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Citation for published version (APA):

Moody, M. R., Genderen, van, M. H. P., & Buck, H. M. (1990). Thermodynamics of polymolecular duplexes between phosphate-methylated DNA and natural DNA. *Biopolymers*, 30(5-6), 609-618.
<https://doi.org/10.1002/bip.360300513>

DOI:

[10.1002/bip.360300513](https://doi.org/10.1002/bip.360300513)

Document status and date:

Published: 01/01/1990

Document Version:

Publisher's PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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- The final published version features the final layout of the paper including the volume, issue and page numbers.

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Thermodynamics of Polymolecular Duplexes between Phosphate-Methylated DNA and Natural DNA

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SYNOPSIS

Phosphate-methylated (P.M.) DNA possesses a very high affinity for complementary natural DNA, as a result of the absence of interstrand electrostatic repulsions. In this study, a model system phosphate-methylated d[C_n] with natural d(G_k) ($n < k$) is chosen for an investigation of the thermodynamic properties that determine duplex stability. The enthalpy change of a melting transition is shown to be considerably larger than is observed for corresponding natural DNA duplexes. It is found that ΔH_n^0 of GG/CC nearest neighbor pairwise interaction equals -15.6 kcal/mol, compared to -11.0 kcal/mol for the natural analog. The entropy change is strongly dependent on the length of the natural DNA strand and the number of phosphate-methylated DNA oligomers hybridized. The results are explained by means of a model in which a cooperative effect for subsequent hybridizations of phosphate-methylated DNA oligomers is assumed, thus giving additional stability.

INTRODUCTION

The use of antisense DNA or RNA has proven to be a powerful tool for the manipulation of gene expression.¹ The technique is based on blocking the informational flow from DNA via RNA to protein by hybridizing an oligonucleotide with a complementary target sequence.¹⁻⁵ The effectiveness of antisense oligomers can be increased when modifications are introduced that serve to improve the efficiency of cellular uptake,^{6,7} the resistance to enzymatic degradation,⁸⁻¹² or the interaction with polynucleotides.^{6,13-18} Recently, very promising results were obtained with phosphate-methylated (P.M.) DNA oligomers,^{14,19} which have been shown to possess a very high affinity for natural DNA.¹⁵⁻¹⁸ Because of the absence of phosphate charges in the sugar-phosphotriester backbone, a duplex with a complementary sequence can be formed in which no interstrand phosphate-phosphate repulsions are present. Therefore, a major duplex destabilizing

force has been eliminated,^{15,20} leading to very strong association. No destabilizing forces resulting from steric hindrance are added, because the methyl group introduced at the phosphates is small-sized and fits into the groove of the DNA (for both configurations of the chiral phosphorus atom).^{14,18}

P.M. DNA has been subjected to several nucleases, and has been studied in *E. coli* cell lysate and intact *E. coli* cells with ³¹P-NMR. During these experiments, no chemical or enzymatic breakdown was detected, showing that the stability in vitro and in vivo is high enough to maintain its biological function during a long period of time.²¹

An antisense P.M. 18 mer was able to block the replication of the *E. coli pabB* gene, inserted in a single-stranded M13mp18 phage, precisely at the hybridization site.¹⁴ In prokaryotic cells sense P.M. oligomers of length 22 and 21 were able to block the transcription of selected genes within 20 min of incubation by hybridization to duplex DNA (β -galactosidase in *E. coli*, *alr* and *dadB* racemase in *Salmonella typhimurium*).^{19,22} Thus both replication and gene expression can be blocked by interaction of P.M. DNA with natural DNA.

In order to get a good impression of the blocking potential of a certain P.M. oligomer, it is necessary, however, to have a detailed thermodynamic model

describing the P.M. DNA-natural DNA hybridization process. Unfortunately, only few investigations have been carried out that deal with these thermodynamics so far. The study of Van Genderen et al.^{17,18} has revealed that a duplex of P.M. d[A₃] with oligo(dT) is more stable when more P.M. DNA molecules are hybridized with the same natural DNA strand, suggesting that there is a cooperative effect involved in the hybridization process. The results were explained by means of a simple hybridization model, in which it was assumed that the natural DNA adopts a suitable conformation for duplex formation, whereas the relatively rigid P.M. DNA remains unchanged. For the description of this process, and adaptation entropy ΔS_k^0 was introduced. To our knowledge, no other DNA or RNA duplex structures have been reported in literature that possess similar properties.

