Sequence dependent effects in methylphosphonate deoxyribonucleotide double and triple helical complexes

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ABSTRACT

Deoxyribooligonucleotides containing 19 repeating bases of A, T or U were prepared with normal phosphodiester (dA₁₉, dT₁₉, dU₁₉) or methylphosphonate (dA^{*}₁₉, dT^{*}₁₉, dU^{*}₁₉) linkages. Complexes of these strands have been investigated at 1:1 and 1:2 molar ratios (purine:pyrimidine) by thermal melting and gel electrophoresis. There are dramatic sequence dependent differences in stabilities of complexes containing methylphosphonate strands. Duplexes of dA^{*}₁₉ with dT₁₉ or dU₁₉ have sharp melting curves, increased T_m values, and slopes of T_m versus log (sodium ion activity) plots reduced by about one half relative to their unmodified 'parent' duplexes. Duplexes of dA₁₉ with either dT^{*}₁₉ or dU^{*}₁₉, however, have broader melting curves, reduced T_m values at most salt concentrations and slopes of less than one tenth the values for the unmodified duplexes. Duplex stabilization due to reduced phosphate charge repulsion is offset in the pyrimidine methylphosphonate complexes by steric and other substituent effects. Triple helical complexes with dA₁₉ + 2dT₁₉ and dA₁₉ + 2dU₁₉, which can be detected by biphasic melting curves and gel electrophoresis, are stable at increased Na⁺ or Mg⁺² concentrations. Surprisingly, however, no triple helix forms, even at very high salt concentrations, when any normal strand(s) is replaced by a methylphosphonate strand. Since triple helical complexes with methylphosphonates have been reported for shorter oligomers, inhibition with larger oligomers may vary due to their length and extent of substitution.

INTRODUCTION

One of the goals of successful chemotherapeutic treatment of a number of diseases is the development of nucleic acid interactive agents which can exert highly selective effects on target cells or organisms¹. Several promising methods for development of such

highly selective drugs include the use of synthetic nucleic acid sequences which could 1) disrupt translation by forming double-stranded complexes with target mRNA sequences²⁻¹⁸, 2) completely disrupt an array of viral biological processes via complex formation with the genomic RNA of RNA viruses¹⁹⁻²⁷, or 3) affect gene expression and other regulatory and replicative processes of target cells through triple helix formation of the synthetic segments with cellular duplex DNA²⁸⁻³³. In addition to chemotherapeutic uses, such synthetic nucleic acid complexes with natural DNA samples have a number of other potential applications. Highly selective cleavage of chromosomal DNA, for example, can be affected by coupling Fe(II)-EDTA³⁴ or the Cu(I) complex of 1,10-phenanthroline³⁵ to a synthetic DNA strand which can engage in triple helix formation.

For synthetic nucleic acids to be effective in chemotherapy, methods must be found which can engineer nuclease resistance and membrane permeability into their molecular structure. Nuclease resistant nonionic oligomers were envisaged by Halford and Jones³⁶ as more effective antisense inhibitors of the function of mRNA based on the assumptions that these neutral molecules would be better taken up by cells, and form more stable duplexes with negatively charged target sequences than normal phosphodiester oligomers. DNA analogs with internucleoside methylphosphonate linkages have been extensively studied by Ts'o and Miller^{2,8,13,14,17,22,37-39} as sequence-specific inhibitors of gene expression which could possibly serve this purpose. Applications of this 'antisense' approach to basic research and drug development⁴⁰ have recently led many other investigators to consider methylphosphonate analogs^{11,12,18,20,21,24,41,42} as well as alternative types of nonionic linkages, such as phosphotriesters^{12,17,43-45}, phosphoramidates^{46,47}, carbamates^{48,49}, carbonates^{50,51}, carboxymethyls⁵², and dialkyl and diphenylsilyl groups^{53,54}.

Because of these many promising applications of nucleic acids with modified backbones, it is of critical importance to understand the effects of alterations, such as methylphosphonate substitution, on the formation of double and triple helical structures. The

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stability of these helical structures is critical in determining the level of biological activity of a particular agent. Although an early impetus for replacement of the normal phosphodiester linkage with that of a methylphosphonate was that an increase in duplex stability would be obtained, the observed effects have more frequently suggested a destabilizing influence on DNA complexes^{55,56}. A thorough understanding of the factors which affect stability is crucial for design of drugs which will form stable complexes with cellular nucleic acids.

There is very little information available concerning the effects of methylphosphonate substitution on triple helix formation. Triple-stranded structures have been observed with very short methylphosphonate oligonucleotides⁵⁷⁻⁵⁹ and a molecular dynamics investigation has indicated that a triplex of poly d(A)·2poly d(T) containing methylphosphonates should be quite stable⁶⁰. It is necessary to have a good understanding of these effects since regulation of gene expression at the DNA level by nucleic acid drugs is dependent on formation of triple-stranded complexes with duplex DNA^{61,62}.

The chirality of singly-substituted phosphodiester linkages introduces stereochemical complexities⁶³ which, in the case of methylphosphonates, have been studied with simplified model systems^{56,64-66} but not *in vivo*. Stereo-controlled synthesis of homochiral methylphosphonates⁶⁴⁻⁶⁶ with chain lengths relevant to biological applications are not yet practical. Consequently, to our knowledge, all of the reported antisense studies of methylphosphonates have used stereo-random heterochiral oligomers. Such compounds with *Rp* and *Sp* configurations exist as a mixture of 2ⁿ diastereomers, where *n* is the number of methylphosphonate linkages. Additional results on the sequence dependence of stabilities of double and triple stranded complexes containing such diastereomeric mixtures is essential for rational design of antisense drugs.

We have initiated a series of studies to investigate the thermodynamics and structure of complexes containing modified phosphate linkages^{1,40,56}. As one system of study, we have chosen nonalternating normal (dA₁₉) or methylphosphonate modified (dA*₁₉) oligodeoxyriboadenylate in combination with complementary normal (dT₁₉ or dU₁₉) or methylphosphonate modified (dT*₁₉ or dU*₁₉) oligodeoxyribopyrimidates. We have synthesized oligomers containing 19 bases because this length gives approximately two turns of a double helix and is, thus, a good model for sequences in larger nucleic acids. With only three sequences and their methyl phosphonate analogs, we can, thus, investigate several important factors in nucleic acid chemistry and the potential use of methylphosphonates as medicinal agents: 1) the effects on duplex and triple helix stabilities of switching dA·dT base pairs for dA·dU base pairs, 2) triple helix formation in oligonucleotides and their methylphosphonate analogs of discrete length (e.g. not synthetic polymers), 3) stability effects of duplexes with methylphosphonate linkages at different positions in the same sequence, 4) environmental effects on duplexes with the full normal DNA anionic charge, approximately one half the normal charge (one methylphosphonate and one normal chain) or approximately no charge.

