropriate ratios.

TABLE I: Characterization of Hybrid Oligonucleotides.

Oligonucleotide	Chromatographic Data				Products from Chemical or Enzymatic Degradation
	$\bar{V}^a$ (ml)	$\bar{V}^b$ (ml)	$\bar{V}^c$ (ml)	$R_F{}^d$	
pdT-A		56			pdT:pA, 1.06:1.00[e]
pdT-dT-A	31	67		0.81	pdT-dT-A,[f] dT-dT-A;[g] pdT:pA, 2.07:1.00[e]
pdT-dT_2-A	40	75		0.63	dT-dT_2-A;[g] pdT:pA, 2.97:1.00[e]
pdT-dT_3-A	52	89		0.50	pdT-dT_3-A;[f] dT-dT_3-A;[g] pdT:pA, 3.98:1.00[e]
pdT-dT_4-A	68		68	0.38	dT-dT_4-A[g]
pdT-dT_5-A	86		83	0.29	dT-dT_5-A[g]
pdT-dT_6-A	103				
dT-dT-A		71		1.04	
dT-dT_2-A		86		0.92	dT-dT_2-A;[f] dT:pdT:pA, 1.00:1.76:1.00[e]
dT-dT_3-A				0.89	
dT-dT_4-A			79	0.85	dT:pdT:pA, 1.00:3.94:1.04[e]
dT-dT_5-A		108		0.81	dT:pdT:pA, 1.00:4.92:1.07[e]
pdT-dT_2-A-Ame		92			
pdT-dT_3-A-Ame		118			pdT-dT_3-Ap, Ame[f]
pdT-dT_4-A-Ame			90		
pdT-dT_5-A-Ame			103		
dT-dT_2-A-Ame		112			dT-dT_2-Ap, Ame[f]
dT-dT_4-A-Ame			105		
pdT-dT_3-A_2-Ame		133			pdT-dT_3-Ap + Ame:Ap, 1.00:1.11[f]
dT-dT_2-A_2-Ame		130			dT-dT_2-Ap + Ame:Ap, 1.00:1.06[f]
pdT-dT-A-A	60	95			
pdT-dT-A_2-A	84				pdT:pA, 2.00:3.02[e]
pdT-dT-A_3-A	108				
pdT-dT-A_4-A	130				
pdT-dT-A_5-A	150				
pdT-dT-A_6-A	160				
pdT-dT-A_7-A	168				
pdT-dT_2-Ap					dT:A, 2.77:1.00[h]
dT-dT_2-Ap					dT:A, 2.93:1.00[h]
pdT-dT_3-Ap					pdT-dT_3-Ap;[f] dT:A, 3.80:1.00[h]
dT-dT-A-A		93			
dT-dT-A_2-A		109			
dT-dT-A_3-A		120			
pdT-dT_2-A-A		100			
dT-dT_2-A-A		117		0.75	
dT-dT_2-A_2-A		132		0.53	
dT-dT_2-A_3-A		144		0.36	
pdT-dT_2-A-dC-dC		104			pdT-dT_2-Ap + dC-dC[f]

[a] Retention volumes on Dowex 1-X2, −400 mesh (100 × 0.4 cm), using 200 ml of 40% ethanol containing a linear gradient of 0.3–0.5 M ammonium chloride (pH 8) at 12 ml/hr. [b] Retention volumes on Dowex 1-X2, −400 mesh (40 × 0.4 cm), using 200 ml of 20% ethanol containing a linear gradient of 0.1–1.0 M ammonium chloride (pH 8.5) at 17 ml/hr. [c] Retention volumes on Dowex 1-X2, −400 mesh (40 × 0.4 cm), using 200 ml of 20% ethanol containing a linear gradient of 0.4–0.8 M ammonium chloride (pH 8.5) at 17 ml/hr. [d] R_F values relative to that of thymidine 5′-phosphate on Whatman 3MM paper using 1-propanol–concentrated ammonia–water (55:10:35, v/v) as solvent. [e] Products and molar ratios of products obtained by digestion with snake venom phosphodiesterase. [f] Products and molar ratios of products obtained by alkaline hydrolysis. [g] Products obtained by degradation with alkaline phosphatase. [h] Products and molar ratios of products obtained by degradation with snake venom phosphodiesterase and alkaline phosphatase.