The work presented in this paper was conducted (1) to learn whether the cooperativity model can be used also to describe the stability of P.M. DNA-natural DNA duplexes with C-G base pairs; (2) to examine the importance of interactions between P.M. DNA strands that were not taken into account in the preliminary studies; and (3) to give a full thermodynamic description of the hybridization process that includes the calculation of nearest-neighbor pairwise enthalpy and entropy interactions. For this purpose, we had to choose a number of short oligos that form duplexes with complementary DNA, whose stability is not too low and not too high. Best results can be obtained if all T_m values are within a measurable range, that is, 10–80°C. Longer oligos (which might show more resemblance to those used for the biological studies) would be rather impractical, as the expected duplex stabilities with natural DNA would be too high. Thus we selected the P.M. oligomers d[C₃], d[C₄], and d[C₅] and examined their hybridization ability with natural d(G_k) ($k = 10, 18, 30$, and 600). The cooperative effect, as already seen for P.M. d[A₃]·oligo(dT), was observed again and could be described by the same model. The enthalpy term ΔE_{inter} , describing the P.M. d[C_n] interactions, appeared to be negligible compared to the other interactions present in the duplex. The other enthalpy and entropy values calculated were compared to those of all-natural DNA duplexes, and it could be concluded that the ΔH^0 of an association process becomes more negative as a result of phosphate-methylation, whereas ΔS^0 is strongly dependent on the length of the natural DNA and the number of P.M. oligomers hybridized. Control experiments indicate that for natural analogs no cooperative effect occurs. All duplex

structures investigated were considerably more stable than corresponding all-natural DNA duplexes.

MATERIALS AND METHODS

¹H-NMR and ³¹P-NMR spectra were obtained using a Bruker AC-200 spectrometer. Proton chemical shifts, recorded at 200 MHz, were referenced against TMS (δ 0 ppm) or HDO (δ 4.68 ppm). Phosphorus chemical shifts, recorded at 80.9 MHz, are relative to 85% H₃PO₄ (δ 0 ppm); they are designated positive if downfield from the standard. UV spectra and UV melting curves were recorded on a Perkin-Elmer 124 spectrophotometer. Poly(dG) with an average chain length of 600 nucleotides was purchased from Pharmacia. The oligomers d(G₁₀), d(G₁₈), d(G₃₀), and (natural) d(C₅) were synthesized on an Applied Biosystems 381A DNA synthesizer. Solutions of d(G_k) were prepared by dissolving the solid in 1 M NaOH (ultrasonic bath), followed by neutralization with Tris/HCl pH 7 (final Tris concentration, 50 mM). This procedure was necessary, because long dG sequences tend to form insoluble aggregates when they are brought into a neutral solution directly. P.M. d[C₃] was synthesized as described by Van Genderen et al.¹⁸ No separation of diastereoisomers [$R_P/S_P - P(\text{OMe})$] was carried out, because it is known that all diastereoisomers form equally stable duplexes.¹⁸

P.M. d[C₄]

Essentially the same coupling procedure as used for the synthesis of P.M. d[C₃] was performed to add another cytidine residue at the 5' position, thereby yielding P.M. d[C₄] with 9-fluorenyl methoxycarbonyl (Fmoc) as a base-protecting group on the cytidine residues. The product was purified by column chromatography on Woelm silica gel (chloroform/ethanol 95 : 5 as eluent; $R_f = 0.20$). ¹H-NMR (CDCl₃): δ 2.07 (3H, s, CH₃ acetyl), 2.2–2.5 (4H, m, H(2') of dC), 2.6–2.9 (4H, m, H(2'') of dC), 3.7–3.9 (11H, m, H(5')/H(5'') of 5'-terminal dC and POCH₃), 4.1–4.5 (22H, m, H(4') of dC, H(5')/H(5'') of non-5'-terminal dC and CH/CH₂ of Fmoc), 5.1–5.4 (4H, m H(3') of dC), 6.1–6.4 (4H, m, H(1') of dC), 7.2–7.4 (20H, m, aromatic Fmoc and H(5) of dC), 7.55 (8H, m, aromatic Fmoc), 7.74 (8H, m, aromatic Fmoc), 7.8–8.4 (4H, m, H(6) of dC). ³¹P-NMR (CDCl₃) exhibited very distinct clusters of signals. δ –0.98 (2P), –0.88 (1P), –0.34 (3P), –0.26 (6P), –0.10 (9P), +0.25 (3P). Theoretical number of signals: $2^3 \times 3 = 24$.

