Effect of Ionic Strength on the Hybridization of Oligodeoxynucleotides with Reduced Charge Due to Methylphosphonate Linkages to Unmodified Oligodeoxynucleotides Containing the Complementary Sequence†

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ABSTRACT: A 12-mer oligodeoxynucleotide containing 10 methylphosphonate bonds and 1 phosphodiester bond was shown to bind specifically to the restriction endonuclease fragment containing complementary DNA in a Southern blot. This 12-mer as well as 14-mer oligodeoxynucleotides containing 3 methylphosphonate and 10 phosphodiester bonds was used to examine the effect of reduced charge on the thermodynamics of binding to complementary DNA or complementary oligodeoxynucleotides with additional nucleotides overlapping both the 3′ and 5′ ends. The 14-mer oligodeoxynucleotides were synthesized with one methylphosphonamidite (A, C, G, or T). Melting profiles were examined by spectrophotometry for the 14-mers and by a gel-shift assay for the 12-mer. Nearest-neighbor free energy values were compiled for predicting concentration-dependent melting temperatures for all oligodeoxynucleotide hybridizations, including those involving adjacent dG residues. The free energy contribution to duplex formation from the dangling ends was about 1 kcal/mol. The free energy decrement due to introduction of each methylphosphonate linkage was −0.75 kcal/mol in high salt independent of the methylphosphonamidite used for synthesis of the oligodeoxynucleotide. However, the change in charge per nearest-neighbor base pair decreased from 0.26 to 0.0 when the nearest-neighbor base pair contained one methylphosphonate. Thus at very low salt, methylphosphonate-substituted oligodeoxynucleotides form more stable hybrids than analogous phosphodiester sequences. The 12-mer with 10 methylphosphonate bonds outcompetes the analogous phosphodiester 12-mer below 0.01 M NaCl. The temperature of 50% dissociation of bound oligodeoxynucleotide after being washed for 30 min was measured with a dot-blot assay. These results, together with the thermodynamic results, indicate that the substitution of methylphosphonate linkages at high salt only affects the reverse rate constant.

Oligodeoxynucleotide analogues containing modifications in their backbones are of particular interest for applications where nuclease resistance is important. For example, phosphorothioate deoxynucleotides have been used to examine the function of various enzymes and proteins that act on or associate with DNA (Eckstein, 1985), and oligophosphorothioate deoxynucleotides have been tested for antisense activity in vivo (Marcus-Sekura et al., 1987; Matsukara et al., 1987). In addition to being nuclease resistant and exhibiting antisense activity (Miller et al., 1985; Murakami et al., 1985; Marcus-Sekura et al., 1987; Matsukara et al., 1987), oligodeoxynucleotide analogues containing nonionic methylphosphonate linkages have decreased overall charge compared to their phosphodiester or phosphorothioate counterparts. The purpose of the work presented here with methylphosphonate-substituted oligodeoxynucleotides was to examine the effect of charge reduction on the thermodynamic behavior of helix–coil transitions of DNA.

The study was complicated by the chiral methylphosphonate bonds, which exist in two isomeric configurations (*R*, pseudoaxial; *S*, pseudoequatorial) (Miller et al., 1979). Helix–coil transitions of self-complementary, singly substituted (*R*)- and (*S*)-methylphosphonate oligodeoxynucleotides have been investigated (Bower et al., 1987). These diastereoisomeric forms differed in ability to hybridize to a complementary DNA sequence, bonds of the *R* type contributing to more stable base pairing than those of the *S* type. In spite of these complications due to chirality, methylphosphonate linkages were chosen for study because they are more efficiently synthesized than nonchiral linkages such as carbamates (Stirchak & Summerton, 1987) and because no studies have been reported concerning the biological properties of oligodeoxynucleotides with nonchiral linkages.

For this study, several standard phosphodiester as well as one fully (10 of 11 linkages) and several partially methylphosphonate substituted oligodeoxynucleotides were synthesized. All studies involved hybridizations of these oligodeoxynucleotides to complementary phosphodiester oligodeoxynucleotides which extended beyond the duplex region in both the 3′ and 5′ directions, just as is the case for hybridization to single-stranded DNA. The fully substituted oligodeoxynucleoside methylphosphonate was shown to be specific for its phosphodiester complement by Southern hybridization analysis.

By use of known thermodynamic data for heteropolymeric DNA, natural DNAs, and oligodeoxynucleotides, a single set of nearest-neighbor thermodynamic parameters was obtained that fit the helix–coil transition for all oligodeoxynucleotides, including those containing adjacent guanosine residues. These results provided a quantitative framework for assessing the effect of the 3′ and 5′ tails on helix stability as well as the effect of methylphosphonate linkages at all ionic strengths on helix stability. Concentration-dependent melting temperatures for phosphodiester and partially methylphosphonate substituted oligodeoxynucleotides were measured optically as a function of ionic strength. A gel migration assay was employed for studying duplex formation by the fully methylphosphonate

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substituted oligodeoxynucleotide. Conditions for preferential binding of methylphosphonate-substituted oligodeoxynucleotides to single-stranded DNA are described. Finally, for each duplex type, the ionic strength dependence of the time-dependent dissociation temperature was determined by examining elution from dot blots. These results, together with the thermodynamic results, indicate that substitution of methylphosphonate linkages at high salt only affects the reverse rate constant.

Materials and Methods

Oligodeoxynucleotides and Plasmid DNA. All oligodeoxynucleotides (Table I) were synthesized on an Applied Biosystems Model 380B DNA synthesizer. Phosphodiester linkages were generated by standard phosphoramidite chemistry, and methylphosphonate bonds were introduced by the coupling of methylphosphonamidite monomers. Hydrolysis of base-protecting groups and cleavage from the support for phosphodiester oligodeoxynucleotides were accomplished by NH_4OH treatment, which was followed by ethanol precipitation. Fully and partially methylphosphonate modified oligodeoxynucleotides were released and deprotected in ethylenediamine–ethanol (1:1) for 7 h at room temperature (Miller et al., 1986). Use of this alternate deprotection method with a phosphodiester oligodeoxynucleotide led to a product indistinguishable from that produced by using NH_4OH deprotection. The methylphosphonate oligodeoxynucleotide containing the single 5′ phosphodiester linkage was purified by NH_4HCO_3 elution from a DEAE-cellulose column (Miller et al., 1986), and the oligodeoxynucleotides containing mixed phosphodiester and methylphosphonate bonds were purified by 4 M ammonium acetate elution from NACS Prepac columns (Bethesda Research Labs). All oligodeoxynucleotides were 5′-labeled with ^{32}P by using T4 polynucleotide kinase, subjected to polyacrylamide gel electrophoresis on 20% acrylamide–8 M urea gels, and visualized by autoradiography. The unmodified oligodeoxynucleotides had the correct mobility for their sizes. Oligodeoxynucleotides with methylphosphonate substitutions have lower mobilities than their phosphodiester analogues. Both fully and partially substituted oligodeoxynucleotides were predominantly one large product. Minor, shorter, presumably failure and/or cleavage sequences could also be detected. Plasmid pALA-D is a pUC9 expression vector containing the cDNA sequence of human δ-aminolevulinic acid dehydratase (ALA-D), a heme biosynthetic enzyme (Wetmur et al., 1986).