The results are quite surprising and informative: methyl phosphonate substitution on the A chain is generally stabilizing relative to the unsubstituted chain, but substitution on the T chain is destabilizing except at very low salt concentrations; the effects of salt on the two duplexes of identical charge (dA*₁₉·dT₁₉ and dA₁₉·dT*₁₉) are quite different; no triple helix was observed with any of the methylphosphonate strands.

EXPERIMENTAL

Description of oligomers

The unmodified 'parent' compounds used were of the type d[N(pN)₁₈] where N = adenine, thymine, or uracil and p is a normal phosphodiester linkage. The corresponding methylphosphonate analogues employed were of the type d[NpN(pN)₁₆pN] where p = 5'-O-P(O)CH₃-O-3'. All but one phosphate group are replaced with methylphosphonate groups to reduce the charge on each single strand to -1. Abbreviations used for methylphosphonate derivatives of dA₁₉, dT₁₉, and dU₁₉ are dA*₁₉, dT*₁₉, and dU*₁₉, respectively.

Synthesis of dA₁₉, dT₁₉ and dU₁₉

Homooligomers of dA and dT were synthesized automatically on a 1-μmol scale with *O*-β-cyanoethyl phosphoramidites⁶⁷ and purified by HPLC as the 5'-DMT derivatives according to recommended protocols⁶⁸. The homopolymer of dU was synthesized and purified similarly but utilized *O*-methyl phosphoramidites, which demonstrably (G. Zon, unpublished data) required extended phosphate deprotection with thiophenol-triethylamine⁶⁹ (or an improved alternative reagent, 2-mercaptobenzothiazole⁷⁰). All of the final 5'-HO oligomers were precipitated in their sodium form as described previously⁷¹.

Synthesis of deoxynucleoside-3'-(N,N-diisopropylamino) methylphosphonamidites

5'-Dimethoxytrityl (5'-DMT) derivatives (American BioNuclear) of dT, 2'-deoxyuridine (dU), and N-protected dA^{NBz} (1 mmol) were made anhydrous by repeated co-evaporation with anhydrous pyridine (Aldrich) and subsequent drying under high-vacuum. In an atmosphere of nitrogen (AtmosBagTM, Aldrich), distilled anhydrous diisopropylamine (4 mmol for 5'-DMT-dA^{NBz}, 6 mmol for 5'-DMT-dT, and -dU) was added dropwise (exothermic) to a magnetically stirred solution of methylchlorophosphine (Alfa, used as received; 2 mmol for 5'-DMT-dA^{NBz}, 3 mmol for 5'-DMT-dT, and -dU) in CHCl₃ (10 mL), which had been washed with water, refluxed with CaCl₂, and then distilled from CaCl₂. The resultant mixture was stirred at ambient temperature for 5–10 min. during which time the protected deoxynucleoside was dissolved in a mixture of CHCl₃ (10 mL) and distilled anhydrous diisopropylethylamine (4.4 mmol for 5'-DMT-dA^{NBz}, 6.6 mmol for 5'-DMT-dT, and -dU). This solution of the protected deoxynucleoside was added dropwise to the above-prepared phosphorylating reagent, and after 10–15 min. of additional stirring the reaction mixture was quenched, first by the addition of anhydrous CH₃OH (0.5 mL) and then, after 10–15 min., by addition to ice-cold CHCl₃. The resultant solution was extracted quickly with an ice-cold, saturated solution of NaHCO₃ and then dried with anhydrous MgSO₄ for rotary evaporation followed by standing under a high-vacuum to give a 'foam'. This crude product was dissolved in anhydrous CH₂Cl₂ (Aldrich; 3–4 mL) and then added dropwise to vigorously stirred, ice-cold, low-boiling petroleum ether (300–400 mL), which resulted in the formation of a precipitate which was collected by suction-filtration, washed with petroleum ether, and then dried over CaCl₂ in a vacuum desiccator.

Each of the resultant powders was characterized by ³¹P NMR⁷², which also established purity (>95%) with regard to phosphorus-containing species. The performance of these products, as judged by coupling yields, was found to vary from

good to poor, on a batch-to-batch basis, and became unacceptable (>85%) upon prolonged storage (1–2 months) of the materials at room temperature in a desiccator. To achieve a high-level of coupling efficiency, the low-performance precipitated powders and degraded powders were purified (*ca.* 1-g scale) by elution from a column (*ca.* 2.5×25 cm) of silica gel (E.M. Science; Kiessgel 60, 230–400 mesh) with low-boiling petroleum ether-benzene-Et₃N (5:20:2 v/v): R_f (Analtec silica gel plates, 250 m) = *ca.* 0.3. In each case, the pooled product fractions were evaporated to give a 'foam' which was precipitated as described above.

Synthesis of dA*₁₉, dT*₁₉ and dU*₁₉

Automated syntheses were carried out on a 1-μmol scale in 1, 2, or 3 columns using long-chain alkylamino controlled pore glass support (Applied Biosystems) and a commercially available instrument (Applied Biosystems, Model 380B). The derivatives of the deoxynucleoside methylphosphonamidites, which were either made as described above or purchased (Applied Biosystems), were prepared as 0.1 M solutions in anhydrous CH₃CN, as were the 5'-DMT derivatives of the corresponding, commercially available (Applied Biosystems) *O*-methyl and *O*-β-cyanoethyl phosphoramidites. The oxidation step (30 s) was carried out either with the standard mixture of I₂-H₂O-lutidine in THF, or with 2 M *t*-butyl hydrogen peroxide in CH₃CN. The other steps in the cycle as well as the reagents and solvents were identical to those employed in the synthesis of unmodified oligomers, per the instrument manufacturer recommendations. Methylphosphonate 19-mers were synthesized as their 5'-DMT derivatives using methylphosphonamidite reagents and an *O*-alkyl phosphoramidite reagent for only the last cycle of coupling. The solid support was dried in the column with a stream of nitrogen and then transferred to a 4-mL vial, which was placed in a vacuum-centrifuge at room-temperature for several hours to remove traces of water. The vial was fitted with a Teflon™ lined screw-cap during the following cleavage and deprotection step: 7h at room temperature with distilled ethylenediamine-absolute EtOH (1:1 v/v, 0.4 mL total). The resultant solution was transferred by pipet to a test tube, and the support was washed thoroughly with absolute EtOH (3×1 mL). The washings were added to the same test tube as above for evaporation to dryness under a stream of nitrogen. The support was dried in a vacuum-centrifuge at room temperature for several hours, washed with 50% aqueous EtOH (3×1 mL), and the washes were added to the same test tube as above; the apparent pH was adjusted, if necessary, to a value of *ca.* 7 by very careful addition of 3% aqueous HOAc. (Caution: addition of HOAc can cause detritylation if the resultant concentration of hydronium ion is too high.) The solution was filtered, if necessary, using a Millex-GV filter-unit (Millipore), and then subjected to HPLC (*vide infra*). [Attempts to use a saturated solution of NH₃ in absolute EtOH (*ca.* 7 M, 2 mL) at 0° C for cleavage from the support and deprotection of the base and phosphate residues led to extensive degradation of the product based on HPLC analysis.] The HPLC columns, equipment, and general principles for HPLC purification of synthetic oligonucleotides and backbone-modified analogues have been reported and discussed elsewhere^{73,74}. The crude 5'-DMT-bearing products were eluted from a poly(styrene-divinylbenzene) column (Hamilton PRP-1, 7×305 mm) with the following gradient of CH₃CN (eluent A) vs. water (eluent B) at 3 mL/min., which are defined by time (min.) points and the ratio

