Fluorescence Studies of Nucleotides and Polynucleotides

II. 7 DEAZANEBUtARIN: CODING AMBIGUITY IN TRANSCRIPTION WITH BASE PAIRS CONTAINING FEWER THAN TWO HYDROGEN BONDS*

(Received for publication, June 7, 1971)

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SUMMARY

Deazanebularin, a cytotoxic nucleoside, is fluorescent at ambient temperature and at neutral pH. Some of its fluorescence properties and those of its phosphorylated derivatives are reported here. Deazanebularin nucleotides are substrates for Escherichia coli polynucleotide phosphorylase and RNA polymerase. With RNA polymerase deazanebularin triphosphate acts as an ambiguous substrate in that it functions, with almost equal efficiency, as an analogue of either ATP or GTP. The relevance of this observation to the mechanism of base selection by RNA polymerase is discussed. The synthesis, characterization, and properties of polymers containing deazanebularin are also described. Some of these polymers form unique ordered structures with base pairs possessing only a single hydrogen bond; these permit an assessment of the contribution of hydrogen bonding to helix stability. These polymers also exhibit some unusual fluorescence properties, including excited state energy transfer.

In a preceding communication (1) we have described the fluorescence properties of three purine nucleoside analogues, and of their nucleotide and their polynucleotide derivatives. These compounds were shown to be fluorescent at physiological conditions of temperature, solvent, and pH, and some applications of the fluorescence properties to the study of conformational transitions in polynucleotides were presented.

In this paper we outline the characteristics of a fourth fluorescent purine ribonucleoside analogue, 7-deazanebularin (DN)

* This work was supported in part by Research Grant CA 08290-04 from the National Institutes of Health. Paper I in this series is Reference 1.

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‡ The abbreviations used are: DN, 7-deazanebularin, DNMP, DNDP, DNTP, the mono-, di-, and triphosphate of 7-deazanebularin; TP, the triphosphate of pseudouridine, poly(rDN), poly(rBrU), poly(dC), poly(dT), the homopolymers of deazanebularin, 5-bromouridine, 2'-deoxyctydine, and 2'-deoxythymidine, respectively; poly[r(A-U)], poly[r(DN-U)], and poly[r(5FU-U)], perfectly alternating double stranded ribo- and uridylate, deazanebularin and uridine, and 2,6-diaminopurine and uridine, respectively; poly[r(DN,A)], poly[r(DN,G)], random ribo- and uridylate of deazanebularin and adenine and of deazanebularin and guanine, respectively, poly[d(A-T)], an alternating deoxyribo- and uridylate of deoxyadenosine and 2'-deoxythymidine; poly[r(2AP-U)], alternating copolymer of 2-amino- and uracil; poly[r(F-U)], alternating copolymer of formycin and uracil.
is described by Gerster et al. (2).

The synthesis of this nucleoside is 7-deazanebularin (pyrrolo(4,3-d)-pyrimidine-3-β-n ribofuranoside). The synthesis of this nucleoside is described by Gerster et al. (2).

\[ \text{FIN. 2. Structure of the 7-deazapurine-uracil base pair.} \]

\[ \text{Fig. 1. Structure of 7-deazanebularin (pyrrolo(4,3-d)-pyrimidine-3-β-n ribofuranoside). The synthesis of this nucleoside is described by Gerster et al. (2).} \]

\[ \text{Fig. 2. Structure of the 7-deazapurine-uracil base pair.} \]

The 5'-monophosphates of deazanebularin and the pyrimidine nucleosides were prepared by the POCl₃ method previously described (3). [α-²³²P]Nucleotides were prepared by the same procedure with [³²P]phosphorous oxychloride obtained from New England Nuclear Corp. The corresponding di- and triphosphates were chemically synthesized by the methods of Michelson (4) and Smith and Khorana (5). Commercially available nonradioactive nucleotides were purchased from P-L Biochemicals, Freehold, N. J. Ribonuclease T₁ was the generous gift of Dr. Herbert Sober.

Escherichia coli B RNA polymerase was prepared and assayed according to the method of Chamberlin and Berg (6). The enzyme fraction eluted from DEAE-cellulose was used throughout; the specific activity of different preparations was in the range 2000 to 6000 units per mg of protein. Polynucleotide phosphorylase was isolated from E. coli B as previously described (3).

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Bovine pancreatic RNase, bacterial alkaline phosphatase, venom phosphodiesterase, spleen phosphodiesterase, micrococcal nuclease, and pancreatic DNase were purchased from Worthington Biochemicals, Freehold, N. J. Ribonuclease T₁ was the generous gift of Dr. Herbert Sober.

Routine uncorrected fluorescence analyses were done at a temperature of 20 ± 0.5°C on a Hitachi MFP-2A recording spectrofluorimeter. Corrected excitation spectra were obtained on the spectrofluorimeter described by Stryer (7). We thank Dr. L. Stryer for the use of this instrument. Quantum yield determinations, fluorimetric thermal denaturation profiles, and pH titrations were performed as previously described (1).

Optical rotatory dispersion spectra were obtained on a Cary 60 spectropolarimeter in a thermostated, water-jacketed cell with a 1-cm light path. The observed rotations, after correction for solvent blanks, are expressed in terms of the molar rotation (°) as described by T'so et al. (8).

The conditions used for synthesizing the various alternating ribocopolymers were as follows. Each 1-ml reaction contained Tris-HCl buffer, pH 7.9, 40 mM; MgCl₂, 1.0 mM; MgCl₂, 4.0 mM; 2-mercaptoethanol, 3.0 mM; poly[d(A-T)], 0.04 mM; 0.4 mM [³²P]ATP or DNTP (³²P) or [³²P]GTP at specific activities of 10³ to 10⁶ cpm per pmole; and one of the following nucleoside triphosphates at 0.4 mM: UTP, BUTP, rTTP, or ΨTP; RNA polymerase, 5 to 15 units. The reactions were incubated at 37°C and the extent of synthesis determined by measuring the acid-precipitable radioactivity in 50-μl aliquots on Millipore filters (9).

For the large scale synthesis of poly[³²P]-poly(DN-U)] the reaction conditions were modified as follows. The reaction mixtures (20 ml) contained only 0.2 mM triphosphates and they were incubated at 10°C. An alternative procedure for the synthesis of poly[³²P]-poly(DN-U)] was to incubate the reactions at 15°C in the presence of 2 to 4 × 10⁴ x 10⁴ cpm per pmole. Aliquots were removed at approximately 8-hour intervals until the rate of polymerization started to plateau (48 to 72 hours). Purified pancreatic DNase (10), 25 μg, was then added and the incubation continued for a further hour. The reaction mixture was deproteinized by shaking with an equal volume of redistilled phenol. The aqueous layer was concentrated by vacuum dialysis to approximately 3 ml and the high molecular weight polymer fraction isolated by gel filtration on a column (20 × 2 cm) of Sephadex G-100 with 0.05 M Tris-HCl, pH 7.9, as the eluting buffer. The material which emerged at the void volume was used for subsequent experiments.

