Synthesis and physicochemical properties of two analogs of poly(dA): poly(2-aminopurine-9-β-D-deoxyribonucleotide) and poly 2-amino-deoxyadenylic acid

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SUMMARY

Polymerization of chemically synthesized dn^2h^6ATP and dn^2ATP by deoxynucleotidyl transferase from calf thymus furnished poly(dn^2h^6A) and poly(dn^2A) respectively. The synthetic polynucleotides were characterized by spectroscopic, ultracentrifugation and enzymatic methods. In polynucleotide-polynucleotide interaction, poly(dn^2h^6A) and poly(dn^2A) behaved like analogs of poly(dA).

INTRODUCTION

2-Aminopurine- and 2-aminoadenine nucleotides possess interesting biological as well as physicochemical properties (1,2,3,4).Worth to be emphasized are the luminescence of the two purines in the ultra violet. 2-Aminopurine functions as a powerful mutagen (5,6,7,8). It is incorporated into DNA (9,10) and might be ambiguitive with respect to base pairing. A special kind of ambiguity of 2-aminopurine has been established in transcription employing DNA-dependent RNA polymerase from E.coli and synthetic templates (11). The substrate analog n^2h^6ATP was exclusively utilized as substitute for ATP, whereas n^2h^6A bases in templates were recognized as guanine analogs. 2-Aminoadenine is likewise extensively incorporated into DNA and seems to affect fidelity of replication (12). However, this could not be proven in in vitro experiments (11).

Quite a number of synthetic polynucleotides containing 2-aminopurine or 2-aminoadenine have been prepared with the aim to study their specificity in polynucleotide- polynucleotide interactions as well as the biological properties (1,11,13,15,16); their spectroscopic features have been reported. Especially polydeoxynucleotides containing n^2h^6A or n^2A should be useful, because their luminescence properties might be employed in studies of protein-polynucleotide interactions. Hitherto the syntheses of the alternating synthetic DNA's poly[d(n^2h^6A-T)] (17) and poly[d(n^2A-T)] (13) have been described. In this paper we report the syntheses of the poly(dA) analogs

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poly(dn^2h^6A) and poly(dn^2A), their characterization by various physical techniques as well as their specificities in polynucleotide-polynucleotide interactions.

EXPERIMENTAL PROCEDURES

Chemicals. Poly(dT), poly(U) and $(pT)_3$ were purchased from Boehringer (Mannheim). 2'-Deoxy-2-aminoadenosine and 2'-deoxy-6-thioguanosine were obtained from PL-Biochemicals (Milwaukee,USA). The latter was converted to 2-aminopurine-9-(β-D-2'-deoxyriboside) according to the procedure of Fox et al.(18).

Enzymes. Phosphodiesterase from Crot.terr.terr. (EC 3.1.4.1) was a commercial preparation from Boehringer (Mannheim). Terminal deoxynucleotidyl transferase (EC 2.7.7.31) from calf thymus was the general gift of Dr.T.M.Jovin.

UV-absorption spectroscopy.Absorption measurements,determinations of temperature-absorption profiles as well as spectrophotometric titrations were carried out as described earlier (19,20).

Fluorescence spectroscopy. The fluorimeter employed in this study was that described by Baehr et al.(21). Continous fluorimetric titrations were carried out with the above fluorimeter in connection with a Fabritek model 1074 instrument computer as described (21). Instead of a fluorescence emmision monochromator, a 340 nm-cut off filter was used in all measurements.

Synthesis of deoxynucleotides. The deoxyribonucleosides were rendered anhydrous by repeated co-evaporations with anhydrous pyridine. The residual gum was dissolved in 2 ml of pyridine. After addition of one equivalent of diphenyl phosphorochloridate, the reaction mixture was kept one hour at room temperature. Then 2 ml of water were added and the resulting mixture was evaporated to dryness. The residue was dissolved in the minimal volume of methanol and the solution subjected to preparative thin layer chromatography on silica gel employing $CHCl_3$-CH_3OH (85:15/v/v) as solvent. The ultra violet absorbing band containing the desired triester was eluted from silica gel with methanol. The eluate was taken to dryness and the residue dissolved in 10 ml of concentrated NH_4OH. After 15 h at room temperature the NH_4OH was evaporated. The remainder was dissolved in 1 ml of 10 mM Tris-HCl pH 8 and 0.1 mg of snake venom phosphodiesterase were added. The mixture was incubated for 10 h at 37°C. The enzymatic reaction was stopped by heating the mixture for 2 min at 100°C. Water was then added to give a final volume of 50 ml. The solution was applied to a DEAE Sephadex A 25 column (1x10 cm) and elution

performed with a linear gradient of triethyl ammoniumbicarbonate from 0 - 0.2 M. Fractions containing the triethylammonium salts of 5 and 6 respectively were pooled and evaporated to dryness. Further details are given in Table 1.

