2026.
Mackey, J. K., and Gilham, P. T. (1971), *Nature (London) 233*, 551.
Mandeles, S., and Kammen, H. O. (1966), *Anal. Biochem. 17*, 540.
Rottman, F., and Heinlein, K. (1968), *Biochemistry 7*, 2634.
Sadtler Standard Spectra (1967), Philadelphia, Pa., Sadtler Research Laboratories, Inc., No. 2949.
Shaw, S. J., Desiderio, D. M., Tsuboyama, K., and McCloskey, J. A. (1970), *J. Amer. Chem. Soc. 92*, 2510.
Singer, M. F. (1966), *Procedures Nucleic Acid. Res.*, 245.
Sonnenbichler, J., Feldmann, H., and Zachau, H. G. (1963), *Z. Physiol. Chem. 334*, 283.
Tazawa, I., Tazawa, S., Alderfer, J. L., and Ts'o, P. O. P. (1972), *Biochemistry 11*, 4931.
Torrence, P. F., Waters, J. A., and Witkop, B. (1972), *J. Amer. Chem. Soc. 94*, 3638.
Ts'o, P. O. P., Schweizer, M. P., and Hollis, D. P. (1969), *Ann. N. Y. Acad. Sci. 158*, 256.
Zmudzka, B., Janion, C., and Shugar, D. (1969), *Biochem. Biophys. Res. Commun. 37*, 895.
Zmudzka, B., and Shugar, D. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 8*, 52.

Synthesis and Characterization of Potential Interferon Inducers. Poly(2′-azido-2′-deoxyuridylic acid)†

Paul F. Torrence,‡ Albert M. Bobst, James A. Waters, and Bernhard Witkop*

ABSTRACT: 2′-Azido-2′-deoxyuridine (I) was converted in a series of steps to 2′-azido-3′-acetyl-2′-deoxyuridine (IV) which was phosphorylated with 2-cyanoethyl phosphate to give 2′-azido-2′-deoxyuridine 5′-monophosphate (V) from which the 5′-diphosphate (VI) was prepared by the morpholidate procedure. Polynucleotide phosphorylase polymerized VI in the presence of Mg^{2+} or Mn^{2+} to poly(2′-azido-2′-deoxyuridylic acid) [poly(dUz)] which was completely resistant to degradation by 0.3 N KOH and pancreatic ribonuclease A, as well as DNase I. Circular dichroism studies indicate that at 2.5°, poly(dUz) assumes a secondary structure similar to that of poly(U) (most likely a single hairpin). Ultraviolet and circular dichroism–temperature profiles in a number of buffer systems indicate that this structure has a thermal stability which is somewhat greater than poly(U). This finding indicates that neither a 2′-OH group nor a 2′-oxygen atom is necessary for the formation of ordered structure in poly(U). The construction of mixing curves at the isochromic wavelengths for this system revealed that poly(dUz) can form both double- and triple-stranded complexes with poly(A), *i.e.*, poly(dUz)·poly(A) and 2poly(dUz)·poly(A). A study of the course of the thermal dissociation of these complexes appeared to indicate that both the doubly and triply stranded complexes undergo monophasic transitions directly to constituent homopolymers (3 → 1 and 2 → 1, respectively). If the assignment of the above transitions (3 → 1 and 2 → 1) is correct, then the introduction of the azido group into the C-2′ position of poly(U) does not have a significant effect on the stabilities of both the triple- and double-stranded complexes when compared with the corresponding complexes in the poly(A)–poly(U) system.

Perhaps the most rewarding approach to the elucidation of the role of specific functional groups in the physical and biological properties of nucleic acids has been the synthesis of modified nucleosides and their conversion to 5′-diphosphates, followed by polymerization by the enzyme polynucleotide phosphorylase (Grunberg-Manago, 1963). In contrast to the chemical modification of a preformed polynucleotide, such an approach has the advantages of widening the possibilities for structural changes and leads to chemically well-defined high molecular weight homopolymers. In most instances, the substrate specificity of polynucleotide phosphorylase is the only limitation to this approach, but, fortunately, the nonspecific enzyme accepts a relatively wide variety of substrate modifications which can be extended even further by the use of manganous ion as cofactor (Babinet *et al.*, 1965; Thang *et al.*, 1965; Zmudzka *et al.*, 1969a,b; Rottman and Heinlein, 1968; Chou and Singer, 1971; Torrence and Witkop, 1972; Mackey and Gilham, 1971) or possibly through the use of matrix-bound enzyme (Brentnall and Hutchinson, 1972). By such techniques, a number of polynucleotides containing 2′-fluoro (Janik *et al.*, 1972; Hendler *et al.*, 1971), 2′-amino (Hobbs *et al.*, 1972a), 2′-chloro (Hobbs *et al.*, 1971, 1972b), 2′-*O*-methyl (Zmudzka *et al.*, 1969a,b; Rottman and Heinlein, 1968; Zmudzka and Shugar, 1970; Tazawa *et al.*, 1972), and 2′-*O*-ethyl (Khurshid *et al.*, 1972; Tazawa *et al.*, 1972) substituents have been introduced and studied recently. Such 2′-modified polynucleotides can be powerful tools in the elucidation of the role of the 2′-hydroxyl group in RNA and the factors responsible for the clinically promising phenomenon of the induction of interferon by synthetic polynucleotides (Colby, 1971; Kleinschmidt, 1972). In this paper[1] we describe the synthesis and characterization of poly(2′-

