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Polynucleotides Containing 2′-Amino-2′-deoxyribose and 2′-Azido-2′-deoxyribose†

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ABSTRACT: 2′-Azido-2′-deoxycytidine 5′-diphosphate, 2′-amino-2′-deoxycytidine 5′-diphosphate, and 2′-amino-2′-deoxyuridine 5′-diphosphate are substrates for polynucleotide phosphorylase from *Micrococcus luteus*. Poly(2′-azido-2′-deoxycytidylic acid) [poly(Cz)], poly(2′-amino-2′-deoxycytidylic acid) [poly(Ca)], and poly(2′-amino-2′-deoxyuridylic acid) [poly(Ua)] are obtained with *s* values of 8.6, 4.8, and 3.1, respectively. All these polymers are stable to alkali and pancreatic ribonuclease. They are degraded by snake venom phosphodiesterase and micrococcal nuclease at rates considerably slower than for poly(rC) and poly(rU).

The need to understand the way in which a substituent at the 2′ position of the ribose ring influences the structure and function of the corresponding nucleosides, nucleotides, and polynucleotides has led to the synthesis and study of a number of compounds of this type, some of which have not been found in nature. Thus, polynucleotides have been reported which contain 2′-*O*-methyl (Zmudzka and Shugar, 1970; Zmudzka *et al.*, 1969) and 2′-*O*-ethyl substituents (Kusmierek *et al.*, 1973; Khurshid *et al.*, 1972), 2′-fluoro (Janik *et al.*, 1972), and 2′-chloro (Hobbs *et al.*, 1972b) substituents, and also the 2′-azido (Hobbs *et al.*, 1972a; Torrence *et al.*, 1972) substituent. A further spur to the investigation of such compounds has been the wish to determine the structure–function relationships of potential interferon inducers analogous to the homopolynucleotide duplex, poly(rI·rC). The data available at present indicate that the presence of a 2′-hydroxyl group in the polynucleotide is an absolute requirement for interferon inducers (Black *et al.*, 1972; DeClercq *et al.*, 1972). However, the compounds which have been tested have lacked the ability of the 2′-hydroxyl group to act as an electron donor, while simultaneously possessing a hydrogen atom suitable for the formation of hydrogen bonds. Both these properties are likely to be important in determining the structure of the polynucleotides and their complexes. The amino group would thus be a substituent of interest, since it possesses these properties. We wish to report the syntheses of poly(2′-azido-2′-deoxycytidylic acid) [poly(Cz)][1] and poly(2′-amino-2′-deoxycytidylic acid) [poly(Ca)], and to describe some of their properties, along with some of those of the previously reported poly(2′-azido-2′-deoxyuridylic acid) [poly(Uz)] (Hobbs *et al.*, 1972a; Torrence *et al.*, 1972) and poly(2′-amino-2′-deoxyuridylic acid) [poly(Ua)].

Experimental Section

Materials and Methods. Synthesis of Nucleosides. 2′-Azido-2′-deoxyuridine was synthesised by the method of Verheyden *et al.* (1971). It was converted to 2′-azido-2′-deoxy-3′,5′-diacetyluridine as described by the same authors, and 2′-azido-2′-deoxycytidine was prepared from this latter compound as described below. 2′-Amino-2′-deoxyuridine and 2′-amino-2′-deoxycytidine were prepared from the corre-

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[1] Abbreviations used are: poly(Cz), poly(2′-azido-2′-deoxycytidylic acid); poly(Ua), poly(2′-amino-2′-deoxyuridylic acid); poly(Ca), poly(2′-amino-2′-deoxycytidylic acid); poly(Ucl), poly(2′-chloro-2′-deoxyuridylic acid); poly(Ccl), poly(2′-chloro-2′-deoxycytidylic acid); poly(Uz), poly(2′-azido-2′-deoxyuridylic acid).

sponding 2′-azido-2′-deoxynucleosides by reduction as described below.

Synthesis of Nucleotides. Phosphorylation of nucleosides was carried out using $POCl_3$ as described for ribonucleosides by Yoshikawa *et al.* (1967), and the method of Michelson (1964) employing diphenyl phosphorochloridate was used to prepare the corresponding 5′-diphosphates. For the synthesis of the labeled nucleotides, $^{32}POCl_3$ was obtained from the Radiochemical Centre, Amersham, England.

Polynucleotide phosphorylase from *Micrococcus luteus* (specific activity 30), bovine pancreatic ribonuclease, spleen phosphodiesterase (1 mg/ml), snake venom phosphodiesterase (1 mg/ml), and alkaline phosphatase (1 mg/ml) were purchased from Boehringer, Mannheim, Germany. Polynucleotide phosphorylase from *Escherichia coli*, specific activity 200, was generously supplied by Dr. Lehrach, Max-Planck-Institut für biophysikalische Chemie, Göttingen. Snake venom phosphodiesterase used for the nucleoside analyzer work was purchased from Worthington, Freehold, N. J. [^{14}C]Poly(rU) was purchased from Miles Laboratories, Elkhart, Ind.; [^{14}C]-poly(rC) was synthesized from [^{14}C]CDP using polynucleotide phosphorylase from *M. luteus*.

Thin-layer chromatography (tlc) was carried out using 0.2-mm layer SiO_2 plates (PF-254) supplied by E. Merck and Co., Darmstadt, Germany, and preparative tlc using similar plates of 2 mm thickness, from the same supplier. Paper chromatography was carried out by the descending method using Schleicher & Schüll 2043b (washed) paper in system A (ethanol–1 M ammonium acetate, 7:3, v/v) or as specified. Electrophoresis was carried out on the same paper either at pH 7.5 (0.1 M triethylammonium bicarbonate) or at pH 3.5 (0.05 M ammonium formate) for 90 min with 30 V/cm.