Southern Hybridization. The Southern (1975) blot was prepared according to a standard procedure (Maniatis et al., 1982), and the Zetabind filter (AMF Cuno) was treated as recommended by the manufacturer. A 2-h prehybridization was carried out in 5× SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 10× Denhardt's solution [1% of each: Ficoll, poly(vinylpyrrolidone), and bovine serum albumin], 500 μg/mL sonicated and boiled salmon sperm DNA, and 0.05 M sodium phosphate buffer, pH 7. The hybridization solution contained 5× SSC, 1% Denhardt's solution, 100 μg/mL salmon sperm DNA, and 0.02 M sodium phosphate buffer, pH 7. The oligodeoxynucleotide was 5′-labeled with ^{32}P by using T4 polynucleotide kinase and added to the hybridization solution at a concentration, based on A_{260}, of 5–10 ng/mL. Hybridization was carried out as is described for the dot-blot analyses.

Dot-Blot Analyses. Dots of plasmid DNA were made by spotting 0.5-μg quantities of plasmid pALA-D onto Zetabind filters, drying at room temperature, denaturing for 15 min in 0.5 M NaOH and 1.5 M NaCl, and neutralizing for 15 min in 1 M Tris-HCl (pH 8.0). Once dried at room temperature, filters were washed in 0.1× SSC and 0.5% SDS (sodium dodecyl sulfate) at 65 °C for 1 h to decrease the background during hybridization. The filters were prehybridized for 2–3 h at 37 °C in the same solution described for the Southern hybridization. ^{32}P-Labeled oligodeoxynucleotide was added to the hybridization solution (also described above) at concentrations ranging from 4 to 20 ng/mL. These conditions facilitate dot-blot hybridization for methylphosphonate oligodeoxynucleotides. Hybridization was carried out at 37 °C for $^1/_2$ h and then at room temperature for 1.5 h. Each filter was washed at the specified salt and temperature for 30 min.

Melting Temperature Analyses. Complementary oligodeoxynucleotides were mixed 1:1 in various concentrations of salt: 6× SSC, 100, 20, or 2 mM NaCl in 2 mM Tris-HCl, and 0.2 mM EDTA. Solutions were heated at 0.3 °C/min in a 1-cm quartz cuvette in a Beckman Model 25 spectrophotometer equipped with a water-jacketed cell holder. Temperature was monitored with a thermistor attached to the cell holder. Hyperchromicities for all 14-mer plus 18-mer duplexes were 21%. The concentration-dependent melting temperatures ($T_m = t_m + 273.16$) were calculated according to the method of Marky and Breslauer (1987). T_m values reported are reliable to ±1 °C.

Gel Migration Analyses. The phosphodiester recipient oligodeoxynucleotide 18-P3 was 5′-labeled with ^{32}P by using T4 polynucleotide kinase and purified with a spun column (Maniatis et al., 1982) of Sephadex G-50 in NTE (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA) and TE/5 (2 mM Tris-HCl, 0.2 mM EDTA). To determine binding stoichiometry for the fully substituted oligodeoxynucleotide 12-Me^{10}, it was mixed with 5′-labeled complementary oligodeoxynucleotide 18-P3 at various ratios. Annealing was carried out at room temperature for at least 15 min prior to electrophoresis. To examine the stability of the 12-Me^{10} plus 18-P3 duplex under the conditions used for electrophoresis, the complex was formed at a stoichiometric ratio, cooled to 4 °C, and incubated with 12-P1 as a competitor. To measure competition of 12-P1 and 12-Me^{10} for 18-P3 at various salt concentrations under equilibrium conditions, both 12-mers were added to the 18-mer, incubated at 70 °C for 5 min, and cooled slowly to room temperature. Samples were loaded onto a 20% acrylamide gel in either 10% glycerin plus bromophenol blue and xylene cyanol or 2.5% Ficoll 400. Gel electrophoresis was performed at 4 °C at 250–400 V, 5–15 mA, in TBE (89 mM Tris-HCl, 89 mM borate, 1 mM EDTA) buffer. The gels were dried, and the results were obtained by autoradiography.

Results and Discussion

All of the oligodeoxynucleotides used in this study are listed in Table I. There are three "recipient" oligodeoxynucleotides, each of which is an 18-mer with a phosphodiester backbone. Oligodeoxynucleotide 18-P3 is complementary to the noncoding strand of the ALA-D gene (Wetmur et al., 1986) in the region of the start of the protein sequence, corresponding to the cDNA positions of −4 to +14. Oligodeoxynucleotides 18-P1 and 18-P2 are complementary to each other and correspond to the cDNA positions of 627–644. For the purposes of this study, the use of sequences in the human ALA-D cDNA was fortuituous.

Each recipient oligodeoxynucleotide has a set of complementary oligodeoxynucleotides, identical in length and sequence but differing in backbone structure. 12-mer sequences complementary to 18-P3 include a phosphodiester sequence (12-P1) and a sequence containing 10 methylphosphonate bonds and 1 phosphodiester bond at the most 5′-position

Table I: Oligodeoxynucleotides Used in This Study

recipient		complement	
no.	sequence	no.	sequence[a]
18-P1	GGA TGC AGC TAA GTC AAG	14-P1	TGA CTT AGC TGC AT
		14-A	TGA CTT AGC TGC AT
		14-C	TGA CTT AGC TGC AT
18-P2	CTT GAC TTA GCT GCA TCC	14-P2	ATG CAG CTA AGT CA
		14-G	ATG CAG CTA AGT CA
		14-T	ATG CAG CTA AGT CA
18-P3	CGC CAT GCA GCC CCA GTC	12-P1	TGG GGC TGC ATG
		12-Me[10]	TGG GGC TGC ATG
none		12-P2	CAT GCA GCC CCA

[a] Underlines indicate base with methylphosphonate linkage to 3′ side.