The base-protecting Fmoc groups were removed by treatment with triethylamine/pyridine. After evaporation in vacuo, a crude product was obtained that was purified by crystallization on the separation layer of $\text{H}_2\text{O}/\text{CHCl}_3$ (5% EtOH). $^1\text{H-NMR}$ (D_2O): δ 2.02 (3H, s, CH_3 acetyl), 2.1–2.4 (4H, m, H(2') of dC), 2.4–2.6 (4H, m, H(2'') of dC), 3.6–3.8 (11H, m, H(5')/H(5'') of 5'-terminal dC and POCH_3), 4.1–4.4 (10H, m, H(4') of dC and H(5')/H(5'') of non-5'-terminal dC), 4.9–5.3 (4H, m, H(3') of dC), 5.8–6.0 (4H, m, H(5) of dC), 6.0–6.2 (4H, m, H(1') of dC), 7.4–7.8 (4H, m, H(6) of dC). $^{31}\text{P-NMR}$ (D_2O): δ 1.9–2.3. UV (H_2O): $\lambda_{\text{max}} = 266 \text{ nm}$, $A_{250}/A_{266} = 0.84$, $A_{290}/A_{266} = 0.29$.

P.M. d[C₅]

The P.M. oligomer d[C₅] was synthesized according to the procedure of Moody et al.,¹⁴ which consists of four steps: (1) synthesis of natural d(C₅) on a DNA synthesizer; (2) base protection, using Fmoc; (3) methylation; and (4) base deprotection. After the base deprotection, all apolar side products were removed by preparative thin-layer chromatography (Merck silica gel 60 F254 plates; methanol as eluent). The polar side products (such a pyridinium salts) were removed by an 18-h extraction of the silica gel with water. Subsequently, the P.M. d[C₅] was separated from the silica gel by extraction with methanol. UV (H_2O): $\lambda_{\text{max}} = 267 \text{ nm}$, $A_{250}/A_{267} = 0.80$, $A_{290}/A_{267} = 0.29$.

Melting Curves

The stability of the duplexes between phosphate-methylated DNA and natural DNA was determined by measuring the melting temperatures (T_m) of the duplexes. The T_m is defined as the temperature at which half of the single strands is in the duplex state. P.M. DNA and natural DNA were added to a buffer of 70 mM Tris/HCl pH 8.0, in a final nucleotide concentration of 20 μM , with respect to the natural DNA and 10 μM or 20 μM , with respect to the P.M. DNA. (No salt was added, as the stability of this type of duplex is independent of the ionic strength of the solution¹⁴). Control experiments with natural d(C₅) and natural d(G_k) were carried out with the same DNA concentrations and the same buffer solutions as mentioned above, but it was necessary to add also 1 M NaCl, because otherwise no duplex formation would have occurred. All the mixtures were heated to 70–80°C and cooled down during 30–40 min in order to allow complete duplex formation. The melting behavior was followed by measuring the UV hyperchromicity effect at 260 nm. T_m values were determined with an accuracy of 0.5°C by fitting a melting curve through the data points (see Fig. 1).

EQUATIONS FOR THE CALCULATION OF THERMODYNAMIC DATA

Concentration Dependence of the T_m Value

The equation that shows the concentration dependence of T_m of a polymolecular duplex structure

Figure 1. A typical absorbance versus temperature melting curve of a polymolecular duplex structure recorded for P.M. d[C₅] · d(G₃₀); nucleotide ratio equals 1 : 1.

Figure 2. Schematic representation of a polymolecular duplex structure consisting of p identical, short strands (of n nucleotides each) that are hybridized with one other, long strand (of k nucleotides).

consisting of non-self-complementary strands is given by Marky and Breslauer:²³

$$\frac{1}{T_m} = \frac{(m-1)R}{\Delta H^0} \ln c_T + \frac{\Delta S^0 - (m-1)R \ln 2m}{\Delta H^0}$$

where m = the total number of strands forming a complex; c_T = the total strand concentration; ΔH^0 = the association enthalpy; ΔS^0 = the association entropy. For the hybridization of p identical, short strands with one other, long strand (as shown in Fig. 2), this expression can be simplified. We introduce $p = m - 1$ and $c_0 = c_T/m$ (so c_0 = the concentration of the long strand). Upon rearrangement we get

$$T_m = \frac{\Delta H^0}{pR \ln(c_0/2) + \Delta S^0}$$

Because every hybridization of an identical strand involves roughly the same enthalpy and entropy effect, we may also use ΔH_n^0 and ΔS_n^0 , which are the enthalpy and entropy change of one single hybridization: $\Delta H_n^0 = \Delta H^0/p$ and $\Delta S_n^0 = \Delta S^0/p$. Substituting these expressions into the equation given above yields