Stepwise Enzymatic Addition of Adenosine Phosphate to the Oligonucleotide Series, pdT-dT_n-A and dT-dT_n-A. It has recently been shown that, in the presence of 2′-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates, polynucleotide phosphorylase is capable of adding a single nucleotide unit to an acceptor oligoribonucleotide (Mackey and Gilham, 1971; Bennett *et al.*, 1973). The reaction is catalyzed by Mg^{2+} or Mn^{2+} ions although, in the synthesis of oligonucleotides of specific sequence, the latter ion is preferred since it tends to restrict the amount of transnucleotidation that can occur during the addition reaction. It is now shown that, while the oligodeoxyribonucleotides pdT-dT-dT and pdT-dT_4-dT do not serve as acceptor molecules in this system, the hybrid series pdT-dT_n-A (n = 2–5) react readily with the substituted nucleoside diphosphate in the presence of Mn^{2+} ions. For example, pdT-dT_2-A and 2′-*O*-(α-methoxyethyl)adenosine 5′-diphosphate can be converted to pdT-dT_2-A-Ame, a pentanucleotide containing a 2′-*O*-(α-methoxyethyl) group at its

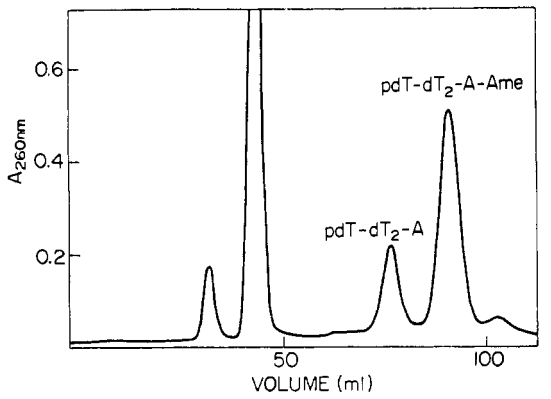

FIGURE 2: Elution pattern from the separation of the products resulting from the reaction of pdT-dT$_2$-A with 2′-*O*-(α-methoxyethyl)adenosine 5′-diphosphate and polynucleotide phosphorylase. The column of Dowex 1-X2 (−400 mesh) ion-exchange resin had dimensions of 40 × 0.4 cm, and the elution was carried out at 17 ml/hr, under pressure, with 200 ml of 20% ethanol solution containing a linear gradient of 0.1–1.0 M ammonium chloride which had been brought to pH 8.5 with ammonia.

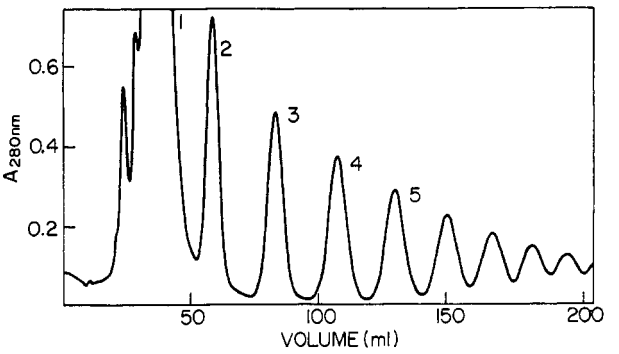

FIGURE 3: Elution pattern from the separation of the products resulting from the reaction of pdT-dT-A with adenosine 5′-diphosphate and polynucleotide phosphorylase. The column of Dowex 1-X2 (−400 mesh) ion-exchange resin had dimensions of 100 × 0.4 cm, and the elution was carried out at 12 ml/hr, under pressure, with 200 ml of 40% ethanol solution containing a linear gradient of 0.3–0.5 M ammonium chloride which had been brought to pH 8 with ammonia. Peaks 2, 3, 4, 5, ... correspond to the series pdT-dT-A$_n$-A, where n = 1, 2, 3, 4, ..., respectively.