The progress of synthesis of nonradioactive polynucleotides was monitored visually by applying aliquots of the reaction solution to paper chromatograms (2 × 14 cm, Whatman 3MM paper) and developing by the descending technique using ethanol–1 M ammonium acetate (1:1). In synthetic experiments involving radioactive nucleotides or degradation experiments of polynucleotides, summarized in Tables II–V, either this technique for [^{14}C]poly(rU), [^{32}P]poly(Ua), and [^{32}P]poly(Uz), or that of Bollum (1966) using Whatman 3MM filter disks for [^{32}P]poly(Cz), [^{32}P]poly(Ca), and [^{14}C]poly(rC), was used to separate the polymeric material. In the former, the origin of the chromatogram was excised and counted, a constant area being removed each time. Radioactivity counting was performed in a liquid scintillation counter (Tri-Carb Model 4312) using a toluene-based scintillation solution.

pK_a determinations in sodium citrate buffer (pH 7.5, 0.1 M Na^+) were carried out as described recently (Hobbs *et al.*, 1972b).

Phosphate determinations were carried out as described by Hurst and Becking (1963).

Circular dichroism (CD) curves were obtained on a Cary 61 spectrometer.

Ultraviolet (uv) absorption–temperature profiles were recorded on a Gilford Model 2000 recorder coupled to a Beckman Model DUR spectrophotometer.

Sedimentation coefficients were determined by analytical ultracentrifugation in a Spinco Model E ultracentrifuge equipped with uv absorption optics in sodium citrate buffer, pH 7.5 (0.05 Na^+).

Spectrophotometric titrations were performed in a Zeiss PMQ II spectrophotometer using a flow cell and an Orion Research digital pH meter (Model 201).

Nucleoside analyses were carried out using an analyzer (Uziel *et al.*, 1968) incorporating a column of Beckman M71 ion exchange resin (0.5 × 35 cm) operated at 50°. Prior to each analysis the column was washed with 0.4 M ammonium acetate (pH 8.8) and equilibrated with 0.4 M ammonium formate (pH 4.75). Nucleosides were eluted with 0.4 M ammonium formate (pH 4.75) and 2′-amino-2′-deoxynucleosides with 0.4 M ammonium acetate (pH 7.8) at a flow rate of 0.3 ml/min.

Nuclear magnetic resonance spectra were determined on a Bruker Physik HFX 60 spectrometer using TMS as an inner standard. All shifts are reported in τ values.

Synthesis of 2′-Azido-2′-deoxycytidine. 2′-Azido-2′-deoxy-3′,5′-diacetyluridine (0.9 g) was dissolved in 12.5 ml of ethanol-free chloroform, and dry dimethylformamide (0.125 ml) and thionyl chloride (2 ml) were added. The solution was heated under reflux for 6 hr, cooled to room temperature, evaporated, and dissolved in methanol (30 ml) which had been presaturated with ammonia. The solution was stirred at room temperature for 5 days. Tlc (methanol–chloroform, 40:60, v/v) showed the major product to have R_F 0.62. The solution was evaporated, and the product was separated by preparative tlc in the above system, the required band eluted with methanol, and the material thus isolated reapplied to 2-mm tlc plates, developing with benzene–acetone–water (2:8:1, v/v), and again eluting the desired band with methanol. The residue after evaporation of the methanol was triturated with dry pyridine (3 × 5 ml) and filtered to remove traces of silica gel. The pyridine solution was evaporated, traces of pyridine being removed by addition and reevaporation of water. The resulting material was a pale yellow foam which did not crystallize, but showed a single spot on tlc in methanol–chloroform (40:60, v/v) and a uv spectrum typical of a cytidine derivative. The yield was 8385 A_{270} units, and the material was phosphorylated without further purification.

An analytical sample was prepared by treating the material obtained as described above with activated charcoal, and applying the colorless gum thus obtained to a tlc plate which had previously been run in analytical grade methanol and then air-dried. The plate was developed in the normal way; the only uv-absorbing band was excised and eluted with methanol, silica gel traces were eliminated with pyridine as described above, and the material thus obtained was dissolved in a little methanol and precipitated with a large excess of dry ether. The white amorphous solid was filtered off and stored in a desiccator for 1 week; upon addition of methanol, spontaneous crystallization was found to have occurred. The crystalline 2′-azido-2′-deoxycytidine melts with decomposition and rapid evolution of gas at 215° ($\lambda_{max}^{H_2O}$ 270 nm (ϵ 9.3 × 10^3); ν_{max}^{KBr} 2140 cm^{-1} ($-N_3$ asymm.str.)); nmr (CD_3OD) 3.86 (m, 2, C_5H_2) 4.07 (m, 2, C_2′H and C_4′H), 4.43 (t, 1, $J_{2',3'} = J_{3',4'} = 5.5$ Hz, C_3′H) 5.97 (d, 1, $J_{1',2'} = 4.0$ Hz, C_1′H), 5.97 (d, 1, $J_{5,6} = 7.5$ Hz, C_5H), 8.05 (d, 1, $J_{5,6} = 7.5$ Hz, C_6H).

Anal. Calcd for $C_9H_{12}N_6O_4$: C, 40.30; H, 4.51; N, 31.33. Found: C, 40.32; H, 4.54; N, 31.38.

Synthesis of 2′-Amino-2′-deoxyuridine 5′-Diphosphate. 2′-Azido-2′-deoxyuridine 5′-diphosphate (860 A_{260} units) was dissolved in methanol (9 ml) and 10% palladium/charcoal (35 mg) added. The vessel was filled with hydrogen at a slight excess above atmospheric pressure (50 cm of H_2O) and hydrogenation carried out for 1 hr. The catalyst was filtered off, and a sample of the product was examined by electrophoresis at pH 3.5. The major product ran with mobility 15.4 cm, slightly slower than a CDP standard (17.4 cm) and markedly slower than UDP (24.8 cm). Traces of impurity were visible at 24.2 cm (unreduced starting material) and 3.7 cm.

TABLE I: Optical Properties of Polymers.