Synthesis of deoxynucleoside 5'-triphosphats. The tri-n-butylammonium salts of the corresponding 5'-monophosphates were dried by repeated coevaporations with anhydrous pyridine. In a glove-box, under anhydrous conditions, the residual gum was dissolved in 1 ml of anhydrous DMF. After addition of 1 equivalent of diphenyl phosphorochloridate, the reaction was kept in the glove-box for 3 h. Then 5 equivalents of tri-n-butyl ammonium pyrophosphate dissolved in 1 ml of anhydrous DMF were added. The resulting mixture was kept at room temperature for 15 h. After dilution by two volumes of water, the mixture was applied to a DEAE Sephadex A 25 column (1x10 cm). Elution was performed by a linear gradient of triethyl ammoniumbicarbonate from 0 - 0.4M. Fractions containing the 5'-triphosphates were pooled and evaporated. Triethylammonium salts were converted to ammonium salts by repeated co-evaporations with NH_4OH. Further details are given in Table 1.

Enzymatic synthesis of polydeoxynucleotides. The incubation mixture contained in one ml: 1 umol dn^2h^6ATP or dn^2ATP respectively, 0.2 mmoles cacodylate pH 7.1, 1 umol $MgCl_2$, 0.1 µmol dithiothreitol, 40 µmol KCl, 0.1 A_{260}-units $(pT)_3$ and 0.45 µg terminal deoxy- nucleotidyl transferase. The mixture was incubated for 15 h at 37°C. After deproteinization by chloroform-isoamylalcohol, the resulting mixture was dialyzed at 4°C against several portions of a buffer containing 0.01 M Tris-HCl pH 7.5, 0.01 M KCl and 0.1 mM EDTA. Poly(dn^2h^6A) : yield 75%; poly(dn^2A): yield 55%. Ultraviolet absorption spectra are given in Table 1.

TABLE 1 Chemical synthesis of nucleotides

Product	starting material (mmoles)	yield (mmoles)	deoxynucleoside/ phosphate
dn^2h^6AMP	dn^2h^6A (0.25)	0.05 (20%)	1.1
dn^2AMP	dn^2A (0.25)	0.09 (36%)	0.9
dn^2h^6ATP	d^2h^6AMP (0.05)	0.012 (23%)	2.9
dn^2ATP	dn^2AMP (0.05)	0.007 (14%)	2.9

Experimental details of chemical syntheses are described in Methods. Abbreviations: dn^2h^6AMP, 2-aminopurine-9-(β-D-2'-deoxyriboside)5'-phosphate; dn^2AMP, 2'-deoxy,2-aminodenosine 5'-phosphate; dn^2h^6ATP, 2-aminopurine-9-(β-D-2'-deoxyriboside)5'-triphosphate; dn^2ATP, 2'deoxy,2-aminoadenosine 5'triphosphate.

