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Deoxydodecanucleotide heteroduplex d(TTTTATAATAAA). d(TTTATTATAAAA) containing the promoter Pribnow sequence TATAAT. I. Double-helix stability by UV spectrophotometry and calorimetry

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ABSTRACT

Thermally induced structural transition in the d(TTTTATAATAAA) d(TTTATTATAAAA) heteroduplex is characterized by UV-spectroscopy and differential scanning calorimetry. At low salt(<0.1 M)the occurrence of a cooperative transition in the lower temperature range, followed by a broad transition connected with small increase in absorbance is observed. At high salt (≥0.2 M)a single, monophasic transition appears. Linear dependence of the latter on log of salt concentration ($dTm:d\log M=14.2^{o}C$) and of 1/Tm on log of oligomer concentration [derived therefrom ΔH(v.H.) =77.1 kcal/mole (duplex)] allows relating it to the melting of the heteroduplex helix. The non-cooperative transition, independent of oligomer concentration and similar to that of the single chain, was attributed to melting of short hairpin helices upon heteroduplex dissociation. Calorimetric enthalpy: 75.6 kcal/ /mole(duplex) proved significantly lower than predicted from known calorimetric data for poly[d(AT)] and poly d(A)· poly d(T).

INTRODUCTION

Initiation of the transcription in procaryotic systems is preceded by the formation of transient "closed" complex between template DNA and RNA polymerase, which transforms subsequently into an "open" very stable form characterized by a local unwinding and melting of the DNA helix at a promoter site (1). The extent of the opening of the DNA duplex structure is temperature dependent, as would be expected for an enthalpy controlled process, and can attain up to 17 ± 1 base-pairs at $37^{o}C$(2). The melting begins several base-pairs before the transcription starting point (3,4) within a promoter region called Pribnow box, common to all promoters and thus believed to be essential for their specific recognition by the enzyme. The mechanism of the melting process and the role of Pribnow box sequence therein remains to be elucidated. One possible way of doing this is to study complexes formed between DNA fragments containing this sequence and the enzyme. With this aim in mind we synthesized two deoxydodecanucleotides d(TTTTATAATAAA) and d(TTTATTATAAAA),containing the simplest form of the Pribnow sequence TATAAT. In the present paper we report the results of investi-

gations on the thermodynamic stability of the heteroduplex dodecamer formed by these two oligomers.

MATERIALS AND METHODS

Preparation of deoxydodecanucleotides

The two deoxydodecanucleotides: 5'd(TTTTATAATAAA)3' and 5'd(TTTATTATAAAA)3', were synthesized by a phosphotriester method (5-8), as described in detail elsewhere (9). The synthesis was carried out according to the sequential scheme shown for d(TTTTATAATAAA) in Chart 1. The protecting groups used are specified in the scheme.1-(2,4,6-Triisopropylbenzenesulfonyl)-tetrazole was employed as the condensation activating agent. After each condensation the protected oligonucleotide blocks were separated by column chromatography and the overall yield of the synthesis was determined, starting with tetranucleotides (see Table 1). The dimethoxytrityl group was removed from the final products, dodecanucleotides, with benzenesulfonic acid in a chloroform/methanol mixture, and the products were chromatographed on a silica gel column. The benzoyl and p-chlorophenyl protecting groups were removed thereafter by treatment with concentrated ammonia. The completely deprotected oligomers were purified on a Sephadex G-75 (superfine) column using 0.1 M triethylammonium bicarbonate, pH 8.0, as a buffer.