The homopolymers of deazanebularin, uridine, adenosine, and 5-bromouridine were synthesized with E. coli polynucleotide phosphorylase using the following reaction conditions: Tris-HCl buffer, pH 8.3, 0.1 M; MgCl₂, 0.25 M; EDTA, pH 8.3, 3 mM; the appropriate nucleoside diphosphate, 0.05 mM; and approximately 1 to 2 units of enzyme per ml. The synthesis of nonradioactive polymers was followed by measuring the release of inorganic phosphate colorimetrically (11). Radioactive polymer synthesis was monitored by acid precipitation on Millipore filters. Radioactive random copolymers of deazanebularin and guanosine or deazanebularin and adenine were prepared as above. Although the nucleoside diphosphate ratios were varied the total nucleotide concentration was maintained at 0.05 M. The base composition of these copolymers was determined directly by double labeling techniques with [³²P]GDP and [³²P]ADP or [³²P]GDP (specific activities of 2 to 5 × 10⁴ cpm per pmole were used routinely).

Enzymatic degradation of the synthetic polymers was monitored at 25°C with one or more of the following procedures: (a) measuring the decrease in acid precipitability of radioactive polymers; (b) determining the increase in fluorescence intensity and changes in energy transfer efficiency upon digestion; and (c) measuring the increase in ultraviolet absorption. The reactions (0.25 to 1.0 ml) were incubated (with and without 0.02 M MgCl₂ in the following buffers: for RNase T₁ and pancreatic RNase, 0.1 M sodium acetate buffer, pH 5.5; for spleen phosphodiesterase, 0.1 M Tris-HCl buffer, pH 7.9; for venom phosphodiesterase, 0.1 M Tris-HCl buffer, pH 7.0. The enzyme and polymer concentrations used in any given experiment are shown under “Results.”

**RESULTS**

**Fluorescence Properties of DN and Phosphorylated Derivatives.**

**Absorption, Excitation, and Emission Spectra: Effects of pH**

The absorption, excitation, and emission spectra of DN in water are illustrated in Fig. 3. The absorption and excitation
spectra are practically identical; comparable superposition of these spectra was found for the corresponding 5′-mono-, di-, and triphosphates. The spectral identity and the homogeneous chromatographic behavior of these compounds suggest that they are free from fluorescent impurities. Although the main absorption profile of DN, with λ\text{max} at 270 nm, is similar to that of normal nucleic acid constituents, its spectra are noteworthy in two respects. First, the value of its molar extinction coefficient is extremely low, 3800;\textsuperscript{3} in addition there is an unusually broad tailing of the absorption spectrum toward longer wave lengths in acidic solution (Fig. 4). The wave length of maximum absorption of DN (270 nm) is closer to that of the normal nucleotides than was found for the other fluorescent purine analogues we have studied. However, selective excitation of the fluorophore in the presence of a large excess of normal bases can still be achieved by exciting at longer wave lengths (up to 300 nm); in this case, owing to the lower extinction coefficient, the fluorescence intensity is appreciably reduced. In contrast, the emission maximum occurs at a longer wave length (400 nm) than observed with the other analogues.

The effect of pH on the fluorescence intensity of deazanebularin is shown in Fig. 5. This fluorimetric pH titration yields excited state pK values of 4.2 and 11.8. The pK for protonation in the ground state, obtained by spectrophotometric titration is 4.3.

Quantum Yield Values—The quantum yields of DN and of its 5′-phosphorylated derivatives were determined according to the method of Parker and Rees (12) and are given in Table I. The quantum yield of DN itself (0.08) is intermediate between those of formycin and 2-aminopurine, and is in a useful range for fluorescence studies of oligo- and polynucleotides. Introduction of one or more phosphoryl groups at the 5′-position of DN does not alter the absorption spectrum but does produce minor

\textsuperscript{3} The extinction coefficient of deazanebularin reported in the paper of Gerster et al. (2) was misprinted.
TABLE I
Quantum yields of 7-deazanebularin and its 5'-nucleotides at 25° in 0.01 M sodium phosphate buffer, pH 7.0

| Compound      | Quantum yield |
|---------------|---------------|
| Deazanebularin| 0.080         |
| DNMP          | 0.070         |
| DNDP          | 0.073         |
| DNTP          | 0.076         |

Quantum yields of phosphorylated derivatives are determined by comparing their fluorescence to that of the nucleoside before and after treatment with bacterial alkaline phosphatase.

![Graph showing the effect of NaCl and MnCl₂ on fluorescence intensity of 7-deazanebularin.](image)

**FIG. 6.** The effect of NaCl (○) and MnCl₂ (□) on the fluorescence intensity of 7-deazanebularin. The solvent is 0.01 M Tris-HCl buffer, pH 7.9. Nucleoside concentration, excitation, and emission wave lengths were as stated for Fig. 5.

TABLE II
Effect of solvent variation on fluorescence intensity of deazanebularin

The nucleoside concentration used was 1.2 × 10⁻⁴ M; excitation and emission spectra were monitored at 280 and 400 nm, respectively.

| Solvent mixture | Relative fluorescence intensity | Solvent mixture | Relative fluorescence intensity |
|-----------------|--------------------------------|-----------------|--------------------------------|
| Water           | 100                            | Water           | 100                            |
| Ethanol 80      | 135                            | Propylene glycol| 100                            |
| Ethanol 60      | 180                            |                 | 205                            |
| 20% 40% 60%     |                                |                 |                                |
| 20% 80% 100%    |                                |                 |                                |
| 100%            |                                |                 |                                |

changes in quantum yield. These changes are qualitatively similar to those which were observed with the formycin, 2-amino-purine, and 2,6-diaminopurine nucleotides (1).

The fluorescence intensity of deazanebularin compounds decreases as a function of increasing ionic strength (Fig. 6). Salts such as sodium phosphate, Tris, KCl, MgCl₂, and sodium citrate quench to the same degree as NaCl. The effect of ionic strength on the fluorescence of previously studied nucleosides (1) was less pronounced.

**Excited State Lifetime**—The value for the excited state lifetime of DN in water was determined by Dr. L. Stryer and was found to be 6.0 nsec.

**Effect of Temperature**—The fluorescence intensity of DN in water decreases with increasing temperature (Fig. 7), but there is no change in the absorption or emission spectra. The absorbance is minimally affected by heating, showing that the decrease in fluorescence reflects changes in quantum yield. These effects of changing temperature are fully reversible, and apply also to the 5'-phosphorylated derivatives of DN.

**Effect of Solvent**—Examination of solvent effects on the fluorescence of DN were restricted by the limited solubility of the nucleoside and its derivatives in nonaqueous media. Studies of DN fluorescence in alcohol-water mixtures gave the following results (Table II). Increasing concentration of ethanol (or methanol) and propylene glycol cause progressive and small increases in quantum yield, without detectable changes in the excitation spectrum. In absolute ethanol, or methanol, there was a decrease in fluorescence intensity, whereas in propylene glycol there was a 4-fold increase. Under these anhydrous conditions the emission spectrum was shifted by approximately 10 nm toward the blue.

In summary, the fluorescence properties of DN are qualitatively very similar to those of the formycin, 2-amino-purine ribose, and 2,6-diaminopurine ribose. The quantum yield of fluorescence of DN (0.08) and the excited state lifetime (6 nsec) are in a range which is convenient for several types of fluorescence studies, including polarization. In its response to changes in solvent, temperature, pH, and ionic strength, the fluorescence of DN recalls that of the pyrimidine ribonucleoside analogues previously described.