(12-Me[10]). 14-mer sequences complementary to either 18-P1 or 18-P2 included phosphodiester sequences (14-P1 and 14-P2) and sequences synthesized by employing one methylphosphonamidite (14-A, -C, -G, or -T). The strand to be synthesized was dictated by a requirement to scatter the methylphosphonate bonds throughout the oligodeoxynucleotide. All the complementary oligodeoxynucleotides were shorter than the recipient oligodeoxynucleotides with duplex formation resulting in equal-length single-stranded 3′ and 5′ recipient dangling ends.

Specificity of Binding of Methylphosphonate Oligodeoxynucleotides. In order to demonstrate the degree of specificity that a methylphosphonate oligodeoxynucleotide has for its complementary sequence versus unrelated sequences, Southern (1975) hybridization was performed in 1 M sodium ion on restriction endonuclease digested pALA-D, as well as control DNAs by using 5′-^{32}P-labeled oligodeoxynucleotide 12-Me[10]. This is the fully substituted oligodeoxynucleotide, having the most diastereoisomeric forms of all of the methylphosphonate oligodeoxynucleotides in this study. Hybridization was carried out at 37 °C for 30 min and continued for 2 h while the temperature was decreased to room temperature. This temperature range was chosen because the dissociation temperature had previously been determined to be between 30 and 35 °C (see dot blots below).

Figure 1a shows the *Eco*RI/*Hin*dIII/*Rsa*I restriction enzyme digest of pALA-D DNA separated on a 1% agarose gel. The indicated (796-bp) fragment contained the sequence complementary to oligodeoxynucleotide 12-Me[10]. Figure 1b shows the autoradiogram of the Southern hybridization, demonstrating that ^{32}P-labeled 12-Me[10] bound specifically to the 796-bp fragment of pALA-D. In addition, no binding of this oligodeoxynucleotide was observed to either of the digested phage DNAs. A search of the bacteriophage λ DNA sequence revealed four regions of complementarity with only two mismatches, in one case with an uninterrupted homology of 10 nucleotide pairs. Thus, a fully substituted oligodeoxynucleotide maintains a high degree of specificity for its complementary DNA sequence.

Characteristic Temperatures and Thermodynamics of DNA Melting. There are three temperatures characteristic of oligodeoxynucleotide binding to complementary DNA:

T_m^∞. The DNA melting temperature is defined to be the temperature at which an infinitely long DNA molecule is half-denatured. T_m^∞ depends on DNA base composition and on the properties of the solvent, including the ionic strength.

T_m. The oligodeoxynucleotide melting temperature is defined to be the temperature at which 50% of the nucleotides of an equimolar mixture of complementary oligodeoxynucleotides are in the duplex state. T_m depends upon oligodeoxynucleotide *concentration* in addition to the nucleotide sequence and on the properties of the solvent.

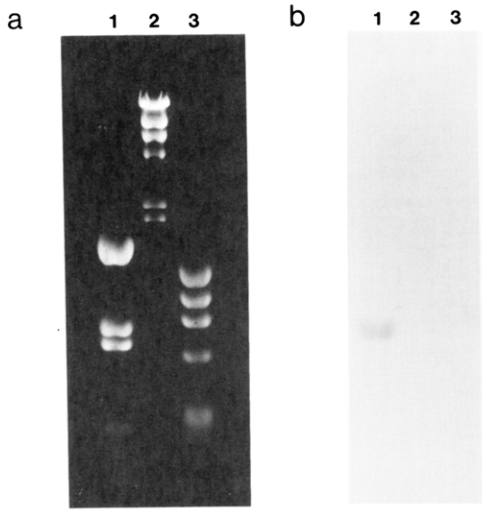

FIGURE 1: Specificity of binding of fully modified methylphosphonate oligodeoxynucleotide 12-Me[10] to complementary DNA. Panel a: Ethidium bromide stained 1% agarose gel. Lane 1 = *Eco*RI-, *Hin*dIII-, and *Rsa*I-digested plasmid DNA containing the complementary sequence. Lane 2 = *Hin*dIII-digested λ DNA. Lane 3 = *Hae*III-digested φX174 DNA. Panel b: Autoradiograph of Southern hybridization of the same gel.

T_d. The dissociation temperature ($T_d = t_d + 273.16$) is defined as the temperature at which 50% of the labeled oligodeoxynucleotide is dissociated from complementary DNA after a specified time. T_d depends on *time* and the nucleotide sequence and the properties of the solvent but is independent of concentration. T_d is related to a kinetic and not a thermodynamic property of the duplex. The specified time for 50% dissociation (half-time for the reaction) is equal to ln 2 divided by the rate constant for the helix to coil transition at T_d.

Breslauer et al. (1986), using the methods described in Marky and Breslauer (1987), have analyzed the melting of DNA and complementary oligodeoxynucleotides in terms of nearest-neighbor contributions to the enthalpy of base pair formation. For a long homo- or heteropolymeric DNA, the helix–coil transition enthalpy thus determined, ΔH°_{av}, is the average of the contributions of the various nearest-neighbor enthalpies. Letting $T^\circ = 298.16$ K be the reference temperature, the avearage nearest-neighbor free energy of the helix–coil transition is given by

$$\Delta G^\circ_{av} = \Delta H^\circ_{av}(1 - T^\circ/T_m^\infty) \quad (1)$$

A similar analysis was carried out for two-state helix–coil transitions involving complementary oligodeoxynucleotides by assuming that no enthalpy is associated with the formation of the first base pair. Then all thermodynamic parameters may still be associated with nearest-neighbor interactions. Defining ΔG° and a temperature-independent ΔH° to be the standard free energy and enthalpy for the sum of all near-

est-neighbor contributions to the helix–coil transition and letting C be the total concentration of both of the complementary oligodeoxynucleotides

$$\Delta G^\circ = \Delta H^\circ(1 - T^\circ/T_m) - RT^\circ \ln (C/4) \quad (2)$$

As the molecules become large, the last term becomes increasingly insignificant. ΔH° becomes very large and T_m approaches T_m^∞. For self-complementary oligodeoxynucleotides, the $RT^\circ \ln (C/4)$ term becomes $RT^\circ \ln (C/2)$.

Calculation of Nearest-Neighbor Free Energies. Breslauer et al. (1986) stated that their results failed to predict T_m values correctly for oligodeoxynucleotides containing GG nearest neighbors. Because one of the oligodeoxynucleotides involved in the study of the effect of methylphosphonate linkages contained three adjacent GG interactions, we decided to reexamine the thermodynamic free energy parameters involved in helix–coil transitions to see if such molecules were indeed exceptions. All enthalpy parameters used were those determined by Breslauer et al. (1986).