$$T_m = \frac{\Delta H_n^0}{R \ln(c_0/2) + \Delta S_n^0}$$

An Additional Entropy Term That Influences the T_m Value

For the polymolecular duplex structures studied in our work it is not possible to fit the experimental results with the equation given. The melting temperature does not only depend on the concentration, but also on the length of the natural DNA and the degree of occupancy by P.M. DNA strands. For this type of polymolecular structures, therefore, we use the extension of the original formula, as was first proposed by Van Genderen et al.¹⁷ It is assumed that an additional entropy term $\Delta S_k^{0'}$ is necessary for a

full description of the association process. ΔS_n^0 would be the entropy change if the natural DNA and the P.M. DNA possessed equal helix geometries before duplex formulation, whereas $\Delta S_k^{0'}$ accounts for the additional entropy change in case the natural DNA strand has to adopt its conformation. For the phosphate-methylated DNA, we do not add another entropy term. Consequently, association of one phosphate-methylated DNA molecule with a complementary sequence can be described by the equation:

$$T_m = \frac{\Delta H_n^0}{\Delta S_n^0 + R \ln(c_0/2) + \Delta S_k^{0'}}$$

Association of a second phosphate-methylated DNA molecule will involve the same ΔH_n^0 and ΔS_n^0 terms, but the value of $\Delta S_k^{0'}$ is thought to be different. It is assumed that the conformational change caused by the hybridization of the first phosphate-methylated DNA molecule, has not been restricted to the site of hybridization. Instead, the natural DNA is supposed to have changed its conformation over a longer distance, in such a way that hybridization of following phosphate-methylated DNA molecules is facilitated. Thus for the second phosphate-methylated DNA molecule only a term $\alpha \Delta S_k^{0'}$ is involved, with $\alpha \in [0, 1]$. The parameter α is a measure of cooperativity, ranging from no effect ($\alpha = 1$) to a complete conformational change after the first hybridization ($\alpha = 0$). With this assumption, the T_m value for two hybrids of identical phosphate-methylated DNA molecules on the same natural DNA strand may be expressed as:

$$T_m = \frac{\Delta H_n^0}{\Delta S_n^0 + R \ln(c_0/2) + \Delta S_k^{0'} \times (1 + \alpha)/2}$$

In the model, each subsequent hybridization involves another factor α , resulting from a continuing adaptation of the geometry of the natural DNA strand. We suppose that every hybridization is equally effective in changing the natural DNA conformation, which means that α has a constant value during the whole process. Therefore, the T_m value belonging to p hybrids of identical phosphate-methylated DNA molecules is given by the equation:

$$T_m = \frac{\Delta H_n^0}{\Delta S_n^0 + R \ln(c_0/2) + \sum_{i=0}^{p-1} \alpha^i \Delta S_k^0 / p}$$

which can also be written as:

$$T_m = \frac{\Delta H_n^0}{\Delta S_n^0 + R \ln(c_0/2) + \Delta S_k^0 \times (1 - \alpha^p) / (1 - \alpha)p}$$

This general expression indicates that hybridizing more molecules of phosphate-methylated DNA with the same natural DNA strand raises the T_m value of the association complex, when $\alpha < 1$.

Calculation of ΔH_n^0 from the Slope of a Melting Curve

The transition enthalpy for a polymolecular duplex structure is given by:²³

$$\Delta H^0 = -(2 + 2m)RT_m^2 \left(\frac{\partial f}{\partial T} \right)_{T=T_m}$$

where f = the fraction of dissociated strands and

$$\left(\frac{\partial f}{\partial T} \right)_{T=T_m}$$

equals the slope of the melting curve at the T_m . For our structures consisting of one natural DNA strand and p P.M. DNA strands we may write

$$\Delta H_n^0 = \frac{-(4 + 2p)}{p} RT_m^2 \left(\frac{\partial f}{\partial T} \right)_{T=T_m}$$

Calculation of $\Delta H_{GG/CC}^0$, $\Delta S_{GG/CC}^0$ and ΔS_i^0

According to the model of Breslauer et al.,²⁴ the ΔH_n^0 and ΔG_n^0 values of natural, non-self-complementary DNA sequences are given by:

$$\Delta H_n^0 = \sum_x \Delta H_x^0$$

$$\Delta G_n^0 = \sum_x \Delta G_x^0 + \Delta G_i^0$$

where $\sum_x \Delta H_x^0$ and $\sum_x \Delta G_x^0$ represent all nearest-neighbor pairwise interactions and ΔG_i^0 is an initiation free energy. Using the standard thermody-