**Interaction of DN Nucleotides with Polymerizing Enzymes**

**RNA Polymerase**—The utilization of DNTP by E. coli RNA polymerase was studied under a variety of conditions and with...
several templates. DNMP is incorporated into RNA with both natural and synthetic DNA templates, and with native as well as denatured DNA (Tables III to VI). The data in Table III show that DNTP can substitute for either ATP or GTP (but not for CTP or UTP) in reactions directed by RNA polymerase. It is of interest that DNTP replaces ATP and GTP (but not both) with almost equal effectiveness in reactions requiring four nucleotides (Table III). However, the total amount of RNA product which can be formed in this way is only a small fraction of that produced with the normal substrates (1 to 4%) although the reaction is linear for up to 45 min. The polymerizations were performed with both radioactive and non-radioactive DNTP, and the results of these and of the appropriate control experiments show clearly that DNTP is incorporated into RNA by RNA polymerase and that its coding behavior with respect to the DNA template is ambiguous. In reactions directed by natural DNAs, the requirement for three additional nucleoside triphosphates, and the sensitivity to actinomycin all indicate that the incorporation of DNMP into RNA is mediated by the normal catalytic function of RNA polymerase, and not by some contaminating enzymatic activity.

The results of a more quantitative test of the ability of DNTP to substitute for either ATP or GTP are illustrated in Fig. 8. In these experiments native calf thymus DNA was the template, and DNTP replaced, respectively, ATP (Curves A and B) or GTP (Curves C and D). The progress of RNA synthesis was monitored by following the incorporation of two radioactive nucleotides, namely [3H]UTP and [3H]CTP. When DNTP is substituting for ATP, somewhat more DNMP is incorporated into product than CMP. In fact, the ratio DNMP:AMP in the newly formed RNA is 1.27, which is exactly equal to the ratio dTMP:dGMP in the template. The RNA product therefore faithfully reflects the complementary base composition of the template, just as it does when all four normal triphosphate substrates are used, and DNMP is being polymerized at precisely

| TABLE III |
| DNTP replaces ATP and GTP in RNA polymerase reactions |

The reactions (0.25 ml) were incubated under standard conditions (6) for 15 min. Calf thymus DNA concentration, 0.15 mM. Specific activities (counts per min per pmole): [3H]UTP, 1.1 × 10⁶; [3H]CTP (used in Experiments 1, 6, 7, 8, and 10) 4.3 × 10⁵; [3H]ATP (Experiment 9), 5.5 × 10⁵.

| Experiment No. | Triphosphate substrates | Radioactive NMP polymerized |
|---------------|--------------------------|----------------------------|
|               |                          | nmoles/ml                  |
| 1             | Complete: ATP, GTP, CTP, [3H]UTP | 0.54                       |
| 2             | ATP + [3H]DNTP           | 0.40                       |
| 3             | GTP + [3H]DNTP           | 0.21                       |
| 4             | CTP + [3H]DNTP           | <0.01                      |
| 5             | UTP + [3H]DNTP           | <0.01                      |
| 6             | ATP                       | <0.01                      |
| 7             | GTP                       | <0.01                      |
| 8             | CTP                       | 0.01                       |
| 9             | UTP                       | 0.01                       |
| 10            | Complete: + actinomycin (5 μg per ml) | 0.05                       |
| 11            | ATP + [3H]DNTP + actinomycin | 0.02                      |
| 12            | GTP + [3H]DNTP + actinomycin | 0.02                      |

| TABLE IV |
| Incorporation of DNMP as analogue of AMP in RNA polymerase-catalyzed syntheses of poly(A) using heat-denatured calf thymus DNA template |

The reactions (1.0 ml) were incubated under standard conditions (6) and 200-μl aliquots removed and analyzed at the indicated times. DNA concentration, 0.13 mM. Specific activities, (counts per min per pmole), [32P]DNTP, 7.4 × 10⁵; [3H]ATP, 4.9 × 10⁵.

| Experiment No. | NTP added | Substrate concentration | Nucleotide polymerized |
|---------------|-----------|--------------------------|------------------------|
|               |           |                          | 10 min                 |
|               |           |                          | DNMP       | AMP       | DNMP       | AMP       | DNMP       | AMP       |
|               |           |                          | nmoles/ml | nmoles/ml | nmoles/ml | nmoles/ml | nmoles/ml | nmoles/ml |
| 1             | [32P]DNTP | 645                      | <0.005    | 12.2      | <0.005    | 32.3      | <0.005    | 66.5      |
| 2             | [3H]ATP   | 400                      |           |           |           |           |           |           |
| 3             | [32P]DNTP | 160                      | 0.28      | 5.15      | 0.79      | 14.7      | 1.71      | 32.4      | 20:1      |
| 4             | [32P]DNTP | 320                      | 0.21      | 0.82      | 0.58      | 2.05      | 1.14      | 4.80      | 4:1       |

| TABLE V |
| Incorporation of DNMP as analogue of AMP in RNA polymerase reactions with poly(dT) template |

Standard reaction conditions (6); 1.0-ml reaction. Poly(dT) concentration, 0.09 M. Specific activities (counts per min per pmole); [32P]DNTP, 1.7 × 10⁶; [3H]ATP, 5.3 × 10⁵.

| Experiment No. | NTP added | Substrate concentration | Nucleotide polymerized |
|---------------|-----------|--------------------------|------------------------|
|               |           |                          | Product NMP ratio A:DN |
|               |           |                          | DNMP | AMP | DNMP | AMP | DNMP | AMP | DNMP | AMP |
|               |           |                          | nmoles/ml | nmoles/ml | nmoles/ml | nmoles/ml | nmoles/ml | nmoles/ml | nmoles/ml | nmoles/ml |
| 1             | [32P]DNTP | 420                      | <0.005    | 278      |           |           |           |           |           |           |
| 2             | [3H]ATP   | 400                      |           |           | 4.02      | 114       | 35:1      |           |           |           |
| 3             | [32P]DNTP | 180                      | 4.02      | 114       | 35:1      |           |           |           |           |           |
| 4             | [3H]ATP   | 80                       | 4.02      | 114       | 35:1      |           |           |           |           |           |
Incorporation of DNMP as analogue of GMP in RNA polymerase reactions with poly(dC) template

Reactions (1.0 ml) were incubated under standard conditions (6) and 200 µl aliquots removed and analyzed at the indicated times. Poly(dC) concentration 0.05 mM. Specific activities (counts per min per µmole): [32P]DNTP, 5.6 × 10^6; [3H]GTP, 5.3 × 10^6.

| Experiment No. | NTP added | Substrate concentration | Nucleotide polymerized |
|----------------|-----------|-------------------------|------------------------|
|                | [3P]DNTP | µmoles/ml               | DNMP GMP DNMP GMP DNMP GMP |
| 1              | 590       | <0.005                  | 18.0 33.2 38.4 |
| 2              | 400       | 0.36                    | 9.6 17.5 23.4 30:1 |
| 3              | 472       | 0.41                    | 16.1 35.7 38.6 56:1 |
| 4              | 293       | 0.29                    | 17.1 29.8 36.0 100:1 |
| 5              | 117       | 0.30                    | 30.8 36.0 100:1 |

DNMP is not utilized by RNA polymerase in the absence of a normal substrate. This is seen in the homopolymer reactions directed by denatured DNA (Table IV), poly(dT) (Table V), poly(dC) (Table VI), poly(rC) and poly(rU). In all of these cases, no detectable acid-precipitable product is formed in the presence of DNMP alone; no experiments were performed to test for the formation of short oligonucleotides in these reactions. However, in poly(dT)- and poly(dC)-directed reactions which contain mixtures of ATP and DNTP or GTP and DNTP, respectively, DNAIP is incorporated (Tables V and VI) the amount incorporated depends on the input nucleotide ratio. These homopolymer reactions thus conclusively show the ambiguity of DN in its ability to replace either ATP or GTP. The data in Tables IV to VI also indicate that DNTP inhibits the polymerization of AMP more than that of GMP.