The basis set used for determining nearest-neighbor free energy parameters is given in Table II together with references to the sources of the data. The basis set included approximately equal weighting for oligodeoxynucleotides, including several with adjacent GG nearest neighbors, heteropolymers, and natural DNAs of various base compositions. Finally, a penalty was imposed for each nearest-neighbor interaction for deviation from simple dependence on base composition. These data were entered into the MGLH program of SYSTAT, Inc. (Evanston, IL), for calculation of nearest-neighbor free energies and the intercept, ΔG°_i, the negative of the free energy for helix initiation, which best fit all of the data for both oligodeoxynucleotide duplex formation and melting of long DNAs as described by eq 1 and 2 above. The symmetry term for self-complementary oligodeoxynucleotides was treated in the same manner as by Breslauer et al. (1986). ΔG°_{nn}, the nearest-neighbor free energies, are listed at the top of Table II. Standard errors for all ΔG°_{nn} were approximately 0.04. ΔG°_i was determined to be −2.2 kcal/mol (−2.6 kcal/mol if self-complementary). Identical values have been determined for polymers containing dG and dC nucleotides (Pohl, 1974). Unlike the calculations of Breslauer et al. (1986), no assumption about the length of the cooperative unit for melting of large DNAs was built into the model. The resulting low calculated contribution of the initiation term to the free energies for oligodeoxynucleotide helix–coil transitions may derive from a dependence of the enthalpy on temperature.

The standard error for calculated and experimental ΔG° is 7%. The calculated and experimental T_m^∞ values (DNAs) differ by no more than 1 °C. The ΔG° values for oligodeoxynucleotides of known concentration are easily converted into T_m values by using a rearranged form of eq 2. The standard error for calculated and experimental T_m values is 2 °C. Thus, a single formulation fits well for all types of DNA melting analyses, including helix–coil transitions of oligodeoxynucleotides containing adjacent GG nearest neighbors.

The calculated T_m^∞ for poly[d(AT)·d(AT)], taken as an unknown, agreed with experiment. The calculated t_m^∞ for poly(dG·dC) was 104 °C in 1 M NaCl. This value is lower than experimental values extrapolated to 1 M NaCl. Poly(dG·dC) thermodynamic data may be anomalous, and its inclusion in the data used for interpreting other DNA hybridization experiments may be erroneous.

Effect of Dangling Ends. Both oligodeoxynucleotides used for this study were hybridized to recipient molecules overlapping at both the 5′ and 3′ ends. The contribution of dangling ends was found to be about 1 kcal/mol (Table III), leading to an increase in T_m of 3–4 °C. The 14-mer + 18-mer duplex with 5′ and 3′ adjacent dangling purines was slightly (1 °C) more stable than the duplex with 5′ and 3′ adjacent dangling pyrimidines. The magnitude of the result is in line with other studies of dangling ends (Senior et al., 1988).

Effect of Ionic Strength on the T_m for Phosphodiester or Partially Methylphosphonate Substituted Oligodeoxynucleotides Bound to Complementary Oligodeoxynucleotides with 5′ and 3′ Dangling Ends. Table III lists melting temperatures determined for phosphodiester and partially methylphosphonate substituted oligodeoxynucleotides at various ionic strengths. At 100 mM NaCl methylphosphonate and phosphodiester oligodeoxynucleotides have equivalent hyperchromicities, indicating that all of the diastereoisomers for each of the methylphosphonate-substituted oligodeoxynucleotides form duplexes with their respective recipients. The width of the helix–coil transition for 14-A + 18-P1 was 3 °C greater than that for 14-P1 + 18-P1. This difference can be explained by the 2.5 °C difference in T_m values for the R and S diastereoisomers at each chiral center (Bower et al., 1987).

The t_m values reported in Table III include those for 18-P1 plus either 14-P1, 14-A, or 14-C as well as 18-P2 plus either 14-P2, 14-G, or 14-C. The oligodeoxynucleotides 14-A, -C, -G, and -T contained three nonadjacent methylphosphonate linkages 3′ to the A, C, G, or T nucleotides. In 1 M NaCl, substitution of these methylphosphonate linkages resulted in a decrease in t_m of 7–11 °C. There does not appear to be a large sequence specificity for the destabilization due to nonadjacent methylphosphonate bonds.

The free energy of destabilization associated with one such bond is defined as ΔG°_d. An additional free energy term may be introduced to account for the effect of decreasing ionic strength on T_m, ΔG°_s. ΔG°_s is taken to be 0 in 1 M NaCl. ΔG°_e, the dangling ends term, is taken to be +1 kcal/mol, and ΔG°_i, the initiation term, is taken to be −2.2 kcal/mol. Calculated T_m values in Table III were increased by 2 °C to correct for the difference between experimental and theoretical results for 14-P1 + 14-P2 shown in Table II.

In summary, calculated melting temperatures were obtained from

$$T_m = T^\circ\Delta H^\circ/[\Delta H^\circ - \Delta G^\circ - RT^\circ \ln (C/4)] \quad (3)$$

where

$$\Delta H^\circ = \sum_{nn}(N_{nn}\Delta H^\circ_{nn})$$

and

$$\Delta G^\circ = \sum_{nn}(N_{nn}\Delta G^\circ_{nn}) + \Delta G^\circ_i + \Delta G^\circ_e + N_{Me}\Delta G^\circ_d + \Delta G^\circ_s$$

ΔG°_d was found to be −0.75 kcal/mol, leading to a substantial decrease in T_m for methylphosphonate-substituted oligodeoxynucleotides at 1 M NaCl. This result is compatible with those of Bower et al. (1987).

The ionic strength data were fit to

$$\Delta G^\circ_s = (n_h - n_c)RT^\circ\{\ln [M/(0.3 + M)] + \ln (1.3)\} \quad (4)$$

where $n_h - n_c$ is the change in total charge difference between the helix and coil forms and M is the sodium ion concentration.

For each nearest-neighbor base pair in phosphodiester DNA

$$(n_h - n_c)/(\sum_{nn}N_{nn}) = 0.26$$

This value is greater than predicted by Record and Lohman (1978) and indicates the need for more theoretical work aimed toward explaining the association of counterions with oligodeoxynucleotides.

