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Influence of Different Levels of 2-Thiocytidine on Physical and Template Properties of Cytidine–2-Thiocytidine Copolymers†

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ABSTRACT: Copolymers containing cytidine (C) and various amounts of 2-thiocytidine (s^2C) were prepared using polynucleotide phosphorylase from *Micrococcus luteus*. Both CTP and s^2CTP polymerized equally well. The copolymers were used as templates for DNA-dependent RNA polymerase from *Escherichia coli* in the presence of Mn^{2+}, and it was found that, in contrast to the homopolymer poly(s^2C), the (C,s^2C) copolymers were active as templates in this transcriptional assay. When the amount of s^2C did not exceed approximately 10%, no difference in GMP incorporation compared to poly(C) was noted under the standard conditions of 2-h incubation. When the amount of s^2C was greater than approximately 10%, the decrease in template activity was proportional to the increase in amount of s^2CMP. The changes in template activity were paralleled by the change in the melting temperature of the "self-complementary" double strand. With increasing time of transcription the depressing effect of increasing amounts of s^2C in copolymers was diminished, and after 8-h incubation, transcription was similar to that of poly(C). When the template copolymer contained [^{14}C]C the [^{14}C]C–[^{3}H]G ratio of the double-stranded polymer changed with the amount of s^2C present, as expected, thus establishing the fact that base pairing between s^2C and G occurred. It is assumed that the presence of sulfur instead of oxygen causes the observed slower rate in enzymatic polymerizations with both polynucleotide phosphorylase and RNA polymerase but that this is not a critical factor for base recognition. A model is given to explain the stereochemistry of s^2C–G base pairing resulting in the formation of only two hydrogen bonds.

Our continuing study of the effects of alkylation on coding properties of pyrimidines in polynucleotides (Singer & Fraenkel-Conrat, 1970; Singer et al., 1978) is based on the assumption that there are very few modified bases in an in vivo modified nucleic acid. Enzymes involved in transcription would therefore generally recognize such nucleic acids as being structurally unchanged, and normal transcription would proceed until a modified base is reached. The decision as to whether or not a nucleoside becomes incorporated, and if so, which nucleoside, should depend only on the properties of the individual modified base. This is in contrast to the parameters for base pairing in modified homopolymers where the effect of secondary structure contributed by the modified base can

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be paramount. Consequently, in studies related to mutagenesis, copolymers with a very limited number of modified bases are better models than homopolymers.

As a simple model for minor base modification, we chose 2-thiocytidine[1] (s^2C), a base which differs from its parent base primarily in its geometry. Polymers containing 2-thiocytidine were of particular interest since there are conflicting data in the literature as to whether 2-thiocytidine is capable of forming a base pair with guanosine: the homopolymer, poly(s^2C), does not serve as a template for DNA-dependent RNA polymerase and also is not a messenger in a cell-free system (Rackwitz & Scheit, 1977), which suggests that 2-thiocytidine cannot base-pair with guanosine. On the other hand s^2CTP is, under certain conditions, a substrate for RNA polymerase, but only if a double-stranded polymer rather than DNA is used as template (Faerber, 1974; Rackwitz & Scheit, 1977). In codon–anticodon binding experiments, s^2C has a limited, but detectable, capacity for replacing cytidine (Vormbrock et al., 1974).

We have studied transcription of poly(C,s^2C) copolymers containing 2–50% s^2C, and our results enable the previously obtained data to be interpreted in a uniform manner.

Materials and Methods

Chemicals. [^{3}H]ATP (26 Ci/mmol), [^{3}H]CTP (21 Ci/mmol), [^{3}H]GTP (15 Ci/mmol), and [^{3}H]UTP (18 Ci/mmol) were products of Schwarz/Mann; [^{14}C]CDP (0.43 Ci/mmol) and [α-^{32}P]CTP (initially 7.3 Ci/mmol) were products of New England Nuclear. [α-^{32}P]CDP was obtained as an impurity of [α-^{32}P]CTP and purified by TLC on MN Polygram 300 PEI cellulose (Machery-Nagel, Germany) using 0.9 M LiCl as solvent system. [2-^{14}C]Iodoacetic acid (28 mCi/mmol) was obtained from Amersham/Searle. 2-Thiocytidine 5′-diphosphate (s^2CDP) and poly(s^2C) were kindly given by Dr. O. Saiko (Merck AG, Darmstadt). All other chemicals used were of highest purity from commercial sources.