Because the amount of synthesis in reactions with calf thymus DNA as template was small, no determination of K_m was carried out. A K_m value was established from reactions directed

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Because the amount of synthesis in reactions with calf thymus DNA as template was small, no determination of K_m was carried out. A K_m value was established from reactions directed
by poly[d(A-T)] (Fig. 9). With this template, relatively substantial and prolonged synthesis occurs when DNTP serves as the only "purine" nucleotide substrate. The $K_m$ value found for DNTP ($1.0 \times 10^{-4}$ M) does not differ markedly from that of ATP ($6.6 \times 10^{-5}$ M) with the same template. No $K_m$ value has been determined for DNTPi when it is acting as an analogue of GTP.

The synthesis with poly[d(A-T)] template of alternating copolymers containing DN and a pyrimidine was studied in some detail. Analysis of the RNA product using radioactive double labeling techniques reveal that DN and uridine are incorporated at equal rates as is required by the base composition of the template. In addition, alkaline hydrolysis of the product synthesized with $\alpha$-32P-labeled DNTP (and nonradioactive UTP) as substrate show quantitative transfer of $[^32P]$ to UMP (Table VII). The reciprocal experiment using $[^32P]$UTP shows quantitative label transfer to DNMP. These facts show that the copolymer contains a perfectly alternating sequence of DN and uridine residues. In this case, DN is replacing adenosine during the synthesis and in the structure of the product. Similar results were obtained from the analysis of poly[r(DN-P)] (Table VII).

With poly[d(A-T)] template the incorporation of DNMP in the presence of $\alpha$ is very poor; even with an input ratio DNTP:ATP = 10 the incorporation ratio is at best $\Delta$DNMP:AMP = 0.05. Thus the DN content of alternating copolymers containing both DN and adenosine has been confined to the range of 0.1 to 5%.

Although the RNA product faithfully reflects the template composition and sequence the kinetics of synthesis are unusual in several respects.

1. At $37^\circ$, the polymerization of UMP and DNMP proceeds at a reasonable rate for about 1 hour. During the initial phase of the reaction the rate is approximately one-tenth that achieved with AMP and UMP under identical conditions. The reaction then stops abruptly although only a small fraction of the input nucleotide has been polymerized. In contrast, the synthesis of poly[r(A-U)] continues at a rapid rate for extended periods (up to 12 hours) until more than 50% of the substrate has been utilized.

2. The pattern of synthesis at $37^\circ$ was the same when the 5'-triphosphates of 5-fluorouridine, 5-methyluridine, or 5-bromo-uridine were used to replace UTP. However, with $\gamma$TP as the pyrimidine substrate polymerization proceeded at the initial rate for prolonged periods (Fig. 10).

3. The arrest in synthesis at $37^\circ$, which occurred typically

### TABLE VII

Nearest neighbor analysis of alternating copolymers synthesized using poly[d(A-T)] template

Reactions (5.0 ml) were incubated at $37^\circ$ under standard conditions (see "Materials and Methods") for 60 min. The reactions were terminated, processed, and analyzed by electrophoresis as previously described (3). The electrophoretic mobility of DNMP is like that of AMP and therefore easily resolved from UMP. Specific activities (counts per min per umole) were: $[^32P]$DNTP, $6.1 \times 10^6$; $[^32P]$UTP $1.3 \times 10^5$; $[^32P]$TP $3.0 \times 10^5$.

| Substrates | Radioactivity in isolated mononucleotides |
|------------|------------------------------------------|
|            | $[^32P]$-DNMP | $[^32P]$-UMP | $[^32P]$-UTP |
| $[^32P]$DNTP + UTP | 9,372 | 3,940 |
| DNTP + $[^32P]$UTP | 10 | 12,052 |
| $[^32P]$DNTP + $[^32P]$UTP | 63,210 | 21 |

Fig. 9. The effect of DNTP concentration on the rate of synthesis in RNA polymerase reactions with poly[d(A-T)] as template. The reactions (0.25 ml) were incubated for 30 min at $37^\circ$ as described under "Materials and Methods." $[^32P]$DNTP specific activity, $5.8 \times 10^6$ cpm per umole; poly[d(A-T)] concentration, 0.08 M. The $K_a$ plot (inset) yields a value of $1.0 \times 10^{-4}$ M.

Fig. 10. The kinetics of synthesis of alternating ribonucleotides containing deazanebularin and one of the following pyrimidines: uridine (■), ribothymidine (○), bromouridine (△), and pseudo uridine (●). The reaction conditions are as described under "Materials and Methods" for the synthesis of poly[r(DN-U)] at $37^\circ$. $[^32P]$DNTP specific activity was $7.3 \times 10^5$ cpm per umole.
Analysis of the experimental variable—no explanation was found spermine could be established only by performing a titration variable in different experiments; the effective concentration of a very narrow range of concentrations—which was found with pyrimidines. The effect of spermine was seen only in the absence (O) and presence (X) of 1.0 X 10\(^{-4}\) M spermine. Synthesis of poly[r(DN-U)] in the presence (O) of 1.0 X 10\(^{-4}\) M spermine. Synthesis of poly[r(DN-U)] was determined as described under “Materials and Methods.”

Specific activities (counts per min per pmole): \([^3H]ADP, 9.3 \times 10^6; [3H]ATP, 4.2 \times 10^6\].

| Substrate | Polymer synthesized |
|-----------|---------------------|
|           | 0.5 hr | 1.0 hr | 2.0 hr |
| [3H]ADP  | 647    | 1210   | 2306   |
| [3H]DNDP | 181    | 350    | 680    |

After approximately 60 min could be eliminated by the addition of spermine (Fig. 11). In the presence of the polyamine the kinetics of synthesis with all pyrimidines resembled that which was found with ATP. The concentration of spermine required to give maximal stimulation was variable from experiment to experiment (see text), and outside this narrow concentration range (2- to 3-fold) spermine was either without effect or inhibited synthesis. The standard (37°C) reaction conditions were used (see “Materials and Methods”). Specific activities (counts per min per pmole); \([^3H]ADP, 9.3 \times 10^6; [3H]ATP, 4.2 \times 10^6\].

Properties of Polymers Containing Deazaebularin

Alternating Copolymer of Deazaebularin and Uridine:poly[r(DN-U)]—Poly[r(DN-U)] was isolated from a large scale incubation as described under “Materials and Methods.” The minimum molecular weight, judged by gel filtration and end group analysis, was in excess of 75,000. As isolated (in 0.05 M Tris-HCl buffer, pH 7.9) poly[r(DN-U)] possesses none of the spectral characteristics of multistrandedness. No sharp or reversible optical transitions are observed on heating to 70°C; however, a variable and substantial number of chain breaks are introduced during the heating cycle. These are manifest as slight irreversible increments in fluorescence and absorption, and as corresponding decreases in acid precipitability of the polymer.

