Duplex Formation of a Nonionic Oligo(deoxythymidylate) Analogue [Heptadeoxythymidylyl-(3′–5′)-deoxythymidine Heptaethyl Ester (d-[Tp(Et)]7T)] with Poly(deoxyadenylate). Evaluation of the Electrostatic Interaction†

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ABSTRACT: The heptaethyl ester of heptadeoxythymidylyl-(3′–5′)-deoxythymidine (d-[Tp(Et)]7T or d-T8·Et) has been prepared by chemical methods. The material, consisting of a mixture of diastereoisomers, forms a 1:1 complex with $(dA)_n$ in neutral aqueous buffer; this interaction is virtually independent of ionic strength. The octamer triester does not bind to $(dA)_n$·$(dT)_n$, and it interacts with $(rA)_n$ only at low temperatures. By cochromatography with $(dA)_n$ on Sephadex G-50, d-T8·Et fractions with different binding affinities for the polyadenylates were obtained. This heterogeneity in binding affinity is ascribed to the diastereoisomerism of d-T8·Et. Enthalpies of duplex formation were determined by the concentration variation method. At 0.1 M sodium ion concentration, the enthalpy of binding of the various d-T8·Et fractions to $(dA)_n$ is essentially invariant (−8.1 kcal/mol of base pairs at 0 °C to −8.6 kcal at 25 °C) and 1.6 kcal/mol of base pairs more negative than the enthalpy of binding of the phosphodiester analogue, d-$(Tp)_7$T, to $(dA)_n$ (−6.8 kcal/mol of base pairs at 11 °C). This difference is the electrostatic contribution to the enthalpy of duplex formation, arising from the interstrand electrostatic repulsion and the intrastrand repulsion in d-$(Tp)_7$T. The entropy of binding to $(dA)_n$ is more negative for the octamer triesters than for the diester analogue, and is different for the various d-T8·Et fractions. This is interpreted in terms of varying degrees of restriction of rotational freedom for the ethyl substituents upon double helix formation.

The interaction of nonionic polynucleotide analogues with complementary polynucleotides has been studied in various laboratories. Poly(1-vinyluracil) forms a complex with $(rA)_n$ (Pitha et al., 1970) as does poly(1-vinylcytosine) with $(rG)_n$ or $(rI)_n$ (Pitha and Michelson, 1970), but the stoichiometry of the complexes is ill defined, the solubility of the vinyl polymers is very low, and the stereochemical arrangements with the complementary polynucleotides are not in register, resulting in only a small extent of interaction as reflected by little change in absorbance. More substantial optical effects were realized with the use of poly(acrylic acid) hydrazide derivatized with adenine groups to a low degree of substitution (Boulton et al., 1971). Homopolymers in which deoxyadenosine or deoxycytidine is linked in 3′–5′ fashion by oxoethylene bridges have a base spacing which is regular and approximately correct for hybridization with complementary polynucleotides, and they form better defined complexes with $(rU)_n$ and $(rI)_n$, respectively (Jones et al., 1973; Bleaney et al., 1975).

The methyl and ethyl esters of deoxyadenylyl-(3′–5′)-deoxyadenosine have been found to form 1:2 complexes with $(rU)_n$ with a T_m higher than that of dApdA·$2(rU)_n$ (Miller et al., 1971). While this system illustrates the importance of electrostatic effect, it does not serve for a quantitative evaluation of the influence of electrostatic interactions on the stability of polynucleotide complexes, first because of the small oligomer length, and second because in the model system interstrand electrostatic repulsion is not entirely eliminated, as the triplex still contains two charged polyuridylate strands. A more fertile object for study would be the interaction of an oligonucleotide triester of moderate chain length with complementary polynucleotides to yield complexes containing only one charged strand. The present is a report on the synthesis of heptathymidylyl-(3′–5′)-thymidine[1] heptaethyl ester (d-[Tp(Et)]7T = d-T8·Et)[2] and its interaction with polyadenylates.

Results

Synthesis of d-T8·Et. The synthetic scheme adopted in the preparation of the octamer triester, d-[Tp(Et)]7T, is based on earlier work from this laboratory (DeBoer et al., 1973; Miller et al., 1974). The salient feature is the formation of the internucleotide linkage in the phosphoric acid diester form, to ensure high yields of chain elongation, and subsequent ethylation of the phosphate moiety to the triester form. This approach has been used successfully by Smrt and coworkers (Smrt, 1973a,b; Zielinski and Smrt, 1974; Mikhailov and Smrt, 1975) in the triester type synthesis of oligonucleotides, both with chain extension by monomeric nucleotide units and in blockwise fashion.

In order to reduce the number of required reactions, the octamer triester was prepared by successive condensation of blocks of dimers. The syntehsis is outlined in Scheme I. 5′-*O*-Mono-*p*-methoxytritylthymidine and 3′-*O*-acetylthymidine

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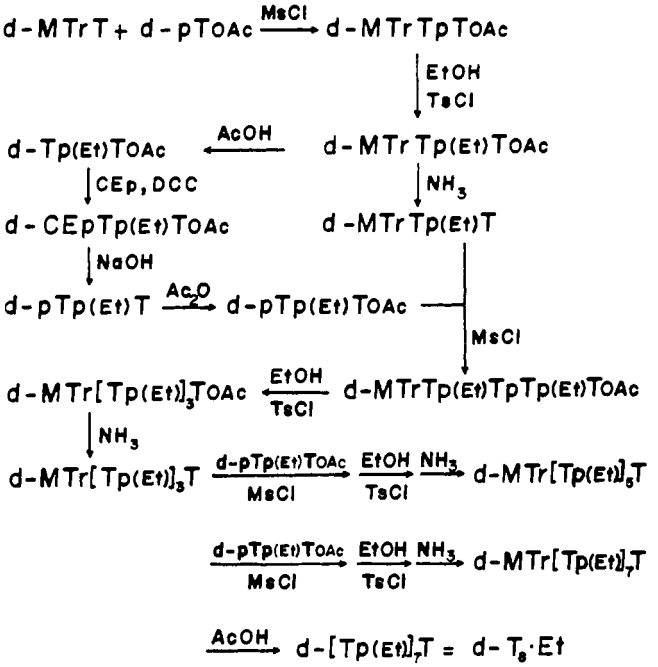
[1] Throughout the text the term thymidine will refer to the 2′-deoxyribonucleoside.

[2] Np(Et)N indicates ethylation of the 3′–5′ internucleotide phosphate linkage. The fully triesterified octamer will be denoted either by d-[Tp(Et)]7T or by d-T8·Et. Abbreviations used are: MTr, *p*-anisyldiphenylmethyl = *p*-methoxytrityl; CE, β-cyanoethyl; TosCl, *p*-toluenesulfonyl chloride; MsCl, mesitylenesulfonyl chloride; H_4furan, tetrahydrofuran.

Scheme I



5′-phosphate are condensed in pyridine solution, activated by mesitylenesulfonyl chloride. The resulting diester is converted to the ethyl triester in an *N,N*-dimethylformamide solution containing 2,6-lutidine, *N*-methylimidazole, *p*-toluenesulfonyl chloride, and ethanol. Part of the triester, d-MTrTp(Et)TOAc, is deacetylated with ammonia in pyridine–water to yield d-MTrTp(Et)T, which constitutes the 5′-terminal block in the octamer synthesis. The rest of the fully protected dimer is detritylated to d-Tp(Et)TOAc, which is then condensed with pyridinium cyanoethyl phosphate to give d-CEpTp(Et)TOAc. After alkaline removal of the cyanoethyl and acetyl groups, the 3′-hydroxyl group is reacetylated with acetic anhydride to yield d-pTp(Et)TOAc, the dimer block to be added iteratively in the octamer synthesis. Condensation of d-MTrTp(Et)T with d-pTp(Et)TOAc produces d-MTrTp(Et)TpTp(Et)TOAc, which is ethylated to the fully protected tetramer, d-MTr[Tp(Et)]$_3$TOAc. The 3′ position is deacetylated, and the cycle is repeated twice, to yield d-MTr[Tp(Et)]$_7$T. After acid-catalyzed cleavage of the 5′-methoxytrityl group, the desired octamer triester is obtained. The use of appropriate protecting groups throughout the synthesis ensures that the internucleotide linkages are exclusively of the 3′–5′ type.

Table I summarizes the scale and the yields of each reaction starting with the synthesis of the tetramer. In the chain extension steps, the reaction mixture was worked up by simple extraction procedures, and the materials obtained were contaminated by traces of side products. For this reason, the calculated yields should be slightly too high. Rigorous purification of the desired products by ilica gel column chromatography was undertaken after the ethylation and deacetylation steps. Examination of the product mixtures by thin-layer chromatography revealed, apart from the major band, small amounts of closely comigrating species. The isolation of the major peak free from the minor species required repeated careful column chromatography using step gradients of ethyl acetate–tetrahydrofuran mixtures. This process was fruitful only up to a point, as the relative enrichment of the minor components and the depletion of the major species made its isolation in pure form more difficult and less rewarding. In the case of oligonucleotide β,β,β-trichloroethyl esters, Werstiuk and Neilson

TABLE I: Reaction Yields in the Synthesis of d-T$_8$·Et.

	$n = 4$	$n = 6$	$n = 8$
Chain Extension			
mmol of d-MTr[Tp(Et)]$_{n-3}$T	2.8	1.00	0.33
mmol of d-pTp(Et)TOAc	3.7	1.49	0.66
Reaction time (h)	23	8	7
Yield (%)	86[a]	92[a]	98[a]
Ethylation			
mmol of d-MTr[Tp(Et)]$_{n-3}$TpTp(Et)-TOAc	2.4	0.86	0.30
Yield (%)	51[a]	68[a]	59[a]
Deacetylation			
mmol of d-MTr[Tp(Et)$_{n-1}$TOAc	1.1	0.46	0.13
Yield (%)	90[a]	80[a]	65[a]

[a] For a proper interpretation of the yields see text.