3′ terminus (Figure 2). Similarly, pdT-dT$_3$-A yields the hexanucleotide, pdT-dT$_3$-A-Ame, and this product has been characterized by alkaline degradation to 2′-*O*-(α-methoxyethyl)adenosine and pdT-dT$_3$-Ap, with the structure of the latter product being assigned on the basis of its degradation with alkaline phosphatase and snake venom phosphodiesterase to thymidine and adenosine in the molar ratio, 4:1 (Table I).

The dephosphorylated series, dT-dT$_n$-A (n = 2–4), are also acceptors in this reaction and the products can be characterized in a similar fashion. For example, the product dT-dT$_2$-A-Ame yields 2′-*O*-(α-methoxyethyl)adenosine and dT-dT$_2$-Ap on alkaline hydrolysis. The structure of the latter product was assigned, as before, by its degradation with phosphatase and phosphodiesterase to yield thymidine and adenosine in the appropriate molar amounts. In order to demonstrate the technique of effecting a second single addition, two of the products described above were subjected to mild acid treatment to remove their methoxyethyl groups and the resulting oligomers were reacted again with the substituted nucleoside diphosphate. Thus, pdT-dT$_3$-A-Ame was hydrolyzed to pdT-dT$_3$-A-A which was then converted to pdT-dT$_3$-A$_2$-Ame. The structure of this product was proved by alkaline degradation to the methoxyethyladenosine, adenosine 2′(3′)-phosphate and a pentanucleotide that was identical with pdT-dT$_3$-Ap above described. Similarly, dT-dT$_2$-A-Ame was converted to dT-dT$_2$-A$_2$-Ame which was characterized by alkaline hydrolysis to methoxyethyladenosine, adenosine 2′(3′)-phosphate, and the tetranucleotide, dT-dT$_2$-Ap, also above described.

Enzymatic Polymerization of Adenosine Phosphate onto the Oligonucleotide Series, pdT-dT$_n$-A and dT-dT$_n$-A. In the presence of polynucleotide phosphorylase, adenosine 5′-diphosphate and Mn^{2+} ions the oligonucleotide series, pdT-dT$_n$-A and dT-dT$_n$-A (n = 1–2), can be converted to the series, pdT-dT$_n$-A$_m$-A and dT-dT$_n$-A$_m$-A (n = 1–2, m = 1, 2, 3, 4, ...), respectively. The conditions employed for these reactions are similar to those established by Thach and Doty (1965a,b) for the limited polymerization of nucleoside diphosphates onto oligoribonucleotide primers, except that Mn^{2+} ion is used in place of Mg^{2+} ion. When Mg^{2+} ion is the sole divalent cation added to the reaction mixture there is essentially no synthesis of hybrid oligonucleotides. The chromatographic elution pattern of the separation of the products corresponding to the conversion of pdT-dT-A to pdT-dT-A$_m$-A (m = 1, 2, 3, ...) is shown in Figure 3. The reaction products obtained from pdT-dT$_2$-A, dT-dT-A, and dT-dT$_2$-A yield similar elution patterns. The product pdT-dT-A$_2$-A (peak 3, Figure 3) has been characterized by the conventional method: degradation with phosphodiesterase to give the 5′-phosphates of adenosine and thymidine in the molar ratio of 3:2. Further characterization of these products has resulted from a study of their cleavage with deoxyribonucleases and the results of this study will be reported in a future communication.