namic relationship, $\Delta G^0 = \Delta H^0 - T\Delta S^0$, an expression for ΔS_n^0 is obtained

$$\Delta S_n^0 = \sum_x \Delta S_x^0 + \Delta S_i^0$$

where $\Delta S_i^0 = -\Delta G_i^0/298$. For hybrids between p oligo(dC) strands and one oligo(dG) strand, the expression for ΔH_n^0 is not complete. We have to add a term describing *interstrand oligo(dC) interactions*, such as stacking interactions and electrostatic repulsions. Thus, we write

$$\Delta H_n^0 = (n - 1)\Delta H_{GG/CC}^0 + \Delta E_{inter}$$

$$\Delta S_n^0 = (n - 1)\Delta S_{GG/CC}^0 + \Delta S_i^0$$

where $\Delta H_{GG/CC}^0$ and $\Delta S_{GG/CC}^0$ represent the interaction of a GG/CC pair, ΔE_{inter} is the energy term that is added to include interstrand oligo(dC) interactions and n is the number of nucleotides of an oligo(dC) strand. Identical formulas as given here were used to describe the behavior of polymolecular duplex structures consisting of p P.M. d[C_n] strands and one natural d(G_k) strand. It should be mentioned, however, that for these structures ΔS_i^0 is not the whole initiation entropy of a hybridization event. The term $\Delta S_k^0 \times (1 - \alpha^p) / (1 - \alpha)p$, which is needed to give a full description of the hybridization process, is also part of the total initiation entropy.

RESULTS AND DISCUSSION

In Table I an overview is given of the observed T_m values of duplexes between P.M. d[C_n] and natural d(G_k). It can be seen that the stability of these kinds of duplexes strongly depends on the length of the natural DNA (shown graphically in Fig. 3). For example, if d[C₃] is hybridized with d(G₁₈) at a nucleotide ratio of 1 : 1, then the observed melting temperature of the duplex is over 40°C lower than the T_m when d[C₃] is hybridized with poly(dG) (at the same nucleotide ratio). Apart from this clear influence of the natural DNA chain length, the duplex stabilities are also dependent on the degree of occupancy of the natural DNA. The latter effect may seem smaller, but it should be kept in mind that only differences between fully and half-occupied strands have been examined. Comparing nucleotide ratios of, for example, 1 : 4 or 1 : 8 with a ratio of 1 : 1 probably would have resulted in a much clearer difference. Unfortunately, however, for such nu-

Table I Stability of Duplexes between Phosphate-Methylated d[C_n] (*n* = 3–5) and natural d(G_k)

P.M. Oligomer	Nucleotide Ratio Oligo : Natural DNA	<i>T_m</i> Values (°C)				ΔH_n^0 (kcal/mol)
		<i>k</i> = 600	<i>k</i> = 30	<i>k</i> = 18	<i>k</i> = 10	
d[C ₃]	1 : 1	54.5	32.0	13.0	^a	33 ± 2
d[C ₃]	1 : 2	53.0	31.0	12.0	^a	31 ± 2
d[C ₄]	1 : 1	79.0	60.0	52.0	^c	43 ± 3
d[C ₄]	1 : 2	75.5	54.5	^c	^c	45 ± 3
d[C ₅]	1 : 1	^b	71.5	66.0	61.5	59 ± 4
d[C ₅]	1 : 2	^b	68.5	63.5	60.0	63 ± 4

^a *T_m* value too low to be determined.^b *T_m* value too high to be determined.^c No clear *T_m* value observed.

cleotide ratios the UV hyperchromicity effect is too small to determine a reliable *T_m* value.

We have performed some control experiments also with *natural* d(C₅) and *natural* d(G_k), in order to find out whether any of the unusual properties of the P.M. DNA · *natural* DNA are present with the all-natural analog, too. As could be expected, no such properties were detected. The data presented in Table II show only minor differences between the var-

ious *T_m* values measured, and those can be explained without introducing an adaptation entropy ΔS_k^0 .

Calculation of ΔH_n^0 and $\Delta H_{GG/CC}^0$

Describing our measurements with the cooperativity model implies that we have to calculate a value for ΔH_n^0 , that is, the enthalpy change caused by the hybridization of one molecule of P.M. d[C_n] with *natural* d(G_k). ΔH_n^0 can be calculated from the slope of a melting curve near the melting temperature, at least if there is only *one* distinct melting transition in that particular temperature area. Transitions where *p* is not equal to a whole number usually do not yield appropriate ΔH^0 values, because in these cases the melting curve is composed of *two different parts*, which both possess a different *T_m* value. For example, for P.M. d[C₄] · *natural* d(G₁₈), nucleotide ratio 1 : 1, *p* equals 4.5, which means that one molecule of d(G₁₈) is associated with either 4 or 5 molecules of phosphate-methylated DNA. Such complexes are not suitable for ΔH_n^0 calculations.