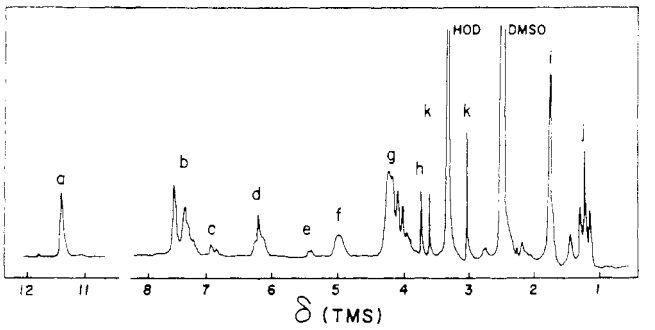


FIGURE 1: ^{1}H NMR spectrum (100 MHz) of d-MTr[Tp(Et)]$_7$T in dimethyl-d_6 sulfoxide at 23 °C.

(1972) have shown that the chirality introduced at the phosphate moiety by triester formation influences the chromatographic mobility. It is thus likely that the minor species observed in this synthesis constitute those diastereoisomers of the desired material which differ in mobility from the bulk. Fractions containing the minor species, in many cases mixed with considerable amounts of the major product, were not included in the calculation of the yields for ethylation and deacetylation listed in Table I. This, in conjunction with the losses attendant on repeated chromatography, provides an explanation for the relatively low yields of ethylation and of deacetylation reported in the table.

In the interest of an unequivocal synthesis, only the purified major peak was carried on to the next step. It is therefore probable that many of the possible diastereoisomers were eliminated at the various stages of the synthesis.

Chromatographic mobilities for various intermediates in the synthesis are listed in Table II. For equivalent species, R_f values for adsorption chromatography as well as elution volumes for gel filtration chromatography decreased with increasing degree of polymerization, as anticipated. The chromatographic data from the Sephadex G-25 and G-50 columns are the most direct demonstration of the different oligomer sizes; however, they do not serve to establish uniformity of chain length for the various materials, as the peaks were quite broad. Complete separation of hexamer and octamer was achieved only for the unprotected oligomers, d-[Tp(Et)]$_{n-1}$T, on silica gel thin-layer chromatography in tetrahydrofuran and on paper chromatography in solvent I′.

Figure 1 shows the ^{1}H NMR spectrum of d-MTr[Tp(Et)]$_7$T in perdeuterated dimethyl sulfoxide. Chemical shifts (δ values, relative to tetramethylsilane internal standard), assignments,

TABLE II: Chromatographic Data for the Oligonucleotide Ethyl Esters.

	Silica Gel TLC, R_f Values			Column Chromatogr., $K_{av} = v_e - v_o/v_t - v_o$,[a] Seph. G-50, Me_2SO
	H_4furan–EtOAc (1:1)	H_4furan	$CHCl_3$–MeOH (9:1)	
d-MTrTp(Et)TOAc	0.50	0.63	0.60	0.77
d-MTr[Tp(Et)]$_3$TOAc	0.18	0.52	0.53	0.63
d-MTr[Tp(Et)]$_5$TOAc	0.06	0.46	0.43	0.52
d-MTr[Tp(Et)]$_7$TOAc	0.00	0.42	0.30	0.40
d-MTrTp(Et)T	0.37	0.57	0.44	
d-MTr[Tp(Et)]$_3$T	0.07	0.46	0.36	
d-MTr[Tp(Et)]$_5$T	0.02	0.42	0.31	
d-MTr[Tp(Et)]$_7$T	0.00	0.36	0.24	

	Silica Gel TLC, R_f		Paper Chromatogr., R_f		Column Chromatogr., $K_{av} = v_e - v_o/v_t - v_o$[b]	
	Acetone	H_4furan	Solvent H	Solvent I′	G-25, H_2O	G-50, H_2O
d-Tp(Et)T	0.25	0.43	0.31	0.66		0.93
d-[Tp(Et)]$_3$T	0.15	0.25	0.12	0.56		0.87
d-[Tp(Et)]$_5$T	0.02	0.14	0.07	0.41	0.43	
d-[Tp(Et)]$_7$T	0.00	0.06	0.03	0.31	0.36	0.73

[a] v_o, v_t, and v_e; elution volumes of India ink, N^3-methylthymine, and oligomer, respectively. [b] v_o, v_t, and v_e; elution volumes of $(rA)_n$, N^3-methylthymine, and oligomer, respectively.

and integration of the peaks are as follows: (a) 11.37 ppm, 8 N^3-H, 7.9 protons; (b) 7.48 and 7.31 ppm, 8 H-6 plus 14 MTr-H, 23.0 protons; (c) 6.88 ppm, 2 MTr-H, 2.1 protons; (d) 6.17 ppm, 8 H-1′, 8.0 protons; (e) 5.42 ppm, 1 3′-OH, 1.1 proton; (f) 4.96 ppm, 7 H-3′, 6.8 protons; (g) 4.18 ppm, 1 3′-terminal H-3′ plus 8 H-4′ plus 14 H-5′,5″ (all except the 5′-terminal group) plus 7 ethyl CH_2 groups, 38.2 protons; (h) 3.69 ppm, CH_3O group, 3.0 protons; (i) 1.75 ppm, 75-CH_3 groups, 20.8 protons; (j) 1.41 and 1.22 ppm, 1 5′-terminal 5-CH_3 group plus 7 ethyl CH_3 groups, 24.2 protons; (k) water sidebands.

Considerable care was taken to ascertain that the thymine rings were not ethylated in the triester forming steps, as this would interfere with hydrogen bonding and affect the interaction of the octamer triester with the polyadenylates. Extensive treatment of the model compound 5′-*O*-isobutyloxycarbonyl-3′-*O*-mono-*p*-methoxytritylthymidine (Ogilvie and Letsinger, 1967) with the standard ethylation mixture left the nucleoside unaltered. Within the error margin of several percent, the agreement between the number of N^3 protons calculated and measured in the NMR experiment excludes ethylation at the base moiety. N^3-Ethylation, specifically, was shown not to occur by treatment of d-MTr[Tp(Et)]$_5$TOAc with acetic acid/concentrated aqueous HCl (2:1) at 100 °C for 4 h (Hoffer, 1960). Paper chromatography of the hydrolysate indicated quantitative liberation of thymine; no N^3-ethylthymine was detectable. Under these hydrolysis conditions, N^3-methylthymidine (prepared by the method of Markiw and Canellakis, 1968) was cleanly converted to N^3-methylthymine. This test does not serve to exclude the possibility of O^4-ethylation, since under strongly acidic conditions the 4-ethoxy-5-methyl-2-pyrimidinone moiety would be hydrolyzed to thymine. Therefore, to ensure elimination of any degree of O-ethylation undetected by the NMR technique, the reaction time for detritylation in 80% aqueous acetic acid was extended from several hours to 3 days. These conditions proved sufficient to completely hydrolyze 1-β-D-ribofuranosyl-4-methoxy-2-pyrimidinone to uridine.

For neutral aqueous solutions of d-T_8·Et, the absorption maximum occurs at 264 nm and the minimum at 234 nm. The values of A_{250}/A_{260} and A_{280}/A_{260} are 0.69 and 0.59, respectively. On changing the solvent to 0.1 M aqueous NaOH, the absorption at λ_{max} is reduced by 14.9%. On the assumption that for the fully ionized octamer the extinction coefficient (per monomer unit) is the same as that for thymidine in alkali, and using the published extinction coefficients for thymidine in neutral and alkaline aqueous solutions, a value of $\epsilon_{264} = 8.7 \times 10^3$ M^{-1} cm^{-1} (per monomer unit) is calculated for d-T_8·Et in neutral aqueous medium, identical with the value reported by Cassani and Bollum (1969) for octathymidylate, d-pT_8. In agreement with the findings of Rich (1960) on oligo(deoxythymidylates), the absorption spectrum of d-T_8·Et is independent of temperature in the range examined (−2 to 60 °C).

The octamer triester is quite soluble in methanol and ethanol, but only moderately soluble in water, with a solubility limit of 1.5×10^{-3} M (in monomer units) at 30 °C. It also shows a propensity to adhere to glass. A single transfer of a 10^{-4} M aqueous solution of d-T_8·Et using a glass pipet results in a 3% loss of optical density. The use of siliconized glass or of plastic implements aggravates this problem.

Interaction of d-T_8·Et with Polyadenylates. Throughout this section, numbers of moles, concentrations, and input ratios are given in terms of monomeric nucleotide units. In the temperature–absorbance profiles, the quantity plotted on the ordinate, $A_{260}(exptl)/A_{260}(calcd)$, is the ratio of the measured absorbance at 260 nm to the value calculated as the sum of the absorbances at 260 nm of the separate components at that temperature. Thus, a value of $A_{260}(exptl)/A_{260}(calcd)$ of 0.90 indicates the mixture to be 10% hypochromic with respect to the constituents. All temperature curves shown were obtained by slow cooling from a temperature well above the transition. Where the corresponding melting curves were determined, they were found to coincide with the annealing curves.

Preliminary experiments established that in 0.15 M NaCl–0.04 M potassium phosphate (pH 6.9) an equimolar mixture of $(dA)_n$ and d-T_8·Et at 10^{-4} M total concentration exhibited a fairly broad transition with a midpoint in the proximity of 18 °C. A mixing curve for this system is shown in Figure 2. At all wavelengths examined, the minimum absorbance occurs at 52% octamer, with hypochromicities of 22,

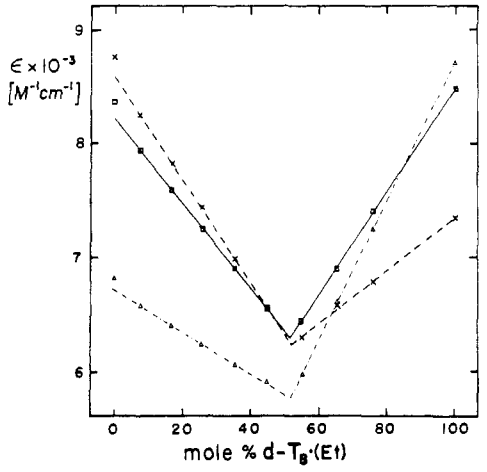


FIGURE 2: Mixing curve for $(dA)_n$ + d-T_8·Et at 0 °C, in 0.15 M NaCl-0.04 M potassium phosphate (pH 6.9); total concentration, 10^{-4} M: (×- -×) ε 255 nm; (□—□) ε 260 nm; (△···△) ε 265 nm.