of A:B at those times: 0 (inject), 10:90; 10, 27:73; 20, 48:52; 25, 10:90 (re-equilibrate). 5'-Hydroxyl-bearing oligomers gave rise to a fast-eluted 'sawtooth' pattern of peaks. For dA*₁₉, this was followed by a relatively broad 'hump' of peaks presumed to be N⁶-β-aminoethyl-bearing oligomer byproducts⁷⁵. The desired 5'-DMT material was eluted next, as the proportion of CH₃CN was increased to values of 27–48%, and was collected for subsequent concentration to dryness in a vacuum-centrifuge, after most of the CH₃CN had been evaporated under a stream of nitrogen. The resultant residue was detritylated in a Pyrex™ test tube with 80% (v/v) HOAc in water (1 mL) for 6 min. at room temperature. This reaction mixture was frozen quickly in dry-ice for concentration to dryness in a vacuum-centrifuge. The resultant material was dissolved in 50% aqueous EtOH (1 mL) and applied to a commercially available (Pharmacia) size-exclusion column (Sephadex G-25, 9-mL bed volume), which had been pre-washed with 50% aqueous EtOH (30–50 mL); 1-mL fractions were collected and the final product, which was located (tubes 3–5 or 6) by measurement of Abs₂₆₀ values and pooled, was concentrated to dryness in a vacuum-centrifuge.

dA*₁₉ = d[A_pA(_pA)_{16p}A], 99.7% average coupling yield, 5'-DMT oligomer elution time 14.5 min., final product yield 80 OD₂₆₀ units.

dT*₁₉ = d[T_pT(_pT)_{16p}T], 98.4% average coupling yield, 5'-DMT oligomer elution time 23.5 min., final product yield 69 OD₂₆₀ units.

dU*₁₉ = d[U_pU(_pU)_{16p}U], 98.5% average coupling yield, 5'-DMT oligomer elution time 15.8 min., final product yield 49 OD₂₆₀ units.

Oligomer stock solutions

Stock solutions were made from lyophilized samples of oligomers which were dissolved in small amounts of triple distilled deionized water and kept frozen when not in use. Concentrations of oligomer stock solutions were determined on a per nucleotide basis at 25°C using the following extinction coefficients (per mole of nucleotide unit): ε(dA₁₉) = 8,600 L/mol-cm; ε(dT₁₉) = 8,700 L/mol-cm; ε(dU₁₉) = 9,200 L/mol-cm. These are extinction coefficients (per nucleotide) previously reported⁷⁶ for poly d(A) at a wavelength of 257 nm, poly d(T) at 265 nm, and poly r(U) at 260 nm, respectively. The same extinction coefficients were used to calculate concentrations of stock solutions containing methylated derivatives.

Sample preparation and buffer

Purine:pyrimidine 1:1 and 1:2 molar ratio mixtures of nucleic acid oligomers were prepared for uv spectroscopy experiments by addition of a concentrated aqueous stock solution to a one ml volume of PIPES buffer (0.01 M [piperazine-N, N'-bis {2-ethane sulfonic acid}], 0.001 M disodium EDTA adjusted to pH 7.0 with NaOH and filtered through a 0.45 micron teflon filter prior to use). The desired ionic strength was obtained for individual samples by additions of either solid or a 4 M solution of NaCl. Salt concentrations are referred to as follows: PIPES 00 (no NaCl added; 0.019 M Na⁺), PIPES 05 (0.050 M NaCl added; 0.069 M Na⁺), PIPES 10 (0.100 M NaCl added; 0.119 M Na⁺), PIPES 20 (0.200 M NaCl added; 0.219 M Na⁺), PIPES 40 (0.400 M NaCl added; 0.419 M Na⁺), PIPES 60 (0.600 M NaCl added; 0.619 M Na⁺), PIPES 80 (0.800 M NaCl added; 0.819 M Na⁺), and PIPES 100 (1.000 M NaCl added; 1.019 M Na⁺).

UV spectral and T_m measurements

Oligomer uv spectral analyses and melting temperature experiments were performed using a Cary 219 spectrophotometer interfaced to an Apple IIe microcomputer. Up to four samples were concurrently monitored via a five-position rotatable cell turret; buffer solution of the appropriate ionic strength was placed in a reference cuvette occupying one position of the cell turret. Sample solutions were contained in matched, reduced volume quartz cuvettes with teflon stoppers and a pathlength of 1 cm.

T_m measurements were initiated near 0°C and the temperature was increased at a rate of 0.5°C per minute until complete melting curves were obtained for all samples. Identical results were obtained when the heating rate was reduced to 0.25°C per minute. Examination of melting reversibility (duplex formation) was accomplished by following this procedure in reverse. Temperature control of sample solutions was maintained, using a Haake A81 constant temperature circulator with heating and refrigeration capabilities which was connected to the jacketed cell compartment and regulated with a Haake PG 20 temperature programmer. Temperatures were measured using a Varian thermister unit connected to the Cary 219. The thermister probe was fitted snugly through a hole in the teflon stopper into a cuvette containing the buffer solution. At temperatures below 20°C, nitrogen gas was continuously passed through the sample compartment to prevent formation of condensate.