Polymer	λ_{max} (nm)	ϵ ($\times 10^{-3}$)	Hyperchromicity (%)
Poly(Ua)	260	8.6	9.2
Poly(Uz)	259	8.7	14.1
Poly(Ca)	270	8.1	1.2
Poly(Cz)	268	6.8	37.0

The product was applied to two sheets of paper (35-cm wide) for preparative electrophoresis, which was performed for 2 hr at pH 3.5, using the conditions described above. After drying, the product-containing bands were excised, and the product was eluted from the paper with methanol–water (1:1, v/v). The solution was evaporated and applied to a DEAE-Sephadex column (23 × 1.5 cm) and elution performed with a linear gradient of triethylammonium bicarbonate solution, 0.1–0.3 M in 2 l. A single peak was obtained, containing 515 A_{260} units.

The product had mobility 15.5 cm on electrophoresis at pH 3.5 (CDP 17.8 cm, UDP 26.2 cm) and 21.8 cm at pH 7.5 (CDP 21.5 cm, UDP 21.9 cm).

A sample of product (*ca.* 7 A_{260} units) was dissolved in 0.05 ml of Tris-HCl (pH 8.9), and alkaline phosphatase (0.1 ml, 1 mg/ml) was added. The mixture was incubated 30 min at 37°, and then examined by paper chromatography in system A. A single spot was obtained, R_F 0.63 (2′-aminouridine, 0.63; uridine, 0.68; 2′-azidouridine, 0.78). Phosphate analysis, nucleoside:phosphorus = 1:1.92.

Preparation of 2′-Amino-2′-deoxycytidine 5′-Diphosphate. 2′-Azido-2′-deoxycytidine 5′-diphosphate was reduced in a similar manner to that described for 2′-azido-2′-deoxyuridine 5′-diphosphate, but using platinum dioxide as catalyst, and triethylamine equimolar with the quantity of nucleotide was added. Paper electrophoresis of the product was performed at pH 4.5, and the product bands were eluted with water and purified on DEAE-Sephadex as before. The product gave a single spot on electrophoresis at pH 7.5, mobility 20.7 cm (CDP 21.3 cm, CMP 17.1 cm), and also at pH 3.5, mobility 7.6 cm (CMP 7.1 cm, CDP 15.1 cm).

Examination by alkaline phosphatase digestion and paper chromatography as described above for UDP gave a spot of R_F 0.57 (2′-aminocytidine, 0.58, 2′-azidocytidine, 0.78, cytidine, 0.68). Phosphate analysis, nucleoside:phosphorus = 1:1.81.

Radioactive Substrates. The labeled nucleotides [^{32}P]-5′-UzMP, 5′-[α-^{32}P]UzDP, 5′-[α-^{32}P]UaDP, [^{32}P]-5′-CzMP, 5′-[α-^{32}P]CzDP and 5′-[α-^{32}P]CaDP were prepared according to the standard methods described (Yoshikawa *et al.*, 1967; Michelson, 1964), or as described above, using ^{32}P-labeled phosphorus oxychloride to prepare the monophosphates.

Polymerization of Diphosphates. The polymerization mixtures contained Tris-HCl (pH 8.5, 100 mM), $MnCl_2$ (5 mM), GpU (0.05 mM), nucleoside diphosphate (4.6 mM for uridine derivatives, 5.1 mM for cytidine derivatives), and 45 units of polynucleotide phosphorylase per milliliter of solution. Polymerizations of UaDP and CaDP were generally performed for 4 hr, those of UzDP and CzDP for 6 hr. All incubations were performed at 37°. Aliquots were withdrawn for examination at the times indicated and treated as described in the Experimental Section. The reactions were stopped by freezing in acetone–Dry Ice. Isolation of polymers was carried out by thawing, repeated extraction of protein with chloroform–isoamyl alcohol (5:2, v/v) in the cases of poly(Ua) and poly(Uz), or Genetron 113 (Torrence *et al.*, 1972) in the cases of poly(Ca) and poly(Cz), and successive passages over Sephadex G-25, G-200, and G-25 for the chloroform–isoamyl alcohol treated polymers, and Sephadex G-50 and G-25 for the Genetron 113 treated polymers. The isolated polymers were stored in aqueous solution, frozen at −20°. The yields obtained were rather variable: typical values obtained per milliliter of incubation mixture were: poly(Ua), 9.56 A_{260} units (18.4%); poly(Uz), 4.43 A_{260} units (9%); poly(Ca), 3.1 A_{270} units (7.6%); poly(Cz), 29.2 A_{270} units (41.5%). When *E. coli* polynucleotide phosphorylase (45 units per milliliter of incubation) was used, the yield of poly(Uz) was raised to 43%.

Extinction Coefficients of Polymers. In a 1-ml cuvette were placed 0.1 ml Tris-HCl (1 M pH 8.5) and 0.85 ml of water containing sufficient polymer to give an optical density at the ultraviolet maximum of *ca.* 0.6. The spectrum was recorded, snake venom phosphodiesterase (5–10 μg) in the same buffer (0.05 ml) was added, and the cuvette was incubated at 37°. The spectrum was then rerecorded, and the hyperchromicity calculated, corrected for the dilution introduced by addition of the enzyme solution. The values of λ_{max}, ϵ, and the hyperchromicity on digestion are given in Table I.

Analysis of Polynucleotides. In each case *ca.* 0.9 OD_{270} of polynucleotide was dissolved in water (5 μl) and 1 M Tris-HCl (pH 8.75) (2 μl), 50 mM $MgCl_2$ (5 μl), snake venom phosphodiesterase (10 mg/ml; 5 μl), and alkaline phosphatase (1 mg/ml; 5 μl) were added. The mixtures were incubated 1 hr at 37° and applied to the nucleoside analyzer. Samples of the corresponding nucleosides were incubated in similar solutions (but without enzymes) for the same length of time, and also analyzed.

Circular Dichroism Spectra. These were determined in 10^{-1} M phosphate solution (pH 7.5) which had an ionic strength of 5×10^{-3}. Spectra of samples at a concentration of *ca.* 10^{-4} M were recorded, and corrected for solution absorbance.