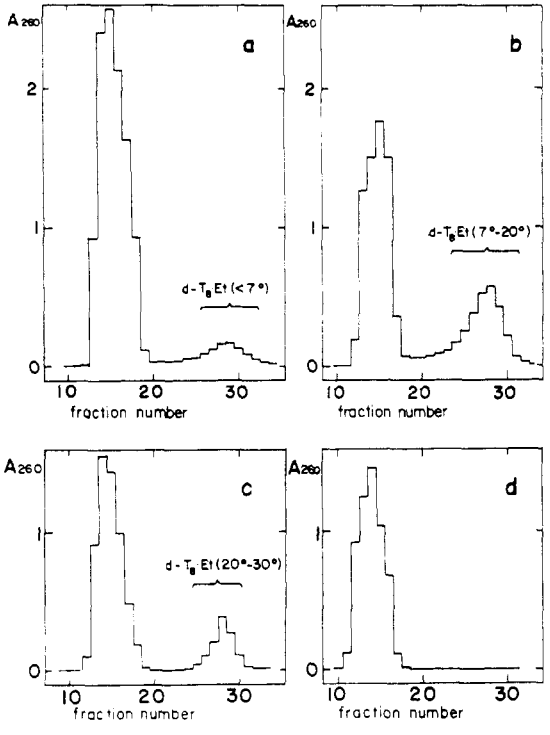


FIGURE 3: Fractionation of d-T_8·Et by cochromatography with $(dA)_n$ on Sephadex G-50; temperature: (a) 7 °C; (b) 20 °C; (c) 30 °C; (d) 40 °C.

25, 26, 11, and 5% for 255, 260, 265, 280, and 285 nm, respectively. The ultraviolet circular dichroism spectrum of the 1:1 mixture, measured at 1 °C, showed the pattern typical for the $(dA)_n$·$(dT)_n$ duplex (Wells et al., 1970), with peaks at 258 and 282 nm and a trough at 267 nm.

Since the sample of d-T_8·Et should consist of a large number of diastereoisomers, the annealing curve observed might result from the superimposition of a multitude of oligomer-polymer transitions with different stabilities. To address this possibility, an attempt was made to fractionate the octamer triester according to its ability to comigrate with the deoxyadenylate polymer on a Sephadex G-50 column at different temperatures. The eluent used was 0.001 M sodium cacodylate (pH 6.9). The use of the dilute buffer would later obviate the need for desalting, and subsequent experiments confirm that the oligomer

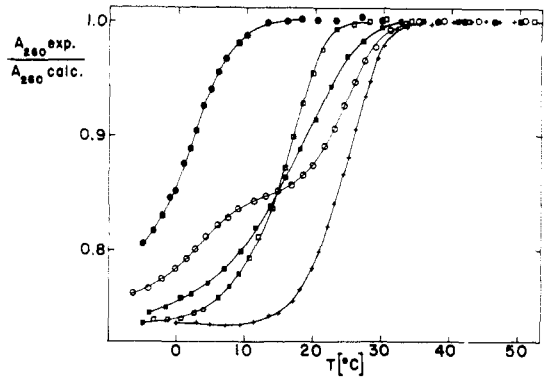


FIGURE 4: Annealing of $(dA)_n$ with various fractions of d-T_8·Et in 0.1 M sodium cacodylate (pH 6.9); total concentration, 10^{-4} M: (■) $(dA)_n$ + d-T_8·Et (unfractionated) (1:1); (●) $(dA)_n$ + d-T_8·Et (<7 °C) (1:1); (□) $(dA)_n$ + d-T_8·Et (7-20 °C) (1:1); (+) $(dA)_n$ + d-T_8·Et (20-30 °C); (○) $(dA)_n$ + d-T_8·Et (<7 °C) + d-T_8·Et (20-30 °C) (2:1:1).

triester-polymer interaction is insensitive to ionic strength.

A solution of 3.4 μmol of $(dA)_n$ and 3.2 μmol of d-T_8·Et in 5.2 mL of 0.001 sodium cacodylate (pH 6.9) was incubated at 4 °C for 1 h and then chromatographed on a Sephadex G-50 column (137 mL) equilibrated at 7 °C. The elution profile is shown in Figure 3a. The high molecular weight peak contains $(dA)_n$·d-T_8·Et complexes. The second peak exhibits the same absorption spectrum as d-T_8·Et and will be termed d-T_8·Et (<7 °C), i.e. the d-T_8·Et fraction which does not comigrate with $(dA)_n$ at 7 °C; this fraction constitutes 13% of the total d-T_8·Et introduced. The large molecular weight peak was lyophilized and rechromatographed on Sephadex G-50 in 0.001 M sodium cacodylate at 20 °C (Figure 3b). The low molecular weight peak makes up 47% of the total d-T_8·Et and is termed d-T_8·Et (7-20 °C), i.e. the d-T_8·Et fraction which comigrates with $(dA)_n$ at 7 °C but does not comigrate at 20 °C. Rechromatography of the high molecular weight fraction on Sephadex G-50 at 30 °C (Figure 3c) yielded d-T_8·Et (20-30 °C), 22% of the input d-T_8·Et. The high molecular weight peak in this chromatography had an absorption spectrum of pure $(dA)_n$, and its rechromatography at 40 °C did not liberate any more oligomer (Figure 3d). In these chromatographies the octamers eluted just prior to the salt peak, as established by conductivity measurements.

Annealing curves for equimolar mixtures of the various d-T_8·Et fractions and $(dA)_n$, at 10^{-4} M total concentration, in 0.1 M sodium cacodylate (pH 6.9) are shown in Figure 4. At temperatures below 10 °C, the $(dA)_n$ + d-T_8·Et mixture exhibits a hypochromicity of 26.6% at 260 nm. The mixtures of $(dA)_n$ with d-T_8·Et (7-20 °C) and with d-T_8·Et (unfractionated) approach the same value of hypochromicity at temperatures below 0 °C. The $(dA)_n$ + d-T_8·Et (<7 °C) mixture is almost 20% hypochromic at −5 °C, the lowest temperature examined. The temperatures at which these mixtures attain a hypochromicity of 10% ($T_{0.9}$) are 26.0 (+), 16.9 (□), 19.0 (■), and 2.6 °C (●), respectively; in Figure 4. Thus, the transitions for $(dA)_n$ + d-T_8·Et (<7 °C) and $(dA)_n$ + d-T_8·Et (20-30 °C) are separated by 23 °C. Comparing the transition profiles for $(dA)_n$ + d-T_8·Et (7-20 °C) and $(dA)_n$ + d-T_8·Et (unfractionated), one observes that they have similar midpoint temperatures but different breadths. The transition for the $(dA)_n$ + d-T_8·Et (unfractionated) mixture is considerably broader, reflecting the superimposition of oligomer-polymer transitions with more widely different stabilities.

The annealing profile of the $(dA)_n$ + d-T_8·Et (<7 °C) + d-T_8·Et (20-30 °C) (2:1:1) mixture is bimodal (Figure 4) due

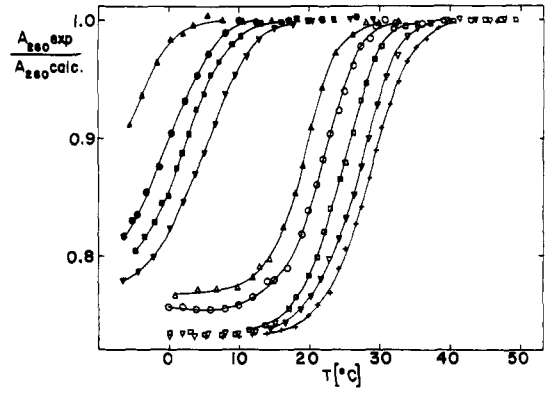


FIGURE 5: Concentration dependence of the annealing of $(dA)_n$ + d-T_8·Et (<7 °C) (1:1) and of $(dA)_n$ + d-T_8·Et (20-30 °C) (1:1) in 0.1 M sodium cacodylate (pH 6.9): (▲) $(dA)_n$ + d-T_8·Et (<7 °C), 0.072 × 10^{-4} M; (●) 0.359 × 10^{-4} M; (■) 1.01 × 10^{-4} M; (▼) 3.86 × 10^{-4} M; (△) $(dA)_n$ + d-T_8·Et (20-30 °C), 0.099 × 10^{-4} M; (○) 0.367 × 10^{-4} M; (□) 1.02 × 10^{-4} M; (▽) 2.54 × 10^{-4} M; (+) 5.35 × 10^{-4} M.