The calculations we carried out on the other complexes yielded values for ΔH_3^0 , ΔH_4^0 and ΔH_5^0 which were independent of the length of the *natural* DNA and the degree of occupancy by P.M. DNA strands (Table I). In principle, one would expect a difference especially for different degrees of occupancy, because of the term ΔE_{inter} , describing the interstand P.M. d[C_n] interactions. The absence of any enthalpy differences suggests that ΔE_{inter} is negligible compared to the other interactions present in the duplex.

Additional evidence for this statement could be obtained by investigating the stability of duplexes between *natural* d(C₅) and *natural* d(G_k). This is a well-defined system, of which the relevant ΔH^0

Figure 3. The stability of a polymolecular duplex P.M. d[C_n] · *natural* d(G_k) (nucleotide ratio equals 1 : 1) as a function of *n* and *k*.

Table II Control Experiments with Natural d(C₅) and Natural d(G_k)*

Nucleotide Ratio		<i>T_m</i> Values (°C)				ΔH_n^0
C : G	<i>k</i> = 600	<i>k</i> = 30	<i>k</i> = 18	<i>k</i> = 10		(kcal/mol)
1 : 1	^a	13.5	15.0	15.5		42 ± 4
1 : 2	^a	13.0	15.5	17.0		46 ± 4

* Carried out in 70 mM Tris/HCl pH 8.0, 1 M NaCl.

^a *T_m* value too low to be determined.

and ΔS^0 terms can be calculated from literature data.²⁴ Therefore, it is possible to determine the magnitude of interstrand oligo(dC) interactions quite accurately. The only difference between d(C₅) interactions and P.M. d[C₅] interactions should be the presence or absence of electrostatic repulsions resulting from charges on the phosphate groups. The magnitude of the electrostatic interactions occurring with natural d(C₅) can be estimated with Coulomb's Law, in which a distance-dependent dielectric constant and a reduced phosphate charge are substituted.²⁵ The distance-dependent dielectric constant accounts for the presence of water molecules, whereas the reduced phosphate charge is used to simulate the presence of salt in the solution. In 1 M NaCl we substitute²⁵ $\epsilon = R_{ij}$ and $q = -0.2$, leading to a repulsion energy of 0.6 kcal/mol for each natural d(C₅) strand in the field of the other d(C₅) strands hybridized. The total interaction energy ΔE_{inter} follows from *T_m* measurements. We found that a duplex d(C₅) · d(G₅) melts at 16.5°C (1 M NaCl, pH 7.0), whereas a polymolecular duplex consisting of 6 d(C₅) strands and one d(G₃₀) strand melts at 13.5°C under the same conditions (Table II). From these results it is calculated that $\Delta E_{\text{inter}} = +0.4$ kcal/mol d(C₅). For P.M. d[C₅] we omit all electrostatic interactions, so we find that $\Delta E_{\text{inter}} = +0.4 - 0.6 = -0.2$ kcal/mol. Comparing this value to the overall ΔH_n^0 values measured confirms our expectations that the interstrand P.M. d[C₅] interactions are negligible.

Using this assumption it is possible to calculate the value of $\Delta H_{\text{GG/CC}}^0$, that is, the nearest-neighbor interaction of a GG/CC pair. Because interstrand P.M. DNA interactions do not have to be taken into account, ΔH_n^0 equals $(n - 1) \Delta H_{\text{GG/CC}}^0$. With the values of ΔH_n^0 that have been measured we find that:

$$\Delta H_{\text{GG/CC}}^0 = -15.6 \pm 1.0 \text{ kcal/mol.}$$

It is interesting to compare this value to the corresponding enthalpy term found for natural DNA duplexes. Breslauer and coworkers have calculated that