† From the Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014 (P. F. T., J. A. W., and B. W.), and from the Department of Chemistry, University of Cincinnati, Cincinnati, Ohio (A. M. B.). *Received April 13, 1973.*

‡ National Institutes of Health Staff Fellow, 1969–present.

[1] A preliminary account of a portion of this work has appeared (Torrence *et al.*, 1972).

azido-2′-deoxyuridylic acid) [poly(dUz)][2] and its complexes with poly(adenylic acid).

Materials and Methods

Synthesis of 2′-Azido-2′-deoxyuridine 5′-Diphosphate (VI). 2′-Azido-2′-deoxyuridine (I) was prepared from 2,2′-anhydro-1-(β-D-arabinofuranosyl)uracil (Verheyden *et al.*, 1971).

2′-Azido-5′-trityl-2′-deoxyuridine (II). To a solution of 2′-azido-2′-deoxyuridine (400 mg, 1.48 mmol) in dry pyridine (10 ml) was added trityl chloride (500 mg, 1.80 mmol). The pyridine solution was warmed at 100° for 1.5 hr, cooled, and slowly added dropwise to ice water with vigorous stirring. The solid that formed was collected and dried. Chromatography on silica gel with benzene–acetone as eluent gave 690 mg (91%) of pure 2′-azido-5′-trityl-2′-deoxyuridine as a glassy solid: mp 89–91°; ν_{max} 2120 cm^{-1} (N_3); pmr ($CDCl_3$) δ 9.60 (s, 1, N*H*), 7.83 (d, 1, J = 8 Hz, H-6), 7.33 (br s, 15, trityl H), 5.94 (d, 1, J = 3 Hz, H-1′), 5.37 (d, 1, J = 8 Hz, H-5), 4.50 (br m, 1, H-3′), 4.12 (br m, 2, H-2′ and H-4′), 3.55 (br s, 2, H-5′), 3.08 (d, 1, J = 6 Hz, 3′-OH). *Anal.* Calcd for $C_{28}H_{25}N_5O_5$: C, 65.75, H, 4.93; N, 13.69. Found: C, 65.48; H, 4.80; N, 13.15.

2′-Azido-3′-acetyl-5′-trityl-2′-deoxyuridine (III). 2′-Azido-5′-trityl-2′-deoxyuridine (600 mg, 1.17 mmol) was dissolved in dry pyridine (5 ml) and after the addition of Ac_2O (0.1 ml) the mixture was allowed to react overnight at 0°. The resulting solution was then added dropwise to a vigorously stirred mixture of ice and water (100 ml) and the resulting solid washed with water and dried. Chromatography on silica gel, with benzene–acetone as eluent, gave 580 mg (90%) of pure 2′-azido-3′-acetyl-5′-trityl-2′-deoxyuridine as a glass: mp 87–89°; ν_{max} 2120 (N_3) and 1740 cm^{-1} (OAc); pmr ($CDCl_3$) δ 9.07 (s, 1, N*H*), 7.75 (d, 1, J = 8 Hz, H-6), 7.36 (br s, 15, trityl H), 6.04 (d, 1, H-1′), 5.45 (d, 1, J = 8 Hz, H-5), 5.25 (br m, 1, H-3′), 4.24 (br m, 2, H-2′ and H-4′), 3.56 (br s, 2, H-5′), 2.24 (s, 3, acetate CH_3). *Anal.* Calcd for $C_{30}H_{27}N_5O_6$: C, 65.09; H, 4.91; N, 12.65. Found: C, 64.88; H, 4.90; N, 12.22.