Stepwise Enzymatic Addition of Deoxycytidine Phosphate to pdT-dT$_2$-A. Polynucleotide phosphorylase has been shown to catalyze the limited addition of thymidine and deoxyadenosine nucleotides to oligoribonucleotides. Kaufmann and Littauer (1969) have found that in the presence of deoxyadenosine 5′-diphosphate the enzyme is capable of adding up to two deoxyadenosine phosphate moieties to A-A and A-U, and Feix (1972) has reported that with thymidine 5′-diphosphate the enzyme can convert A-A$_4$-A to A-A$_5$-dT-dT. More recently, a study carried out in this laboratory has shown that up to three residues of deoxyadenosine or deoxycytidine phosphate and up to two residues of deoxyguanosine or thymidine phosphate may be added to adenosine oligonucleotides in the presence of the *Micrococcus luteus* enzyme, Mn^{2+} ions, and the appropriate deoxyribonucleoside 5′-diphosphate (I. L. Batey and P. T. Gilham, unpublished data). In the present study it was of some interest to test the possible addition of deoxyribonucleotide units to an oligodeoxyribonucleotide containing a ribonucleotide terminus. Such a reaction would then produce an oligodeoxyribonucleotide containing a single internal ribonucleotide residue. The reaction of pdT-dT$_2$-A and deoxycytidine 5′-diphosphate with the enzyme in the presence of Mn^{2+} ion resulted in the complete conversion of the oligonucleotide to the hexanucleotide pdT-dT$_2$-A-dC-dC, and the pentanucleotide, pdT-dT$_2$-A-dC, in the ratio of 4:1. The structure of the di-addition product was proved by subjecting it to alkaline hydrolysis to yield dC-dC and the tetranucleotide, pdT-dT$_2$-Ap, as the only products.

Conclusion. The new procedures described in this paper provide a simple access to a series of hybrid oligonucleotides

of well-defined sequence and chain length that contain either a deoxyribonucleotidyl-(3′–5′)-ribonucleotide linkage or both a deoxyribonucleotidyl-(3′–5′)-ribonucleotide and a ribonucleotidyl-(3′–5′)-deoxyribonucleotide linkage. The synthesis of the starting materials, oligodeoxynucleotides containing a terminal ribonucleotide, by means of the chemical polymerization of two mononucleotides, permits the production of large quantities of these hybrid molecules for the subsequent enzymatic addition reactions. Chemical syntheses of this type, however, produce a variety of different types of products, and the fractionation of these by conventional chromatographic techniques would normally present a formidable task. The separation of these mixtures by a two-step procedure involving DBAE-cellulose chromatography and anion-exchange chromatography on polystyrene resins constitutes a rapid and efficient method for the production of hybrid oligonucleotides. This isolation procedure could also be used with equal effectiveness in the purification of chemically synthesized oligoribonucleotides containing a terminal deoxyribonucleotide.

The discovery that these hybrid oligonucleotides can be used as acceptor molecules for either polymerization reactions or stepwise addition reactions involving polynucleotide phosphorylase and the diphosphate of a nucleoside or modified nucleoside allows the preparation of substrates that are suitable for studies on the mode of action of nucleolytic enzymes on hybrid polynucleotides. In these studies, the use of hybrid molecules containing different nucleotide bases in the ribo and deoxyribo portions of the molecules simplifies the initial analysis of the point of attack preferred by the particular nuclease under examination. Preliminary work on the action of pancreatic deoxyribonuclease on these hybrid molecules has revealed interesting variations in the cleavage patterns depending on the divalent cation used and on the location of the deoxyribo and ribo portions in the hybrid molecule. The details of these experiments constitute the subject matter of a forthcoming publication.

Experimental Section

Materials. Escherichia coli alkaline phosphatase (Grade, BAPF) and snake venom phosphodiesterase (Grade, VPH) were purchased from Worthington Biochemical Corp., Freehold, N. J. The phosphatase was assayed by the method of Garen and Levinthal (1960) and the unit of activity is that quantity of enzyme that liberates 1 μmol of *p*-nitrophenol from *p*-nitrophenyl phosphate per min at 25°. The phosphodiesterase was pretreated according to the procedure of Sulkowski and Laskowski (1971) to remove any contaminating 5′-nucleotidase activity. *M. luteus* polynucleotide phosphorylase (type 15) was obtained from P-L Biochemicals, Milwaukee, Wis., and the unit of activity is that amount of enzyme that catalyzes the incorporation of 1 μmol of ^{32}P into adenosine diphosphate in the presence of poly(A) per 15 min at 37° (Singer, 1966). The chromatographic materials, Dowex 50W-X8, Dowex 1-X2, Dowex 1-X4, and beaded polyacrylamide were purchased as AG 50W-X8 (100–200 mesh), AG 1-X2 (−400 mesh), AG 1-X4 (−400 mesh), and Bio-Gel P-2 (50–100 mesh), respectively, from Bio-Rad Laboratories, Richmond, Calif.