The purity of the deoxydodecanucleotides was checked by homochromatography on thin-layer DEAE-cellulose and also by gel electrophoresis in 20% acrylamide under denaturating conditions (7 M urea) after their labelling with [γ-^{32}P] ATP in the presence of T4 phage polynucleotide kinase. One

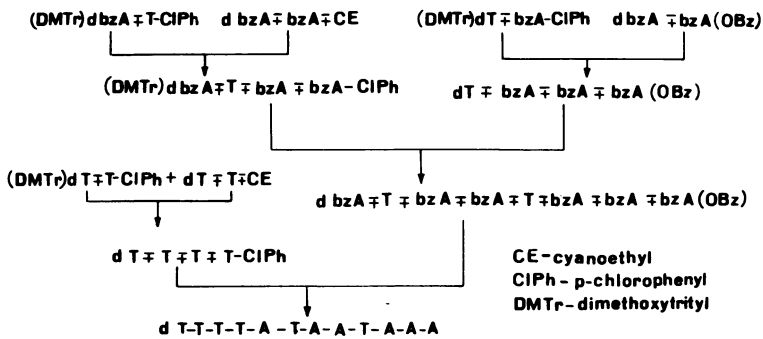


Chart 1. Scheme of synthesis: a phosphodiester bond is represented by (-) and phosphotriester bond by (+). Each internal internucleotidic phosphate is protected with p-chlorophenyl group.

Table 1 Yields of oligonucletides blocks and separation methods

Product	mmole	Overall yield (%)	Column and eluent
DMTr [TTTT] CE	0.140	70	60; 60:40[a]
DMTr [ATAA] CE	0.143	72	60; 65:35[a]
[TAAA] OBz	0.110	56	H; 4[b]
[ATAATAAA] OBz	0.061	61	H; 5[b]
[TTTTATAATAAA] OBz	0.023	47	H; 6[b]
DMTr [TTTA] CE	0.116	58	60; 60:40[a]
DMTr [TTAT] CE	0.056	72	60; 60:40[a]
[AAAA] OBz	0.093	71	H; 4[b]
[TTATAAAA] OBz	0.025	55	H; 5[b]
[TTTATTATAAAA] OBz	0.010	40	H; 6[b]

a, silica gel 60 silanized column, the ratio represents the acetone to water ratio
b, silica gel H, the number refers to the % of CH_3OH in $CHCl_3$

major labelled product was observed for each of the dodecamers. Their sequences were fully confirmed by two-dimensional electrophoresis-homochromatography of venom phosphodiesterase partial digestion products (10).

The concentration of dodecamers in aqueous solution was determined using the extinction coefficient 1.1×10^4, as calculated at 260nm and 25°C according to the nearest-neighbour approximation from the extinction coefficients of the appropiate mononucleotides and dinucleoside phosphates (11).

UV spectroscopy

The absorbance versus temperature profiles were measured at 260 nm using a temperature-programmable, thermoelectricaly controlled Beckman recording spectrophotometer and a Carry 117 recording spectrophotometer equipped with a thermostated cell compartment. The thus obtained A(T)=f(T) profiles were transformed to $A_{rel}(T)=f(T)$ ones by normalization of the absorbance at 50°C to A=1.0. For determination of the melting temperatures, Tm, and slopes of the helix-to-single-strands transition, the latter were converted into f versus temperature transition profiles (where f is the fraction of single strands in the two-chain heteroduplex form) by use of commonly accepted procedure (cf. ref.12). The lower (two-chain helix) and the upper (single strands, partially in hairpin helix form) base lines, employed in the calculations of f, were found by extrapolation of the approximately linear

low- and high-temperature regions of the $A_{rel}(T) = f(T)$ curves. In some instances, when the low-temperature part of a curve (at low salt concentrations) was not fully attainable, it was found by deduction, assuming the slope observed for the next curve within the given family of the recorded profiles.

Calorimetry

Calorimetric experiments were performed on a DASM-1 M model of the Privalov type differential scanning microcalorimeter (13), calibrated with a controlled electric current pulse. Calorimetric heat-absorption curves were recorded at a rate of 0.5°C/min in the temperature range of 5-85°C. They proved fully reversible as indicated by the high reproducibility of repeated heating cycles for the same sample. The melting enthalpies were found from the areas under the heat-absorption curves and the spectrophotometrically determined concentrations of the oligomers.