Table II: Thermodynamic Values

nearest-neighbor values																	
enthalpy	9.10	8.60	6.00	5.80	6.50	7.80	5.60	11.90	11.10	11.00							
free energy	1.55	1.25	0.85	1.15	1.40	1.45	1.15	3.05	2.70	2.30							
entropy (cal)	25.32	24.65	17.27	15.60	17.10	21.30	14.92	29.68	28.17	29.18							
initiation free energy	2.2																
symmetry factor	0.4																
nearest neighbors	AA TT	AT AT	TA TA	CA TG	GT AC	CT AG	GA TC	CG CG	GC GC	GG CC	sym	$\Delta H°$ (calcd)[a]	$\Delta G°$ (exptl)[b]	$\Delta G°$ (calcd)	T_m (exptl)	T_m (calcd)	concn (μM)	ref
oligodeoxyribonucleotides																	
GCGCGC	0	0	0	0	0	0	0	2	3	0	1	57.1	11.1	11.60				*c*
CGTCGACG	0	0	0	0	2	0	2	3	0	0	1	59.9	11.9	11.65				*c*
GAAGCTTC	2	0	0	0	0	2	2	0	1	0	1	56.1	8.7	8.40				*c*
GGAATTCC	2	1	0	0	0	0	2	0	0	2	1	60	9.4	8.65	36	36	18.8	*c, d*
GGTATACC	0	1	2	0	2	0	0	0	0	2	1	84	15.5	55.55				*c*
CGCGAATTCGCG	2	1	0	0	0	0	2	4	2	0	1	107.8		21.65	73	77	88.6	*e*
CGCGTACGCGTACGCG	0	0	2	0	4	0	0	6	3	0	1	142.7	34.1	31.10				*c*
CCCGGG	0	0	0	0	0	0	0	1	0	4	1	55.9		9.65	48	49	100	*f*
CCCAGGG	0	0	0	1	0	1	0	0	0	4	0	57.6		9.60	43	43	100	*f*
GATGGGCAG	0	1	0	2	0	1	1	0	1	2	0	66.7		11.25	46	49	100	*g*
CAAAAAG	4	0	0	1	0	1	0	0	0	0	0	50	6.1	6.60				*c*
CAAACAAAG	4	0	0	2	1	1	0	0	0	0	0	62.3	10.1	9.15				*c*
CAAAAAAAG	6	0	0	1	0	1	0	0	0	0	0	68.2	9.6	9.70				*c*
CAAATAAAG	4	1	1	1	0	1	0	0	0	0	0	64.6	8.5	8.70				*c*
CAAAGAAAG	4	0	0	1	0	2	1	0	0	0	0	63.4	9.5	9.20				*c*
TGGGGCTGCATG	0	1	0	4	0	1	0	0	2	3	0	94.8		17.40	60	58		*h, i*
TGACTTAGCTGCAT	1	1	1	3	1	3	1	0	2	0	0	98.8		17.20	57	55		*i, j*
polymers																		
d(GC)-d(GC)	0	0	0	0	0	0	0	10	10	0	0	230		57.50	123	124		*j*
d(AC)-d(GT)	0	0	0	10	10	0	0	0	0	0	0	123		25.50	101	103		*j*
d(GAT)-d(ATC)	0	7	0	7	0	0	7	0	0	0	0	140		24.85	98	89		*j*
d(AAC)-d(GTT)	7	0	0	7	7	0	0	0	0	0	0	149.8		28.70	97	96		*j*
d(AG)-d(CT)	0	0	0	0	0	10	10	0	0	0	0	134		26.00	96	97		*j*
d(AGT)-d(ACT)	0	0	7	0	7	7	0	0	0	0	0	142.1		25.90	93	91		*j*
d(TCC)-d(GAA)	7	0	0	0	0	7	7	0	0	0	0	157.5		29.05	94	92		*j*
d(AAT)-d(ATT)	7	7	7	0	0	0	0	0	0	0	0	165.9		25.55	79	79		*h*
d(AT)-d(AT)	0	10	10	0	0	0	0	0	0	0	0	146		21.00	75	75		*j*
natural DNAs																		
20% GC	8	4	4	2	2	2	2	0.25	0.25	0.5	0	193.85		33.69	87	88		*k*
40% GC	4.5	2.25	2.25	3	3	3	3	1	1	2	0	195.9		37.50	95	96		*k*
60% GC	2	1	1	3	3	3	3	2.25	2.25	4.5	0	211.15		43.94	102	103		*k*
80% GC	0.5	0.25	0.25	2	2	2	2	4	4	8	0	239.6		53.00	110	110		*k*
nearest-neighbor interactions																		
AT	0	20	0	0	0	0	0	0	0	0	0	172		25.00	75	76		
TA	0	0	20	0	0	0	0	0	0	0	0	120		17.00	75	74		
CA	0	0	0	20	0	0	0	0	0	0	0	116		23.00	101	99		
GT	0	0	0	0	20	0	0	0	0	0	0	130		28.00	101	107		
CT	0	0	0	0	0	20	0	0	0	0	0	156		29.00	96	93		
GA	0	0	0	0	0	0	20	0	0	0	0	112		23.00	96	102		
CG	0	0	0	0	0	0	0	20	0	0	0	238		61.00	123	128		
GC	0	0	0	0	0	0	0	0	20	0	0	222		54.00	123	121		
polymers not in basis set																		
d(A)	20	0	0	0	0	0	0	0	0	0	0	182		31.00	86	86		*j*
d(G)	0	0	0	0	0	0	0	0	0	20	0	220		46.00		104		
no. of times in basis set[l]	256	145	137	192	168	184	180	157	149	158								

[a] Enthalpy ($\Delta H°$) data are from Breslauer et al. (1986). [b] Experimental data (all converted into $\Delta G°$ values). Weighting (to equalize contributions of nucleotides: oligodeoxynucleotides, 4; polymers, 2; nearest-neighbor interactions, 1; natural DNAs, 6. [c] Breslauer et al. (1986). [d] Bower et al. (1987). [e] Marky et al. (1983). [f] Arnold et al. (1987). [g] Woodson and Crothers (1987). [h] Ornstein and Fresco (1983). [i] This work. [j] Wells et al. (1970) (corrected for length effects). [k] Marmur and Doty (1962) (corrected to 1 M NaCl). [l] See text.

Table III: Melting Temperatures (t_m) for Phosphodiester- and Methylphosphonate-Containing Oligodeoxynucleotides

Effect of Dangling Ends in 1.0 M NaCl	
oligodeoxynucleotides[a]	t_m (°C)
12-P1 + 12-P2	60
12-P1 + 18-P3	63
14-P1 + 14-P2	57
14-P1 + 18-P1	61
14-P2 + 18-P2	60

Effect of Ionic Strength	t_m (°C) at salt concn			
oligodeoxynucleotide[a]	1.0	0.1	0.02	0.004
unmodified				
14-P1	61	53	42	31
14-P2	60			
14-P calcd	60	52	42	31
partially substituted[b]				
14-A	52	41	36	30
14-C	50	45	38	32
14-G	53	42		32
14-T	52	43	39	30
14-Me^3 calcd	52	46	38	30

[a] Total strand concentration = 6 μM. [b] Three methylphosphonates on one strand.