Absorbances were measured at 260 nm and plotted as a function of temperature by the Cary 219 chart recorder, while simultaneously being stored to four decimal places (along with the corresponding temperature to two decimal places) in the Apple IIe computer memory. To improve the signal to noise ratio, the computer averaged 10 absorbance readings for each data point of the melting curve. These data were subsequently transferred to disk for storage and later analyses. Averaged absorbance readings were collected every 0.5°C for each sample, and, typically, a total of over 150 points were collected. Sample absorbances were obtained by subtracting absorbances of the reference buffer from measured sample absorbances at the same temperatures. We refer to the 'T_m' for each transition as the temperature at the maximum in the derivative of a plot of sample absorbance versus 1/temperature⁷⁷. 'T_m' values for many of these transitions were also determined by two other methods: 1) taking the temperature at which the derivative of a plot of sample absorbance versus temperature is at a maximum⁷⁸ and 2) taking the temperature at which the fraction of single strands (α) is equal to 0.5, obtained by estimating sloping baselines and taking the ratio at any temperature (T) of the distance between the experimental curve and the lower baseline to that between the two baselines⁷⁹. T_m values obtained from the method using $d\text{Absorbance}/dT^{-1}$ were consistently slightly higher (0°-2°C) than those found using either $d\text{Absorbance}/dT$ or the sloping baseline method, but were essentially within experimental error. It should be noted that since the discrepancies in the methods are systematic, slopes of T_m versus log (sodium ion activity) plots are essentially identical regardless of which of these three methods was employed.

For comparative visualization purposes, melting profiles are shown with absorbances converted to fraction absorbance change, defined as $A_T - A_{LT} / A_{HT} - A_{LT}$ where A_T = the absorbance at some temperature T, A_{LT} = the absorbance at the low temperature extreme investigated, and A_{HT} = the absorbance at the high temperature extreme investigated. Hyperchromicities were calculated from oligomer denaturations by first obtaining

linear sloping baselines in both the high and low temperature regions of each melting curve. When taken at the T_m, the absorbance difference between these lines divided by the upper absorbance value is defined as the hyperchromicity.

Gel electrophoresis

Electrophoresis experiments were conducted using gels containing 20% polyacrylamide (19.5% acrylamide/0.5% bisacrylamide) prepared in a Bio-Rad Protean II gel apparatus with 20×22 cm glass slabs and 0.75 mm spacers. TBM buffer (0.09 M Tris, 0.09 M boric acid, 5 mM MgCl₂, pH 8.3) was used in sample preparations and in the electrophoresis reservoirs. The gel loading buffer contained TBM with 7% sucrose plus 0.025% bromphenol blue tracking dye. Sample volumes ranged from approximately 2 to 10 μ l of diluted stock solution. Experiments were conducted at constant temperature (4°C) and voltage (200 volts) for 15 hours. After electrophoresis was halted, the gel was stained using a Bio-Rad silver staining kit. Photographs were taken using a Fotodyne FCR-10 polaroid camera at 1/4 sec and f-11.

RESULTS

Single strand oligonucleotide melting behavior

Melting profiles of single strand dT₁₉ and dU₁₉, and of each of the two corresponding methylphosphonate analogs, showed essentially no increase in absorbance when heated to 100°C at various salt concentrations. Thus, as expected, no significant base stacking of these oligomers is indicated. Both 'normal' dA₁₉, and its methylphosphonate derivative, however, showed some absorbance increase with increasing temperature as expected for sequences of A bases⁸⁰. There is no significant dependence on salt concentration in the absorbance-temperature plots for any of the single strand oligomers.

Melting behavior of dA₁₉ with dT₁₉ or dU₁₉

Representative melting curves for 1:1 and 1:2 molar ratio mixtures of dA₁₉ + dT₁₉ are shown at two different salt

Figure 1. Melting curves for [dA₁₉]:[dT₁₉] = 1:1 in PIPES 20 (●) and PIPES 100 (■) buffers (8.0×10^{-5} M dA₁₉); and for [dA₁₉]:[dT₁₉] = 1:2 in PIPES 20 (○) and PIPES 100 (□) buffers (4.5×10^{-5} M dA₁₉).

concentrations in Figure 1. Similar curves are shown in Figure 2 at several salt concentrations for $dA_{19} + dU_{19}$. In all cases, absorbance versus temperature plots were identical when samples were heated, cooled, and then reheated under the same experimental conditions. At all salt concentrations considered, curves obtained from 1:1 molar ratio mixtures of oligonucleotides show cooperative *monophasic* melting behavior. At sodium concentrations of less than 0.2 M, curves obtained from 1:2 molar ratio mixtures of $dA_{19} + dT_{19}$ are essentially identical to those obtained from the corresponding 1:1 mixtures. However, absorbance versus temperature profiles for 1:2 mixtures of these same oligomers at sodium concentrations of approximately 0.2 M or greater are *biphasic* and indicate the presence of the triple helix at low temperature and a triple helix to duplex $dA_{19} \cdot dT_{19}$ and single strand dT_{19} transition⁷⁷. Similar results were obtained for 1:2 molar ratio mixtures of $dA_{19} + dU_{19}$ except that the low temperature triple helix to duplex transition was not observed until approximately 0.6 M Na^+ . Melting profiles of similar shape were also obtained when 1 mM Mg^{+2} was substituted for 0.6 M Na^+ in 1:2 molar ratio mixtures of $dA_{19} + dT_{19}$.

T_m values for all observed transitions are listed at various salt concentrations in Table I and are plotted in Figure 3 as a function of $\log$ (sodium ion activity). At all salt concentrations considered, T_m values are lower for complexes containing dU_{19} than for those containing dT_{19} . In all cases, T_m values for the high

temperature transitions in biphasic melting curves resulting from 1:2 molar ratio mixtures of $dA_{19} + dT_{19}$ or dU_{19} are essentially identical to T_m values for transitions from the corresponding 1:1 molar ratio mixtures. T_m values for low temperature transitions, are considerably lower than those of the corresponding 1:1 mixtures. T_m values increase with increasing sodium ion activity and slopes of linear fits shown in Figure 3 are given in Table I. The effects of salt concentration on the high temperature transitions from 1:2 molar ratio mixtures of both $dA_{19} + dT_{19}$ and $dA_{19} + dU_{19}$ are essentially identical to the effects on transitions seen from the corresponding 1:1 molar ratio mixtures. Slopes of T_m versus $\log$ (sodium ion activity) plots for the low temperature transitions are much larger than those for the high temperature transitions. Thus, for 1:2 molar ratio mixtures of both $dA_{19} + dT_{19}$ and $dA_{19} + dU_{19}$, melting temperatures for the two transitions converge as the salt concentration of the sample solution is increased. Hyperchromicities of these transitions at salt concentrations investigated averaged 0.27 ± 0.03 .