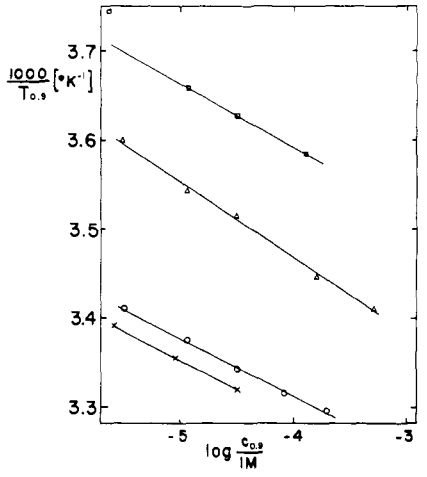


FIGURE 6: Determination of ΔH values in 0.1 M sodium cacodylate (pH 6.9): (□) $(dA)_n$ + d-T_8·Et (<7 °C); (△) $(dA)_n$ + d-T_8; (○) $(dA)_n$ + d-T_8·Et (20-30 °C); (×) $(dA)_n$ + d-T_8·Et [2-20 °C]$_{rA}$.

to the removal of the fraction of octamers with intermediate binding affinity (d-T_8·Et (7-20 °C)) from the d-T_8·Et mixture. The upper portion of the biphasic curve closely follows the course of the $(dA)_n$ + d-T_8·Et (20-30 °C) (1:1) profile. Thus, the interaction of d-T_8·Et (20-30 °C) with $(dA)_n$ is not affected by the addition of d-T_8·Et (<7 °C). This observation indicates that the different binding affinities of d-T_8·Et (<7 °C) and of d-T_8·Et (20-30 °C) for $(dA)_n$ are not due to the presence of an inhibiting contaminant in the d-T_8·Et (<7 °C) fraction. Reexamining the size of d-T_8·Et (<7 °C) by cellulose thin-layer chromatography in solvent I′, it was found to comigrate exactly with d-T_8·Et (unfractionated), clearly separated from d-T_6·Et. Also, the ultraviolet absorption spectra of d-T_8·Et (<7 °C) and d-T_8·Et (20-30 °C) are identical. We, therefore, conclude that the observed differences in binding affinity are the result of the diastereoisomeric heterogeneity of the d-T_8·Et sample.

The octamer triester was also fractionated by cochromatography with poly(riboadenylic acid). Chromatography of an equimolar mixture of $(rA)_n$ and d-T_8·Et (unfractionated) on Sephadex G-50 in 0.001 M sodium cacodylate (pH 6.9), at 2 °C, followed by rechromatography of the high molecular weight peak at 20 °C yielded a small amount (7% of input d-T_8·Et) of d-T_8·Et[2-20 °C]$_{rA}$. Rechromatography of the high molecular weight peak of the second chromatography at 40 °C did not release more octamer. In 0.1 M sodium cacodylate (pH 6.9), an equimolar mixture of $(dA)_n$ and d-T_8·Et[2-20 °C]$_{rA}$ at 10^{-4} M total concentration exhibited a $T_{0.9}$ of 28.1 °C (data not shown), two degrees higher than the $T_{0.9}$ value for the $(dA)_n$ + d-T_8·Et (20-30 °C) mixture.

For a determination of the enthalpy changes in the interaction of $(dA)_n$ with various octamer triester fractions in 0.1 M sodium cacodylate, we followed the method developed by Damle (1970) and by Crothers (1971), which has previously been applied to the oligo(I)·poly(C) system in this laboratory (Tazawa et al., 1972). This method calls for the plotting of the reciprocal of T_m (on the absolute scale) vs. the logarithm of the free oligomer concentration at T_m, and determination of the enthalpy of binding from the slope via the equation $n\Delta H = 2.303R\Delta \log c_m/\Delta(1/T_m)$, where n is the degree of polymerization of the oligomer. In order to be consistent in the determination of ΔH values for this system and the $(dA)_n$ + d-T_8 system to be described below, we chose to obtain the binding enthalpy via $n\Delta H = 2.303R\Delta \log c_{0.9}/\Delta(1/T_{0.9})$ from a plot of $1/T_{0.9}$ (10% hypochromicity) vs. log $c_{0.9}$. The justification for this change from $c_m - T_m$ to $c_{0.9} - T_{0.9}$ is given in the Discussion.

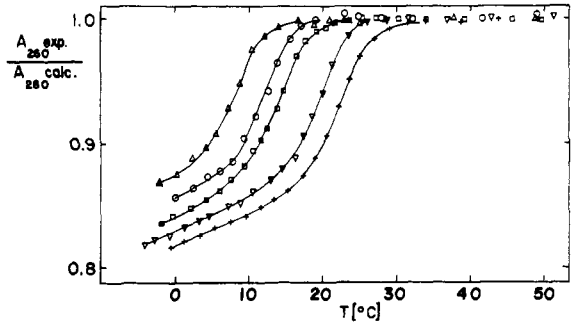


FIGURE 7: Concentration dependence of the annealing of $(dA)_n$ + d-T_8 (1:1) in 0.1 M sodium cacodylate (pH 6.9): (△) 0.097 × 10^{-4} M; (○) 0.368 × 10^{-4} M; (□) 1.02 × 10^{-4} M; (▽) 5.01 × 10^{-4} M; (+) 16.3 × 10^{-4} M.

Figure 5 presents annealing curves for equimolar mixtures of $(dA)_n$ + d-T_8·Et (20-30 °C) in 0.1 M sodium cacodylate at five different total concentrations, from 10^{-5} to 5.3 × 10^{-4} M. The plot of $1/T_{0.9}$ vs. log ($c_{0.9}$/1 M) (Figure 6) gives a straight line yielding a value of $\Delta H = -8.6$ kcal/mol of base pairs. For the interaction of d-T_8·Et [2-20 °C]$_{rA}$ with $(dA)_n$ the concentration dependence of $T_{0.9}$ (determined for 1.00 × 10^{-4}, 0.29 × 10^{-4}, and 0.084 × 10^{-4} M equimolar solutions, data not shown) gave a ΔH value of −8.6 kcal/mol of base pairs (Figure 6). The determination of the enthalpy change for the interaction of $(dA)_n$ + d-T_8·Et (7 °C) was similarly undertaken, using concentrations from 7.2 × 10^{-6} to 3.9 × 10^{-4} M (Figure 5). In a plot of $1/T_{0.9}$ vs. log ($c_{0.9}$/1 M), the point corresponding to $c_{total} = 7.2 \times 10^{-6}$ M falls off the straight line well defined by the other three points (Figure 6). Repeated measurements at this concentration gave identical results. A similar deviation to higher values of $1/T_m$ at low temperatures is seen in a $1/T_m$ vs. log c plot published by Martin et al. (1971). Since this point was measured at −5 °C, this phenomenon may reflect changes in the water structure at temperatures around 0 °C. The straight line in Figure 6 gives a ΔH value of −8.1 kcal/mol of base pairs.

For comparison, the interaction of the octamer diester d-T_8 (= d-Tp)$_7$T) with $(dA)_n$ in 0.1 M sodium cacodylate (pH 6.9) was studied. Annealing curves for five equimolar mixtures, at total concentrations ranging from 9.7 × 10^{-6} to 1.6 × 10^{-3} M, were determined (Figure 7), and a plot of $1/T_{0.9}$ vs. log ($c_{0.9}$/1

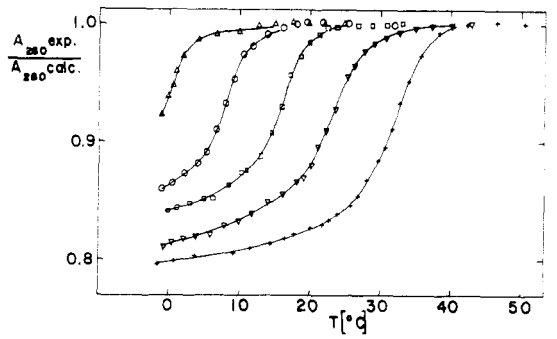


FIGURE 8: Salt dependence of the annealing of $(dA)_n$ + d-T_8 (1:1), 10^{-4} M, pH 6.9: (△) 0.01 M Na^+; (○) 0.04 M Na^+; (□) 0.11 M Na^+; (▽) 0.31 M Na^+; (+) 1.01 M Na^+.

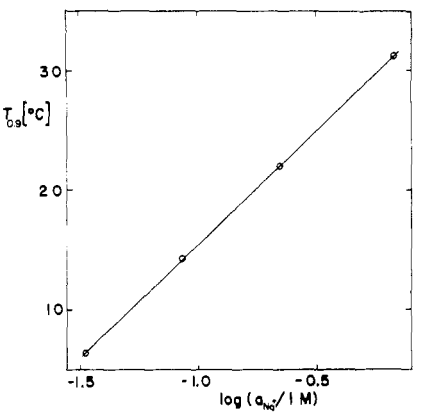


FIGURE 9: Salt dependence of the annealing of $(dA)_n$ + d-T_8 (1:1).

M) gave a ΔH value of −6.8 kcal/mol of base pairs (Figure 6).

The salt dependence of the interaction of d-T_8 with $(dA)_n$ was studied by annealing equimolar mixtures, at 10^{-4} M concentration, in a series of buffers containing 0.01 M sodium cacodylate and varying concentrations of sodium chloride, at pH 6.9 (Figure 8). The calculation of sodium ion activities was based on data given by Robinson and Stokes (1959). Sodium cacodylate at 0.01 M was treated as having the same activity coefficient as sodium chloride, an assumption that was verified by measurements with a sodium ion specific electrode (Beckman No. 39278). Figure 9 presents a plot of $T_{0.9}$ vs. the decadic logarithm of the sodium ion activity for sodium ion concentrations from 0.04 to 1.01 M. The straight line has a slope of 18.8°.

In contrast to the interaction between $(dA)_n$ and d-T_8, the binding of the octamer triester to $(dA)_n$ is hardly affected by changes in ionic strength. As seen in Figure 10, the annealing curves for $(dA)_n$ + d-T_8·Et (20–30 °C) (1:1) at sodium ion concentrations of 0.29 and 0.10 M are superimposable ($T_{0.9}$ = 26.0 °C), while at 0.013 M sodium ion concentration the transition is slightly shifted to higher temperatures ($T_{0.9}$ = 26.4 °C).