Since poly[r(DN-U)] is produced under the direction of a poly[d(A-T)] template, it appeared likely that some sort of base pairing should occur between DN and a complementary pyrimidine. The fluorescence and ultraviolet absorption of the polymer were used to monitor possible physical transitions which might point to the formation of base pairs and structural order. The presence of Mg\(^{2+}\), even in low concentrations (2 X 10\(^{-4}\) M) gives rise to all the spectral characteristics associated with base pairing and double strandedness. Thus, addition of MgCl\(_2\) instantly produces a significant (25%) and reproducible degree of hypochromism at 260 nm. This can be seen in the spectra which are presented in Fig. 12. When the polymer is denatured (45°C) the ultraviolet absorption profile is the same as that which is obtained at lower temperatures in the absence of stabilizing amounts of Mg\(^{2+}\).

Heating the polymer solution in the presence of Mg\(^{2+}\) produces a characteristic melting curve (Fig. 13). This optical transition is sharp, cooperative, and reversible. In addition, it occurs over a narrow range of temperatures (5-8°C), just as in the case of the double stranded poly[r(A-U)].

The midpoint of the thermal transition (T\(_m\)) is a function of the Mg\(^{2+}\) concentration (Fig. 14). Similar T\(_m\) profiles can be observed with the addition of NaCl or KCl, but the concentra-
FIG. 12. Absorption spectra of poly[r(DN-U)] in 0.02 M Tris-HCl buffer, pH 7.9, plus 0.10 M MgCl₂ at 25° and 45°. The extinction coefficient per mole of phosphate (Eₚ) of native poly[r(DN-U)] at λ_max (265 nm) is 5,600; the Eₚ of denatured poly[r(DN-U)] (λ_max of 291 nm) is 7,000.

FIG. 13. Thermal denaturation profile of poly[r(DN-U)] in 0.02 M Tris-HCl buffer, pH 7.9, plus 0.10 M MgCl₂. The Tₘ values are plotted as a function of the negative logarithm of the magnesium ion concentration. The solvent was 0.02 M Tris-HCl buffer, pH 7.9.

FIG. 14. Effects of MgCl₂ concentration on the melting temperature (Tₘ) of poly[r(DN-U)]. The Tₘ values are plotted as a function of the negative logarithm of the magnesium ion concentration. The solvent was 0.02 M Tris-HCl buffer, pH 7.9.

FIG. 15. The uncorrected excitation and emission spectra of poly[r(DN-U)]. Excitation (——) and emission (-----) spectra at 22°. Excitation (——) and emission (-----) spectra at 45°. Solvent: 0.01 M Tris-HCl buffer, pH 7.9, plus 0.02 M MgCl₂. The spectra obtained at 45° are identical in profile with those of poly[r(E-U)] at 22° in the absence of MgCl₂. Polymer concentration: 5.0 × 10⁻⁵ M. The spectra were taken on a Hitachi MPF-2A spectrophotofluorimeter.

Additional evidence for the double stranded structure of poly[r(DN-U)] is as follows.

1. The fluorescence excitation spectra differ qualitatively above and below the region of thermal transition. Above the Tₘ the excitation spectrum corresponds to that of the DNMP monomer, whereas below the Tₘ the excitation spectrum is quite different and provides evidence for energy transfer from uracil to the DN residues (Fig. 15).

2. The kinetics of nuclease digestion of poly[r(DN-U)], seen in Fig. 16 and Table IX, shows that the addition of Mg²⁺ slows the rate of degradation of the polymer by the enzymes tested, all of which have a preference for single stranded structures.

3. The optical rotatory dispersion spectra of poly[r(DN-U)]...
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FIG. 10. Effect of MgCl₂ on the enzymatic degradation of [³H]-poly[r(DN-U)]. RNase T₂ digestion in the presence (○—○) and absence (■—■) of 0.02 M MgCl₂. Venom phosphodiesterase digestion with (●—●) and without (▪—▪) added MgCl₂ (0.02 M). Aliquots (100 μl) were removed from 1.0-M reactions at the indicated times and the acid-precipitable polymer collected on Millipore filters. [³H]poly[r(DN-U)] (labeled with [³H]6??) concentration was 1.7 X 10⁻⁴ M (3.2 X 10⁴ cpm per μmole); RNase T₂, 15 μg per ml; venom phosphodiesterase, 10 μg per ml; for buffers see “Materials and Methods.”

TABLE IX

Enzymatic degradation of poly[r(DN-U)] monitored fluorimetrically

For incubation conditions see “Materials and Methods.” Poly[r(DN-U)] concentration, 1.7 X 10⁻⁴ M. Enzyme concentration (micrograms per ml): RNase T₂, 10; pancreatic RNase, 5; venom and spleen phosphodiesterases, 25. An additional aliquot of each enzyme was added after 2 hours but no further fluorescence changes occurred. Additon of RNase T₂ (10 μg) to limit digests of the other three enzymes gives a rapid increase to 580 ± 10 fluorescence units (see text). Identical results were obtained in the presence of 0.02 M MgCl₂.

| Enzyme                      | Fluorescence intensity (arbitrary units) |
|-----------------------------|------------------------------------------|
|                            | Zero time | After limit digestion                   |
| RNase T₂                   | 130       | 582                                     |
| Pancreatic RNase           | 138       | 380                                     |
| Venom phosphodiesterase    | 135       | 375                                     |
| Spleen phosphodiesterase   | 130       | 370                                     |

Fig. 17. The optical rotatory dispersion spectrum of poly[r(DN-U)] at 22° (---) and 45° (---). The spectra were taken at a polymer concentration of 8.5 X 10⁻⁴ M in 0.02 M Tris-HCl buffer, pH 7.9, plus 0.01 M MgCl₂. Inset, rotational strength of poly[r(DN-U)] at 257 nm as a function of temperature. The mid-point of the rotational transition (37°) occurs at the same temperature as the Tₘ, determined by ultraviolet absorption.

Fig. 18. The optical rotatory dispersion spectrum of poly[r(DN-U)] at 22° (---) and 45° (---). The spectra were taken at a polymer concentration of 8.5 X 10⁻⁴ M in 0.02 M Tris-HCl buffer, pH 7.9, plus 0.01 M MgCl₂. Inset, rotational strength of poly[r(DN-U)] at 257 nm as a function of temperature. The mid-point of the rotational transition (37°) occurs at the same temperature as the Tₘ, determined by ultraviolet absorption.

Table IX shows that the addition of Mg²⁺ ions results in an increased molar rotation which is indicative of an increased structural organization. The spectral changes induced by MgCl₂ can be completely reversed by heating; the variation in rotational strength as a function of temperature exhibits a profile typical of polynucleotide denaturation. Additional considerations relating to the structure of poly[r(DN-U)] are presented under “Discussion.”

Poly[r(DN)] and Random Copolymers Containing DN—Poly[r(DN)] was prepared with polynucleotide phosphorylase under the reaction conditions described under “Materials and Methods.” The minimum molecular weight, as judged by Sephadex gel filtration was 40,000. End group analysis of [³H]poly[r(DN)] gave an average chain length of 160, which corresponds to an average molecular weight of 56,000. The ultraviolet absorption spectrum of the polymer at neutral pH is almost identical with that of the corresponding monomer spectrum (see Fig. 4). Poly[r(DN)] is also virtually devoid of hypochromicity relative to the nucleoside; the extinction coefficient (per mole of phosphate) at λ max being 3600 compared with 3800 for DN, indicating little, if any, structural order for single stranded poly[r(DN)].