Polymerization of a Mixture of Thymidine 5′-Phosphate and N^6,$O^{2'}$,$O^{3'}$-Triacetyladenosine 5′-Phosphate. Sodium thymidine 5′-phosphate (2.0 mmol) was converted to the pyridinium salt with a column of the pyridinium form of Dowex 50W-X8 (100–200 mesh) ion-exchange resin, and then combined with 0.5 mmol of the pyridinium salt of N^6,-$O^{2'}$,$O^{3'}$-triacetyladenosine 5′-phosphate (Rammler and Khorana, 1962). The solution of the two components was evaporated to dryness and the mixture was repeatedly dissolved in dry pyridine followed by evaporation to dryness *in vacuo* to remove traces of water. Finally, the mixture was dissolved in dry pyridine (4.0 ml), and a solution of 2,4,6-triisopropylbenzenesulfonyl chloride (6.0 mmol in dry pyridine (4.0 ml) was slowly added. The mixture was kept at 4° for 48 hr and was then mixed with water (3.0 ml) and allowed to stand at room temperature for 3 hr. The mixture was again rendered anhydrous by repeated evaporation of its pyridine solution. Dry pyridine (10 ml) and acetic anhydride (10 ml) were added to the product and the mixture was shaken for 18 hr. Water (20 ml) was then added and the solution was evaporated to dryness *in vacuo*. The product was then repeatedly dissolved in water and concentrated to dryness *in vacuo* in order to remove pyridinium acetate. Ammonium hydroxide (7 M, 20 ml) was added to the residue and the mixture was shaken for 18 hr at room temperature. The product was concentrated by evaporation *in vacuo* and then extracted with water (20 ml). After filtration, the residue was again extracted with water (2 × 10 ml) and again filtered. The combined filtrates (40 ml) contained a total of 20,500 ODU_{260nm} of polymerized products.

Isolation and Separation of Oligonucleotides Containing Terminal Adenosine. A portion of the above solution of the polymerization reaction product (2.0 ml containing 1025 ODU_{260nm}) was mixed with 3.0 ml of the elution solvent, 0.05 M morpholinium chloride–1 M sodium chloride–0.1 M magnesium chloride (pH 8.7) in 20% dimethyl sulfoxide, and the sample was then applied to a column (50 × 1 cm) of *N*-[*N*′-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose (Weith *et al.*, 1970) which had been packed and washed with the elution solvent beforehand. The oligonucleotide sample was washed through the column with the elution solvent at a flow rate of 4 ml/hr until the A_{260nm} of the effluent dropped below 0.3. At this time the elution solvent was changed to 0.05 M sodium 2-(*N*-morpholino)ethanesulfonate–1 M sodium chloride–0.1 M magnesium chloride (pH 5.5) in 20% dimethyl sulfoxide and elution was continued at 16 ml/hr until the second ultraviolet-absorbing peak appeared in the effluent. This fraction (430 ODU_{260nm}) was dialyzed at 4° against water (2 × 4 l.) for 12 hr, a procedure which removed 64 ODU_{260nm} of mononucleotides and small oligonucleotides as well as most of the salt. The remaining products were concentrated and fractionated by ion-exchange chromatography on a column of Dowex 1-X2 (−400 mesh) ion-exchange resin using a linear gradient of ammonium chloride in 40% ethanol (Ho and Gilham, 1973). The elution pattern and the details of the separation are given in Figure 1, and the retention volumes are listed in Table I. The oligonucleotide corresponding to each peak was desalted by passage through a column (55 × 1.0 cm) of beaded polyacrylamide (Bio-Gel P-2, 50–100 mesh). The distribution of the yields of the species, pdT-dT_n-A where n = 0, 1, 2, 3, 4, 5, 6, and >6, were 7, 21, 26, 21, 13, 7, 3, and 2%, respectively.