Melting behavior of complexes containing dA^*_{19} , dT^*_{19} , and/or dU^*_{19}

Example melting profiles for complexes formed from 1:1 mixtures of dA_{19} or dA^*_{19} with dT_{19} or dT^*_{19} are shown in Figure 4. At any given salt concentration, slightly broader curves of similar shape, but lower T_m values, were obtained when dU_{19} was substituted for dT_{19} . Transition breadths $[dT/d(\text{Fraction absorbance change})]_{T_m}$ from melting profiles of the various duplexes at 0.20 M $[Na^+]$ are: $dA_{19} \cdot dT_{19}$, 13.1; $dA^*_{19} \cdot dT_{19}$, 11.8; $dA_{19} \cdot dT^*_{19}$, 31.5; $dA^*_{19} \cdot dT^*_{19}$, 28.2; $dA_{19} \cdot dU_{19}$, 21.5; $dA^*_{19} \cdot dU_{19}$, 18.1; $dA_{19} \cdot dU^*_{19}$, 23.7 and

Figure 2. Melting curves for (A) $[dA_{19}]:[dU_{19}] = 1:1$ in PIPES 05 (+), PIPES 20 (●), and PIPES 100 (■) buffers (3.4×10^{-5} M dA_{19}); and for (B) $[dA_{19}]:[dU_{19}] = 1:2$ in PIPES 20 (○), PIPES 60 (×), and PIPES 100 (□) buffers (3.3×10^{-5} M dA_{19}). The results are shown on two separate plots because of extensive curve overlap.

Table I. T_m Values ($^{\circ}C$) for Complexes Formed from $dA_{19} + dT_{19}$ or dU_{19} .

$[Na^+]/\log a_{Na^+}$	$dA_{19} + dT_{19}$	$dA_{19} + 2dT_{19}^a$	$dA_{19} + 2dT_{19}^b$
0.05/-1.39	38.2	—	—
0.10/-1.11	44.5	—	—
0.20/-0.84	49.8	—	8.1
0.22/-0.80	—	48.6	—
0.40/-0.56	55.4	—	—
0.41/-0.54	—	55.1	22.1
0.62/-0.38	—	57.0	31.6
0.66/-0.35	—	57.9	35.2
0.82/-0.26	—	58.8	39.8
1.00/-0.18	61.1	—	45.8
1.02/-0.17	—	59.3	43.7
Slope ($^{\circ}C$):	20.7	20.2	56.6
$[Na^+]/\log a_{Na^+}$	$dA_{19} + dU_{19}^d$	$dA_{19} + 2dU_{19}^a$	$dA_{19} + 2dU_{19}^b$
0.02/-1.78	15.0	15.5	—
0.07/-1.25	25.9;26.6	27.2	—
0.12/-1.04	31.0;32.0	31.7	—
0.22/-0.80	36.3;36.6	36.8	—
0.42/-0.54	41.3;41.9	41.8	—
0.62/-0.38	43.7	44.4	12.2
0.82/-0.26	45.3	46.4	18.9
1.02/-0.17	46.4	47.8	24.2
Slope ($^{\circ}C$):	21.0	20.8	58.0

^a Values from high temperature transitions in 1:2 molar ratio mixtures.

^b Values from low temperature transitions in 1:2 molar ratio mixtures.

^c Slopes of T_m versus $\log$ (sodium ion activity) plots from Figure 3.

^d Duplicate values are separated by a semicolon.

Figure 3. The effect of sodium ion activity on T_m for $[dA_{19}]:[dT_{19}] = 1:1$ duplex (●) and for $[dA_{19}]:[dT_{19}] = 1:2$ duplex (▲) and triplex (■); for $[dA_{19}]:[dU_{19}] = 1:1$ duplex (○) and for $[dA_{19}]:[dU_{19}] = 1:2$ duplex (△) and triplex (□). Oligomer concentrations are given in the legends to Figures 1 and 2. Linear fits for duplex to single strand transitions include data from log activities of sodium ion less than -0.3 ; for triplex to duplex + single strand, less than 0. Slopes of linear fits are given in Table I.

$dA^*_{19} \cdot dU^*_{19}$, 36.0. In all cases, absorbance versus temperature profiles for methylphosphonate containing complexes were identical when samples were heated, cooled, and then reheated under the same experimental conditions. Mixtures containing dT^*_{19} or dU^*_{19} were found to have melting curves which are broadened relative to those of their parent non-methylated duplexes at all salt concentrations considered. Melting profiles of mixtures containing dA^*_{19} with dT_{19} or dU_{19} , however, have shapes similar to those of non-methylated duplexes. All complexes displayed cooperative monophasic melting behavior at the salt concentrations investigated. T_m values are shown in Table II.

Melting curves obtained from samples containing methylphosphonate derivative(s) in 1:2 molar ratios (purine:pyrimidine) resulted in the same cooperative *monophasic* transitions as those from the 1:1 mixtures at all salt concentrations investigated. Example curves from samples containing a methylphosphonate derivative in molar ratios of 1:1 and 1:2 (purine:pyrimidine) at 1 M $[Na^+]$ are shown in Figure 5 and are compared to those for 1:1 and 1:2 molar ratio mixtures of the corresponding normal oligonucleotides. Unlike the results for normal phosphodiester samples at high salt concentrations, no evidence was obtained at any salt concentration for an additional low temperature transition in 1:2 molar ratio mixtures containing methylphosphonate derivative(s). Particularly noteworthy is the fact that a 1:1:1 molar ratio mixture of $dA_{19}:dT_{19}:dT^*_{19}$ shows no indication of an additional low temperature transition even at high salt concentrations. This combination of oligomers was investigated in two different experimental situations, as follows: 1) a normal $dA_{19} \cdot dT_{19}$ duplex was allowed to form (by cooling a 1:1 mixture of $dA_{19} + dT_{19}$ to approximately 4°C for several hours) before dT^*_{19} was added and a melting experiment was initiated immediately, and 2) single-stranded dA_{19} , dT_{19} , and

Figure 4. Melting curves for 1:1 ratios of $[dA_{19}]:[dT_{19}]$ (●), $[dA_{19}]:[dT_{19}]$ (×), $[dA_{19}]:[dT^*_{19}]$ (■), and $[dA_{19}]:[dT^*_{19}]$ (▲) all in PIPES 20 buffer. All oligomer concentrations are approximately $8.0 \pm 0.5 \times 10^{-5}$ M.

dT^*_{19} were all allowed to compete for formation of thermodynamically stable complexes (by heating the 1:1:1 mixture of $dA_{19}:dT_{19}:dT^*_{19}$ to 90°C, then cooling to approximately 0°C) before a melting experiment was initiated. Identical cooperative monophasic melting profiles were obtained in both cases. Addition of magnesium ion at 10, 50 or 200 mM concentrations also did not induce triple helix formation with any mixture of oligomers which contained methylphosphonate strands.