Results

Synthesis of Nucleosides. 2′-Azido-2′-deoxyuridine and 2′-amino-2′-deoxyuridine were prepared according to the procedure already described, and a sample of the latter compound was found identical in chromatographic, electrophoretic, and melting behavior to an authentic sample kindly donated by Dr. J. Moffatt, Palo Alto. 2′-Azido-2′-deoxycytidine was prepared from 2′-azido-2′-deoxy-3′,5′-diacetyluridine by utilizing the procedure of Zemlicka and Sorm (1965). The protected nucleoside is treated with the Vilsmeier–Haack reagent thionyl chloride–dimethylformamide in refluxing chloroform, and the intermediate formed, 2′-azido-2′-deoxy-3′,5′-diacetyl-4-chlorouridine (which was not isolated), was treated with methanolic ammonia at room temperature to give the desired product. The material is reduced by platinum dioxide to give 2′-amino-2′-deoxycytidine, which was qualitatively identical on chromatography and electrophoresis with an authentic sample supplied by Dr. Moffatt. 2′-Azido-2′-deoxycytidine is markedly more stable than 2′-chloro-2′-deoxycytidine (Doerr and Fox, 1967), and may be maintained at 90° for several hours without apparent deterioration. The extent to which this failure to cyclize on heating is a function of azide ion as a poorer leaving group or of an altered conformation due to the greater bulk of the substituent is a matter for conjecture.

Phosphorylation of Nucleosides. Both 2′-azido-2′-deoxyuridine and 2′-azido-2′-deoxycytidine may be phosphorylated

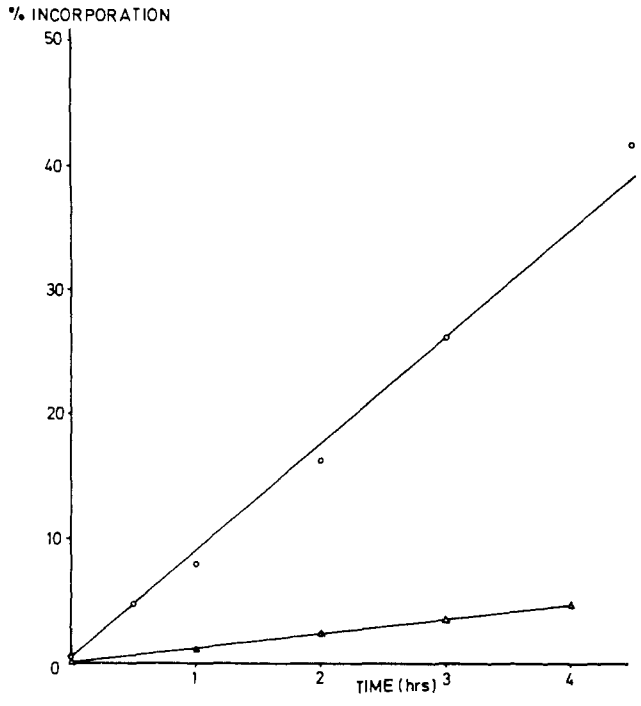

FIGURE 1: Incorporation of [α-^{32}P]CzDP (○) and [α-^{32}P]CaDP (△) into trichloroacetic acid precipitable material. Conditions as described in Methods.

in reasonable yield (57 and 56%, respectively) using the phosphorus oxychloride method of Yoshikawa *et al.* (1967), and further phosphorylated to the diphosphates using the method of Michelson (1964). The 2′-azido-2′-deoxynucleotides are then catalytically reduced to the 2′-amino-2′-deoxynucleotides (Wagner *et al.*, 1972). This method, as opposed to direct phosphorylation of the 2′-amino-2′-deoxynucleosides, was adopted to avoid likely complications owing to reaction of the 2′-amino group with the phosphorylating reagents. The aminonucleoside diphosphates are conveniently purified by preparative electrophoresis at pH where the 2′-amino group is charged; any unreduced material runs ahead and is thus easily eliminated. Any adventitious dihydronucleoside diphosphates should also be separated by proper choice of pH. The products were purified by passage over DEAE-Sephadex. No dihydronucleoside contaminants could be demonstrated with 4-dimethylaminobenzaldehyde spray and the nucleoside/phosphate ratios on analysis were consistent with the required diphosphates. This is important, since it has been demonstrated that dihydrouridine 5′-diphosphate may be incorporated into polymers (Torrence and Witkop, 1972).

Polymerization Experiments. All four polymers were substrates for polynucleotide phosphorylase from *M. luteus*, on incubation at pH 8.5 in the presence of Mn^{2+} ions and GpU as primer. Figure 1 shows the incorporation of labeled CaDP and CzDP into polymeric material: a figure for UaDP and UzDP was given in a previous publication (Hobbs *et al.*, 1972a). Although CzDP is very readily polymerized in high yield, UzDP was a very poor substrate in our hands. However, others have reported differently (Torrence *et al.*, 1972). UzDP was a good substrate for *E. coli* polynucleotide phosphorylase, however, a high degree of incorporation into polymer being obtained after only 30 min (Figure 2). Both UaDP and CaDP are polymerized, but, particularly with the latter, the high-molecular weight material in the peak excluded from the Sephadex G-100 column appeared to break down either upon evaporation or on desalting and the yield obtained after