The oligonucleotide fraction (620 ODU_{260nm}) which did not bind to the dihydroxyboryl-substituted cellulose column at pH 8.7 was again passed through the same column in the same pH 8.7 elution buffer without any loss of material. This fraction was then rendered salt free by dialysis at 4° against water (2 × 4 l.) for 12 hr, and then separated on a column (40 × 0.4 cm) of Dowex 1-X2 (−400 mesh) using 200 ml of 20% ethanol containing a linear gradient of 0.1–

1.0 M ammonium chloride (pH 8.5), at a flow rate of 17 ml/hr. The main peaks obtained had retention volumes: 1, 53 ml; 2, 58 ml; 3, 70 ml; 4,82 ml; 5, 95 ml; 6, 105 ml. All of the products had a λ_{max} of 266–267 nm. Each of the oligonucleotide peaks was treated with alkaline phosphatase and the dephosphorylated products corresponding to peaks, 1 and 3, were assigned the structures, dT-dT and dT-dT-dT, on the basis of their degradation with snake venom phosphodiesterase and the subsequent analysis of the products, which gave ratios of thymidine to thymidine 5′-phosphate of 1.00:0.97 and 1.00:1.93, respectively. The material present in peak 2 was resistant to attack by phosphatase and snake venom diesterase and it is presumed to be one of the cyclic oligonucleotides that are always formed in the chemical polymerization of mononucleotides.

Alternative Procedure for the Isolation of Thymidine Oligonucleotides Containing a Terminal Adenosine. A portion of the mixture of polymerized products (8 ml, containing 4100 ODU_{260nm}) was chromatographed on a column (55 × 2.5 cm) of DEAE-cellulose (Whatman DE-23) using 8 l. of 7 M urea–0.05 M Tris-chloride (pH 7.5) containing a linear gradient of 0–0.5 M sodium chloride at a flow rate of 42 ml/hr. The peaks with retention volumes of 1545, 1797, 2091, 2268, and 2419 ml corresponded to a mixture of pdT-dT_n-dT and pdT-dT_n-A, where n = 1, 2, 3, 4, 5, respectively. The mixture of oligonucleotides corresponding to each peak was desalted by passing it through a column (50 × 2.5 cm) of Bio-Gel P-2 (50–100 mesh). The desalted material in each case was then separated into the thymidine oligomer and the corresponding oligomer with terminal adenosine by passing it through the dihydroxyboryl-substituted cellulose column at pH 8.7 as described above. Each oligomer containing the terminal adenosine was then eluted at pH 5.5 and desalted as described above.

Dephosphorylation of Oligonucleotides with Alkaline Phosphatase. Approximately 4 ODU_{260nm} of the oligomer was dissolved in 0.1 ml of 0.04 M Tris-chloride–0.02 M magnesium chloride buffer (pH 8.0) and treated with 0.1 ml of a solution of alkaline phosphatase (0.1 unit) in water. After incubation at 37° for 4 hr the mixture was applied to Whatman 3MM paper and chromatographed for 20 hr in descending fashion with 1-propanol–concentrated ammonia–water (55:10:35, v/v). The R_F values and ion-exchange retention volumes of oligonucleotides and their dephosphorylated products are listed in Table I.