Enzymes. Polynucleotide phosphorylase and pancreatic ribonuclease were products of P-L Biochemicals, RNA polymerase *E. coli* K12 was a product of Miles Laboratories, and ribonuclease T_1 was obtained from Calbiochem (stored in 10 mM Tris, pH 7.4, 1 mM EDTA).

Preparation of Polynucleotides. CDP was copolymerized with varying proportions of s^2CDP as described by Singer et al. (1978). The polymerization was carried out for 15–17 h at 37 °C in a total volume of 90 μL containing 1 mg of nucleoside diphosphate (the molar ratio of s^2CDP–CDP varied from 0 to 5:1), 0.1 M pH 8.2 Tris buffer, 0.5 mM EDTA, 5 mM $MgCl_2$, 0.04 mg/mL of bovine serum albumin, and 20–30 μg of polynucleotide phosphorylase. The incubation mixture was directly spotted on cellulose TLC plates (Eastman Kodak TLC Cellulose, No. 6065) using 1-propanol, concentrated NH_4OH, and water (55:10:35) as solvent (Singer & Kröger, 1978). In some experiments polymers were separated from nucleoside diphosphates using gel filtration on Biogel P-150 (Ikehara et al., 1969; Ikehara & Hattori, 1972; Gerchman et al., 1972) (1.5 × 38 cm; Bio-Rad). The yield of polymer isolated by TLC was 4–7 A_{270} units (50–60%) while the yield from gel filtration was slightly lower. The time course of the polymerization was followed using the cellulose thin-layer plate separation procedure (Singer & Kröger, 1978).

Analysis of Polynucleotide Composition and Molecular Weight. The ratio between absorption at 250 and 270 nm was used to characterize both the polymers and the remaining unpolymerized nucleoside diphosphate. The λ_{max} of poly(C) in water is 268 nm while the λ_{max} of poly(s^2C) is 244 nm (Scheit & Faerber, 1971). Polymers with increasing amounts of s^2C have 250/270 ratios indicative of their composition.

The amount of s^2C could be independently determined by incorporation of [2-^{14}C]iodoacetic acid according to the following procedure (Sprinzl et al., 1974). Polymer (0.05–0.1 A_{270} unit), 50 mM [2-^{14}C]iodoacetic acid, and 40 mM pH 6.0 sodium citrate were mixed in a 50-μL volume and kept at 37 °C for 18 h. The entire sample was spotted on Whatman 3 MM paper disks and the amount of bound radioactivity determined after trichloroacetic acid precipitation.

The average molecular weight was determined for a series of polyribonucleotides by means of 3′-terminal nucleoside end-group analysis of polymers containing [^{14}C]cytidine (Sugiyama & Fraenkel-Conrat, 1961).

Nearest-neighbor analysis was performed with copolymers containing ^{32}P-labeled CMP and different amounts of s^2C. Preparation of the polymer was as described above, except that 10 μL of unbuffered [α-^{32}P]CDP was added to the original 90-μL volume, decreasing the actual buffer concentration as well as the yield. The ^{32}P-containing polymers were hydrolyzed in 0.1 M KOH for 17 h at 37 °C, and the resulting [^{32}P]-nucleoside 2′(3′)-monophosphates were separated on MN Polygram 300 PEI cellulose TLC plates using 0.2 M acetic acid as solvent.

Determination of Melting Temperature. The melting temperature of the helical form of poly(C) at pH 4.0 was determined according to Scheit & Faerber (1971) with 3 mL of polymer solution containing 0.6–0.7 A_{270} unit of polyribonucleotide, 0.112 M sodium citrate (pH 4.0), and 0.05 M sodium chloride using the temperature range 14–86 °C in a Gilford Model 2000 multiple sample absorbance recorder connected with a Beckmann DU Model 2400 spectrophotometer and a Tamson thermostat.

Transcription and Translation of Copolymers of C and s^2C. The incorporation of nucleoside triphosphates into polyribonucleotides using RNA polymerase was performed according to Gleason & Fraenkel-Conrat (1976). The 125-μL incubation mixture contained 0.01–0.05 A_{270} unit of polyribonucleotide, 0.4 mM each ATP, CTP, GTP, and UTP, 0.4 μM appropriate ^{3}H-labeled nucleoside 5′-triphosphate, 80 mM Tris (pH 7.8), 4 mM $MnSO_4$, and 40 mM β-mercaptoethanol. After varying incubation times at 37 °C, 100 μL was spotted on DEAE paper disks and washed seven times with 7% Na_2HPO_4 and briefly twice with water. When kinetic studies were performed, smaller aliquots were taken and treated in the same manner.