Comparing the binding of d-T_8 and of d-T_8·Et with $(dA)_n$ to their interaction with the ribopolymer, $(rA)_n$, considerable differences were found. In 0.1 M sodium cacodylate, at 10^{-4} M total concentration, the equimolar mixtures $(rA)_n$ + d-T_8 and $(rA)_n$ + d-T_8·Et (unfractionated) have $T_{0.9}$ values of 8.6 and ca. −11 °C, respectively (Figure 11). Thus, the octamer triester has a much weaker interaction with the ribopolymer than does the diester. In contrast, $(dA)_n$ + d-T_8 and $(dA)_n$ + d-T_8·Et (unfractionated) had $T_{0.9}$ values of 11.3 and 19.0 °C

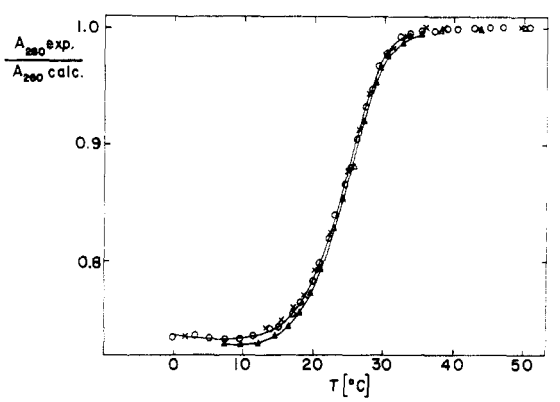


FIGURE 10: Salt dependence of the annealing of $(dA)_n$ + d-T_8·Et (20–30 °C) (1:1), 10^{-4} M, pH 6.9: (△) 0.013 M sodium cacodylate; (○) 0.10 M sodium cacodylate; (×) 0.27 M NaCl–0.02 M sodium cacodylate.

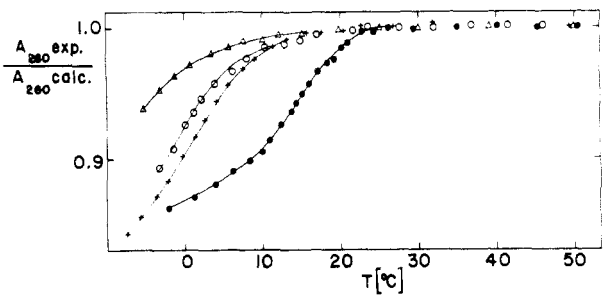


FIGURE 11: Annealing of $(rA)_n$ with d-T_8 and with various fractions of d-T_8·Et in 0.1 M sodium cacodylate (pH 6.9); total concentration 10^{-4} M: (△) $(rA)_n$ + d-T_8·Et (unfractionated) (1:1); (○) $(rA)_n$ + d-T_8·Et (20–30 °C) (1:1); (+) $(rA)_n$ + d-T_8·Et [2–20 °C]$_{rA}$ (1:1); (●) $(rA)_n$ + d-T_8 (1:1).

(Figures 7 and 4), i.e. the octamer triester binds more efficiently to $(dA)_n$ than does the diester. Put in different terms, while the transitions for $(rA)_n$ + d-T_8 and for $(dA)_n$ + d-T_8 occur at approximately the same temperature, the transitions for $(rA)_n$ + d-T_8·Et (unfractionated) and for $(dA)_n$ + d-T_8·Et (unfractionated) are separated by ~30 °C.

Figure 11 also shows the curve for $(rA)_n$ + d-T_8·Et (20–30 °C) and for $(rA)_n$ + d-T_8·Et [2–20 °C]$_{rA}$, with $T_{0.9}$ values of −2.6 and −0.2 °C. Thus, the affinity of the d-T_8·Et fractions for binding to $(rA)_n$ varies parallelly to their affinity for $(dA)_n$, but the latter transitions are shifted by ~30 °C with respect to the former.

Finally, to study the possible addition of the octamer triester to the dA·dT double helix, d-T_8·Et (20–30 °C) was added to preannealed $(dA)_n$·$(dT)_n$ to give a solution 0.33 × 10^{-4} M in each component, in 0.03 M sodium cacodylate (pH 6.9). No hypochromicity was observed for temperatures down to −5 °C and, upon heating, a single transition occurred at 56 °C, the melting temperature of $(dA)_n$·$(dT)_n$ in this buffer. This indicates that the octamer triester cannot participate in forming a dA·dT·dT type triple helix.

Discussion

As shown in the Results section, the octamer triester sample is heterogeneous with respect to its binding affinity for $(dA)_n$, and we ascribed this phenomenon to the diastereoisomerism introduced on ethylation of the phosphate group. The number of diastereoisomers in unfractionated d-T_8·Et should be large (ideally 2^7 = 128, if every ethylation can take place at either of the two phosphate oxygens and if no diastereoisomers were

eliminated in the purification steps during the course of the synthesis). It follows that any of the d-T$_8$·Et fractions obtained by cochromatography with (dA)$_n$ at arbitrarily chosen temperatures should still be a mixture of diastereoisomers, albeit of smaller multiplicity and with a narrower range of affinity for (dA)$_n$. Therefore, it remains to be demonstrated that the method of ΔH determination developed by Damle (1970) and by Crothers (1971) for the interaction of a homopolymer with a homogeneous population of oligonucleotides will yield meaningful ΔH values when applied to our case, where a heterogeneous mixture of complementary oligomers competes for identical sites on the polymer.

For two octamer triester fractions (d-T$_8$·Et (<7 °C) and d-T$_8$·Et (20–30 °C) with widely varying affinity for (dA)$_n$, Damle's method gives average ΔH values of −8.1 and −8.6 kcal/mol of base pairs, respectively. Below, we shall ascribe this small variation in ΔH to the difference in transition temperatures (~0 °C vs. ~25 °C). Therefore, for the octamer triester fractions with their narrow temperature transitions, the ΔH for the various constituent diastereoisomers should be nearly identical. The use of Damle's and Crother's method is therefore warranted, provided that the relative proportion of the diastereoisomers and the input ratio of total d-T$_8$·Et to (dA)$_n$ are invariant for all concentration variation experiments.

A more formalistic approach to this probelem is given by Wyman (1964) in treating the case of two competing ligands. His equation $\Delta H_Y = R(\mathrm{d} \ln y/\mathrm{d}(1/T))_{\overline{XY}}$ is analogous to eq 55 of Damle and eq 62 of Crothers in that the enthalpy of binding of ligand Y is calculated from the slope of the function relating the logarithm of the free ligand activity y to the inverse of absolute temperature at a constant degree of saturation ($\overline{Y}$) of the matrix with ligand Y, but it contains the additional stipulation that the degree of saturation ($\overline{X}$) of the matrix with the competing ligand X also be constant. This requirement is satisfied if the binding enthalpies of both ligands are similar and the input ratios of both ligands to the polymer are kept constant throughout the entire set of binding experiments, i.e. if these conditions are fulfilled, constant ($\overline{X} + \overline{Y}$) implies constant $\overline{X}$ and constant $\overline{Y}$. Furthermore, under these conditions, a constant value of hypochromicity is correlated with a constant ratio of free ligand concentrations; thus, at constant hypochromicity the concentration of any particular free ligand is linearly related to the total ligand input concentration and $\Delta \ln y$ is simply calculated as $\Delta \ln (c_{\mathrm{total}})$.

As seen from Figure 5, the temperature absorbance profiles for the interaction of (dA)$_n$ with a given octamer fraction are reasonably parallel. Therefore, the ΔH value determined from measurements of $T_{0.9}$ is representative for the binding enthalpy for all isomers contained in the fraction.

The above considerations vindicate the use of $T_{0.9}$ instead of T_m in the quantitative examination of the (dA)$_n$ + d-T$_8$·Et transition, since the midpoint of the observed transition loses its usual meaning where a heterogeneous population of oligomers is concerned. We have also used $T_{0.9}$ in the treatment of the (dA)$_n$ + d-T$_8$ transitions, for the following reason. Cassani and Bollum (1969) reported exclusive formation of duplex on interaction of (dA)$_n$ with d-(pT)$_{10}$ or d-(pT)$_{11}$ at 23 °C, even at 1 M LiCl or in magnesium containing buffer. In contrast, Tamblyn and Wells (1975) indicate that in magnesium containing buffer a (dA)$_n$ + (dT)$_{10}$ 1:1 mixture contains both duplex and triplex at 25 °C. Pörschke (1971) made similar observations for (rA)$_{18}$ + (rU)$_{18}$ in 0.5 M NaCl. The low value of hypochromicity and the broad appearance of the lower temperature portion of our (dA)$_n$ + d-T$_8$ transition profiles suggest that partial formation of triplex structures might occur at low temperatures. In order to avoid this possible complication and to limit the measurements to the 2→1 transition, only the steep high-temperature portion of the transition curve (e.g., $T_{0.9}$) was used. The value of $\mathrm{d}T_{0.9}/\mathrm{d} \log a_{\mathrm{Na}^+}$ of 18.8 °C indicates that it is indeed the 2→1 transition that is observed in this region. It should be observed that for a calculation of ΔH, Damle's method requires the correlation of temperature and free ligand concentration at a constant degree of matrix saturation, not necessarily at exactly half-saturation; thus, the use of $T_{0.9}$ instead of T_m is warranted.

In the calculation of $c_{0.9}$ we assumed the hypochromicity attendant on total duplex formation to be 26%, with 10% hypochromicity impling 38% duplex formation. An error in this assumption would leave the calculated ΔH values unaffected, since, at stoichiometric input, the error would add a constant term to the log $c_{0.9}$ values. The same holds true for our substitution of monomeric concentration values instead of octamer concentrations, which results in a constant shift of log 8 in the $1/T_{0.9}$ vs. log ($C_{1/9}$/1 M) plots.