RESULTS AND DISCUSSION

The helix stability by UV spectrophotometry

Figure 1 shows the relative absorbance versus temperature profiles obtained for 1:1 solutions of the two complementary deoxydodecanucleotides of 8.6×10^{-5} M concentration (in terms of base residues) at different selected salt concentrations. Their shape at lower salt concentrations indicates the occurrence of a cooperative transition followed by further slow increase in absorbance extending over a broad range of temperatures. The transition proved to be quickly and fully reversible on cooling of the solutions. As the salt concentration increases the transition shifts towards a higher temperature range, and the melting curve attains a more symmetrical shape characteristic of a monophasic transition. Simultaneously the hypochromicity of the system undergoing heat transition reaches its maximum value of about 25 per cent. This pattern is consistent with the melting of the dodecamer heteroduplex which at salt concentrations higher than 0.2 M K^+ (Na^+) and temperatures below 10°C is the only oligomer species present in solution.

The melting temperature, Tm, of the cooperative transition correlates linearly with the logarithm of the counterion (K^+) concentration up to about 1.0 M (Fig.2). The slope of the Tm versus log M plot, $dTm/d\log M = 14.2$°C is distinctly smaller than 22°C found in ref. (14) for the alternating copolymer poly [d (AT)] , but very close to the value which can be estimated for the $d(TA)_{14}$ linear oligomer in hairpin helix form from the

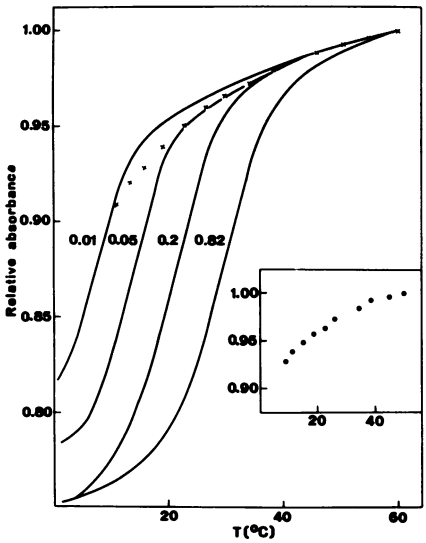


Figure 1. Normalized absorbance vs. temperature profiles of d (TTTTATAATAAA) + d(TTTATTATAAAA) at various (specified) KF concentrations, 1:1 solution 0.085 mM in base residues, 0.01 M phosphate buffer pH 7.0, (x) values for a single chain at 0.05 M (salt). In the insert profiles at 0.006 M Na^+ for d (TTTTATAATAAA) (o) and 1:1 mixture of both oligomers (●) .

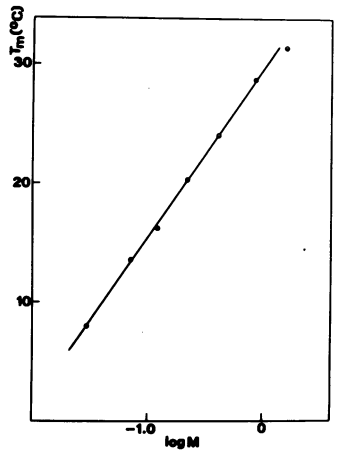


Figure 2. Variation of Tm with concentration of K^+ ions for the cooperative transition in 1:1 solution of d(TTTTATAATAAA) + d(TTTATTATAAAA) : (o) experimental data, (——) least square interpolation curve: Tm=14.2 x log M + 29.7.

Tm (N) =f(log M Na^+) data for $d(TA)_N$ oligomers with N=13 and N=15 (15) .