$\Delta H_{\text{GG/CC}}^0 = -11.0$ kcal/mol in 1 M NaCl, pH 7.0.²⁴ Our present results show that the transition enthalpy for P.M. DNA · natural DNA is 4.6 kcal/mol more negative, even though the formation of natural DNA duplexes is stimulated by high ionic strength. So this is a clear illustration of the effect of eliminated phosphate-phosphate repulsions as a result of phosphate-methylation. One can compare our findings also to the results of Koole et al., who determined the association enthalpy of a P.M. d[A₃] · natural d(T_k) duplex.¹⁵ For ΔH_n^0 , a value of -23.4 kcal/mol was observed, implying that $\Delta H_{\text{AA/TT}}^0 = -11.7$ kcal/mol (interstrand P.M. DNA interactions neglected). Because the corresponding value for the natural DNA duplexes is -9.1 kcal/mol, a difference of 2.6 kcal/mol is found in this case. The results of this study, as well as those of our present work, can be explained reasonably if one assumes that the observed enthalpy difference is purely a consequence of differing electrostatic repulsions between the long DNA strand and the short strands hybridized. After substituting $\epsilon = R_{ij}$ and $q = -0.2$ in Coulomb's Law it is seen that the electrostatic repulsion energy for an all-natural DNA duplex is of the order of magnitude of 2 kcal/mol. For a duplex consisting of one natural DNA strand and *p* P.M. DNA strands, no such repulsion energy is present.

Calculation of α , ΔS_n^0 and ΔS_k^0

In our calculations, the value of α was chosen constant for one particular P.M. DNA oligomer, not only with respect to subsequent hybridizations on one strand, but also with respect to hybridizations on different strands [e.g., poly(dG) and d(G₃₀)]. Despite this restriction, it was still possible to give a good description of the duplex stabilities as obtained experimentally. In Table III, the optimum values of α , ΔS_n^0 and ΔS_k^0 are listed. Using these data with the cooperativity model, *T_m* values can be calculated that in no case deviate more than 1°C from the experimental values.

Table III Calculated Values of α , ΔS_n^0 and $\Delta S_k^{0'}$ *

Oligomer	α	ΔS_n^0	$\Delta S_{600}^{0'}$	$\Delta S_{30}^{0'}$	$\Delta S_{18}^{0'}$	$\Delta S_{10}^{0'}$
P.M. d[C ₃]	0.99 ± 0.01	-58 ± 6	-3	-15	-23	—
P.M. d[C ₄]	0.93 ± 0.03	-97 ± 9	-10	-18	-21	—
P.M. d[C ₅]	0.94 ± 0.02	-135 ± 11	—	-19	-22	-25

* All entropies are given in cal/mol·K. $\Delta S_k^{0'}$ values are ±2 cal/mol·K. $R \ln(c_0/2)$ was -35.6 cal/mol·K for hybridizations with natural d(G₆₀₀); -29.6 cal/mol·K for hybridizations with natural d(G₃₀); -28.6 cal/mol·K for hybridizations with natural d(G₁₈); and -27.5 cal/mol·K for hybridizations with natural d(G₁₀).

The results show that α does not possess the same value for every P.M. DNA oligomer. In the case of P.M. d[C₃], α equals 0.99, so the conformational change of the natural DNA upon duplex formation hardly facilitates the attachment of the next phosphate-methylated DNA molecule. For P.M. d[C₄] and P.M. d[C₅], however, we find that the value of α is 0.93 and 0.94, respectively, which demonstrates that in these cases the conformational change of the natural DNA does have a clear, stimulating effect on the hybridization of following P.M. d[C_n] molecules. Apparently, the conformational changes that occur are not always equally effective. An explanation for this observation could be that a natural DNA conformation can be altered more easily by strongly associated P.M. DNA oligomers than by weakly associated P.M. DNA oligomers.

Perhaps most striking about Table III is that the value of $\Delta S_k^{0'}$ depends so strongly on the length of the natural DNA, to which the P.M. DNA is attached. More specifically, the entropy lost by the natural DNA strand after hybridization of a P.M. DNA strand is larger for short strands (10 mers, 18 mers, 30 mers) than for longer strands (600 mers). Earlier, we proposed that this difference is caused by the ordered/disordered structure of the natural DNA before hybridization.¹⁷ As long DNA strands already possess a slightly ordered structure in the single-strand situation,²⁶ the hybridization of P.M. DNA only results in the transition of one ordered structure into another. The difference in entropy can be relatively small, therefore. Shorter DNA strands usually have a more random conformation, which means that the hybridization of P.M. DNA causes a transition from a disordered into an ordered structure. Consequently, the loss of entropy can be quite considerable.