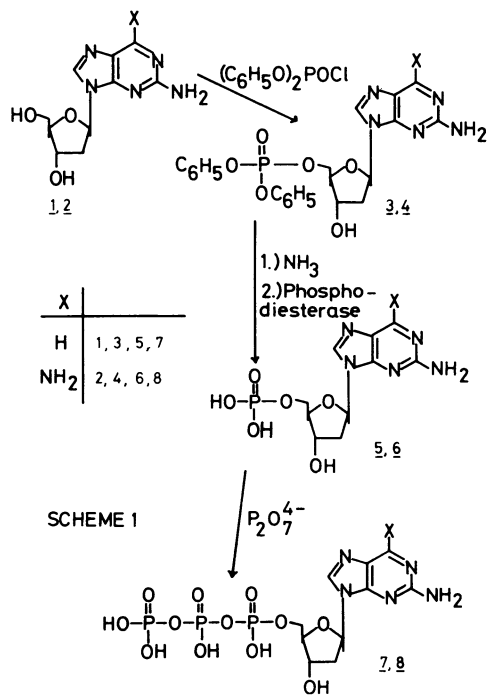
RESULTS AND DISCUSSION

Phosphorylation of 2'-deoxynucleotides is not an easy task because of the highly acid-labile glycosyl bond. We found it convenient to phosphorylate 2'dn^{2}h^{6}A and 2'dn^{2}A by stoichiometric amounts of diphenyl phosphorochloridate (Scheme 1). Due to the bulky diphenyl grouping, the phosphorylation occurred in 5'-position. The resulting triesters (3,4) could be easily isolated by preparative thin layer chromatography. A combination of alkaline hydrolysis by ammonia and enzymatic hydrolysis by snake venom phosphodiesterase led to dn^{2}h^{6}AMP (5,20%) and dn^{2}AMP (6,36%) respectively (see also Table 1). The preparation of the 5'-triphosphates dn^{2}h^{6}ATP (7,23%) and dn^{2}ATP (8,14%) involved activation of the corresponding monophosphates 5 and 6 by diphenylphosphorochloridate. We found the described method especially useful if only small quantities of the starting nucleoside were available.

The chemical syntheses of dn^{2}h^{6}ATP (19) and dn^{2}ATP (13) by different procedures was already described. The synthesis of dn^{2}h^{6}ATP exclusively by enzymatic processes was described by Bessman et al.(10).

Synthesis and characterization of polydeoxynucleotides.

Primer-dependent polymerization of dn^{2}h^{6}ATP and dn^{2}ATP respectively by



SCHEME 1

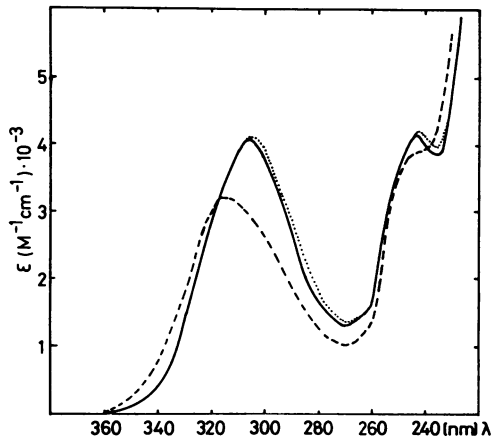


Figure 1. Absorption spectra of poly(dn^2h^6A)
(- - -),water-HCl,pH 3.7;(-), 1mM Tris-HCl, pH 7; (...), 1mM Tris-HCl, pH 10.

calf thymus deoxynucleotidyl transferase furnished poly(dn^2h^6A) as well as poly(dn^2A) in reasonable yields. Because of the primer employed, both polydeoxynucleotides possessed a pTpTpT-sequence at the 5'-termini. Poly(dn^2h^6A) and poly(dn^2A) exhibited sedimentation coefficients of $s_{20,w}$ of 3.5 and 3.1 S respetcively. This indicated a minimal chainlength of

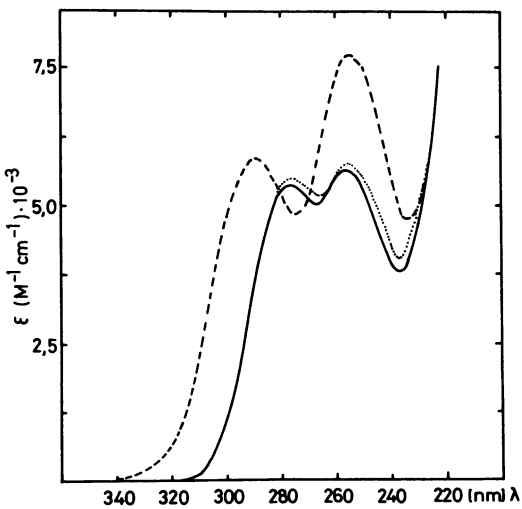


Figure 2. Absorption spectra of poly(dn^2A)
(- - -), water-HCl,pH 3.8;(-), 1mM Tris-HCl,pH 7; (...),1mM Tris-HCl,pH 10.