As demonstrated by Scheffler et al. (16,17), $d(TA)_N$ oligomers ($5 \leqslant N \leqslant 25$) at low counterion concentrations (<0.01 M Na^+) form one-chain, hairpin helices with four unpaired bases in the loop. At higher counterion concentrations and low temperatures the formation of two-chain helices is favoured. With the rise of temperature they are first converted into one-chain hairpins which melt subsequently. This interconversion is accompanied by relatively small change in the absorbance proportional to the fraction of looped out base pairs. The melting behaviour of the hairpin helix formed by the $d(TA)_{14}$ oligomer would thus be expected to simulate closely that of the AT heteroduplex dodecamer investigated. Good agreement between the dTm/dlog M values obtained for the heteroduplex dodecamer and those deduced for the $d(TA)_{14}$ hairpin helix from published data (15) confirms this expectation.

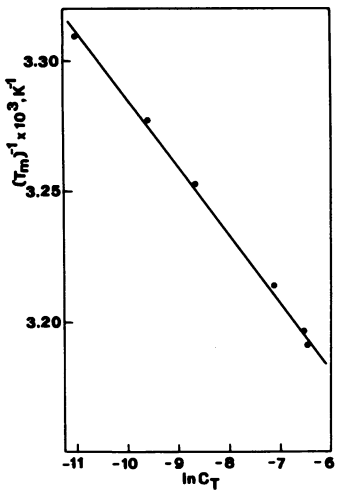


Figure 3. Plot of 1/Tm vs. logarithm of oligomer concentration C_T for the cooperative transition in d(TTTTATAATAAA) + d(TTTATTATAAAA) 1:1 solution, 1.0 M NaCl, phosphate buffer pH 7.0, 0.1 mM EDTA: (o) experimental points, (——) least square correlation curve.

The final proof that the cooperative transition observed at the 1:1 molar ratio of the complementary deoxydodecanucleotide chains concerns the melting of the two-chain helix was obtained from measurements of Tm of the transition in 1.0 M NaCl in function of the total concentration C_T of the single chains. The 1/Tm versus ln C_T plot (Fig.3) shows clearly the characteristic chain-concentration dependence of the thermal stability of the two-chain helices. The relevant relationship is given by the equation:

$$1/Tm = (R/\Delta H) \cdot \ln C_T + S/\Delta H \ ,$$

where the thermodynamic parameters have the following values:
ΔH= 77.1 (σ = 0.06) kcal/mole duplex and ΔS = 656.7 e.u.

In principle there is a possibility that some homoduplex helices form under certain conditions from each of the single chains. The most stable form which can be predicted would contain two 5-base-pair long helical segments with one dangled off base at the end, interrupted by a bulge made of two identical bases on the opposite strands, as shown schematically (IV) in Fig.4.

The stability of this form, compared to that of the perfect dodecamer heteroduplex under similar conditions, is expected to be lower by 6-7 orders of magnitude, however. Indeed, no effect was noted on the $A_{rel}(T) = f(T)$ profiles when at the moderate counterion concentration of 0.06 M Na^+ the concentration of the d(TTTTATAATAAA) single chain was raised 20 fold from 5×10^{-5} to 1×10^{-3}M.

In order to explain the cause of the small increase in absorbance over

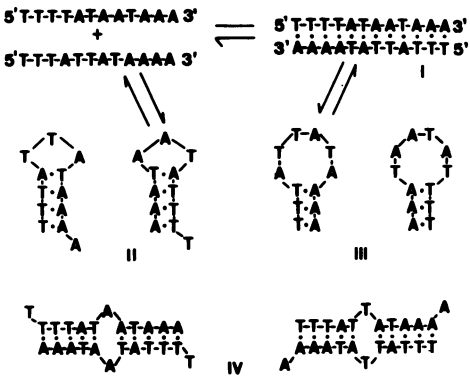


Figure 4. Schemes of various possible structural forms of d(TTTTATAATAAA) and d(TTTATTATAAAA) oligomers in aqueous solutions: I- heteroduplex helix, II, III-hairpin helices, IV-homoduplex helices.