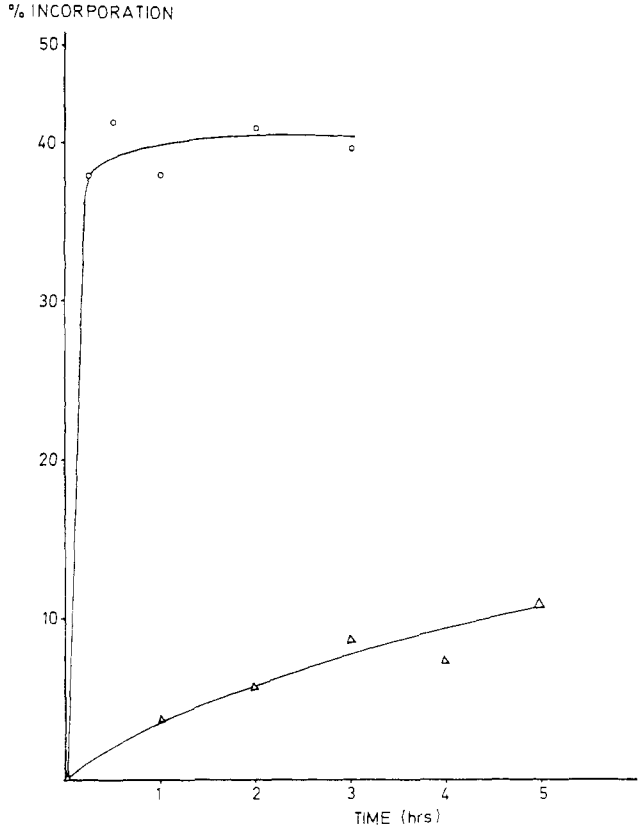

FIGURE 2: Incorporation of UzDP into polymer, followed by phosphate release, using polynucleotide phosphorylase from *E. coli* (○) and *M. luteus* (△). Phosphate was determined by the method of Hurst and Becking (1963). Conditions as described in Methods.

passage over G-25 was markedly depleted, a substantial quantity of low molecular weight material being separated. The

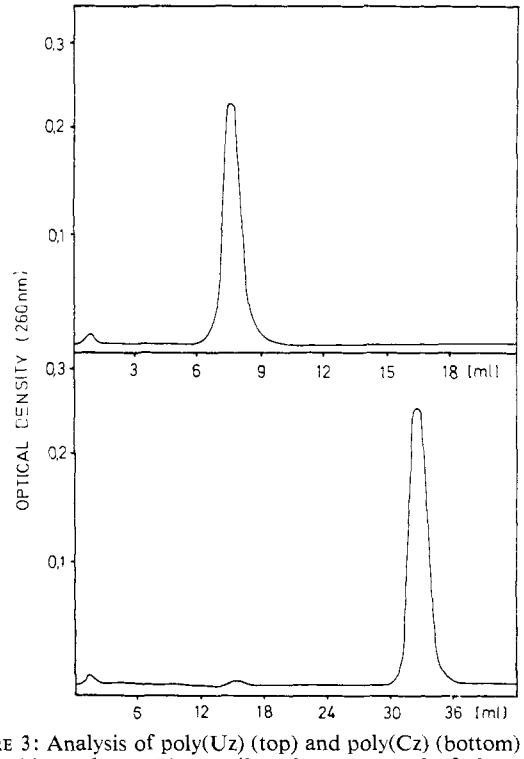

FIGURE 3: Analysis of poly(Uz) (top) and poly(Cz) (bottom) with a nucleoside analyzer. The small peak at *ca.* 1 ml of eluate is the breakthrough volume. Columns were eluted with 0.4 M formate buffer (pH 4.75). The elution volumes for the Uz and Cz standards were identical with those found for the polymer digestion products.

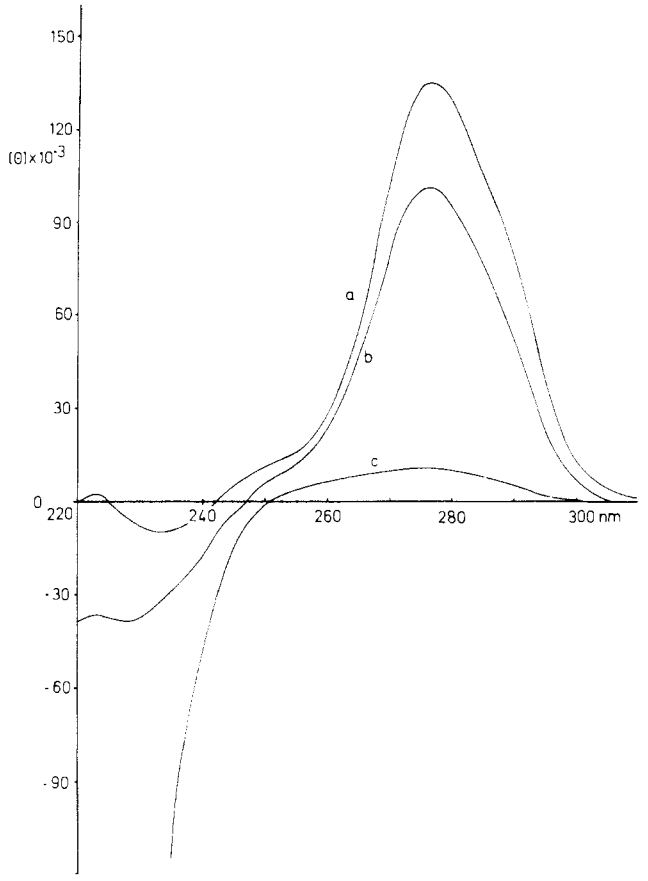

FIGURE 4: CD spectra of poly(rC) (a), poly(Cz) (b), and poly(Ca) (c) in 10^{-4} M phosphate, ionic strength 5×10^{-3}, at 18°.

polymers showed slight shifts in the positions of the uv maxima from those of the corresponding diphosphates. Thus, UaDP has λ_{max} 261.5 nm; UzDP, 265 nm; CaDP, 271 nm; and CzDP 271 nm. The λ_{max} for the corresponding polymers, with their