The ability of the polymers to serve as messengers was determined as described by Gleason & Fraenkel-Conrat (1976).

Enzymatic Digestion of Single-Stranded and Transcribed Polymers. Poly([^{14}C]C,s^2C) in the single-stranded form and the double strand formed after transcription using [^{3}H]GTP (for details of the transcription assay see above) were digested with various enzymes either directly as the single strand or after isolation of the transcribed polymer by gel filtration on Biogel P-150. Digestion was in a 200-μL volume containing 0.06–0.12 A_{270} unit of polymer, 2 × SSC (0.03 M sodium citrate and 0.3 M sodium chloride), and 0.4 μg of ribonuclease

[1] Abbreviations used: s^2C, 2-thiocytidine; s^2CMP, s^2CDP, and s^2CTP, 2-thiocytidine 5′-(mono-, di-, tri)phosphate; 2′(3′)-s^2CMP, 2-thiocytidine 2′(3′)-monophosphate; poly(C,s^2C), polyribocytidylic acid containing 2-thiocytidine 5′-monophosphate randomly in any input ratio; poly(C,-s^2C)(2:1) indicates a 2:1 C-s^2C input ratio; other input ratios are similarly abbreviated; TLC, thin-layer chromatography; SSC, standard sodium citrate buffer; RNase, ribonuclease.

Table I: Evidence for Accurate Transcription of Poly(C,s^{2}C). Enzymatic Prepartion and Digestion of Poly([^{14}C]C,s^{2}C)·Poly([^{3}H]G)

polymer preparation			analysis of transcription products				
amt of starting material			digestion		^{14}C:^{3}H ratio		
CDP (μmol)	s^{2}CDP (μmol)	[^{14}C]CDP (μCi)	+RNase A, % ^{14}C	+RNase T$_1$ % ^{3}H	−RNase A	+RNase A	calcd[a]
3.31	0	0.3	1	0	1.01	1	1
1.65	0.36	0.2	26	5	0.97	0.72	0.82
1.32	0.72	0.2	75	7	2.6	0.65	0.64
1.00	1.07	0.2	76	5	2.5	0.59	0.48

[a] Calculated for the fully transcribed double strand poly(C,s^{2}C)·poly(G) on the basis of the ^{14}C content of the single strand.

A or 4 units of ribonuclease T$_1$. After 30-min incubation at 24 °C 100 μL was spotted on Whatman 3 MM paper disks. The ^{14}C–^{3}H ratio was determined in the trichloroacetic acid precipitable material. For kinetic investigations 25-μL volumes were spotted after various time intervals.

Results

Preparation and Analysis of Polyribonucleotides. Regardless of the amount of s^{2}CDP relative to CDP, the resulting polynucleotides had the same ratio of s^{2}C–C as the input ratio. No significant change in the 250:270 nm absorbance ratio in the polymer as compared to the remaining starting material was observed even at the earliest times of polymerization. The amount of s^{2}C in each polymer was also determined from the reaction with [2-^{14}C]iodoacetic acid and agreed with the s^{2}C–C input ratios.

When the amount of s^{2}CDP was increased, the rate of polymer formation was decreased (see Figure 1, Singer & Kröger, 1978). While only 8 h was necessary for almost complete polymerization of CDP alone or in the presence of up to 15% s^{2}CDP, 24 h was necessary in the presence of 15–50% s^{2}CDP, while 75% s^{2}CDP could only be polymerized to half that extent after 24 h.

End-group analysis was used to compare the molecular weights of polymers prepared after 17-h polymerization of [^{14}C]CDP in the presence of various amounts of s^{2}CDP. The average chain length was similar for polymers with 0–50% s^{2}CDP present but was slightly decreased with higher s^{2}CDP concentration, although still greater than 200 000 daltons. Nearest-neighbor analysis of poly(C,s^{2}C) containing [^{32}P]CMP showed the following distribution between 2′(3′)-[^{32}P]CMP and 2′(3′)-[^{32}P]s^{2}CMP: poly(C,s^{2}C)(3:1), 25 ± 8% 2′(3′)-s^{2}CMP, and poly(C,s^{2}C)(1:1), 60 ± 10% 2′(3′)-s^{2}CMP. This indicates that s^{2}C and C are randomly distributed in the polyribonucleotide.