the broad temperature range, observed at low counterion concentrations after completion of the cooperative transition associated with melting of the heteroduplex dodecamer (cf. Fig.1), the possibility of formation of hairpin helices by d(TTTTATAATAAA) and d(TTTATTATAAAA) single chains should be considered. As demonstrated for the $d(TA)_N$ oligomers (15,16), the single hairpin helices are more stable at low counterion concentrations than the corresponding two-chain helical structures because, owing to the existence under these conditions of long range destabilizing electrostatic interactions, the electrostatic energy contribution to the total free energy of the helix is proportional to the number of base-pairs. Consequently, a biphasic melting profile is observed for the $d(TA)_5$ oligomer (17) of a shape similar to that found for the dodecamer heteroduplex. The biphasic melting curve reveals also at low counterion concentrations the presence of the self-complementary deoxydodecanucleotide d(CGCGAATTCGCG) (18). It should be noted here that both oligomers can form presumably only one stable single-chain hairpin helix.

For each of the two dodecanucleotides studied two hairpin helix structures can be envisaged, as shown schematically in Fig.4: (i) form II with 4 base-pairs in the helix, 3 bases in the loop and one dangling base at the end, and (ii) form III with 3 base-pairs in the helical stem and 6 bases in the loop. A 4-base loop is believed to be most probable among the $d(TA)_N$ hairpin helices (16). The formation of the 3-base loop could result in a somewhat strained conformation. However, the corresponding un-

favourable contribution of free energy would be most probably more than compensated by the formation of an additional stack of AT base-pairs, so that form II can be expected to be somewhat more stable than form III. On the other hand, it could be expected that the unfavourable electrostatic contribution to the free energy of hairpin helix formation is smaller for the form III with a shorter helical segment. We are thus tempted to believe that both forms can be present in solution at concentrations proportional to their statistical weights. The free energy of either of the two hairpin helix forms may be also somewhat different for each of the complementary single chains. Therefore, the two deoxydodecanucleotides in solution can be distributed among four different hairpin helices characterized by similar melting profiles but somewhat shifted one from the other along the temperature scale. Superposition of the profiles would result in a broad experimental melting profile without well defined inflection point, observed on biphasic melting curves of $d(TA)_5$ and d(CGCGAATTCGCG) oligomers, referred to above. In order to confirm,at least indirectly, the validity of our interpretation, we measured the relative absorbance as a function of temperature of the two equimolar in base residues (8.5×10^{-5} M) solutions at 0.006 M Na^+, one of which contained the d(TTTTATAATAAA) single chain and the other both complementary dodecamers in 1:1 molar ratio. The two $A_{rel}(T)=f(T)$ profiles proved to coincide exactly at all temperatures above 8^oC (cf. insert in Fig.1), what means that under these conditions the two-chain heteroduplex does not exist. Also $A_{rel}(T)$ values determined for the d(TTTTATAATAAA) chain at 0.06 M Na^+, i.e. under conditions at which the presence of the two-chain helix in solution is clearly seen on the melting profile, could be well fitted by the high temperature part of the latter (cf. Fig.1, $A_{rel}(T)=f(T)$ curve at 0.06 M counterion concentration).

Owing to the fact that thermal stability of longer helices exhibits a much stronger dependence on the counterion concentration than of shorter ones, the two transitions corresponding to the melting of the two-chain dodecamer helix and to that of the hairpin helices were brought to overlap with the rising salt concentration. As a result of this, the experimental $A_{rel}(T)=f(T)$ profiles attain a more symmetrical shape of an apparently monophasic transition.

All these considerations and observations allow us to summarize the helix-coil equimolar solutions of the d(TTTTATAATAAA) and d(TTTATTATAAAA) deoxydodecanucleotides as shown schematically in Fig.4.

Calorimetry

Figure 5 shows the calorimetric heat-capacity curve for the melting of the dodecamer heteroduplex in 1.0 M NaCl solution. The enthalpy of the transition: $\Delta H(cal) = 75.6$ kcal/mole(duplex) was evaluated from the area under the curve. The value found is very close, indeed, to the van't Hoff enthalpy $\Delta H(v.H.) = 77.1$ kcal/mole (duplex) derived from the chain-concentration dependence of the dodecamer heteroduplex thermal stability in 1.0 M NaCl. This agreement is understandable since in the calorimetric experiment the total enthalpy of the helix-to-coil transition of the heteroduplex has been measured, while the variation of Tm with C_T in the spectrophotometric experiments related the same transition whose midpoint could be but little affected by the appearance of low-amplitude, intermediate hairpin-helix states.