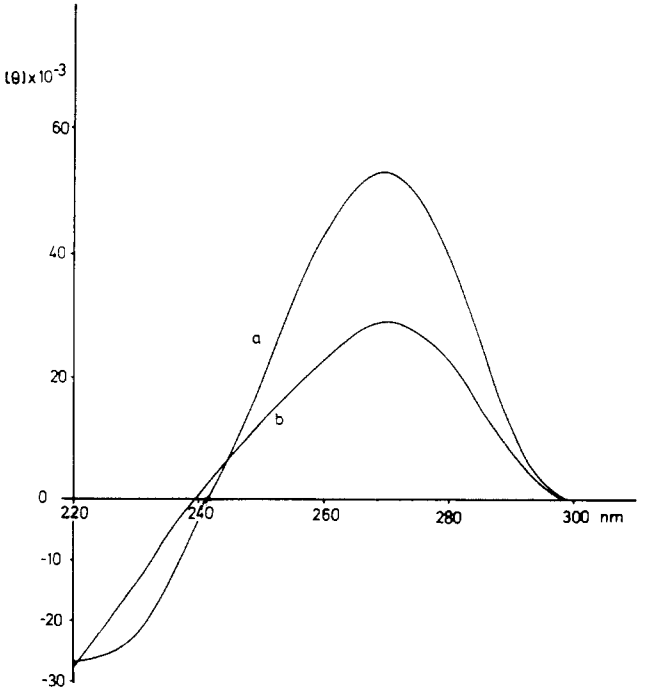

FIGURE 5: CD spectra of CzDP (a) and CaDP (b) in 10^{-4} M phosphate, ionic strength 5×10^{-3}, at 18°.

TABLE II: Degradation using Pancreatic Ribonuclease.[a]

	A			B		
Time (min)	Poly-(rU) (%)	Poly-(Ua) (%)	Poly-(Uz) (%)	Poly-(rC) (%)	Poly-(Ca) (%)	Poly-(Cz) (%)
5	1	98	100	2	99	100
10	1	96	99	2	98	100
20	1	92	98	1	96	100
40	1	84	96	1	92	100

[a] The figures represent the per cent of undegraded polymer, determined as described in the Experimental Section. (A) The incubation solution (total volume 0.16 ml) contained 0.1 M Tris-HCl (pH 7.4) and the following concentrations of polymers and protein: poly(rU), 0.2 mM, 120 ng of protein; poly(Ua) 0.4 mM, 2.4 μg of protein; poly(Uz) 0.4 mM, 2.4 μg of protein. (B) The incubation solution (total volume 0.21 ml) contained 0.1 M Tris-HCl (pH 7.4), 45 ng of protein, and the following concentrations of polymer: poly(rC), 0.33 mM; poly(Ca), 0.26 mM; poly(Cz), 0.35 mM.

extinction coefficients and hyperchromicity on digestion with snake venom phosphodiesterase, are given in Table I.

Characterization of Polymers. To check the integrity of the polymers, each polymer was analyzed by degradation with snake venom phosphodiesterase and alkaline phosphatase at 37°, and the mixture so obtained was separated on a nucleoside analyzer. The elution profile for the analysis of poly(Uz) and poly(Cz) is given in Figure 3. Uridine is eluted at 5.0 ml, cytidine with 24.3 ml with this column. The enzymes used for hydrolysis appear in the breakthrough. Poly(Ua) and poly(Ca) were analyzed in the same way. They were applied to the column and first washed with 42 ml of 0.4 *M* ammonium formate (pH 4.75) followed by 0.4 M ammonium acetate (pH 7.8). Both nucleosides were eluted with the basic buffer, Ua at 54.3 ml and Ca at 56.5 ml. No contamination by other nucleosides could be detected, the lower limit of detection being about 0.02%.

Typical values obtained for the sedimentation coefficients of the polymers were $s_{20,w} = 3.1$ for poly(Ua), $s_{20,w} = 7.8$ for poly(Uz), $s_{20,w} = 4.8$ for poly(ca), and $s_{20,w} = 8.6$ for poly(Cz). The circular dichroism spectra of poly(Ca) and poly(Cz) and the corresponding 5′-diphosphates are shown in Figures 4 and 5, as well as the corresponding ribopolymers for comparison. Spectrophotometric determination of the pK_a value of poly(Cz) is shown in Figure 6. The value obtained was 5.47. No value could be obtained for poly(Ca), owing to precipitation of the polymer as the pH was lowered. The nucleosides Ca and Cz both gave a figure of 3.76 for the pK_a value for the cytidine ring. The value obtained in CaDP was 3.93.

Degradation of Polymers. The kinetics of enzymatic breakdown of poly(Ua), poly(Uz), poly(Ca), and poly(Cz) in comparison to poly(rU) and poly(rC) are described in Tables II–IV. The resistance of the same polymers to alkaline breakdown is shown in Table V.

Discussion

The ability of polynucleotide phosphorylase to accept modified nucleotides as substrates for polymerization has been

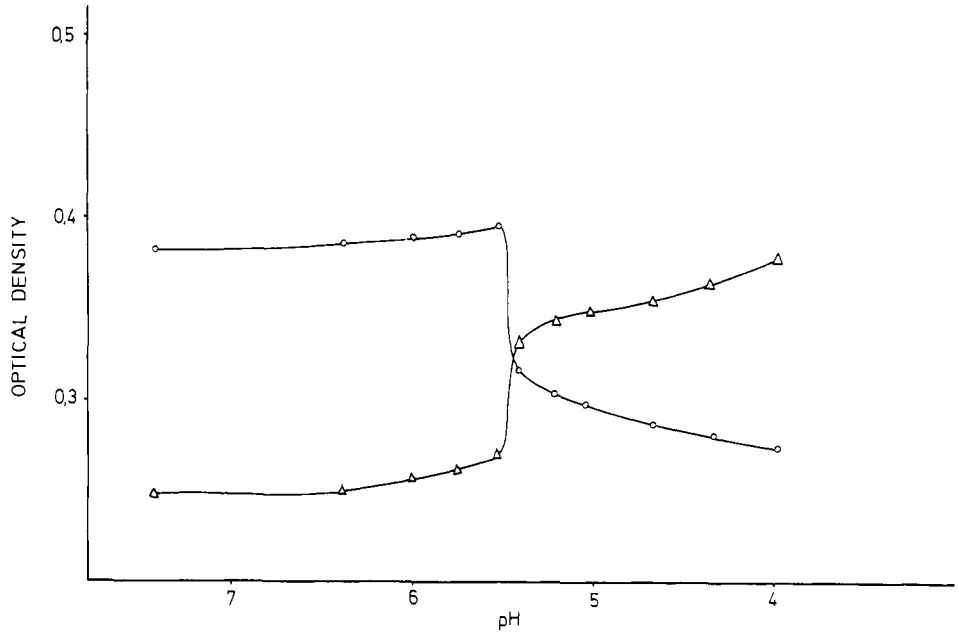

FIGURE 6: Titration curve of poly(Cz) at 285 nm (△) and 252 nm (○).