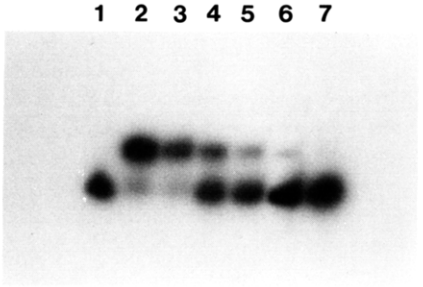

FIGURE 2: Determination of the stoichiometry of binding of fully modified methylphosphonate oligodeoxynucleotide 12-Me^{10} to ^{32}P-labeled complementary 18-P3. Complementary oligodeoxynucleotides were annealed in 100 mM salt at room temperature. Electrophoresis was carried out in a 20% polyacrylamide gel at 4 °C in TBE. Lane 1, 18-P3 only; lanes 2–7, 18-P3 + 12-Me^{10} at 1:20, 1:10, 1:5, 1:2.5, 1:1.25 and 1:0.625 (arbitrary units).

For each nearest-neighbor base pair in methylphosphonate DNA

$$n_h - n_c = 0$$

The calculated t_m values using eq 3 and these $n_h - n_c$ results are compared with experimental t_m values obtained at various ionic strengths in Table III. The fit between experimental and theoretical t_m values is quite satisfactory.

In low salt, a higher relative stability for methylphosphonate-containing oligodeoxynucleotides is seen, where T_m values for either partially (Table III) or fully (Figure 4) methylphosphonate substituted oligodeoxynucleotides become greater than those of the analogous phosphodiesters either at or below 4 mM NaCl.

Determination of Binding 12-Me^{10} to 18-P3 Using the Polyacrylamide Gel Migration Assay. As demonstrated in Figure 2, duplex formation between a phosphodiester oligodeoxynucleotide that has been 5′-^{32}P-labeled (18-P3) and a complementary oligodeoxynucleotide (12-Me^{10}) can be demonstrated by the altered gel mobility of the band containing the labeled oligodeoxynucleotide. In addition, the stoichiometry of hybridization may be quantitated by such an analysis. It is often difficult to determine precise concentrations (micrograms per milliliter) of fully substituted methylphosphonate oligodeoxynucleotides by ultraviolet spectrophotometry. More importantly, no optical melting curve can be obtained for such a large mixture of chiral forms of differing duplex stabilities. With the gel migration assay, a set quantity of recipient oligodeoxynucleotide may be titrated with varying amounts of methylphosphonate-substituted complementary oligodeoxynucleotide to determine the arbitrary absorbance ratio that allows complete hybridization. Figure 2 shows that a ratio of 1:10 for 18-P3:12-Me^{10} is sufficient for complete hybridization, whereas 1:5 is not sufficient. All hybridization mixtures of 18-P3 and 12-Me^{10} for subsequent gel migration studies were prepared by using the 1:10 ratio.

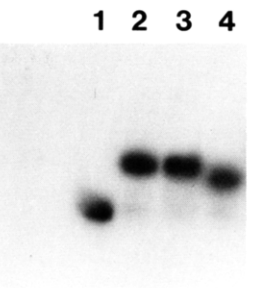

FIGURE 3: Fully modified methylphosphonate oligodeoxynucleotide 12-Me^{10} bound to ^{32}P-labeled complementary 18-P3 is not displaced at 4 °C. Complementary oligodeoxynucleotides were annealed in 100 mM NaCl at room temperature. electrophoresis was performed in a 20% polyacrylamide gel at 4 °C in TBE. Lane 1, 18-P3 only; lane 2, 18-P3 + 12-Me^{10}; lane 3, 18-P3 + 12-Me^{10} followed by 12-P1; lane 4, 18-P3 + 12-P1.

With the gel migration assay, the migration distances of duplexes formed between a recipient and either complementary phosphodiester or methylphosphonate sequences may be compared. Figure 3 shows a distinct difference between the migration for the 18-P3:12-P1 (lane 2) and 18-P3:12-Me^{10} (lane 4) duplexes. The duplex containing the reduced-charge oligodeoxynucleotide has a lower mobility than the duplex containing the equivalent phosphodiester oligodeoxynucleotide.

The gel migration assay was also used to determine the extent to which duplexes with labeled recipient strands, once formed, would exchange complementary strands. As demonstrated in lane 3, Figure 3, the addition of 12-P1 to the annealed 18-P3:12-Me^{10} hybrid does not displace oligodeoxynucleotide 12-Me^{10} (100 mM NaCl, TE/5 at 4 °C). Thus the gel migration assay may be used to quantitate the products of hybridization reactions carried out at elevated temperatures in various salt solutions.

Finally, the gel migration assay was used to determine the ionic strength dependence of the preference for binding of 12-P1 versus 12-Me^{10} to 18-P3. Mixtures were made containing a 1:10 ratio of 18-P3:12-Me^{10} and a 1:1 ratio of 18-P3:12-P1 at various salt concentrations: TE/5, 10 mM NaCl in TE/5, 100 mM NaCl in TE/5, and 1 M NaCl in TE/5. After a 5-min incubation at 70 °C to assure complete denaturation of any transiently formed duplexes, the mixture was slowly cooled to room temperature, cooled to 4 °C, and assayed by gel electrophoresis. The results in Figure 4 show that 18-P3 hybridizes preferentially to 12-P1 in the 1 M and 100 mM conditions, is divided between 12-P1 and 12-Me^{10} in 10 mM NaCl, and binds preferentially to 12-Me^{10} in TE/5. Thus, at low salt, the fully substituted methylphosphonate-containing oligodeoxynucleotide forms more stable hybrids than analogous phosphodiester sequences.