In view of the fairly symmetric shape of the calorimetric curve, indicative of the practical independence of the melting enthalpy of temperature within the temperature range investigated, a melting curve was derived therefrom, drawn as the fraction f of the oligomers in heteroduplex form as a function of temperature (cf. Fig.5). From the slope of this curve at Tm the van't Hoff enthalpy of the transition 65.7 kcal/mole (duplex) was again calculated according to the eqution: $\Delta H(v.H.) = 6RTm^2(df/dT)_{Tm}$.

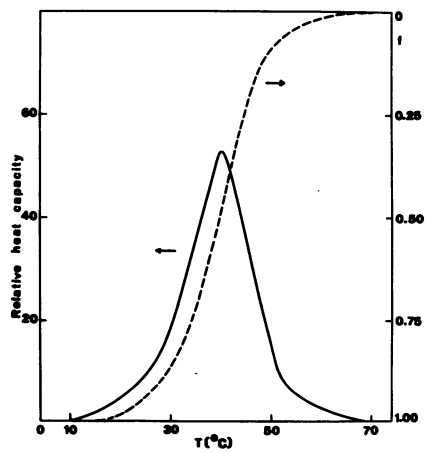
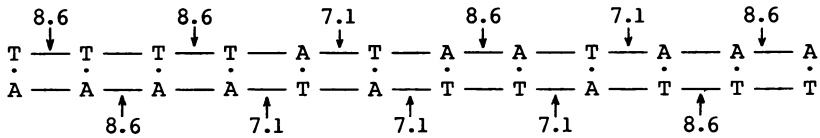


Figure 5. Calorimetric heat capacity-temperature curve(——) for the helix-to-single strands transition of the d(TTTTATAATAAA)· d(TTTATTATAAAA) heteroduplex helix, and melting profile (—) derived therefrom; 1.0 M NaCl, 0.01 M phosphate buffer pH 7.0, 0.1 mM EDTA, C_T=1.43 mM (base residues).

A similar mean value of ΔH(v.H.) was calculated from the slopes of the spectrophotometric melting curves recorded at 1.0 M NaCl and various oligomer concentrations. Since the slope of such a melting curve of the dodecamer heteroduplex can be expected to be influenced by the formation and disappearance of the intermediate hairpin-helix states within the same temperature range, the lower value of ΔH(v.H.) thus derived reflects simply the occurrence of such processes.

The calorimetric transition enthalpies accompanying the duplex-to-single-strands transition of poly [d(AT)] and poly d(A) · poly d(T) (as well as of some other polynucleotides containing G and C) complexes were recently determined and the average basestacking enthalpies derived therefrom were used successfully to predict the transition enthalpies of some oligomeric DNA duplexes (19). It seemed thus worthwhile to calculate the transition enthalpy of the AT heteroduplex investigated by use of the reported base-stacking enthalpies and to compare it with the experimental calorimetric enthalpy. The calculation based on the assumption of the nearest-neighbour approximation of stacking interactions is illustrated by the scheme below.



The numbers in the scheme refer to the base-stacking enthalpies: 7.1 kcal/stack for the AT/TA and TA/AT stacks, and 8.6 kcal/stack for the AA/TT stack (19). The enthalpy of the transition ΔH(pred) = 87.12 kcal/mole (duplex) calculated in this way is distinctly larger than the experimental enthalpy ΔH(cal) = 75.6 kcal/mole (duplex). The difference between the two values could even be larger if the 8.6 kcal/stack enthalpy measured for the poly d(A) · poly d(T) in 0.01 M NaCl appeared to be higher in 1.0 M NaCl, at which salt concentration the enthalpy of the AT heteroduplex has been determined. The transition enthalpy of poly [d(AT)] is known (20) to increase linearly with the logarithm of the Na_2SO_4 molal activity up to about 0.6 molal salt concentration and then decreases abruptly. As a result the 7.1 kcal/stack (TA/AT, AT/TA) enthalpy obtained for poly [d(AT)] as a mean value of enthalpies measured in 0.01 and 1.0 M NaCl solutions (19) agrees reasonably with that calculated from the data referred to above. For poly d(A) · poly d(T) the dependence of the melting enthalpy on salt concentration, which has not been studied yet, may take a different course. The dif-