Attempts were made to prepare homopolymer pair complexes of poly[r(DN)] with either poly(rU) or poly(rG+U). No convincing evidence was obtained for the formation of such complexes at room temperature by either absorption or fluorescence measurements although equimolar amounts of polymers were mixed in solutions of high ionic strength (up to 3.0 m KCl and NaCl or 0.02 M MgCl₂; MgCl₂ concentration higher than 0.02 M cause precipitation of poly[r(DN)]). The effect of DN on the stability of double stranded RNA helices was therefore studied by using random copolymers containing DN and either adenosine, guanosine, or cytidine in various base ratios. A detailed spectral and fluorimetric study of the polymers will be presented elsewhere, however, two points deserve mention here. As shown in Fig. 18, the Tₘ values of poly[r(DN, A)] + poly(U) complexes are considerably lower than the Tₘ of poly[r(A+U)], although a relatively small amount of DN is present in these polymers. The degree of hyperchromicity observed on denaturation and the cooperativeness of the melting profiles are also considerably reduced. The fact that denaturation curves obtained photometrically yield the same Tₘ and occur over the same temperature range as the absorption measurements suggests that there is no selective early denaturation of the DN-containing regions.

The second point of interest is that deazaadenosine-adenosine copolymers exhibit pronounced energy transfer (14, 15) from

4 D. C. Ward and E. Reich, manuscript in preparation.
adenosine to deazanebulamin residues (Fig. 19). No such energy transfer is seen in deazanebulinine-guanosine or deazanebularine-cytidine copolymers. Some of the fluorescence properties of these polymers are presented below.

Nuclease Susceptibility of DN Polymers—Poly[r(DN-U)] is susceptible to degradation by all the enzymes tested (Table IX, Fig. 16), although the rate of degradation is, in every case, slower in the presence of added MgCl₂. The decreased nuclease susceptibility in MgCl₂ is most likely a consequence of an increase in the double stranded character of poly[r(DN-U)]; the enzymes used preferentially degrade single stranded polymers. Complete degradation of poly[r(DN-U)] by RNase T₂ results in a 5-fold increase in fluorescence intensity and yields the expected mononucleotides on electrophoresis at pH 3.5. Limit digests with pancreatic RNase or with either of the phosphodiesterases renders the polymer totally acid soluble but gives only a 3-fold increase in fluorescence. The full 5-fold increase in fluorescence is obtained only after addition of RNase T₂ to the digests. Since the major product of these enzyme reactions, prior to RNase T₂ addition, has the same electrophoretic mobility at pH 3.5 as ApT₆p, it is assumed that the end products in these limit digests are dinucleotides. Although DNpU₆p is the expected product of pancreatic RNase digestion, the results suggest that the phosphodiesterases cleave certain DN-containing dinucleotides slowly, if at all.

The four nucleases described above were also tested for their ability to degrade poly(rDN). The polymer was resistant to pancreatic RNase and susceptible to RNase T₂ as anticipated from the known substrate specificity of these enzymes. Poly(rDN) was, however, unexpectedly resistant to digestion by both spleen and venom phosphodiesterases. High enzyme concentrations (50 μg per ml) and extended incubation times (up to 24 hours) were required to obtain partial hydrolysis of the polymer. In contrast, an identical concentration of poly(rA) was totally depolymerized in 10 min when incubated with 10 μg per ml of these phosphodiesterases.

Fluorescence Properties of DN Polymers—The fluorescence properties of poly(rDN) resemble qualitatively those of other fluorescent polynucleotides. The excitation and emission spectra are the same as those of the monomer (see Fig. 3), but the fluorescence intensity is decreased to one-fifth that of DNMP. This degree of quenching is much less than that observed with other polynucleotide fluorophores and can be correlated with the low hypochromicity of the polymer. Both of these observations suggest that poly(rDN) has very little structural order in the single stranded state.

The fluorescence of copolymers of DN and adenosine differs from that of poly(rDN). The fluorescence emission spectrum is virtually identical with that of the monomer; however, the excitation spectrum is modified in two ways. Compared with poly(rDN) and DNMP, the wave length of maximum excitation is shifted by up to 30 nm toward the blue. The copolymers that contain high adenosine to DN ratios exhibit the largest spectral changes (Fig. 19). Since the λ<sub>max</sub> of adenosine and of DN also differ by 10 nm (290 nm for adenosine, 270 nm for DN) it is likely that energy transfer is taking place between the AMP and DNMP residues in the polymer. A second change in the excitation spectrum is the appearance of a prominent shoulder at approximately 300 nm, which has no counterpart in the corresponding absorption spectrum. It is likely, therefore, that this shoulder represents a transition involving only residues which
are in the excited state; this could represent a charge transfer complex of adenosine and DN in the excited state. Treatment of these copolymers with RNase T1 abolishes both of these spectral characteristics; the resultant excitation spectrum is identical with that of DN monomers.

The addition of poly(U) to poly[r(DN, A)] under conditions which lead to the formation of a 1:1 ordered homopolymer pair structure also rapidly converts the excitation spectrum to one resembling that of poly(rDN) and reduces the fluorescence intensity to approximately one-fifth of that of the single stranded copolymer. When the newly formed double stranded structure is heated, a fluorescent Tm is obtained and the excitation spectrum reverts to that of single stranded poly[r(DN, A)]. However, the maximal fluorescence intensity after melting is only one-half the value found for the copolymer in the absence of an equal concentration of poly(U). The fluorescent Tm, which decreases with increasing DN content of the copolymer is identical with that obtained by monitoring the ultraviolet absorption (Fig. 18).

The fluorescence emission and excitation spectra of copolymers containing DN and guanosine are qualitatively the same as those of poly(DN) and DNMP. There appears to be no detectable energy transfer between these two nucleosides.

The fluorescence properties of the alternating copolymer poly[r(DN-U)] differ from those of the other polymers containing DN. In the single stranded state (i.e. in the presence of Mg++ at temperatures above Tm), the excitation and emission spectra are like those of DNMP, although the fluorescence intensity is quenched to one-fourth that of the monomer. However, in the ordered form, at temperatures below Tm, the excitation spectrum is altered so that it resembles qualitatively that of the single stranded random copolymer, poly[r(DN, A)]. That is, compared with the monomer or with the denatured polymer, the wave length of maximum excitation of double stranded poly[r(DN-U)] is shifted by about 10 nm toward the blue, and a prominent shoulder appears at approximately 300 nm (Fig. 15). Since the λmax for ultraviolet absorption of uridine is at 200 nm, whereas that of DN is at 270 nm the blue shift in the excitation spectrum suggests that energy transfer is occurring between uridine and DN. As in the case of poly[r(DN, A)], the shoulder in the activation spectrum at 300 nm is not matched by any corresponding change in the absorption spectrum and therefore probably represents a property only of residues in the excited state. This change in the shape of the excitation spectrum which is a property of double strandedness, is associated also with a change in the emission spectrum (Fig. 15). All these facts suggest that the native polymer contains an emitting species which is absent in DNMP, and in single stranded poly[r(DN-U)]. The position of the 300-nm shoulder in the excitation spectrum could be consistent with the existence of a charge transfer complex in the excited state.

As mentioned earlier, poly[r(DN, A)] exhibits energy transfer in the single stranded state but not when complexed with poly(U). Poly[r(DN-U)], on the other hand, exhibits energy transfer only in the double stranded form. These observations taken together imply that the energy transfer which is observed in the copolymer poly[r(DN-U)] (see below) is the product of the interaction between DN and the adjacent uracil residues in the same strand.