Template and Messenger Activity. Using various s^{2}CMP-containing poly(cytidylic acids) prepared under the same set of conditions as templates for transcription by RNA polymerase, the following observations were made. Poly(cytidylic acid) and copolymers of C with up to 10% s^{2}C were transcribed equally well and incorporated [^{3}H]GMP in a complementary strand. Increasing the amount of s^{2}CMP in copolymers decreased the amount of coprecipitable poly([^{3}H]G), while the homopolymer, poly(s^{2}C), did not act as template in accordance with Rackwitz & Scheit (1977). Figure 1A shows the relationship between the amount of s^{2}C in poly(C) and the relative template activity after 2-h incubation.

Only GMP was incorporated into a complementary polynucleotide. When labeled ATP or UTP or CTP was present, in addition to unlabeled GTP, no detectable radioactivity was found to be incorporated.

Poly(C), poly(C,s^{2}C)(5:1), and poly(C,s^{2}C)(2:1) were used as messengers in the cell-free system, and all directed the incorporation of proline with similar efficiency (data not shown).

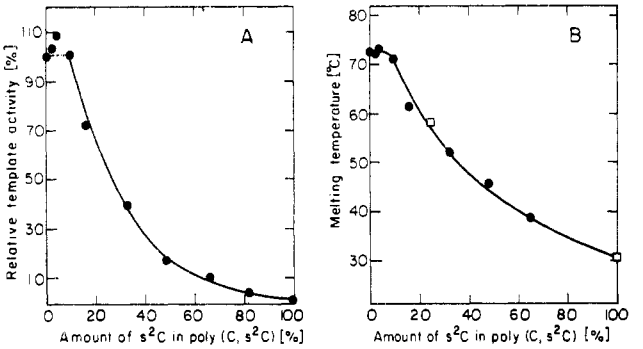

FIGURE 1: Effect of s^{2}C content in poly(C,s^{2}C) on template activity and melting temperature. (A) Relative template activity. The amount of [^{3}H]GMP incorporation into different poly(C,s^{2}C) is shown relative to the incorporation into poly(C). The incubation time was 2 h under the conditions given under Materials and Methods. (B) Melting temperature. The melting temperature (T_M) is determined as described under Materials and Methods using conditions where poly(C) forms a self-complementary double helix (Akinrimisi et al., 1963; Scheit & Faerber, 1971). (●) T_M determined in this study; (□) T_M value reported by Scheit & Faerber (1971) and by Faerber et al. (1972).

Proof for Base Pairing between s^{2}C and G. In order to establish whether double strands are really formed, [^{14}C]-CMP-containing copolymers were transcribed with [^{3}H]GTP in a preparative scale under the same conditions as used for analytical transcription. After isolation by gel filtration on Biogel P-150, aliquots were digested with two single-strand-specific nucleases. In all cases, whether starting with poly(C) or poly(C,s^{2}C) with C–s^{2}C ratios of 5:1, 2:1, and 1:1, polymers containing ^{14}C and ^{3}H radioactivity were obtained after ribonuclease A digestion. Due to the different transcription rates (as illustrated in Figures 1A and 2), the primary products contained some untranscribed single-stranded ^{14}C-labeled sections which were removed by RNase A, while the absolute amount of ^{3}H radioactivity was not affected by either ribonuclease A or T$_1$. This indicates that the poly(G) formed was firmly base-paired. After digestion with RNase A the ^{14}C:^{3}H ratio decreased with increasing amount of s^{2}C, showing that s^{2}C was present in the base-paired segment. For detailed results see Table I.

Kinetics of Transcription by RNA Polymerase. The transcription rate of copolymers was found to differ. Therefore poly(C) was compared with poly(C,s^{2}C)(2:1 and 5:1) for the absolute amount of [^{3}H]GMP incorporation at 2, 4, and 8 h. Representative data are shown in Figure 2, indicating considerable differences during the early periods of incubation.

Influence of Polymer Structure. The decreased rate of transcription of poly(C) containing increasing amounts of s^{2}C (compared to a poly(C) template) correlated with the progressive decrease in the melting temperature of these polymers determined at pH 4.0, near the pK_a of 2-thiocytidine (Figure 1B) (Scheit & Faerber, 1971; Faerber et al., 1972).