TABLE 2 Ultraviolet absorption spectra

Substance	λ_{max} (nm)	λ_{min} (nm)
d^2h^6AMP	303 ($6.5x10^3$)	262 ($1.04x10^3$)
poly(dn^2h^6A)	303 ($3.92x10^3$)	266 ($4.46x10^3$)
	246 ($3.79x10^3$)	236 ($0.66x10^3$)
dn^2AMP	278 ($9.8x10^3$)	264 ($7.5x10^3$)
	255 ($9x10^3$)	238 ($5x10^3$)
poly(dn^2A)	277 ($5.25x10^3$)	268 ($4.85x10^3$)
	256 ($5.75x10^3$)	238 ($3.96x10^3$)

Spectral data were obtained at pH 7 and 0.1M NaCl. Numbers given in parentheses are extinction coefficients. Abbreviations: poly(dn^2h^6A), poly(2-aminopurine deoxynucleotide); poly(dn^2A), poly(2-aminoadenine deoxynucleotide).

approximately 50 nucleotides. The absorption spectra of both polynucleotides are depicted in Figs.1 and 2. Both polynucleotides displayed large hyperchromicities in their absorption spectra as detected by enzymatic hydrolysis (Table 2).

The absorption spectra of the enzymatic hydrolysates of poly(dn^2h^6A) and poly(dn^2A) were similar to those of the corresponding nucleotides: A_{303}/A_{260} [poly(dn^2h^6A)-hydrolysate]=5.53 compared to A_{303}/A_{260}[dn^2h^6AMP]=6.02 ; A_{278}/A_{255}[poly(dn^2A)- hydrolysate]=1.054, compared to A_{278}/A_{255}[dn^2AMP]=1.11 . These data indicate a reasonable chemical purity of the prepared polynucleotides. This is essential in the case of poly(dn^2h^6A) since it is our experience that dn^2h^6AMP is of limited stability in aqueous solution (22). 2-Aminopurine nucleotides possess luminescence spectra, characterized by appreciable quantum yields, which have been thouroughly investigated. This holds likewise for dn^2h^6AMP, whose fluorescence properties are similar to n^2h^6AMP and will not be discussed here. The fluorescence quantum yield of dn^2h^6AMP-residues in poly(dn^2h^6A) was largely reduced upon polymerization. When this polynucleotide was hydrolyzed by snake venom phosphodiesterase, the fluorescence signal drastically increased; a ratio of fluorescence intensities F poly(dn^2h^{6A})/ F dn^2h^6AMP = 0.05 was determined. This phenomenon was also observed in the case of poly(n^2h^6A), although the ratio F poly(n^2h^6A)/ F n^2h^6AMP = 0.01 was significantly smaller (23). If the proposal by Ward et al.(1) holds that only terminal n^2h^6A bases in such polynucleotides fluoresce, then the difference in fluorescence ratios simply reflects significantly higher average chainlength of poly (n^2h^6A) compared to poly(dn^2h^6A).

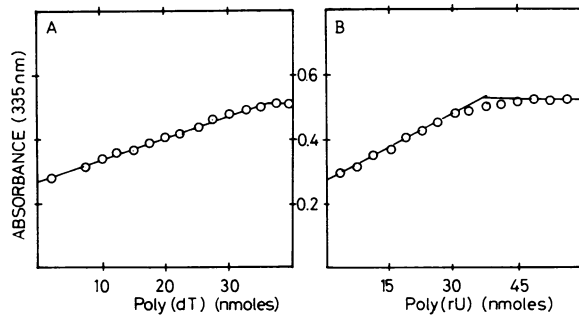


Figure 3. UV-spectrophotometric titration of poly(dn^2h^6A) with poly(dT) and poly(U).

The reaction mixture contained 34.5 nmoles poly(dn^2hA) in one cuvette and buffer in the other cuvette. Titration was carried out by adding aliquots of suitably concentrated solutions of either poly(d^2h^6A) (A) or poly(U) (B) to both cuvettes. After each addition the absorption was monitored until no further change could be noticed.Solvent: 0.1M NaCl, 10mM KH_2PO_4 (pH 7.5) (A); 0.1M NaCl,50 mM $MgCl_2$,10 mM KH_2PO_4 (pH 7.5) (B). Temperature in experiment A) was 25°C, in experiment B) 15°C.

Interaction of polynucleotides.