T_m values for complexes containing normal and methylphosphonate oligomers are listed at various salt concentrations in Table II and are shown as a function of log (sodium ion activity) in Figure 6. At salt concentrations containing greater than 0.07 M $[Na^+]$, melting curves from mixtures containing methylphosphonate dU_{19} have T_m values which are reduced relative to those of their normal 'parent' complexes, although this is not the case at concentrations below 0.07 M $[Na^+]$. Melting curves from mixtures containing methylphosphonate dT_{19} have T_m values which are reduced relative to those of the unmodified complexes at all salt concentrations investigated. However, by extrapolation to a salt concentration of approximately zero, it can be seen that duplexes containing either dT^*_{19} or dU^*_{19} would be more stable than the parent duplexes. Melting transitions from mixtures containing methylphosphonate dA_{19} , however, have T_m values which are higher than those of their non-methylated counterparts at salt concentrations up to around 1 M $[Na^+]$, where T_m values for the two related complexes approach each other.

Slopes of linear fits from T_m versus log (sodium ion activity) plots for methylphosphonate derivatives are given in Table II. From these data it can be seen that all complexes containing methylphosphonates are less affected by salt concentration than their unmodified parent duplexes. The duplexes containing dA^*_{19} with either dT_{19} or dU_{19} , however, have slopes 5–10 times larger than those for duplexes containing dA_{19} with either dT^*_{19} or dU^*_{19} , even though they have the same charge and sequence (Table II). Duplexes in which anionic charges on both oligonucleotide strands are neutralized by methylphosphonate

Table II. T_m Values (°C) for Complexes Containing Normal or Methylphosphonate dA₁₉ with Normal or Methylphosphonate dT₁₉ or dU₁₉.

[Na ⁺]/Log a _{Na⁺}	dA ₁₉ +dT ₁₉ ^a	dA* ₁₉ +dT ₁₉ / ^b dA* ₁₉ +2dT ₁₉	dA ₁₉ +dT* ₁₉ / dA ₁₉ +2dT* ₁₉	dA* ₁₉ +dT* ₁₉ / dA* ₁₉ +2dT* ₁₉
0.05/-1.39	38.2	49.8/-	37.8/-	37.0/-
0.10/-1.11	44.5	52.8/-	37.8/-	37.0/-
0.20/-0.84	49.8	54.9/55.3	38.1/40.0	37.0/37.7
0.40/-0.56	55.4	57.5/-	39.0/-	37.0/-
1.00/-0.18	61.1	62.0/61.7	40.5/41.5	37.8/37.8
Slope (°C) ^c :	20.7	9.1	1.4	0

[Na ⁺]/Log a _{Na⁺}	dA ₁₉ +dU ₁₉ ^{a,d}	dA* ₁₉ +dU ₁₉ / dA* ₁₉ +2dU ₁₉	dA ₁₉ +dU* ₁₉ / dA ₁₉ +2dU* ₁₉	dA* ₁₉ +dU* ₁₉ / dA* ₁₉ +2dU* ₁₉
0.07/-1.25	25.9;26.6	36.9/-	29.9/-	26.9/-
0.12/-1.04	31.0;32.0	40.6/-	30.2/-	26.9/-
0.20/-0.84	-	42.9/43.4	30.7/-	-/-
0.22/-0.80	36.3;36.6	42.5/-	30.8/-	26.9/-
0.40/-0.56	-	45.5/-	31.1/-	-/-
0.42/-0.54	41.3;41.9	44.6/45.5	31.2/34.1	26.9/26.3
1.00/-0.18	-	49.6/48.5	32.2/-	28.6/-
1.02/-0.17	46.4	-/47.2	-/34.4	-/26.3
Slope (°C) ^c :	21.0	10.9	1.8	0

^a T_m values listed for these complexes are those for duplexes only.^b A slash (/) separates results of 1:1 from those of 1:2 molar ratio mixtures.^c Slopes of T_m versus log (sodium ion activity) plots from Figure 3.^d Duplicate values are separated by a semicolon.

substitution show essentially no dependence of T_m on salt concentration. Average hyperchromicities of complexes containing methylphosphonates at all salt concentrations investigated are: dA*₁₉·dT₁₉ (0.32), dA₁₉·dT*₁₉ (0.26), dA*₁₉·dT*₁₉ (0.32), dA*₁₉·dU₁₉ (0.32), dA₁₉·dU*₁₉ (0.22), and dA*₁₉·dU*₁₉ (0.24), and for the parent complexes, 0.27 ± 0.03.

Gel electrophoresis of single-, double-, and triple-stranded oligomers

The equilibrium between single-, double-, and triple-stranded species of dA₁₉ and dT₁₉ can be directly monitored by polyacrylamide gel electrophoresis (Figure 7). This method also provides a qualitative check on the mixing ratios used to obtain double and triple helical species in solution studies. The results in Figure 7 show that the single-, double-, and triple-stranded species can be separated (e.g., exchange among species is relatively slow) with good resolution on a polyacrylamide gel at low temperature. As can be seen from the gel results, the double helix is quantitatively formed at a 1:1 molar ratio of dA₁₉:dT₁₉ and the triple helix at a 1:2 molar ratio. Note that under conditions where the triple helix is quantitatively formed at a 1:2 molar ratio (lane 9), no triple helix is detected at a 1:1 molar ratio (lane 6). This result is in complete agreement with T_m results for this DNA sequence (Figure 1), and indicates that disproportionation of the duplex to triple helix does not occur under these conditions.

Similar experiments were performed with dA*₁₉ + dT₁₉ and with dA₁₉ + dT*₁₉ (not shown). At a 1:1 molar ratio of dA*₁₉:dT₁₉, a single band, somewhat broader relative to that seen for dA₁₉:dT₁₉ was observed. The experiment with dA₁₉·dT*₁₉ produced an even broader duplex band, but no triple helix band at high ratios of dT*₁₉ to dA₁₉. At ratios of up to 1:3, dA*₁₉:dT₁₉, no triple helix band could be detected. This agrees completely with the T_m results which indicate that no triple

Figure 5. Melting curves for [dA]:[dT] = 1:1 (■), [dA]:[dT] = 1:2 (□), [dA*]:[dT] = 1:1 (+), and [dA*]:[dT] = 1:2 (△) all in PIPES 100 buffer (oligomer concentrations are 8.0×10⁻⁵ M dA₁₉ + 8.0×10⁻⁵ M dT₁₉; 4.5×10⁻⁵ M dA₁₉ + 9.7×10⁻⁵ M dT₁₉, 8.0×10⁻⁵ M dA*₁₉ + 8.0×10⁻⁵ M dT₁₉, and 4.5×10⁻⁵ M dA*₁₉ + 9.0×10⁻⁵ M dT₁₉, respectively).

helix forms when either of the DNA strands contains methylphosphonate linkages to this extent.