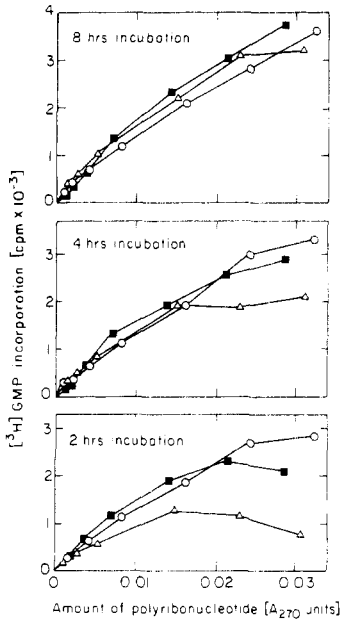

FIGURE 2: Influence of incubation time on the [^{3}H]GMP incorporation into three representative polynucleotides—(○) poly(C), (■) poly(C,s^{2}C)(5:1), and (△) poly(C,s^{2}C)(2:1). The [^{3}H]GMP incorporation is determined at several different template concentrations as described by Singer et al. (1978) and Ludlum & Wilhelm (1968) (see Materials and Methods). The lowest panel shows data after 2-h, the middle panel after 4-h, and the top panel after 8-h incubation.

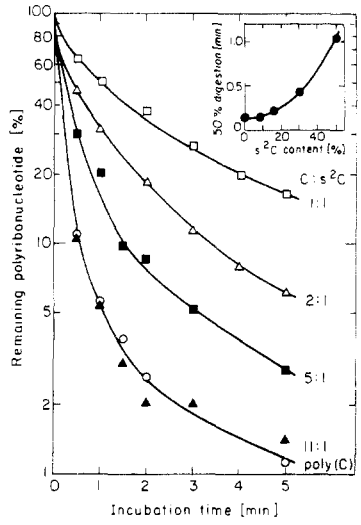

FIGURE 3: Effect of s^{2}C content on initial RNase A digestion of poly(C,s^{2}C). The reaction conditions are described under Materials and Methods. The inset shows the dependence of the 50% digestion time upon the s^{2}C concentration, determined from these data. The composition of each polymer is shown in the figure as C:s^{2}C ratio.

The initial rate of RNase A digestion of single-stranded polymers containing s^{2}C was decreased markedly (Figure 3). However, after 30 min, RNase A digestion was complete, even with poly(C,s^{2}C)(1:1).

Discussion

The purpose of this investigation was twofold: (1) to study base pairing when a small number of modified bases are in a polymer and (2) to attempt to clarify the apparent discrepancies regarding the ability of s^{2}C to base-pair with guanosine (Faerber, 1974; Vormbrock et al., 1974; Rackwitz & Scheit, 1977).

In contrast to the inability of homopolymers of s^{2}C to be transcribed (Rackwitz & Scheit, 1977), copolymers containing C and up to 10% s^{2}C are transcribed as well as poly(C), and our data show that the s^{2}C residues are base-paired with guanosine. When higher amounts of s^{2}C are present in copolymers, the rate of transcription decreases, but not the ability of s^{2}C to base-pair with guanosine. Concerning the influence of secondary structure, almost the same dependence of T_M upon the s^{2}C content was found as in the biological transcription experiment. Since the melting experiments were done under conditions favoring self-complementarity, we interpret these experimental facts as due to an increasing influence of disturbed base pairing between s^{2}C and C or s^{2}C, rather than to disturbed base stacking. It may be possible that only the process of destacking is observed in the remarkably sharp melting profile of poly(s^{2}C) (Scheit & Faerber, 1971). It appears that in both transcription and secondary structure poly(C) is able to tolerate a certain amount of s^{2}C without changing its properties. All evidence is that s^{2}C is incorporated randomly into copolymers, and we are not observing an effect due to clustering.