Spectrophotometric titrations of poly(dn^2h^6A) with poly(dT) or poly(U) at 25°C respectively revealed complex formation.As depicted in Figs.3 A and B,the stoichiometry of interaction was one. Formation of the complex poly(dn^2h^6A)·poly(dT) could be also demonstrated by continous fluorimetric titration, because complex formation was accompanied by a significant decrease in fluorescence emission of n^2h^6A residues involved in base pairing (Fig.4). The apparent fluorescence efficiencies of n^2h^6A moieties differed in the following manner: Fdn^2h^6AMP/ Fpoly(dn^2h^6A)/Fpoly(dn^2h^6A) (polydT)=1:0.05:0.022 . As proposed by Ward et al. (1) for the fluorescence properties of poly(n^2h^6A-U) the residual fluorescence in this helical polynucleotide should result from terminal n^2h^6A bases due to the fact that internal n^2h^6A bases are in a hydrophobic environment where their quantum yields of fluorescence decrease to values in the order of 0.01. The fluorescence properties of the complex poly(dn^2h^6A)·poly(dT) fit into this concept(1). poly(dn^2A) formed stoichiometric complexes with poly(dT) and poly(U) respectively as demonstrated by spectrophotometric titrations (Fig.5 A and B). Attempts to demonstrate interactions of polydn$^2h^6A$) or poly(dn^2A) with either poly(dC) or poly(C) by spectrophotometric or fluorimetric titrations failed.This is in agreement with reports from other researchers who likewise were unable to

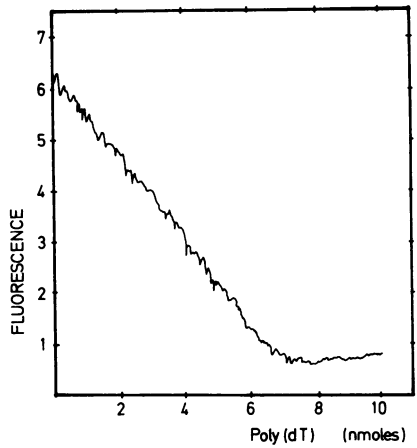


Figure 4. Fluorimetric titration of poly(d^2h^6A) with poly(dT)
A suitably concentrated solution of poly(dT) was continously added to a solution of 7.75 nmoles poly(d^2h^6A) in 1 ml of 0.1M NaCl,10mM KH_2PO_4 (pH 7.5) as described in methods. The experiment was performed at 25°C. The total volume change was less than 2% and was therefore ignored.

detect ambiguitive behaviour of n^2h^6A or n^2A bases in polynucleotide-poly nucleotide interactions (11,14,15,16).

Absorption temperature profiles.

The polynucleotide complexes formed by spectrophotometric titrations:poly(dn^2h^6A)·poly(dT), poly(dn^2h^6A)·poly(U),poly(dn^2A)·poly(dT) and poly(dn^2A)·poly(U) underwent helix-coil transitions as judged by narrow sigmoidal shapes of the respective absorption-temperature profiles. The hybrid poly(dn^2h^6A)·poly(U) had a T_m-value of 28°C in 0.2 M NaCl containing 50 mM $MgCl_2$. Thus, the latter complex could only be attained by titration below 10°C. The thermal absorption-difference spectra of the complexes are given in Fig.6 . The complexes poly(dn^2h^6A)·polyd(T) and poly(dn^2A)·poly(dT) showed a normal dependence of T_m-values versus ionic strength (Fig.7); the observed slopes T_m/ log Na^+ were 17°C and 16°C respectively.

To evaluate the effect of substitution of adenine by adenine analogs on polynucleotide-polnucleotide interactions a table of T_m-values including those of normal polynucleotide complexes is given (Table 3).

Replacement of adenine bases by 2-aminoadenine in polynucleotide complexes generally stabilizes the helical structures significantly. The stabilization is assumed to result from the formation of an adenine-uracil base pair analog possessing three instead of two hydrogen bonds

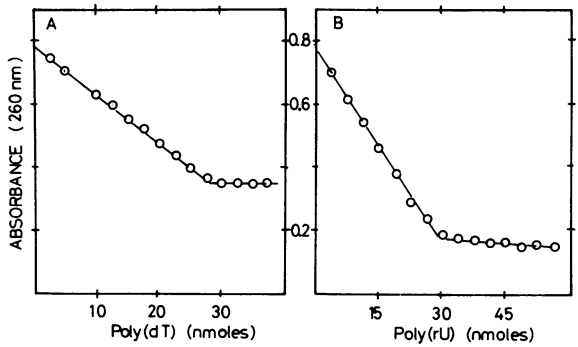


Figure 5. UV-spectrophotometric titration of poly(dn^2A) with poly(dT) and poly(U).