By use of eq 3 and 4, predicted t_m values for 12-Me^{10} range from 34 °C in 1 M NaCl to 31 °C in 0.002 M NaCl. These results predict that t_m for 18-P3:12-Me^{10} and 18-P3:12-P1 hybrids would not become identical until the salt concentration was reduced to 0.002 M, whereas the results in Figure 4 indicate equivalent t_m values in 0.01 M NaCl plus TE/5. One

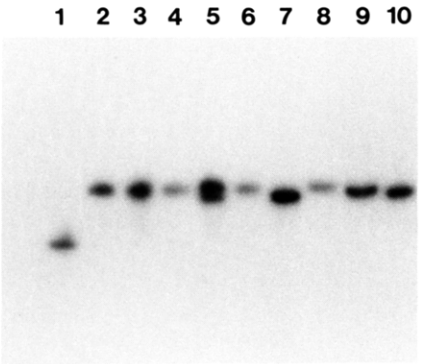

FIGURE 4: Fully modified methylphosphonate oligodeoxynucleotide 12-Me[10] and unmodified oligodeoxynucleotide 12-P1 compete for ^{32}P-labeled complementary oligodeoxynucleotide 18-P3. Oligodeoxynucleotides were heated to 70 °C and slow cooled to room temperature at various ionic strengths. Electrophoresis was performed as described in Figure 3. Competing oligodeoxynucleotide and sodium ion concentration were as follows: lane 1, none, 0.1; lane 2, 12-Me[10], 0.002; lane 3, 12-P1, 0.002; lane 4, 12-Me[10], 0.01; lane 5, 12-P1, 0.01; lane 6, 12-Me[10], 0.1; lane 7, 12-P1, 0.1; lane 8, 12-Me[10], 1.0; lane 9, 12-P1, 1.0; lane 10, 12-P1, 0.1.

explanation for the large apparent relative affinity of 12-Me[10] for 18-P3 is that the chiral mixture which constitutes 12-Me[10] contains many diastereoisomers with smaller detrimental $\Delta G°_d$ values than the −0.75 kcal/mol average value used for the calculations. A corollary to this explanation is that only a subset of the diastereoisomers present in *fully* methylphosphonate substituted oligodeoxynucleotides may be capable of participating in duplex formation.

Effect of Ionic Strength on the Dissociation Temperature of Normal and Backbone-Modified Oligodeoxynucleotides Bound to Complementary DNA. Dot-blot filters were prepared by spotting up to 200 fM denatured pALA-D DNA per dot. Duplexes were formed by incubation of the filters with a 100-fold excess of ^{32}P-labeled oligodeoxynucleotides at 37 °C for 30 min, cooling to room temperature, and incubation for an additional 90 min. On the basis of the specific activity of the oligodeoxynucleotides, no more than 20 fM bound oligodeoxynucleotide could be released from any filter segment containing a dot. That is, at most 10% of the sites on pALA-D contained bound oligodeoxynucleotide. Table IV shows the temperature of 50% dissociation (t_d) of bound oligodeoxynucleotides. Each temperature point of a dissociation curve was determined by using an identical dot which was washed for 30 min into 10 mL or more of the solvent indicated. Either intermittent manual or continuous mechanical stirring was employed during the washing procedure. In no case did the results depend on the method of stirring, although no attempt was made to measure t_d with restricted mixing. Some preliminary experiments had been carried out, with identical results, using the same protocol and manually synthesized (Miller et al., 1986) methylphosphonate oligodeoxynucleotides.

t_d is the temperature of importance for screening plasmid or bacteriophage recombinant DNA libraries with oligodeoxynucleotide probes. Suggs et al. (1981) proposed an empirical rule of thumb for calculating t_d in 1 M NaCl: the sum of 2 °C for each dA·dT base pair and 4 °C for each dG·dC base pair. By use of this rule, t_d for 14-P1 should be 40 °C. The observed value of 41 °C is in good agreement with the rule. On the basis of the model proposed above, the specified time for 50% dissociation at T_d is equal to ln 2 divided by the reverse rate constant for duplex formation, k_r. Thus, k_r is ln 2/1800 at T_d when the specified time is 30 min.

Using the definition of k_2 for DNA duplex formation from Wetmur and Davidson (1968) and defining C_o to be the total nucleotide concentration of non-self-complementary oligodeoxynucleotides, k_2/k_r is equal to $4/C_o$ at T_m. k_2 may thus be determined at T_m if k_r, which is known at T_d, can be determined at T_m by extrapolation using E_r*, the activation energy for k_r. If E_2* is the activation energy for k_2, then

$$\Delta H° = E_r* - E_2* \quad (5)$$

E_2* has been determined for both DNA and oligonucleotide reassociation rates to be very small compared to E_r* [reviewed by Wetmur (1976)]. An appropriate estimate for E_2* would be 4 kcal/mol, approximately that which could be accounted for by a diffusion-controlled rate-determining step depending on T/microscopic solvent viscosity. Thus, for 14-P1 (or 14-A) in 1 M salt, E_r* would be approximately 103 kcal/mol. The relation between T_d and T_m and the reassociation rate constant k_2 is

$$k_2 = \frac{4}{C_o}\left(\frac{\ln 2}{1800}\right) \exp\left[\frac{E_r*}{R}\left(\frac{1}{T_d} + \frac{1}{T_m}\right)\right] \quad (6)$$

The nucleation rate constant for DNA reassociation, k_N', is given by

$$k_N' = k_2 N / L^{0.5}$$

where N is complexity and L is strand length. For non-self-complementary oligodeoxynucleotides, L and N are the same. Wetmur and Davidson (1968) found k_N' for DNA in this same solvent to be 5×10^5 M^{-1} s^{-1} after applying a $^3/_2$ correction to obtain a value for nonpermuted molecules. Lee and Wetmur (1972) observed that k_N' was approximately the same for oligonucleotides and polymers.

During washing at t_d, the half-time for rebinding free oligodeoxynucleotide would be ln $2/k_2C_o$, where C_o for available target pALA-D DNA (10–200 fM in 10 mL) would be $(0.14–2.8) \times 10^{-10}$ M nucleotide. $k_2 = (5 \times 10^5)/14^{0.5}$ at T_d. Thus, the half-time for rebinding could be as short as 5.15 h. However, because the dots were initially saturated with oligodeoxynucleotide, a better estimate of the half-time for rebinding would be in excess of 100 h. In either case, because the rebinding rate is so low, t_d is a measure of k_r and, unlike t_m, is not an equilibrium property. Furthermore, because k_2 decreases dramatically when the ionic strength is decreased, all measurements in salts below 1 M Na^+ would involve even longer rebinding half-times. Clearly, if the concentration of oligodeoxynucleotide were higher during washing, t_d could become a measure of an equilibrium property. When t_d is a kinetic measurement, increasing the washing time, but not the volume, will lead to decreased retention of radioactivity on a dot. If t_d is an equilibrium measurement, increasing the

Table IV: Dissociation Temperatures (t_d)[a] for Methylphosphonate-Containing Oligodeoxynucleotides

t_d in 1 M Sodium Ion	oligodeoxynucleotide	t_d (°C)
unmodified	14-P1	41
partially modified	14-A	32
fully modified	12-Me[10]	30–35
t_d in 0.01 M Sodium Ion	oligodeoxynucleotide	t_d (°C)
unmodified	14-P1	33
partially modified	14-A	32
	14-C	30
	14-G	25–30
	14-T	30
fully modified	12-Me[10]	35–40

[a] t_d = the temperature at which 50% of labeled oligodeoxynucleotide is dissociated from complementary DNA after 30 min of washing.

volume, but not the washing time, will lead to decreased retention of radioactivity on a dot.