To explain the seemingly decreased transcription with increasing s^{2}C content, we tested whether this inhibition was due to the influence of a lower transcription velocity or whether only cytidine nucleosides were transcribed, thus looping out the areas of s^{2}C, as it has been suggested for other systems (Szer & Shugar, 1961; Ikehara & Hattori, 1971; Engel & von Hippel, 1978). The data in Figure 2 clearly showed that only the rate of transcription was affected by the presence of s^{2}C in the template. In addition the effect of s^{2}C on transcription rate was least at lower template concentrations, indicating a critical role of the enzyme capacity rather than the template concentration. To exclude the formation of untranscribed areas (looping out) we studied the ratio between template and the newly synthesized poly(G) strand using a ^{14}C-labeled poly(C,s^{2}C) and [^{3}H]GMP incorporation. Given the same template concentrations (constant number of pyrimidines), a constant amount of [^{3}H]GMP should be incorporated, if 2-thiocytidine were actually transcribed. Thus the ratio between ^{14}C and ^{3}H should decrease with increasing s^{2}C content. As shown in Table I the untranscribed part of the pyrimidine strand was digested by RNase A, while the absolute amount of [^{3}H]GMP was not decreased by RNase T_1, indicating that the formed base pair s^{2}C·G was stable under our conditions. The resulting double-stranded poly(C,s^{2}C)·poly(G) complex gave the expected change in the ^{14}C–^{3}H ratio, again indicating a firm base pair between s^{2}C and G. The unaltered base-pairing capability of s^{2}C was also supported by finding no misincorporation of AMP, CMP, or UMP, nor interference with polyproline formation with the wheat germ translation system.

To reconcile our data and the in part very contradictory data from other laboratories we present the following hypothesis. The s^{2}C·G base-pair geometry must include NH–S as well as NH–O interaction (Donohue, 1969). If the two NH–O hydrogen bonds are in a plane with the heterocyclic ring systems of the bases, the NH–S bonding can be expected to be out of this plane as illustrated in Figure 4. For this interaction only a rotation of the 2-NH$_2$ group of G is necessary. The velocity of base pairing is thus related to the existence of this particular conformation (Engel & von Hippel, 1978). When the number of conformational changes becomes too great, as in poly(s^{2}C), transcription can no longer be observed.

The hypothesis would also explain the high T_M of poly(G,s^{2}C) (Faerber, 1974) and the double-strand formation between poly(s^{2}C) and poly(I) (Faerber et al., 1972). In the first case stacking and base pairing is possible, and in the second case the base pairing is not disturbed by the presence of the sulfur. Sulfur also does not interfere with double-strand

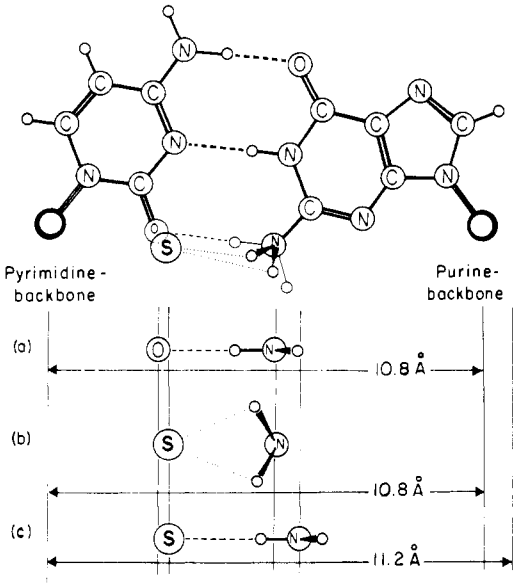

FIGURE 4: Stereochemistry of s^2C·G base pair. The upper part shows the s^2C·G base pair superimposed over the normal C·G base pair. The lower part shows the atoms involved in the altered hydrogen bond if the view is along the plane perpendicular to the helix axis. (a) Normal C·G base pair; (b) s^2C·G base pair with the sulfur located according to the elongated C(2)·S bond (Lin et al., 1971) and the nitrogen of the 2-NH_2 group of G in the identical position. The required distance between sulfur and hydrogen atoms is shown after rotation of the 2-NH_2 group, while neither the position of the N-glycosidic bond nor the other two hydrogen bonds are disturbed; (c) s^2C·G base pair according to Rackwitz & Scheit (1977) with the nitrogen of the 2-NH_2 group of G at the correct distance for S–NH_2 hydrogen bonding. This changes the position of the N-glycosidic bond, and the two other hydrogen bonds are therefore distorted.

formation between poly(s^6G) and poly(C) (Amarnath & Broom, 1976). The inability of s^2CTP to act as substrate for RNA polymerase if calf thymus DNA or T7 DNA is used as template (Rackwitz & Scheit, 1977) may be due to the influence of the strand neighbors of G. The 2-NH_2 group of polymerized G may have here a decreased rotational freedom if a purine is the neighbor, while in alternating poly(G,C) a pyrimidine is always the neighbor.

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