**DISCUSSION**

Many of the observations described in this paper are relevant for problems of polymer synthesis and structure, and these require comment and encourage speculation.

**Coding in Transcription and Interaction with RNA Polymerase**—The data in Figs. 8 to 10 and Tables III to VI leave little doubt that DNTP is an ambiguous substrate for transcription, that it functions as an analogue of either ATP or GTP, and with almost equal efficiency in the two cases. This finding is significant for theories concerning the mechanism of base selection by RNA polymerase. It is important to note that aside from the related structure, nebulin,9 DN is the only base analogue with demonstrable coding ambiguity in transcription. Although the mutagenicity of 2-aminopurine has often been considered to result from pairing ambiguities of the kind observed here (16, 17), no detectable evidence of such behavior has been obtained in vitro for 2-aminopurine with either DNA polymerase or RNA polymerase.5, 6 Thus the obvious ambiguity of DN is a phenomenon which merits some consideration.

The ability of DNTP to replace ATP is not altogether surprising for the following reasons. (a) DN functions as an analogue of adenine with several enzymes that do not require template; (b) DN possesses one of the characteristic hydrogen-bonding positions of adenine, namely, the equivalent of the unprotonated N-1 of adenine. DN can easily be visualized as forming a complementary base pair with uracil or its derivatives (see Fig. 2). This base pair would possess only a single hydrogen bond but could otherwise conform to all the geometrical requirements of a base pair of the Watson-Crick type.

At first sight, the ability of DNTP to replace GTP appears difficult to understand on the basis of the proposal that RNA polymerase selects the incoming nucleotide through some "allosteric instruction" from the template. DN possesses none of the functional groups characteristic of guanine, and specifically lacks all of the specific hydrogen-bonding functions of guanosine; in addition the chemical and physical properties of DN bear no similarity at all to those of guanosine. The function of DNTP as a GTP analogue during transcription would thus seem to require some other explanation.

The incorporation of DNMP in place of GMP can best be rationalized in one of three ways. The first is to imagine that DN can form a base pair with the rare imino form of cytidine. Under these conditions the base pair should contain a single hydrogen bond, which would make it structurally similar to the DN-U base pair. We regard this as unlikely, because of the rarity of the required cytosine tautomer and because the tautomer in question should also be capable of forming a base pair containing two hydrogen bonds with adenine. Thus ATP would be expected to replace GTP as effectively as DNTP does, and this is not the case. A second possibility is that DN is forming a base pair with the protonated form of cytosine; again this base pair would contain a single hydrogen bond. This is unlikely for at least two reasons: (a) the pK of cytidine in polymers (5.8 to 7.4) (18) is too low to permit adequate protonation at

5 D. C. Ward, A. Cerami, E. Reich, and I. H. Goldberg, manuscript in preparation.

6 A. Cerami and E. Reich, manuscript in preparation.

7 B. Brdar and E. Reich, unpublished results. These enzymes include adenosine kinase, myokinase, and tRNA pyrophosphorylase.
the pH at which DN replaced guanosine (up to pH 9.0); and (b) if the required degree of protonation of cytidine did in fact occur, it should enable 2-aminopurine to form two hydrogen bonds with cytidine and also to function as an analogue of guanosine, which is not, in fact, observed.6

The third possibility, which we consider the most attractive, is that the primary nucleotide selection of RNA polymerase is mediated through recognition of the proper geometrical arrangement of a base pair in a helical configuration; the normal specificity of this recognition being directed by hydrogen bonding. For this purpose, RNA polymerase can be visualized as providing a relatively rigid scaffolding of specific amino acid residues which can bind the incoming ribonucleoside triphosphate and catalyze the synthesis of phosphodiester bonds. The internal dimensions of this polypeptide framework would conform precisely to the geometry of a helical intermediate of the Watson-Crick type, the latter consisting of the template nucleotide residue and the nucleotide to be polymerized. In this model, the essential determinant for diester bond synthesis is the appropriate geometric positioning of the α-phosphoryl group of the incoming triphosphate and the 3'-hydroxyl group of the growing RNA chain. Nucleoside triphosphates which would lead to the formation of base pairs (with or without hydrogen bonds) whose over-all geometry was the same (or slightly smaller) than the normal helix, should be substrates for RNA polymerase since the proper position of nucleotide phosphate and growing chain hydroxyl group are maintained. In contrast, nucleotide analogues which give rise to base pairs that are abnormal due to excessive geometrical dimensions would not be utilized by the enzyme, since they are simply too large to fit into the framework of the active site. These points will be elaborated below.

In the context of the proposed model, the base pair formed between DN and uridine, thought less rigid than that formed between adenosine and uridine, would fit perfectly into a “slot” based on the geometry of the ordinary polynucleotide helices. In the case of the pairing between DN and cytidine, the base pair would be slightly smaller; its formation would therefore be possible within the framework provided by the enzyme structure. The DN-C base pair would still permit normal stacking of adjacent sugar residues in a helix, and thereby allow DNTP to act as an analogue of GTP. This base pair, although devoid of hydrogen bonding, may achieve slight stabilization from interactions with the adjacent nucleotide residue. Indeed, the observed energy transfer between uridine and DN in the double stranded form of poly(r(DN-U)] suggests that such base-base interactions are feasible.

It should be noted, however, that the above argument does not permit the formation of pyrimidine-pyrimidine or purine-purine base pairs. In the former case the two glycosidic bonds would be separated by only 8.4 A, compared to 10.8 A for a normal polynucleotide helix (19). The formation of pyrimidine-pyrimidine base pairs would prevent normal stacking of adjacent nucleotide residues and grossly alter the normal structure. Pyrimidine-purine base pairs would be excluded since such structures exceed the limiting geometry of an ordinary polynucleotide helix and therefore would not be accommodated by the “active site” of the enzyme. In contrast to these base pairs, the displacement of the glycosidic bonds in the DN-C base pair need be at most 0.2 A. This displacement may be an indication of the degree of wobble which the polymerizing system can tolerate when such residues are scattered throughout a polynucleotide chain.

Structure of poly[r(DN-U)]—Another noteworthy aspect of DN concerns the physical properties of polymers into which it is incorporated, and the nature of the structure in the ordered form of the alternating copolymer poly[r(DN-U)]. The evidence for significant interaction leading to multistranded structures is weak for the homopolymer pairs, poly(rDN) + poly(rU) or poly(rBrU). On the other hand, the evidence that the alternating copolymer poly[r(DN-U)] can assume an ordered structure appears convincing. What is the nature of the structure? Is the structure a multistranded one with base pairing of some type, or is it single stranded? We propose that under the appropriate conditions (“Results”), poly[r(DN-U)] assumes a structure which is double stranded, probably based on hairpin loops resembling those in poly[r(A-T)] (20); and we propose further that this structure contains base pairs of the kind shown in Fig. 2, which contain a single hydrogen bond between the DN and the uridine residues. Our reasoning is as follows. Two other lines of evidence can be interpreted as support for this view. The first is the qualitative fluorescence changes which accompany the thermal transitions. These changes are observed as alterations of the fluorescence excitation spectrum and are suggestive both of energy transfer between the DN and uridine and of the existence of an excited state complex in the polymer; and these changes are properties only of the ordered structure below the Tm. If the transition were to reflect two different degrees of single stranded order, one might expect gradual, quantitative changes in this fluorescence parameter, rather than the abrupt, all-or-nothing presence of energy transfer. The second line of evidence concerns the correlation between structure and nuclease susceptibility of poly[r(DN-U)], and particularly the susceptibility to pancreatic RNase and RNase T2. The relative protection against pancreatic RNase which is associated with the formation of the ordered form of poly[r(DN-U)] could not be reconciled with any form of single stranded order, because the functional groups of the uracil residues which interact with RNase would still be free to bind to the enzyme and to promote catalysis. In contrast, a base pair of the type we propose would block one of the hydrogen bond groups in the uracil base which appears essential for RNase action (21, 22), in this way partial protection of the polymer against RNase would result. Similar considerations apply to the comparable results obtained with RNase T2, which is known to degrade single stranded polymers preferentially.