We shall examine the salt dependence of duplex formation according to the method of Manning (1972). For the interaction of (dA)$_n$ with d-T$_8$·Et (20–30 °C) we found that $\mathrm{d}T_{0.9}/\mathrm{d} \log a_{\mathrm{Na}^+}$ is zero (Figure 10). According to Manning's eq 28 ($\mathrm{d}T_m/\mathrm{d} \log m_{\mathrm{Na}^+} = 1.15\eta RT_m^2/\Delta H$) we find $\eta = 0$. For the reaction (dA)$_n$·d-T$_8$·Et → (dA)$_n$ + d-T$_8$·Et, the proper form of η is $0.5\xi_A^{-1} - 0.5\xi_{A\cdot TEt}^{-1}$ so that $\eta = 0$ implies that at 26 °C the average linear charge spacings are the same in single-stranded (dA)$_n$ and in (dA)$_n$·d-T$_8$·Et. Our circular dichroic (CD) results indicate the (dA)$_n$·d-T$_8$·Et duplex to have the B-type conformation. With a rise per residue of 3.3 Å for the (dA)$_n$·(dT)$_n$ double helix (Arnott and Selsing, 1974), the average charge spacing in the (dA)$_n$·d-T$_8$·Et duplex is 3.3 Å (not 1.65 Å, since the T strand is electroneutral). We can therefore state that at 26 °C the linear phosphate spacing in (dA)$_n$, which is the same as the linear charge spacing, is 3.3 Å. This value can be compared with the rod length of 3.2 Å/nucleotide for single-stranded (rA)$_n$ at low temperature determined from light scattering measurements (Stannard and Felsenfeld, 1975).

The value of $\mathrm{d}T_{0.9}/\mathrm{d} \log a_{\mathrm{Na}^+}$ obtained for the interaction of (dA)$_n$ with d-T$_8$, 18.8 °C, is close to the $\mathrm{d}T_m/\mathrm{d} \log a_{\mathrm{Na}^+}$ values of 20.5 °C found for (dA)$_n$ + (dT)$_n$ (Riley et al., 1966; Burd et al., 1975) and 18.8 °C found for (dA)$_n$ + (dT)$_{25}$ (Burd et al., 1975). Using ΔH = −6.8 kcal/mol of base pairs (changed to 3.4 kcal/mol of nucleotide, to conform to Manning's notation), T_m = 284 K, and $\mathrm{d}T_m/\mathrm{d} \log m_{\mathrm{Na}^+}$ = 18.8 °C, we obtain a value of η = 0.34 via Manning's eq 28. The proper form of η to use in this case is $0.5\xi_A^{-1} + 0.5\xi_T^{-1} - \xi_{A\cdot T}^{-1}$ (see Record et al. (1976) for the case of (A)$_n$·(U)$_n$ → (A)$_n$ + (U)$_n$). Using linear phosphate spacings of 3.3 Å for (dA)$_n$ and 1.65 Å for (dA)$_n$v2d-T$_8$, and the $e^2/\epsilon kT$ value of 7.1 Å, a phosphate spacing of 4.9 Å is calculated for single-stranded d-T$_8$, somewhat higher than the values of 4.4 Å for (rU)$_n$ (Record et al., 1976) and 4.7 Å for single-stranded DNA (Rix-Montel et al., 1974) calculated by analogous procedures. It should be emphasized that at the high sodium ion concentrations (0.04 to 1 M) used in our determination of $\mathrm{d}T_m/\mathrm{d} \log a_{\mathrm{Na}^+}$, the length of the octamer is much greater than the Debye screening length, so that the use of polyelectrolyte theory is justified.

The enthalpy of duplex formation was calculated to be −8.6 kcal/mol of base pairs for (dA)$_n$. d-T$_8$·Et (20–30 °C) and for (dA)$_n$ + d-T$_8$·Et [2–20 °C]$_{rA}$, while for (dA)$_n$ + d-T$_8$·Et (<7 °C) it was found to be −8.1 kcal/mol of base pairs. This dif-

ference in reaction enthalpy (0.5 kcal) can largely be attributed to the difference in the transition temperatures (~25 °C as opposed to 0 °C), since at lower temperature the stacking process in the $(dA)_n$ single strand will be further advanced and therefore the change in stacking enthalpy attendant on duplex formation will be smaller. For instance, for $(rA)_n + (rU)_n$ in 0.1 M Na^+ Ross and Scruggs (1965) measured ΔH values of −6.0 and −6.5 kcal/mol of base pairs at 24 and 37 °C, respectively.

A considerably lower value, −6.8 kcal/mol of base pairs, was measured for the enthalpy of duplex formation from $(dA)_n$ + d-T_8. The difference in ΔH of about 1.6 kcal/mol of base pairs (−6.8 kcal/mol at 11 °C vs. −8.4 kcal/mol, interpolated for 11 °C) is the enthalpic effect of changing the charged octamer to the electroneutral octamer. For comparison, Delisi and Crothers (1971) calculated an electrostatic enthalpy effect of 0.8 kcal/mol of base pairs for formation of double-stranded helices from polynucleotides.

Since the transitions occurred at comparable temperatures, the large difference in enthalpy change between $(dA)_n$ + d-T_8·Et and $(dA)_n$ + d-T_8 must be compensated by a correspondingly large difference in entropy change. ΔS values were calculated for all observed transitions using the relation $\Delta S = \Delta H/T_m$, giving values on the order of −200 cal (mol of octamer)$^{-1}$ deg^{-1}. Since the entropy change for oligomer-polymer interactions depends on the oligomer concentration, it was necessary to subtract the cratic entropy term to arrive at comparable values. ΔS_{cratic} was calculated as $R \ln (V_N^f C_{0.9})$ setting V_N^f equal to 10 Å^3/molecule of octamer. From the oligomer-polymer data reviewed by Blake (1972), this is a reasonable estimate of V_N^f for octamer-polymer interactions.

After subtraction of the cratic entropy values (ranging from 29 to 40 cal (mol of octamer)$^{-1}$ deg^{-1}) and division by eight, we obtain ΔS values of −25.0, −24.4, −24.3, and −19.5 cal (mol of base pair)$^{-1}$ deg^{-1} for duplex formation of $(dA)_n$ with d-T_8·Et (<7 °C), d-T_8·Et (20–30 °C), d-T_8·Et[2–20 °C]$_{rA}$, and d-T_8, respectively. These values fall into the range of entropy changes usually observed for duplex formation from polynucleotides. The error introduced by the estimation of V_N^f will only insignificantly affect the comparison of the ΔS values, as we used the same V_N^f value for the various complexes.

Neglecting the minor variations in ΔS for the interaction of $(dA)_n$ with the various fractions of d-T_8·Et, the ΔS value for the association of $(dA)_n$ with d-T_8 is about 5 cal (mol of base pairs)$^{-1}$ deg^{-1} more favorable than the ΔS value for the association of $(dA)_n$ with d-T_8·Et. This difference in ΔS can arise from either $\Delta S_{config.}$ or $\Delta S_{elec.}$ or both. Simple reaoning would suggest that the electrostatic entropy change upon duplex formation from two negatively charged polyelectrolytes would be less favorable than the electrostatic entropy change attendant on duplex formation from one charged polyelectrolyte and one nonionic polymer. If this reasoning is correct, the $\Delta S_{config.}$ for $(dA)_n$·d-T_8·Et formation should compare even more unfavorably to that for $(dA)_n$·d-T_8 formation than indicated by the value of 5 cal (mol of base pairs)$^{-1}$ deg^{-1}. As calculated in the following paragraph, the difference for $\Delta S_{config.}$ would be about 7 cal (mol of base pairs)$^{-1}$ deg^{-1}.

For an estimate of the $\Delta S_{elec.}$ values we used Manning's (1972) eq 23: $\Delta S - \Delta S° = -R\mathrm{d}(\alpha T/\mathrm{d}T) - 1.15R \log m_{Na^+}$. The required $\mathrm{d}\alpha/\mathrm{d}T$ erms were calculated from the observed difference of enthalpies of duplex formation, 1.6 kcal/mol of base pairs, according to Manning's eq 22: $\Delta H - \Delta H° = R\mathrm{d}\alpha/\mathrm{d}(1/T)$; this means that we use the octamer triester as the proper model for uncharged octamer diester, at least as far as enthalpy changes are concerned. Values for α were calculated according to Manning's eq A8 in the proper form, using the linear charge spacings reported above and values of a = 10 Å, a_1 = 13 Å, and a_2 = 14 Å for both duplexes and for $(dA)_n$, and a = 5 Å, a_1 = 8 Å, and a_2 = 9 Å for single-stranded d-T_8. The a values are radial parameters for the cylindrical DNA models used in Manning's calculation. The results are $\Delta S_{elec.} = -1.6$ cal (mol of base pairs)$^{-1}$ deg^{-1} for $(dA)_n$ + d-T_8 and $\Delta S_{elec.} = 0$ for $(dA)_n$ + d-T_8·Et, both values calculated for 0.1 M salt. For comparison, we find $\Delta S_{elec.} = -2.7$ cal (mol of base pairs)$^{-1}$ deg^{-1} from the ΔH_{el} and $\Delta G_{elec.}$ values calculated by Nagasawa and Muroga (1972) for T_2 phage DNA in 0.115 M NaCl. After subtracting the electrostatic entropy terms, we obtain $\Delta S_{config.}$ values of ca. −18 and −25 cal (mol of base pairs)$^{-1}$ deg^{-1}, respectively.

This difference of roughly 7 eu represents the difference between the configurational entropy changes for the $(dA)_n$ + d-T*n*8 and $(dA)_n$ + d-T_8·Et transitions. Upon duplex formation, the octamer triester has one potential configurational restriction which is absent in the diester case: confinement of the rotation of the ethyl group. We shall distinguish inward phosphate groups, directed toward the helix axis in the duplex (with a Cahn-Ingold-Prelog *S* configuration around the phosphorus atom, assuming a P=O double bond), and outward phosphate ethyl groups, directed outward (*R* configuration). Examining a Kendrew molecular model of the B-type duplex we found that for various rotamers the distances of closest approach between the β carbon of the inward ethyl group and C-2′ (0.2 Å), H-3′ (0.4 Å), 5-methyl (0.7 Å), and H-6 (0.3 Å) are much smaller than the sum of the van der Waals radii (4.0 Å for C↔C; 3.2 Å for C↔H). The outward ethyl group showed possible overlap of the β-methyl group with H-3′ (1.2 Å) and H-5′ (0.8 Å). Considering the closest approach between the β carbons of consecutive ethyl substituents, and counting from the 5′ end to the 3′ end, we find little or no contact for the sequences outward ethyl-outward ethyl (2.5 Å) and inward-outward (3.9 Å) but considerable overlap for the inward-inward (1.1 Å) and outward-inward sequences (1.0 Å).