Degradation of Oligomers with Snake Venom Phosphodiesterase. The oligonucleotide (3–5 ODU_{260nm}) was dissolved in 0.1 ml of 0.2 M Tris-chloride–0.04 M magnesium chloride buffer (pH 9.0) and mixed with 50 μl of a solution of snake venom phosphodiesterase (1.0 mg/ml). After 2 hr at room temperature the mixture was treated with 0.25 ml of 0.25 M sodium hydroxide and the products of the reaction were analyzed on an ion-exchange column using solvents containing 20% ethanol (Ho and Gilham, 1973). The column of Dowex 1-X4 (−400 mesh) had dimensions of 100 × 0.2 cm, and the following additions, in order, were made *via* a sample injection loop: 0.4 ml of 1 M sodium hydroxide, 2 × 0.5 ml of 20% ethanol, enzyme hydrolysate, and 2 × 0.5 ml of 20% ethanol. Separation was carried out under pressure at 8.5 ml/hr with 200 ml of 20% ethanol solution containing a linear gradient of 0–0.5 M ammonium chloride which had been adjusted to pH 10 with ammonia. In this system, the retention volumes of adenosine, thymidine, adenosine 5′-phosphate, and thymidine 5′-phosphate are 30, 33, 67, and 72 ml, respectively. The results of the analyses of various oligonucleotides are listed in Table I.

In the case of oligonucleotides containing phosphomonoester groups at both ends of the chain alkaline phosphatase (0.1 ml containing 0.1 unit) was also added to the phosphodiesterase digestion and the reaction was allowed to proceed for 5 hr at 37°.

Alkaline Hydrolysis of Oligonucleotides. The salt-free aqueous solution of the oligonucleotide (3–5 ODU_{260nm}) was treated with 0.25 M sodium hydroxide (0.25 ml) and the mixture was then concentrated *in vacuo* to about 0.25 ml and kept at 37° for 20 hr. The products were analyzed by ion-exchange chromatography as described above for the products from phosphodiesterase digestion. In this system 2′-*O*-(α-methoxyethyl)adenosine, adenosine 2′-phosphate, adenosine 3′-phosphate, and deoxycytidylyl-(3′–5′)-deoxycytidine have retention volumes of 11, 65, 71, and 40 ml, respectively. The results of these analyses are listed in Table I.

Single Addition of Adenosine Phosphate to Hybrid Oligonucleotides. A typical reaction mixture contained the buffer, 1 M sodium tris(hydroxymethyl)methylaminopropanesulfonate, pH 9 at 20° (10 μl), 0.1 M manganese chloride (10 μl), 20 μl of 25 mM 2′-*O*-(α-methoxyethyl)adenosine 5′-diphosphate (Bennett *et al.*, 1973), oligonucleotide (5–10 ODU_{260nm} in 40 μl of water), and 20 μl of *M. luteus* polynucleotide phosphorylase (10 units/ml). The reaction was allowed to proceed for 8 hr at 37° and was then fractionated by chromatography on a column (40 × 0.4 cm) of Dowex 1-X2 (−400 mesh) under pressure at a flow rate of 17 ml/hr. For those reaction mixtures corresponding to the addition to oligonucleotides of chain length 5 or less the solvent system was 200 ml of 20% ethanol containing a linear gradient of 0.1–1.0 M ammonium chloride (pH 8.5) and, in the case of those reaction mixtures corresponding to the addition to oligonucleotides of longer chain lengths, the system was 200 ml of 20% ethanol containing a linear gradient of 0.4–0.8 M ammonium chloride (pH 8.5). The oligonucleotides that have been tested in this reaction are pdT-dT_n-A, where n = 2, 3, 4, 5; dT-dT_n-A, where n = 2, 4; dT-dT_2-A-A, and pdT-dT_3-A-A, and in each case the single addition product was obtained in 60–70% yield. The retention volumes and analytical data corresponding to these products are listed in Table I and a typical elution pattern for the analysis of the addition reaction is shown in Figure 2.

The removal of the methoxyethyl group was effected by adjusting the pH of the appropriate fractions from the ion-exchange column to 2.0 with hydrochloric acid and allowing the mixture to stand at room temperature for 30 min. The pH of the solution was then adjusted to 8.0 with sodium hydroxide prior to desalting the product on a column (55 × 1.0 cm) of Bio-Gel P-2 (50–100 mesh).