A) Titration with poly(dT); B) titration with poly(U). The solvent in both experiments was 0.1 M NaCl, 10mM KH_2PO_4 (pH 7.5). The titration was performed at 25°C. The concentration of poly(dn^2A) in both experiments was 34.5 nmoles poly(dn^2A)/ml.

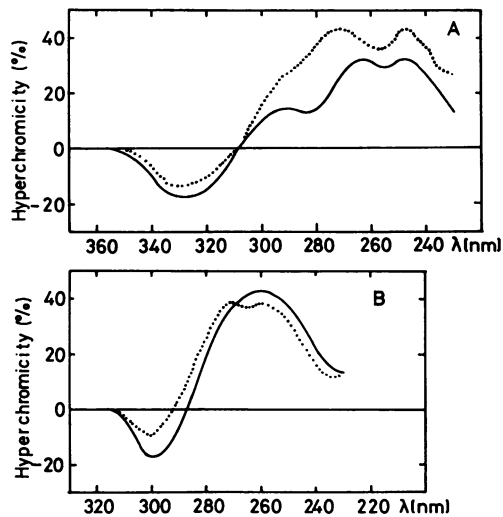


Figure 6.Thermal absorption-differerence spectra of polynucleotide complexes.

All complexes contained stoichiometric amounts of polynucleotides. The solvent was 0.1M NaCl, 0.01M KH_2PO_4,pH 7.5.
A),(-),poly(dn^2h^6A) poly(dT);(- - -),poly(dn^2h^6A) poly(U);
B),(-),poly(dn^2A) poly(dT);(- - -),poly(dn^2A) poly(U).

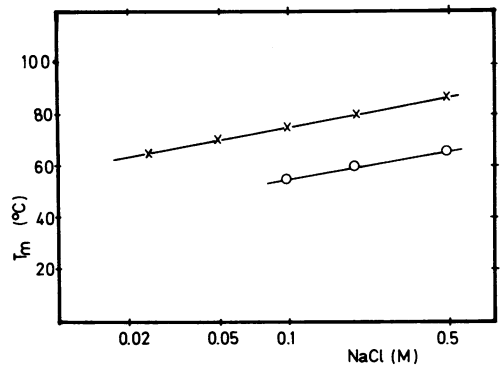


Figure 7. Ionic strength dependence of T_m-values (x-x), poly(dn^2h^6A) poly(dT): (o-o), poly(dn^2A) poly(dT)

(1,11,14,16). Inspection of the data depicted in Table 3 makes evident that substitution of adenine by 2-aminopurine bases weakens helical polynucleotide structures. The destabilization is expressed by an average decrease of T_m-values compared to the normal complexes by 10°C. An exceptional

TABLE 3 T_m-values of polynucleotide complexes

Complex	NaCl-conc. (M)	T_m (°C)	Ref.
poly(dA)·poly(dT)	0.1	68.8	24
poly(A)·poly(dT)	0.1	64.1	24
poly(A)·poly(U)	0.1	56.8	24
poly(dA)·poly(U)	0.1	45.2	24
poly(dn^2h^6A)·poly(dT)	0.1	59.5	
poly(n^2h^6A)·poly(dT)	0.1	53	
poly(n^2h^6A)·poly(U)	0.1	47	15
poly(dn^2h^6A)·poly(U)	0.1	28	
poly(dn^2A)·poly(dT)	0.1	77	
poly(n^2A)·poly(dT)	0.05	80.5	
poly(n^2A)·poly(U)	0.2	86	14
poly(dn^2A)·poly(U)	0.1	65.5	
poly(dG)·poly(dC)	0.001	63	24
poly(G)·poly(dC)	0.001	88	24
poly(G)·poly(C)	0.001	97	24
poly(dG)·poly(C)	0.001	71	24

destabilization as judged by a decrease of 18°C of the respective T_m-values displayed the hybrid structure poly(dn^2h^6A)·poly(U). In this context it is interesting to recall that attempts to synthesize this hybrid by transcription of poly(dn^2h^6A) employing E.coli RNA polymerase and UTP failed (11).

Although interaction between poly(dn^2h^6A) and poly(C) could not been demonstrated, poly(dn^2h^6A) in the presence of CTP, but not UTP, served as template for E.coli RNA polymerase leading to the synthesis of poly(C) (11). These results suggest that the biological property of 2-aminopurine-ambiguity may be an effect which requires the participations of polymerases, because at the level of polynucleotide interactions, this ambiguity could not be observed.

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