On account of the fluid character of the geometry of single strands we have not examined the rotational freedom of their ethyl groups with the aid of Kendrew models. However, the fixation of geometry upon double helix formation should result in a considerable reduction in rotational freedom, and this effect should be more pronounced in the case of the inward ethyl groups, as relates to interaction with 5-methyl and H-6 of the thymine ring, which is locked into the anti conformation in the duplex, and to contacts between inward ethyl groups, which should be more severe in the duplex than in the more extended single strand. One can thus interpret the observed differences in stability of the $(dA)_n$·d-T_8·Et duplexes on the basis of a variation in the relative proportions of inward and outward ethyl substituents (d-T_8·Et (20–30 °C) and d-T_8·Et [2–20 °C]$_{rA}$ having more outward ethyl groups than does d-T_8·Et (<7 °C) and to differences in sequence of ethyl positions (outward-outward, inward-outward, etc.); i.e. the difference in helix stability has entropic reasons, in accord with our observed variation of ΔS values from −24.3 to −25.0 cal (mol of base pairs)$^{-1}$ deg^{-1}.

We have observed that the stability of $(rA)_n$·d-T_8·Et complexes is markedly lower than that of the corresponding $(dA)_n$·d-T_8·Et complexes. We have furthermore found no evidence for formation of dA·dT·dT type triplexes involving the octamer triesters, either in the mixing curve from $(dA)_n$ and d-T_8·Et (unfractionated) or in the $(dA)_n$·$(dT)_n$ + d-T_8·Et

(20–30 °C) experiment. As already discussed, such triplex structures from poly(dA) and oligo(dT) have been observed at low temperatures and in our experiments their formation should have been favored: in the mixing experiments because the two oligomer strands are uncharged and in the $(dA)_n$·$(dT)_n$ + d-T_8·Et experiment because only one, uncharged, strand would be oligomeric. Since recent x-ray crystallographic studies have shown the $(dA)_n$·$2(dT)_n$ triplex to have A-type geometry (Arnott and Selsing, 1974) we attribute the low stability of $(rA)_n$·d-T_8·Et and the failure to form triple-stranded structures to a bias of the octamer triester against participation in A-type helical complexes. Inspection of a Kendrew model for a double helix in A′ conformation again indicates severe restriction to rotation for the inward ethyl group. In many rotamers, the outward ethyl group has closer contact with H-5′ than it would have in the B conformation. The distances of closest approach of β carbons of adjacent ethyl groups show moderate contact in the inward–outward sequence (C↔C = 2.0 Å) and severe contact in all other combinations (outward–outward, 0.6 Å; inward–outward, overlap by 0.3 Å; outward–inward, overlap by 0.7 Å). Thus, the outward ethyl position again appears to be more favorable for duplex formation, but both inward and outward substituents are rotationally more hindered in the A′ helix than in the B form. This would explain the parallel downward shifting of the T_m values for the various d-T_8·Et fractions on changing from $(dA)_n$ to $(rA)_n$.

These observations are not in contradiction with earlier results (Miller et al., 1971) on the formation of A-type 2:1 complexes between $(rU)_n$ and alkylated deoxyadenosine dimers. Kendrew models indicate that in the A′ conformation the contact of the inward ethyl group with the adenine H-8 (C↔H = 1.1 Å) is less severe than with the thymine 5-methyl group (C↔C = 0.6 Å). Furthermore, since the deoxyadenosine dimers (e.g., dAp(Et)dA) were not terminally phosphorylated, the restriction per base pair is reduced by a factor of two, and interference between contiguous phosphate ethyl groups is entirely absent. It must also be pointed out that with the ethyl ester a lower T_m was observed than with the methyl ester, although both T_m values exceed the T_m for dApdA·$2(rU)_n$.

Precedent for destabilization of the helix by minor changes in substituent size can be found in the literature. Kulikowski and Shugar (1974) measured T_m values of 78 and 66 °C, respectively, for $(rI)_n$·$(5\text{-Me-rC})_n$ and $(rI)_n$·$(5\text{-Et-rC})_n$ in 0.1 M NaCl. Similar reductions in T_m on increasing the size of the 5-pyrimidine substituent from methyl to ethyl were reported for $(rA)_n$·$2(rT)_n$ (Swierkowski and Shugar, 1970), $(dA)_n$·$2(rT)_n$ (Barszcz and Shugar, 1968), and $(rX)_n$·$(rT)_n$ (Fikus and Shugar, 1969). In the case of $(dAT)_n$·$(dAT)_n$, a decrease in T_m of 4 °C was observed on changing the pyrimidine 5-methyl group to a hydroxymethyl group (Cassidy et al., 1965). As previously stated (Swierkowski and Shugar, 1970), the ethyl group can be accommodated without distortion of the double helix; in this process, however, it will lose much of the rotational freedom which it possesses in the single-stranded state. For instance, the distance of closest approach between the β carbon and the plane of the 5′-neighboring pyrimidine is 1.5 Å, while the sum of the van der Waals radii is 3.85 Å. The presence of the ethyl substitutuent should therefore contribute an additional negative term to the entropy of the coil-to-helix transition.

In conclusion, the electrostatic interaction of double helical nucleic acids has been the focus of considerable interest (Schildkraut and Lifson, 1965; Delisi and Crothers, 1971; Krakauer, 1974; Record et al., 1976). In the present investigation, a novel approach to this problem was adopted: transformation of one negatively charged strand (d-T_8 in this case) into a nonionic strand. Measurements of the enthalpy changes by the concentration variation method indicate that in 0.1 M salt, at 11 °C, there is a difference of 1.6 ± 0.4 kcal/mol of base pairs between duplex formation from two charged strands ($(dA)_n$ + d-T_8, $\Delta H = -6.8$ kcal) and duplex formation from one charged strand and one nonionic strand ($(dA)_n$ + d-T_8·Et, $\Delta H = -8.4$ kcal). This value of 1.6 kcal is a measure of the electrostatic enthalpy attendant on formation of the double helix in B conformation. It should consist of a term for the contraction of one of the two charged strands (d-T_8) from a linear phosphate spacing of about 4.9 to 3.3 Å upon duplex formation, and a term for the interpenetration of the two polyelectrolytes, since the electrostatic effects of these two processes were eliminated on substituting the octamer triester for the diester. The term corresponding to the contraction of the second strand is not included. In the specific instance of formation of $(dA)_n$·d-T_8 this term should be negligible, since the single-stranded $(dA)_n$ is already fully contracted (linear phosphate spacing of 3.3 Å) in 0.1 M salt at 26 °C. In duplex formation from two extended single strands, however, the contraction of the second strand is expected to contribute a further term to the electrostatic enthalpy change, and $\Delta H_{elec.}$ could be higher than 1.6 kcal/mol of base pairs.

The stability of the duplex containing the phosphotriester oligomer, $(dA)_n$·d-T_8·Et, is lowered by a special entropic effect, which is not present in the interaction of $(dA)_n$ with d-T_8. The ΔS value characterizing this effect was estimated to be about -7 cal (mol of base pairs)$^{-1}$ deg^{-1}. The study of molecular models suggests that this phenomenon is due to a loss of rotational freedom of the phosphate ethyl groups upon duplex formation, and that this effect is more pronounced in the formation of the double helix in the A form than in the B form. In this context, the presence of the thymine 5-methyl group may be of major importance. Oligo(2′-*O*-methyluridylate) ethyl esters are currently being synthesized in this laboratory. These compounds lack the pyrimidine 5-methyl substituent, and their conformation in the single-stranded state may differ from that of the octadeoxythymidylate triesters. The study of their interaction with polyadenylates should therefore allow the assessment of the importance of these structural features for the stability of the complexes described in this paper.

Experimental Section

Materials

5′-*O*-Mono-*p*-methoxytritylthymidine (Schaller et al., 1963), 3′-*O*-acetylthymidine 5′-phosphate (Khorana and Vizsolyi, 1961), and 5′-*O*-isobutyloxycarbonyl-3′-*O*-mono-*p*-methoxytritylthymidine (Ogilvie and Letsinger, 1967) were synthesized by published methods. 1-β-D-Ribofuranosyl-4-methoxy-2-pyrimidinone was kindly donated by Dr. M. Robins. Mesitylenesulfonyl chloride and *p*-toluenesulfonyl chloride were recrystallized from hexane and pentane, respectively, after Norit treatment. Pyridine, 2,6-lutidine, *N*,*N*-dimethylformamide, and ethanol were rendered anhydrous as previously described (Miller et al., 1974). *N*-Methylimidazole (Aldrich Chemical Co.) was distilled at reduced pressure before use. Anhydrous dioxane was obtained by distillation from lithium aluminum hydride onto Linde Molecular Sieves 4A. Poly(deoxyadenylic acid) ($s_{20,w}$ = 6.5), poly(adenylic acid) ($s_{20,w}$ = 9.5) and poly(deoxythymidylic acid) ($s_{20,w}$ = 7.0) were purchased from P-L Biochemicals, Inc.

Methods

Descending paper chromatography on Whatman No. 3MM was performed in the following solvent systems: solvent A, 2-propanol–concentrated ammonia–water (70:10:20); solvent C, 1 M ammonium acetate–ethanol (30:70), pH 7.5; solvent F, 1-propanol–concentrated ammonia–water (55:10:35); solvent H, 1-butanol–water (86:14); solvent I′, 2-propanol–water (85:15). Thin-layer chromatography was carried out by the ascending technique on silica gel (Eastman 13181) or cellulose (MN polygram CEL 400). Paper electrophoresis was performed on a Savant flat plate apparatus using 0.05 M triethylammonium bicarbonate (pH 8.5) as a buffer, at a voltage gradient of 40 V/cm. For high-pressure liquid chromatography a Laboratory Data Control liquid chromatograph was used. The ^{1}H NMR spectrum was measured at room temperature on a Jeolco FX 100 spectrometer in Fourier transform (FT) mode. The sample was a 7 mM solution of d-MTr[Tp(Et)]$_7$T in dimethyl-d_6 sulfoxide (99.5%, Diaprep Inc.).