The double stranded structure and the base pair envisioned for poly[r(DN-U)] seem appropriate also from other points of view. The low thermostability in Na+ calculated8 from the

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6 This calculation is based on the thermostabilities of alternating copolymers containing different proportions4 of two purines (e.g. adenine and 2,6-diaminopurine, or adenine and toyocamycin)
$T_m$ of the mixed alternating copolymers, and the low $T_m$ actually observed in the presence of Mg$^{2+}$ appear appropriate for a base pair containing a single hydrogen bond. The decrease (50°) in $T_m$ compared with poly[r(A-U)] appears reasonable for the loss of one hydrogen bond since an increase of one hydrogen bond, as in poly[r(cn-Pu-U)] or poly[r(cn-Pu-rU)] (23) or poly[d(mP-T)] (24) leads to an increase of approximately 45°; an extra destabilizing influence can be expected from the presence of only a single hydrogen bond, namely, an increased tendency to rotation at the glycosyl bond. This can be anticipated because two coplanar hydrogen bonds per base pair would tend to fix a plane in a cooperative fashion, which is not possible with a single hydrogen bond.

Finally, the structure proposed for poly[r(DN-U)] is also consistent with the synthetic patterns observed in the RNA polymerase reaction. In the absence of spermine, the newly formed poly[r(DN-U)] would be single stranded. As shown by Krakow (25) single stranded RNA quickly inhibits RNA polymerase, and the same would be expected of poly[r(DN-U)]. The addition of spermine would tend to maintain the product as a double stranded structure thereby permitting continued synthesis as occurs normally with poly[r(A-U)]. The fact that no spermine is needed for the synthesis of poly[r(DN-Ψ)], is also consistent with the known pattern of thermostability of base pairs which contain uracil and its analogues; base pairs containing pseudouridine are very thermostable (26), and presumably poly[r(DN-Ψ)] remains in the helical form even without spermine.

**Fluorescence of Polymers Containing DN**—The general fluorescence properties of DN are qualitatively similar to those of other fluorescent nucleotides previously described (1, 27, 28). However, the fluorescence characteristics of nucleotides containing DNMP differ appreciably in several respects from the comparable polymers containing formycin, 2-aminopurine, or fluorescent guanine derivatives. The degree of fluorescence quenching which occurs on polymerization of DNMP is much less than that seen with formycin and 2-aminopurine, although the relative differences in the fluorescence intensity of single and double stranded polymers is approximately the same for all. It might be reasoned to consider that the limited extent of fluorescence quenching of DN in polynucleotides is due to the relatively low stability of structural order which seems to be characteristic of them. However, this explanation cannot suffice since the thermostability of poly[r(DN-U)] in the presence of Mg$^{2+}$ is in the same temperature range as that of poly[r(2AP-U)] or poly[r(F-U)] in solutions of sodium chloride and the difference in fluorescence quenching of the different polymers persists under these conditions (1, 26).

and a single pyrimidine. The copolymers thus consist of mixtures of two base pairs of differing thermostability. We have observed that the $T_m$ is a perfectly linear function of the fraction of the respective purines (or type of base pairs). For example, the $T_m$ values of poly[d(A-T)] and poly[d(nPu-T)] fall at the extremes of a straight line given by a plot of $T_m$ of a series of mixed copolymers of the type poly[d(A,nPu-T)] containing varying proportions of each purine. This makes it possible to predict the $T_m$ of either alternating copolymer containing only a single base pair by extrapolating the line obtained from the $T_m$ values of the mixed alternating copolymers. The $T_m$ of poly[r(DN-U)] has been calculated in this manner by extrapolating the $T_m$ of a series of mixed copolymers of the type poly[r(DN,A-U)] to a value corresponding to an adenine content of zero. The $T_m$ of poly[r(DN-U)] given by this calculation is approximately 55° below that of poly[r(A-U)] under identical ionic conditions.

Several facts suggest that the low degree of fluorescence quenching of DN polymers is an intrinsic property of DN residues in both single and double stranded polynucleotides. Thus, it appears likely that the fluorescence of DN polymers is due to the emission from residues throughout the polymer, and not simply from terminal residues as it appears to be in polymers containing 2-aminopurine or formycin. The most persuasive fact is the relatively high fluorescence intensity of the polymer compared with that of the monomer. To account for the emission of polymers (either single or double stranded) entirely in terms of the fluorescence of the ends would require a quantum yield in excess of unity for the terminal residues. The behavior of the copolymers poly[r(DN, A)] is also especially revealing in this respect. The relative magnitude of the two main components of the excitation spectrum directly reflect the respective adenine and DN content of the polymers; this would be difficult to understand if the only fluorescent residues were DN molecules at the termini of the polymer.

The most noteworthy aspect of DN fluorescence in polymers concerns the changes in the fluorescence spectrum observed in copolymers, and the spectral modifications which occur as a function of secondary structure. The excitation spectrum of the alternating copolymer poly[r(DN-U)] indicates that energy transfer from uridine to DN is taking place only in the double-stranded form. In poly[r(DN, A)], on the other hand, energy transfer from adenosine to DN is observed only in the single stranded state and is eliminated on complexing with poly(U).

Taken together, these observations show that the energy transfer from uridine to DN which occurs in poly[r(DN-U)] is a consequence of the interaction between DN and adjacent uridine residues in the same strand, and not between the bases which constitute a base pair. Another conclusion which can be drawn from these findings is that energy transfer in both cases appears to depend on interactions between adjacent bases in the same strand, and that the geometrical requirements for those interactions cannot be satisfied in both single and double stranded polymers. Our preferred explanation for these effects is to visualize slight differences in pitch between single and double stranded polynucleotide helices. This would produce corresponding differences in the physical overlap of adjacent residues in the two types of polymer structure, and thereby influence energy transfer between them. However, there are no good grounds as yet for excluding several other, equally reasonable, interpretations.

As noted above, the changes on the blue side of the fluorescence excitation spectrum and the slight alterations in the excitation spectrum which accompany the appearance of the energy transfer band suggest that in both poly[r(DN, A)] and in poly[r(DN-U)] charge transfer complexes or "excimers" (14) may be formed in the excited state. Further work will be required to establish or to reject this possibility with more confidence.

**Applications**—Several potentially useful applications of DN should be mentioned. From its ambiguity in base pairing DN should be an excellent mutagen for all systems which incorporate it into polynucleotide. As shown in the accompanying report (29), DN is effectively incorporated into the nucleic acids of animal cells and of both DNA and RNA viruses. Another series of potential applications derives from its fluorescence properties; in particular, its ready incorporation into nucleotide terminus of tRNA can be of specialized value in studying structural
transitions accompanying interactions in which tRNA participates.

Acknowledgments—We wish to thank Miss Annie Chin and Mr. Lou DeNicola for their excellent technical assistance.

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