Multiple Addition of Adenosine Phosphate to Hybrid Oligonucleotides: 4 M sodium chloride (100 μl), 0.1 M manganese chloride (100 μl), 0.08 M adenosine 5′-diphosphate (100 μl), pdT-dT-A (125 ODU_{260nm}) in water 0.4 ml, 1 mM cupric sulfate (100 μl), and 100 μl of *M. luteus* polynucleotide phosphorylase (10 units/ml). After incubation at 37° for 24 hr the mixture was fractionated on a column (100 × 0.4 cm) of Dowex 1-X2 (−400 mesh) using 200 ml of a 40% ethanol solution containing a linear gradient of 0.3–0.5 M ammonium chloride (pH 8) at a flow rate of 10–12 ml/hr. The elution pattern is shown in Figure 3 and the retention volumes of the products are listed in Table I. The yields of the species pdT-dT-A_n-A, where n = 1, 2, 3, and 4 were 26, 18, 15, and 12 ODU, respectively. The oligonucleotides dT-dT-A and dT-dT_2-A were subjected to similar addition reactions and, in these cases, the products were separated on a column (40 × 0.4 cm) of Dowex 1-X2 (−400 mesh) using 200 ml of a 20%

ethanol solution containing a linear gradient of 0.1–1.0 M ammonium chloride (pH 8.5) at a flow rate of 17 ml/hr. All the products of these addition reactions were desalted on columns of Bio-Gel P-2 polyacrylamide gel, as above described.

Addition of Deoxycytidine Phosphate to pdT-dT$_2$-A. The reaction mixture consisted of the buffer, 1 M sodium tris(hydroxymethyl)methylaminopropanesulfonate, pH 9 at 20° (10 μl), 0.1 M manganese chloride (10 μl), 0.05 M deoxycytidine 5′-diphosphate (10 μl), pdT-dT$_2$-A (4.0 ODU$_{260nm}$) in water (50 μl), and 20 μl of *M. luteus* polynucleotide phosphorylase (10 units/ml). After incubation at 37° for 20 hr the mixture was separated on a column (40 × 0.4 cm) of Dowex 1-X2 (−400 mesh) using 200 ml of a 20% ethanol solution containing a linear gradient of 0.1–1.0 M ammonium chloride (pH 8.5). The elution pattern showed that all of the starting material had been converted to products and that the ratio of products pdT-dT$_2$-A-dC-dC:pdT-dT$_2$-A-dC was 4:1. The retention volume of the hexanucleotide product is listed in Table I.

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Interaction of Guanine Ligands with Ribonuclease T$_1$†

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ABSTRACT: Difference spectral measurements of the binding of ribonuclease T$_1$ with a variety of guanine ligands conducted at pH 5.0, 0.2 M ionic strength, and 25° were used to determine association constants and maximal difference molar extinction coefficients, $\Delta\epsilon$, at 290 nm. The following order of affinities for ribonuclease T$_1$ binding were found: Guo-2′-P > Guo-3′-P > dGuo-3′-P > Guo-5′-P = dGuo-5′-P > Guo > dGuo. In all cases the binding stoichiometry was found to be 1:1 within experimental error. The qualitative nature of the difference spectra was similar for all the ligands tested except for a minor variation found with dGuo-3′-P. Values of $\Delta\epsilon$ showed a considerable variation and were greater than those found for the acid pH-induced difference spectra of Guo-3′-P and dGuo-3′-P. The results are compared with those of previous studies and are contrasted with results from similar studies on ribonuclease A with emphasis on the interaction of the ligand 2′-hydroxyl group with these enzymes.

It is an interesting fact that ribonuclease T$_1$ (EC 2.7.7.26) is an unusually acidic protein (Egami *et al.*, 1964) because its substrate, RNA, is a negatively charged polymer. Since polyanionic inhibitors of ribonuclease A (a basic protein) do not inhibit ribonuclease T$_1$ (Egami *et al.*, 1964) it appears likely that there are few, if any, nonspecific electrostatic interactions contributing to the negative free energy of binding for this enzyme with RNA. This apparent lack of utilization of electrostatic binding in the formation of the ribonuclease T$_1$–RNA complex presents an interesting problem regarding protein–nucleic acid interactions.

In an effort to elucidate the specific enzyme–substrate interactions of ribonuclease T$_1$ many investigations have been

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