DISCUSSION

Analyses of DNA complexes formed with methylphosphonate derivatives of very limited composition and chain length have led to conclusions about these compounds which need to be

Figure 6. The effect of sodium ion activity on T_m for 1:1 ratios of (A) $[dA_{19}]:[dT_{19}]$ (●), $[dA^*_{19}]:[dT_{19}]$ (×), $[dA_{19}]:[dT^*_{19}]$ (■), and $[dA^*_{19}]:[dT^*_{19}]$ (▲), and of (B) $[dA_{19}]:[dU_{19}]$ (○), $[dA^*_{19}]:[dU_{19}]$ (+), $[dA_{19}]:[dU^*_{19}]$ (□), and $[dA^*_{19}]:[dU^*_{19}]$ (△). All oligomer concentrations are around 8×10^{-5} M. Linear fits include data from log activities of sodium ion less than -0.3 . Slopes of linear fits are given in Table II. Results are shown on two separate plots because of extensive curve overlap.

reevaluated in light of our new observations. It is the general view, for example, that methylphosphonate substitution either has little effect on duplex stability or causes duplex stabilization. We find that either significant stabilization or destabilization can be obtained depending on the sequence modified and /or the salt concentration. Observations from previous work with duplexes containing one normal and one methylphosphonate chain suggested that there are no significant salt effects in the thermal transitions. We find appreciable salt effects depending on the DNA sequence, and our results are completely consistent with the counterion condensation theory. Previous observations and theoretical calculations indicated that methylphosphonate nucleic acids can form triple helices with one A and two T chains. No triple helix is seen when any methylphosphonate chain is substituted in $dA_{19} + 2dT_{19}$ or $dA_{19} + 2dU_{19}$ mixtures.

Effects of methylphosphonate substitution on DNA duplexes

All methylphosphonate substituted DNA strands interact with complementary unmodified strands to give duplexes which have cooperative melting transitions. Duplex formation is demonstrated by the cooperative melting transitions and by gel electrophoresis. The most surprising effect of methylphosphonate substitution is the dramatic sequence dependence of duplex stabilization. For

Figure 7. Photograph of a polyacrylamide gel with different ratios of dA_{19} and dT_{19} . Lanes are labeled 1–10 from left to right and the ratios of $dA_{19}:dT_{19}$ in each lane are: (1) 1:0 (only dA_{19}); (2) 1:0.08; (3) 1:0.34; (4) 1:0.68; (5) 1:0.85; (6) 1:1.05; (7) 1:1.26; (8) 1:1.69; (9) 1:2.11; (10) 1:2.53. The A_{19} single strand is clearly seen in lane 1; only the duplex is visible in lane 6; and only the triple helix can be seen in lanes 9 and 10. Gels were stained with silver stain and free dT_{19} can not be visualized with that reagent.

example, in 0.1 M Na^+ , $dA^*_{19} \cdot dT_{19}$ has a T_m value approximately $15^\circ C$ higher than $dA_{19} \cdot dT^*_{19}$, even though both have the same base pair sequence and charge. The order of DT_m values (T_m modified – T_m normal) for duplexes containing a single methylphosphonate chain is: $dA^*_{19} \cdot dT_{19} \sim dA_{19} \cdot dU_{19} >> dA_{19} \cdot dU^*_{19} > dA_{19} \cdot dT^*_{19}$. Transition curves for duplexes containing dA^*_{19} with normal dT_{19} or dU_{19} are sharp (highly cooperative), as are those of the unmodified parent duplexes. This implies that the stability of the 2^{17} possible isomeric derivatives (produced as a result of the R_p and S_p configurations at each methylphosphonate linkage) is not detectably influenced by stereochemical differences at phosphorous. By contrast, transition curves for duplexes containing dT^*_{19} or dU^*_{19} with normal dA_{19} are significantly broader. This suggests that the isomeric states of the methylphosphonate do have a significant influence on stability when the pyrimidine strand is substituted.

These findings with duplexes containing dA^*_{19} , dT^*_{19} , and dU^*_{19} are in agreement with our previous results⁵⁶ involving isomerically pure methylphosphonate duplexes and indicate that the following three primary factors affect methylphosphonate duplex stability: 1) elimination of phosphate charge, 2) electronic and other substituent effects of the methylphosphonate versus normal phosphate group, and 3) the steric effect of the methylphosphonate group. For duplexes containing dA^*_{19} , elimination of charge repulsion appears to be the most significant factor. These duplexes have sharp melting transitions and high

T_m values. For duplexes containing dT*₁₉, we feel that all three effects are significant. Decreasing the charge repulsion will increase stability in duplexes containing any of the methylphosphonate modified strands, but the other two factors must also be significant for duplexes containing pyrimidine methylphosphonates since duplex destabilization is observed.

To develop better ideas about the steric factors which affect methylphosphonate duplex stability, we have conducted two types of preliminary computer molecular graphics analyses with the program MACROMODEL (from Professor Clark Still, Columbia University) on a MICROVAX II computer and E & S PS390 graphics terminal. First, phosphodiester groups in the crystal structure⁸¹ of the DNA sequence d(CGCAAATTTGCG) (coordinates from the Brookhaven Protein Data Bank)^{82,83} were converted to methylphosphonates. The central AT region of this sequence is characterized by a narrow minor groove, an unusually high base pair propeller twist, and a bifurcated hydrogen bond between the A amino group and two cross strand carbonyl oxygens on adjacent T bases⁸¹. In this conformation we find no direct contact between methylphosphonate groups in the AT region and any other atoms. Second, we built a nonalternating A·T sequence with standard fiber diffraction B-form coordinates⁸⁴. The minor groove is not as narrow and base pair propeller twist is less in this conformation. Replacement of phosphodiester groups with methylphosphonates at A and T in this sequence did reveal some slight differences. No steric clash was seen with either methylphosphonate isomer of the dA₁₉ or dU₁₉ strands. But, contact could occur between methyl protons in some rotational states of the T-5 methyl group and the methylphosphonate methyl of the Sp isomer when the methyl groups are rotated to give close contact. Since dA₁₉·dU*₁₉ is less stable relative to the unmodified duplex than is dA*₁₉·dU₁₉, at all salt concentrations, steric clash at the T-5 methyl can clearly not account for all of the observed destabilization of duplexes containing pyrimidine methylphosphonates. Since the T_m values of duplexes containing dT*₁₉ are significantly less than those of duplexes with dA*₁₉, simple arguments based on stereoelectronic effects at the methylphosphonate group⁸⁵ are also not sufficient to explain the observed sequence dependence differences in ΔT_m values. As stated above, several factors account for the differences in duplex stabilities on replacement of phosphodiester groups with methylphosphonates, and the results presented here demonstrate that the importance of the various effects varies with nucleic acid sequence and solution conditions. More study with a greater variety of sequences is clearly necessary to provide a complete explanation of the influence of methylphosphonates on duplex and triplex stability. We have such studies underway, as well as more detailed modeling experiments involving energy minimization.