ference between the measured and predicted transition enthalpies for the AT heteroduplex dodecamer should thus be regarded as the lower limit thereof.

The reason for the transition enthalpy being lower than expected can be connected with specific conformational and dynamic properties of the dodecamer duplex structure built of adjacent alternating and non-alternating helical sequences of A and T residues. Both kinds of sequences are believed (21,22) to adopt different conformations with respect to each other and depart somewhat from the classical B form. Further studies on the structure and conformational dynamics of the AT dodecamer heteroduplex should help to explain its relalively low melting enthalpy.

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REFERENCES

1. Chamberlin,M.J.(1982) Enzymes 15,61-85.
2. Gamper,H.B. and Hearst,J.E. (1982) Cell 29,81-90.
3. Siebenlist,U. (1979) Nature 279,651-652.
4. Siebenlist,U.,Simpson,R.B. and Gilbert,W.(1980) Cell 20,269-281.
5. Itakura,K.,Katagiri,N.,Bahl,C.P.,Wightman,R.H. and Narang,S.A. (1975) J.Am.Chem. Soc.97,7327-7332.
6. Katagiri,N.,Itakura,K. and Narang,S.A.(1975) J.Am.Chem.Soc.97,7332-7337.
7. Stawiński,J.,Hozumi,T.,Narang,S.A.,Bahl,C.P. and Wu,R.(1977) Nucleic Acids Res. 4,353-371.
8. Hsiung,H.M.,Brousseau,R.,Michniewicz,J. and Narang,S.A.(1979) Nucleic Acids Res. 6,1371-1385.
9. Bolewska,K.Ph.D.Thesis Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa,(1984).
10. Jay,E.,Bambara,R.,Padmanabhan,R.and Wu,R.(1974) Nucleic Acids Res. 1,331-353.
11. Cantor,C.,Warshaw,M.M. and Shapiro,H.(1970) Biopolymers 9,1059-1077.
12. Nelson,J.W.,Martin,F.H. and Tinoco,I.Jr.(1981) Biopolymers 20,2509-2531.
13. Privalov,P.L.,Plotnikov,V.V.and Filimonov,V.V.(1975) J.Chem. Thermodynamics 7,41-47.
14. Inman,R.B. and Baldwin,R.L(1964) J.Mol.Biol.8,452-469.
15. Elson,E.L.,Scheffler,I.E. and Baldwin,R.L.(1970) J.Mol.Biol.54,401-415.
16. Scheffler,I.E.,Elson,E.L. and Baldwin,R.L.(1968) J.Mol.Biol.36,291-304.

17. Scheffler,I.E.,Elson,E.L. and Baldwin,R.L.(1970) J.Mol.Biol.48,145-171.
18. Marky,L.A.,Blumenfeld,K.S.,Kozlowski,S. and Breslauer,K.J.(1983) Biopolymers 22, 1247-1257.
19. Marky,L.A. and Breslauer,K.J.(1982) Biopolymers 21,2185-2194.
20. Gruenwedel,D.W.(1975) Biochim.Biophys.Acta 395,246-257.
21. Zimmerman,S.B.(1982) Ann.Rev.Biochem. 51,395-427.
22. Viswamitra,M.A.,Shakked,Z.,Jones,P.G.,Sheldrick,G.M.,Salisbury,S.A. and Kennard,O.(1982) Biopolymers 21,513-533.