Calculation of $\Delta S_{GG/CC}^0$ and ΔS_i^0

The values of $\Delta S_{GG/CC}^0$ and ΔS_i^0 can be deduced easily from our experimental data, using the expression $\Delta S_n^0 = (n - 1)\Delta S_{GG/CC}^0 + \Delta S_i^0$. We found that:

$$\Delta S_{GG/CC}^0 = -38.4 \pm 2.4 \text{ cal/mol} \cdot \text{K}$$

and that:

$$\Delta S_i^0 = +18.7 \pm 1.3 \text{ cal/mol} \cdot \text{K}.$$

Both entropy terms will be compared now to the corresponding values for natural DNA duplexes. According to Breslauer et al.²⁴ $\Delta S_{GG/CC}^0 = -26.6 \text{ cal/mol} \cdot \text{K}$. (More recently, Quartin and Wetmur²⁷ reported that $\Delta S_{GG/CC}^0 = -29.2 \text{ cal/mol} \cdot \text{K}$.) The value that we measured for P.M. DNA · natural DNA duplexes was considerably more negative, indicating that methylation of the phosphates of an oligomer not only has favorable consequences for the process of duplex formation. The cause is probably that a duplex structure with tightly bonded strands possesses relatively few degrees of freedom, which is reflected by a low entropy.

The second entropy term is ΔS_i^0 . This initiation entropy cannot be compared directly to the initiation term for natural DNA, as we now have an additional entropy term $\Delta S_k^{0'} \times (1 - \alpha^p)/(1 - \alpha)p$. Where ΔS_i^0 is a constant, we see that the additional initiation term is dependent on k , p , and (less evidently) on n . Table IV shows the values of $\Delta S_i^0 + \Delta S_k^{0'} \times (1 - \alpha^p)/(1 - \alpha)p$ that were calculated for the investigated duplex structures. It is interesting to notice that influence of the natural DNA chain length and the degree of occupancy on the total initiation entropy. $\Delta S_{i,\text{total}}^0$ apparently increases when more P.M. DNA molecules are hybridized with a longer natural DNA strand. For example, in the case of a hybridization with poly(dG), $\Delta S_{i,\text{total}}^0$ always possesses a large positive value, so duplex formation is stimulated. In contrast, we find negative values for $\Delta S_{i,\text{total}}^0$ in case of hybridizations with d(G₁₈) or d(G₁₀). The initiation entropies for natural DNA duplexes can be deduced from available ΔG_i^0 values. Breslauer et al.²⁴ assume that $\Delta G_i^0 = 5 \text{ kcal/mol}$, which is consistent with $\Delta S_i^0 = -16.8 \text{ cal/mol} \cdot \text{K}$. Quartin and Wetmur,²⁷ however, get better agree-

Table IV The Total Initiation Entropy of Duplexes between P.M. d[C_n] and Natural d(G_n)

P.M. Oligomer	Nucleotide Ratio Oligo : Natural DNA	$\Delta S_{i, \text{total}}^0$ (cal/mol · K) ^a			
		$k = 600$	$k = 30$	$k = 18$	$k = 10$
d[C ₃]	1 : 1	+17	+4	-4	—
d[C ₃]	1 : 2	+17	+4	-4	—
d[C ₄]	1 : 1	+18	+4	0	—
d[C ₄]	1 : 2	+17	+2	-2	—
d[C ₅]	1 : 1	—	+2	-2	-6
d[C ₅]	1 : 2	—	+1	-3	-6

^a All values are ± 1 cal/mol · K.

ment between theory and results if a ΔG_i^0 value of 2.2 kcal/mol is chosen, so the corresponding value for ΔS_i^0 would be -7.4 cal/mol · K. Our present results show that the initiation entropy of polymolecular P.M. DNA · natural DNA duplexes approaches the values found for natural DNA duplexes only if the natural DNA strand is relatively short. In this situation the associating strands are almost equal in length, as is the case for the duplexes that Breslauer et al. and Quartin and Wetmur have described. Otherwise, a clearly higher value is found.

CONCLUDING REMARKS

It has been shown that polymolecular duplex structures consisting of p P.M. DNA strands and one natural DNA strand display a thermodynamic behavior that is quite different from what has been determined for natural DNA duplexes. Not only did we find that the stability of these duplex structures is remarkably high, but we were able also to confirm the assumption that there is a cooperative process involved in duplex formation. Comparisons with other modified, neutral DNAs (e.g., methyl phosphonate or ethyl phosphotriester oligomers) are virtually impossible, because for these systems it has been shown that the configuration of phosphorus has a strong influence on the strength of hybridization.^{27,28} Therefore, it would be necessary to separate these modified oligomers in their diastereoisomers, before the thermodynamics can be studied in detail.

In our future investigations we will continue to study the stability of P.M. DNA · natural DNA duplexes. Further, it is of significance to investigate the thermodynamic properties of P.M. DNA · natural RNA duplexes because of possible interac-

tions with mRNA leading to inhibition or stimulation of translation.²¹

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Received November 6, 1989

Accepted March 8, 1990