Effects of salt concentration on methylphosphonate duplex stability

For unmodified oligomers of dA₁₉ with dT₁₉ or dU₁₉, slopes of T_m versus log (sodium ion activity) plots for duplex to single strand transitions are 20.7°C and 21.0°C, respectively, in good agreement with other results for AT rich duplexes⁸⁶⁻⁸⁸. For oligomers containing dA*₁₉ with unmodified dT₁₉ or dU₁₉, slopes of these plots decrease to 9.1°C and 10.9°C, respectively. According to the simplest polyelectrolyte theories, substitution of dT*₁₉ or dU*₁₉ for 'normal' dT₁₉ or dU₁₉, respectively, should affect a purine·pyrimidine duplex to the same degree as does substitution of dA*₁₉, for 'normal' dA₁₉ under the same

experimental conditions. Regardless of base identity, the duplex charge densities are essentially identical. However, there is a strong sequence dependence in our results. Duplexes containing dT*₁₉ or dU*₁₉ complexed with unmodified dA₁₉ have slopes of only 1–2°C, approximately a factor of 10 less than the values for duplexes containing dA*₁₉. Although these slope variations could indicate a difference in the effects of methylphosphonate substitution on the complex, calculations from the counterion condensation theory of Manning⁸⁹ and Record⁹⁰ indicate that the observed differences are explained quite well by stacking differences in the single strands.

From counterion condensation theory, the effect of sodium ion concentration on T_m can be expressed as follows⁹⁰:

$$\delta T_m / \delta \ln a_{Na^+} = -\alpha(\Delta n/2) \times [R(T_m)^2 / \Delta H^\circ]$$

where a_{Na^+} is the sodium ion activity; α is a correction for nonideality; ΔH° is the enthalpy change for the transition; Δn is the stoichiometric counterion uptake during formation of a complex [expressed as $z(1 - \xi_f^{-1})$ for the final $-z(1 - \xi_i^{-1})_n$ for the initial n state(s) of the complex]; z is the number of phosphates involved; $\xi^{-1} = \epsilon kTb/e^2$; b is the average spacing between phosphates based on modeling the oligonucleotide as a linear array of negative charges; ϵ is the charge on the electron; ϵ is the solution dielectric constant; k is Boltzmann's constant; T is the absolute temperature. The value⁹⁰ of $\epsilon kT/e^2$ for a complex in aqueous solution at 25°C is 0.14 Å⁻¹. A value of 3.3 Å has been calculated for b of single stranded poly (A)⁷⁷. A value of 4.8 Å has been calculated for b of single stranded poly d(AT)⁸⁸. Due to the decreased stacking in single stranded dT₁₉ (e.g. no change in Abs₂₆₀ versus temperature), the b value should be greater than 4.8 Å for this oligomer. Thus there is a significant difference in base stacking in the single strands of dA₁₉ and dT₁₉. To calculate slopes of T_m versus log (sodium ion activity) plots for duplexes containing neutralized single strands, we used a value of 1) 1.64 × 10⁵ kcal/mole duplex⁹¹ for ΔH° of duplex formation, 2) 3.5 Å for b of single stranded dA₁₉, 3) 5.6 Å for b of single stranded dT₁₉ and dU₁₉, 4) 1.63 Å for b of the unmodified duplexes⁹², 5) 3.26 Å for b of duplexes containing one methylphosphonate chain, and 6) our experimental T_m values at an average salt concentration. With these values, the following calculated slopes are in excellent agreement with our experimental values (listed in Table II, and given here in parentheses): 20.5 (20.7) for dA₁₉·dT₁₉, 9.1 (9.1) for dA*₁₉·dT₁₉, 1.4 (1.4) for dA₁₉·dT*₁₉, 19.0 (21.0) for dA₁₉·dU₁₉, 8.4 (10.7) for dA*₁₉·dU₁₉, and 1.4 (1.8) for dA₁₉·dU*₁₉. The dramatic differences in observed slopes with duplexes containing the different methylphosphonate strands can, thus, be explained by differences in base stacking in the charged single strands. For example, when dA*₁₉·dT₁₉ or dA*₁₉·dU₁₉ melts, essentially no Na⁺ is associated with dA*₁₉ and salt is released due to the extended P-P separation in pyrimidine single strands. On the other hand, when dA₁₉·dT*₁₉ melts, no significant amount of Na⁺ is associated with dT*₁₉, and dA₁₉ is much less extended than other denatured strands due to purine-purine stacking in single-stranded dA₁₉. Thus, the b value for the dA₁₉ chain changes little and the slope is very low. It should be emphasized that the low slope is due to unusual stacking of the repeated purines in dA₁₉ chains and that, otherwise, methylphosphonate duplexes will show appreciable salt effects as with dA*₁₉·dT₁₉. Interestingly, use of slopes of T_m versus log (sodium ion activity) plots obtained in these type studies provides the first known method for calculating b of a single

strand homopurine which is totally independent of b for the single strand complementary pyrimidine. However, it is important to realize that these results are based on the above listed assumptions. We have assumed, for example, that since all the oligomers studied have 19 similar base pairs, the same value of DH is appropriate for all transitions.

The slopes of T_m versus log (sodium ion activity) plots for $dA^*_{19} \cdot dT^*_{19}$ or $dA^*_{19} \cdot dU^*_{19}$ are approximately 0. This is as expected since the low charge density of these duplexes prevents ion condensation.

Effects of methylphosphonate substitution on DNA triplexes

An important possibility in methylphosphonate drug development is the use of these oligomers to directly complex with cellular DNA and affect gene expression through triple helix formation²⁸⁻³³. Nonalternating duplex sequences which can form triple helical structures with oligomer strands have been found to occur frequently in eukaryotic genome control regions⁹³⁻⁹⁶. Although formation of the triple helix with A·T·T strands has been suggested from both experimental⁵⁷ and theoretical⁵⁹ methods, we find no evidence of triple helix formation under any conditions either by gel electrophoresis or by T_m analysis. If any of the chains A, T, or U is substituted by the corresponding methylphosphonate, no triple helix is detected by either T_m (no biphasic melting is seen) or gel analysis. Adding a methylphosphonate oligomer strand to a normal DNA polymer duplex also gave no detectable triple helix. The difference between our results and those of other workers suggesting triple helix formation may be due to the length of the oligomers studied. Earlier studies⁵⁷ typically used short segments while we have used 19-mers. Clearly, triple helix formation of complexes containing methylphosphonates needs further investigation. In particular, we are investigating triplex formation with less highly methylated strands to obtain a more detailed understanding of the inhibition of triple helix formation on conversion of a phosphodiester to a methylphosphonate.

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