widely used to prepare polymers substituted in the 2′ position (Hobbs *et al.*, 1972b, and references cited therein). It seems, however, that the bulk of the substituent places limitations on the ability of the nucleotide to be polymerized; thus, the 2′-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates investigated by Mackey and Gilham (1971) will add only one residue to the end of a sequence. Conversely, the 2′-*O*-ethyl substituent does not prevent formation of a polymer (Khurshid *et al.*, 1972; Kusmierek *et al.*, 1973) and the nucleotides modified with a 2′-azido group are also acceptable for polymerization, despite the rigidity of the three nitrogen atom chain in the substituent. In our hands 2′-azido-2′-deoxyuridine 5′-diphosphate (UzDP) was polymerized very slowly by PNPase from *M. luteus*, even in the presence of Mn^{2+} ions (Hobbs *et al.*, 1972a), whereas 2′-azido-2′-deoxycytidine 5′-diphosphate (CzDP) was polymerized rapidly and in high yield. However, others (Torrence *et al.*, 1972) have reported that UzDP is polymerized rapidly and in good yield, even when Mg^{2+} is used, Mn^{2+} being omitted. The source of this difference is unclear. We find UzDP is polymerized very rapidly when PNPase from *E. coli* is used instead (Figure 2) of the *M. luteus* enzyme, all other reaction conditions being unchanged. The 2′-amino-2′-deoxy nucleotides UaDP (Hobbs *et al.*, 1972a) and CaDP are also substrates for the *M. luteus* enzyme in the presence of Mn^{2+}, thereby showing that the different hydrogen-bonding properties of the amino substituent as opposed to the hydroxyl substituent do not exert a strongly disadvantageous effect.

The formation of a 2′-amino polynucleotide introduces the possibility of the chain being very susceptible to breakdown due to attack of the nucleophilic amino group on the 3′,5′-phosphodiester link, with chain rupture and formation of a

TABLE III: Degradation using Snake Venom Phosphodiesterase.[a]

Time (min)	Poly(rC) (%)	Poly(Ca) (%)	Poly(Cz) (%)
5	0	8	87
10	0	7	85
20	0	5	64
40	0	4	17

[a] The figures represent the per cent of undegraded polymer, determined as described in the Experimental Section. The incubation solutions (total volume 0.16 ml) contained 0.1 M Tris-HCl (pH 8.5) and the following concentrations of polymer and protein: poly(rC), 0.54 mM, 0.8 μg of protein; poly(Ca), 0.39 mM, 2 μg of protein; poly(Cz), 0.51 mM, 2 μg of protein. Incubation temperature, 37°.

TABLE IV: Degradation using Spleen Phosphodiesterase.[a]

	A		B	
Time (min)	Poly(rU) (%)	Poly(Uz) (%)	Poly(rC) (%)	Poly(Cz) (%)
5	52	81	86	100
10	35	65	72	100
20	14	53	48	100
40	9	40	21	100
80			9	100
100	2	26		

[a] The figures represent the per cent of undegraded polymer, determined as described in the Experimental Section. (A) The incubation solution (total volume 0.23 ml) contained 0.11 M NaCl, 0.09 mM EDTA, 0.11 M Tris-HCl (pH 8.9), 20 μg of alkaline phosphatase, and 0.13 mM poly(rU) or 0.39 mM poly(Uz), respectively. (B) The incubation solution (total volume 0.19 ml) contained 0.13 M NaCl, 0.11 mM EDTA, 0.13 M Tris-HCl (pH 8.9), 20 μg of alkaline phosphatase, and 0.45 mM poly(rC) or 0.48 mM poly(Cz), respectively. After 10 min at 37°, acetic acid (0.01 ml) was added to bring pH to 5.5 in each case, and spleen phosphodiesterase (2 units/ml) was added: in poly(rU), 0.02 ml, in poly(Uz), 0.07 ml, in poly(rC) and poly(Cz), 0.05 ml. Zero readings were taken at once, and as indicated. Incubation temperature, 37°.

TABLE V: Degradation using Alkali.[a]

Time (min)	Poly(rU) (%)	Poly(Ua) (%)	Poly(rC) (%)	Poly(Ca) (%)	Poly(Cz) (%)
20	68	100	80		99
40	37	100	60	100	98
60	19	100	40	100	96
80	11	100		100	95

[a] The figures represent the per cent of undegraded polymer, determined as described in the Experimental Section. 0.1 M potassium hydroxide solution contained polymers of the following concentrations: poly(rU), 0.2 mM; poly(Ua), 0.49 mM; poly(rC), 0.64 mM; poly(Ca), 0.50 mM; poly(Cz), 0.67 mM. Incubation temperature, 37°.

cyclic phosphoramidate. In this contact it may be significant that the sedimentation coefficients obtained for the 2′-amino polymers [poly(Ua): $s_{20,w} = 3.1$; poly(Ca): $s_{20,w} = 4.8$] were lower than those obtained for the 2′-azido polymers [poly(Uz): $s_{20,w} = 7.8$; poly(Cz): $s_{20,w} = 8.6$] and for many others of the 2′-modified polynucleotides so far reported.