Ultraviolet circular dichroism spectra were measured on a Cary 60 spectropolarimeter equipped with a 6001 CD unit; ultraviolet absorption spectra were measured on a Cary 15 spectrophotometer. Temperature–absorbance profiles were obtained as previously described (Tazawa et al., 1970). In view of the adsorption of the octamer triester to glass surfaces, mixtures of d-T$_8$-Et and polyadenylates were prepared by diluting the octamer to the required absorbance in the cuvette and adding the calculated amount of polyadenylate at the appropriate concentration. This method avoids transfers of the solution after the concentration of the octamer has been determined. Solutions were prepared at 24 °C using the following maximum extinction coefficients: $(dA)_n$, 9100; $(rA)_n$, 10 100; d-(Tp)$_7$T, 8700; d-Tp(Et)$_7$T, 8700 M^{-1} cm^{-1}.

Synthesis

Unless indicated otherwise, all reactions were performed at room temperature.

Preparation of d-MTrTp(Et)TOAc. This material was prepared as reported by Miller et al. (1974) except that in the ethylation step *N*-methylimidazole-2,6-lutidine (10:1 on a molar basis) was substituted for lutidine as the base. The yields for condensation and ethylation were 72 and 78%, respectively.

Deacetylation of d-MTrTp(Et)TOAc. d-MTrTp(Et)TOAc (2.47 g, 2.78 mmol) was dissolved in 160 mL of pyridine–concentrated aqueous ammonia (1:1, v/v). After 26 h, the solvents were evaporated and the residue was chromatographed on a silica gel column (2.8 cm × 50 cm). Ethyl acetate eluted a small amount of starting material; then the desired product was eluted with tetrahydrofuran–ethyl acetate (1:1). The product was precipitated from tetrahydrofuran–hexane: 2.33 g (2.75 mmol, 99% yield) of d-MTrTp(Et)T, homogeneous on silica gel TLC in tetrahydrofuran/EtOAc (1:1) (R_f 0.37) and in $CHCl_3$/MeOH (9:1) (R_f 0.43).

Detritylation of d-MTrTp(Et)TOAc. d-MTrTp(Et)TOAc (7.44 g, 8.37 mmol) was dissolved in 130 mL of 80% aqueous acetic acid. After 4 h, the solution was concentrated to dryness, and the residue was chromatographed on a silica gel column (4.5 cm × 70 cm). A small amount of starting material was eluted with ethyl acetate; then, the detritylated material was eluted with tetrahydrofuran–ethyl acetate (1:1). Judging from UV absorption, the appropriate fractions contained 7.86 mmol (94% yield) of d-Tp(Et)TOAc, chromatographically homogeneous on cellulose TLC in solvents A (R_f 0.76) and C (R_f 0.88). This material was used, without isolation, in the next step.

Cyanoethylphosphorylation of d-Tp(Et)TOAc. A mixture of 7.82 mmol of d-Tp(Et)TOAc and 23 mmol of pyridinium β-cyanoethyl phosphate was dried by evaporation of pyridine and treated with *N,N′*-dicyclohexylcarbodiimide (10 mL, 47 mmol) in 75 mL of pyridine for 31 h. The solution was treated with 30 mL of water at 0 °C, and the dicyclohexylurea was filtered off on glass. The filtrate was chromatographed on a column (2.7 cm × 48 cm) of DEAE-Sephadex, using a linear gradient of triethylammonium bicarbonate in water–ethanol (9:1) from 0.001 to 0.25 M (3 L total). The major peak eluted at 0.16 M salt and was determined to contain 6.68 mmol of d-CEpTp(Et)TOAc (85% yield) by UV absorption. The material was homogeneous on paper electrophoresis at pH 8.4 (R_m^{pT} = 0.28) but showed two ill-resolved bands (presumably the two diastereoisomers) of approximately equal intensity on paper chromatography in solvent A (R_f 0.52 and 0.57). This material was used directly in the next step.

Decyanoethylation–Deacetylation of d-CEpTp(Et)TOAc. d-CEpTp(Et)TOAc (6.68 mmol) was dissolved in 100 mL of 1 M sodium hydroxide in pyridine–ethanol–water (1:1:2). After 0.5 h, the solution was neutralized with pyridinium Dowex 50W-X8. The resin was filtered off and washed with pyridine–water (1:1). The combined filtrate and washings contained 6.68 mmol of d-pTp(Et)T (100% yield), measured by UV absorbance. The material was homogeneous on paper chromatography in solvents A (R_f 0.17) and C (R_f 0.52) and was used without isolation in the next step.

Acetylation of d-pTp(Et)T. d-pTp(Et)T (6.68 mmol) was dried by evaporation of anhydrous pyridine and treated with 50 mL of acetic anhydride in 100 mL of pyridine for 4.5 h. Methanol (50 mL) was added at 0 °C, followed, after 0.5 h, by 100 mL of pyridine–water (1:4). After concentration and repeated evaporation of pyridine, the product was precipitated from pyridine–ether, washed with ether, and dried: 4.82 g (6.21 mmol, 93% yield) of d-Tp(Et)TOAc, homogeneous on paper chromatography in solvents A (R_f 0.18) and C (R_f 0.53).

Synthesis of d-[Tp(Et)]$_{n-1}$T. Since chain elongation, ethylation, and deacetylation at the various stages of the synthesis were carried out under essentially identical conditions, the experimental procedure is given in general terms. Yields and scales for the reactions are listed in Table I.

Chain Elongation. A mixture of the 3′-OH component (d-MTr[Tp(Et)]$_{n-3}$T) and from 1.3 to 2.0 mol equiv (see Table I) of the 5′-phosphate component (d-pTp(Et)TOAc) was rendered anhydrous by evaporation of pyridine and dissolved in anhydrous pyridine to make the input concentration of d-MTr[Tp(Et)]$_{n-3}$T 0.05 M. Mesitylenesulfonyl chloride (4.0 mol equiv based on d-pTp(Et)TOAc) was added. After a reaction time of from 7 to 23 h (see Table I) an equal volume of pyridine–water (1:1) was added at 0 °C. The solution was concentrated to a small volume, diluted with pyridine–water (1:19), and extracted first with ether and then with 1-butanol–pyridine (19:1). The butanol layer was concentrated, and the desired product, d-MTr[Tp(Et)]$_{n-3}$TpTp(Et)TOAc, was obtained by precipitation from pyridine–ether, washing with ether, and drying under reduced pressure. The washings were again subjected to pyridine–ether precipitation to yield a second crop. The product was contaminated by trace impurities, as judged by silica gel TLC. It was used, without further purification, in the next synthetic step.

Ethylation. d-MTr[Tp(Et)]$_{n-3}$TpTp(Et)TOAc, dried by repeated evaporation first of pyridine, then of dioxane, was dissolved in anhydrous *N,N*-dimethylformamide to give a 0.05

M solution of the oligonucleotide. Lutidine (2.7 mol equiv), *N*-methylimidazole (14 mol equiv), anhydrous ethanol (40 mol equiv), and *p*-toluenesulfonyl chloride (11 mol equiv) were added at 0 °C. After 1 h fresh aliquots (identical with those above) of *N*-methylimidazole, ethanol, and *p*-toluenesulfonyl chloride were introduced. After a total of 2 h, the reaction mixture was treated with an equal volume of pyridine–water (1:1) at 0 °C. After concentration, the residue was partitioned between methylene chloride and pyridine–water (1:19). The methylene chloride solution was concentrated and subjected to chromatography on silica gel. The column was eluted with tetrahydrofuran–ethyl acetate mixtures of increasing tetrahydrofuran content. The desired d-MTr[Tp(Et)]$_{n-1}$TOAc eluted at 30 to 50% tetrahydrofuran. Mixed fractions were rechromatographed. The product was precipitated from tetrahydrofuran–hexane, filtered off on glass, washed with hexane, and dried. Further crops were obtained from the filtrate by renewed precipitation.

Deacetylation. d-MTr[Tp(Et)]$_{n-1}$TOAc was treated with pyridine–concentrated ammonia (1:1, v/v) for 4 h. The solution was concentrated, and the residue was chromatographed on a silica gel column using tetrahydrofuran–ethyl acetate step gradients. The desired d-MTr[Tp(Et)]$_{n-1}$T eluted at 50 to 70% tetrahydrofuran. Mixed fractions were rechromatographed. The product was precipitated from tetrahydrofuran–hexane and collected on a glass filter. The filtrates yielded further crops upon reprecipitation.

Detritylation. d-MTr[Tp(Et)]$_{n-1}$T was treated with 80% aqueous acetic acid for 8 h. (In the preparation of the d-T_8-Et sample with which the physical studies were done, this reaction time was extended to 66 h). The solution was evaporated to dryness, and the residue was partitioned between water and ether. The aqueous layer was chromatographed on a column of Sephadex G-25 or Sephadex G-50 using water as the eluent. Appropriate fractions were combined and lyophilized. Yields, determined spectrophotometrically, ranged from 91 to 96%.

Preparation of d-T_8. The method of Khorana and Vizsolyi (1961) was followed. The d-PT_8 peak was rechromatographed on DEAE-Sephadex using a shallow salt gradient. Bacterial alkaline phosphatase cleanly converted this material to a product with higher mobility on paper chromatography in solvent F. The product was completely degraded to nucleoside and mononucleotide by bovine spleen phosphodiesterase and was identified as d-(Tp)$_7$T (= d-T_8) on examination of partial spleen digests by high-pressure liquid chromatography on Pellionex AL WAX. After passage over a Bio-Gel P2 column, the compound exhibited λ_{max} 265 nm, λ_{min} 234 nm, A_{250}/A_{260} = 0.71, and A_{280}/A_{260} = 0.61.

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