From Table III, t_m for 14-P1 is 61 °C and t_m for 14-A is 52 °C when C_o is 84 μM. With a rearranged form of eq 6, t_d for 14-P1 is calculated to be 43 °C and t_d for 14-A is calculated to be 34 °C. In both cases, the predicted t_d is 2–3 °C higher than the experimental value. This small difference could be the result of (1) a temperature dependence of $\Delta H°$ leading to a smaller E_r*, (2) a lower t_d for ^{32}P-labeled DNA than unlabeled DNA used to determine t_m, (3) incomplete equivalence of the dangling ends for the t_m measurement with the extended strands involved in the t_d measurements, or (4) any combination of these factors.

The agreement between the results with the phosphodiester 14-P1 and the methylphosphonate-substituted 14-A is remarkable because the apparent k_r for 14-A reflects elution of the various chiral forms (Bower et al., 1987) of 14-A and is not a true rate constant. Nevertheless, we may conclude that the difference in T_m between phosphodiester and partially methylphosphonate substituted oligodeoxynucleotides reflects primarily a difference in the reverse rate constant k_r.

For 14-P1, T_m decreases 24 °C while T_d decreases 8 °C on going from 1.0 M NaCl to 0.01 M NaCl, indicating that the major effect of changing the ionic strength is on the forward rate constant. Pörschke et al. (1973) found that A_NGCU_N duplex strand separation rates were the same at all temperatures in both 0.05 M NaCl and 1.0 M NaCl for both $N = 2$ and $N = 4$, indicating either a small or no dependence of T_d on ionic strength for these oligoribonucleotides. In the case of partially or fully methylphosphonate substituted oligodeoxynucleotides, no ionic strength effect is seen on T_d. In addition, T_d is not very dependent on the nature of the modified base. Interpretation of these results in terms of rate constants is somewhat complicated by the mixture of chiral forms with different equilibrium constants. The precise rate constants could only be obtained by using a combination of stopped-flow and temperature-jump measurements.

Registry No. 18-P1, 117982-65-7; 18-P2, 117982-64-6; 18-P3, 117982-63-5; 14-P1, 117982-46-4; 14-A, 117982-47-5; 14-C, 117982-48-6; 14-P2, 117982-45-3; 14-G, 117982-49-7; 14-T, 117982-50-0; 12-P1, 117941-33-0; 12-Me^{10}, 117941-31-8; 12-P2, 117941-34-1.

References

Arnold, F. H., Wolk, S., Cruz, P., & Tinoco, I., Jr. (1987) *Biochemistry 26*, 4068–4075.

Bower, M., Summers, M. F., Powell, C., Shinozuka, K., Regan, J. B., Zon, G., & Wilson, W. D. (1987) *Nucleic Acids Res. 15*, 4915–4930.

Breslauer, K. J., Frank, R., Blocker, H., & Marky, L. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3746–3750.

Eckstein, F. (1985) *Annu. Rev. Biochem. 54*, 367–402.

Lee, C. H., & Wetmur, J. G. (1972) *Biopolymers 11*, 1485–1497.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Marcus-Sekura, C. J., Woerner, A. M., Shinozuka, K., Zon, G., & Quinnan, G. V., Jr. (1987) *Nucleic Acids Res. 15*, 5749–5763.

Marky, L. A., & Breslauer, K. J. (1987) *Biopolymers 26*, 1601–1620.

Marky, L. A., Blumenfeld, K. S., Kozlowski, S., & Breslauer, K. J. (1983) *Biopolymers 22*, 1247–1257.

Marmur, J., & Doty, P. (1962) *J. Mol. Biol. 5*, 109–118.

Matsukara, M., Shinozuka, K., Zon, G., Mitsuya, H., Reitz, M., Cohen, J. S., & Broder, S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7706–7710.

Miller, P. S., Yano, J., Yano, E., Carroll, C., Jayaraman, K., & Ts'o, P. O. P. (1979) *Biochemistry 18*, 5134–5143.

Miller, P. S., Agris, C. H., Aurelian, L., Blake, K. R., Murakami, A., Reddy, M. P., Spitz, S. A., & Ts'o, P. O. P. (1985) *Biochemistry 67*, 769–776.

Miller, P. S., Reddy, M. P., Murakami, A., Blake, K. R., Lin, S.-B., & Agris, C. H. (1986) *Biochemistry 25*, 5092–5097.

Murakami, A., Blake, K. R., & Miller, P. S. (1985) *Biochemistry 24*, 4041–4046.

Ornstein, R. L., & Fresco, J. R. (1983) *Biopolymers 22*, 1979–2000.

Pohl, F. M. (1974) *Eur. J. Biochem. 42*, 495–504.

Pörschke, D., Uhlenbeck, O., & Martin, F. H. (1963) *Biopolymers 12*, 1313–1335.

Record, M. T., & Lohman, T. M. (1978) *Biopolymers 17*, 159–166.

Senior, M., Jones, R. A., & Breslauer, K. J. (1988) *Biochemistry 27*, 3879–3885.

Southern, E. M. (1975) *J. Mol. Biol. 98*, 506–517.

Suggs, S. V., Hirose, T., Mikaye, T., Kawashima, E. H., Johnson, M. J., Itakura, K., & Wallace, R. B. (1981) *ICN-UCLA Symp. Mol. Dev. Biol. 23*, 683–693.

Stirchak, E. P., & Summerton, J. E. (1987) *J. Org. Chem. 52*, 4202–4206.

Wells, R. D., Larson, J. E., Grant, R. C., Shortle, B. E., & Cantor, C. R. (1970) *J. Mol. Biol. 54*, 465–497.

Wetmur, J. G. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 337–361.

Wetmur, J. G., & Davidson, N. (1968) *J. Mol. Biol. 31*, 349–370.

Wetmur, J. G., Bishop, D. F., Cantelmo, C., & Desnick, R. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7703–7707.

Woodson, S. A., & Crothers, D. M. (1987) *Biochemistry 26*, 905–919.