The pK_a of the 2′-amino group in the nucleoside Ua has been measured as 6.2 (Verheyden *et al.*, 1971), thus implying that when the pH drops below this value each monomeric unit of a polynucleotide chain containing this nucleoside will be a zwitterion, and the chain as a whole will carry no net charge. It was found that under these conditions the polymer precipitates. A similar observation has been made on poly(rC) when the ring N(3) nitrogen is protonated at *ca.* pH 3 (Hartman and Rich, 1965). Poly(Ca) also precipitates below pH *ca.* 5.6, thereby rendering pK_a determination for the formation of secondary structure, if any, impossible. T_m determination at pH 5 was excluded for the same reason.

We have been unable to demonstrate the formation of any complex between poly(Ua) and poly(rA). On mixing equimolar amounts of these polymers, no hypochromicty at room temperature at pH 9.0 or hyperchromicity suggestive of complex formation on heating from 2 to 70° at pH 7.5 could be observed (Hobbs *et al.*, 1972a). Exhaustive dialysis against a buffer at pH 7.5 (0.1 M sodium phosphate–1 mM EDTA) made no difference to the behavior, and dialysis against a buffer at pH 5.0 (0.05 M citrate–0.1 M phosphate–1 mM EDTA) led to complete precipitation as described above.

Upon mixing equimolar quantities of poly(Ca) and poly(rI) in 0.1 M sodium-citrate buffer (pH 7.5), a melting transition over 10° with a midpoint at 25° and hyperchromicity of 20% was observed. Both values are very low and the melting range large compared with those obtained for the complexes formed between poly(rI) and other modified cytidylate polymers. It suggests that the presence of a 2′-amino group is a considerable hindrance to complex formation compared with the other 2′ substituents examined so far. Poly(Cz) forms a complex with T_m 56° when mixed with an equimolar quantity of poly(rI) in 0.1 M sodum citrate (pH 7.5). It exhibits a sharp pK_a transition at pH 5.5 (Figure 6), the same value as poly(Ccl). The T_m value for poly(Cz) in 0.1 M sodium citrate buffer at pH 5.0 is lower than that for poly(Ccl) and poly(rC), however, at 51°. At pH 4.0 in a buffer of similar composition, it is 81°.

Poly(Cz) has a lower degree of stacking in solution than poly(rC) as seen in Figure 4. Poly(Ca) exhibits a very low degree of stacking, and thus exactly parallels poly(Ua).

The degree of base stacking as evidenced from CD data is borne out by the very low increments in optical density on digesting poly(Ua) and poly(Ca) with snake venom phosphodiesterase (Table I). A similarly low degree of stacking has also been reported for poly(dU) (Rabczenko and Shugar, 1971). In contrast, poly(Cz) gives an increase at the ultraviolet maximum of 268 nm of 37% on digestion with snake venom phosphodiesterase, a figure slightly less than the increments observed for poly(rC) and poly(Ccl). Poly(Uz) gives an increase of 14% at the ultraviolet maximum upon digestion, a figure similar to those obtained for poly(rU) and poly(Ua).

The studies performed with nucleases and phosphodiesterases complement the results reported for poly(Ucl) and poly(Ccl). Neither poly(Uz) nor poly(Cz) is broken down by pancreatic ribonuclease to a significant extent, even at very high enzyme concentration. Under the same conditions poly(Ua) and poly(Ca) were also highly resistant. The difference in susceptibility may reflect the relative difficulty of removing a proton from the amino group, as opposed to the hydroxyl group for the formation of the cyclic phosphate. The marked contrast between breakdown by removal of a proton from the 2′-hydroxyl and 2′-amino groups is shown by the degradation studies using 0.1 M KOH; where poly(rU) and poly(rC) are broken down with reasonable rapidity, poly(Ua) and poly(Ca) are stable. As expected, poly(Cz) was also stable.

Poly(Ca) and poly(Cz) are broken down by snake venom phosphodiesterase, more slowly than poly(rC), particularly in the case of poly(Cz). Poly(Ua) and poly(Uz) are also known to be substrates for this enzyme (Hobbs *et al.*, 1972a; Torrence *et al.*, 1972). These observations reinforce the view that while substitution of the 2′ position may hinder breakdown by snake venom phosphodiesterase, it does not, in general, prevent it (Laskowski, 1971).

Poly(Uz) was broken down by spleen phosphodiesterase under the same conditions as poly(rU), but at a slower rate. However, poly(Cz) was completely resistant under conditions in which poly(rC) was broken down. This behavior exactly parallels that of the chlorinated polymers, and the remarks previously made in this connection (Hobbs *et al.*, 1972b) probably apply here also. No studies with poly(Ua) or poly(Ca) could be performed owing to precipitation of the polymer at the pH at which digestion was to be carried out.

The results reinforce the suggestion that modification at the 2′ position increases the resistance of a polynucleotide to nucleases.

Some interest attaches to these polymers because of their potential role as interferon inducers, in analogy to such proved inducers as poly(rA·rU) and poly(rI·rC). Of the polymers described here, poly(Ua) has been tested as a potential single strand inducer (since it forms no complex) and found ineffective (Black *et al.*, 1972). Poly(rU) is also ineffective, although some single-strand homopolynucleotides are potent in this respect (DeClercq and Merigan, 1969). Poly(Ca) and poly(Cz) and their complexes have yet to be tried, though in view of the negative results obtained with poly(rI·rC) analogs containing 2′-OCH_3 (DeClercq *et al.*, 1972), 2′-Cl (Black *et al.*, 1972), and 2′-H in the cytidylate strand, it would be surprising if these compounds did have strong inducing activity.

There is still little information concerning the influence of the 2′ substituent on polymer conformation. A collation of data available on these polymers does not seem to give any

systematic pointers, and high resolution nuclear magnetic resonance data and fiber X-ray data on the polymers need to be gathered. It should be possible to obtain useful information from other enzyme systems in which the homopolynucleotides could potentially serve as templates, *e.g.*, in protein biosynthesis, and work on this is in hand.

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