2′-Azido-3′-acetyl-2′-deoxyuridine (IV). 2′-Azido-3′-acetyl-5′-trityl-2′-deoxyuridine (500 mg, 0.9 mmol) was dissolved in 80% HOAc (10 ml) and warmed for 10–20 min at 90–100°. After the required time period, the solution was cooled and the trityl alcohol which precipitated was removed by filtration. The acetic acid filtrate was evaporated to dryness (<40°) and the oily residue taken up in ethanol (5 ml) to which ligroin (50 ml) was added. After several minutes a crystalline precipitate began to form. This precipitate was collected and washed with ligroin to give 245 mg (88%) of chromatographically homogeneous 2′-azido-3′-acetyl-2′-deoxyuridine. One recrystallization as above gave prisms: mp 189–191°; ν_{max} 2120 (N_3) and 1750 cm^{-1} (OAc); uv $\lambda_{max}^{CH_3OH}$ 260 nm. *Anal.* Calcd for $C_{11}H_{13}N_5O_6$: C, 42.44; H, 4.21; N, 22.50. Found: C, 42.71; H, 4.28; N, 22.74.

2′-Azido-2′-deoxyuridine 5′-Monophosphate (V). 2′-Azido-3′-acetyl-2′-deoxyuridine (270 mg, 0.875 mmol) was mixed with a stock pyridine solution of β-cyanoethyl phosphate (Tener, 1961; 1.9 ml, 1.9 mmol) and the resulting solution dried by three consecutive additions and evaporations of anhydrous pyridine. To the residue was added anhydrous pyridine (10 ml) and dicyclohexylcarbodiimide (824 mg, 4 mmol). After 46 hr of stirring at room temperature, the solution was evaporated (40°) to a viscous residue to which water (10 ml) was added. The resulting precipitate, dicyclohexylurea, was collected and washed with water. The filtrate was deionized with Dowex-50 (H^+) and, after removal and washing of the resin, the clear solution was adjusted to pH 6.5 with $Ba(OH)_2$. This solution was concentrated to 7 ml *in vacuo* (<40°) and the precipitate that formed was collected and washed with water (2 ml). Addition of absolute ethanol (18 ml) produced a copious precipitate which was centrifuged and washed once with EtOH–H_2O (1:1, 4 ml). The combined supernates were evaporated to remove ethanol and deionized with Dowex-50 (H^+) and the pH of the solution was adjusted to 7 with dilute ammonium hydroxide. This solution was evaporated to near dryness and the residue taken up in 7 N NH_4OH (20 ml) and placed in a water bath at 60–65°. Silica gel tlc (*n*-BuOH–MeOH–H_2O–NH_4OH, 40:30:20:10) indicated that at 105 min all cyanoethyl phosphate intermediate had decomposed to leave only one ultraviolet absorbing spot corresponding to the 5′-monophosphate. This solution was evaporated *in vacuo* (<40°), the residue taken up in water, and the resulting solution deionized with Dowex-50 (H^+). After removal of Dowex, the pH was adjusted to 7.5 with barium hydroxide and the solution concentrated to 5 ml at reduced temperature and pressure. After filtration from a small amount of insoluble matter, absolute ethanol (12 ml) was slowly added to this solution to give a fluffy colorless precipitate which was washed with ethanol, acetone, and ether to give, after drying, the barium salt of 2′-azido-2′-deoxyuridine 5′-monophosphate (240 mg, 55%). A second preparation gave the barium salt in 75% yield. A second ethanol precipitation gave analytically pure material which, however, contained ~1% of the total phosphorus as inorganic phosphate: ν_{max} 2120 cm^{-1} (N_3); $\lambda_{max}^{pH\ 7.6}$ 262 nm (ε 10,000); R_F(UMP) 2.0 (system A). Bacterial alkaline phosphatase digestion of this 5′-monophosphate gave 2′-azido-2′-deoxyuridine as the only detectable product both on tlc and paper chromatography each in two different solvent systems. *Anal.* Calcd for $C_9H_{10}N_5O_8PBa \cdot 1.5H_2O$: C, 21.13; H, 2.56; N, 13.68; P, 6.06. Found: C, 21.12; H, 2.30; N, 13.14; P, 6.37.

2′-Azido-2′-deoxyuridine 5′-Diphosphate (VI). 2′-Azido-2′-deoxyuridine 5′-monophosphate (barium salt, 160 mg, 0.31 mmol) was dissolved in water and deionized by passage through a column of Dowex-50 (H^+). The eluent was evaporated *in vacuo* (<40°) to about 5 ml. To this solution were added *t*-BuOH (5 ml) and morpholine (redistilled, 0.12 ml 1.4 mmol) and the resulting solution was refluxed gently, while a solution (8 ml of *t*-BuOH) of dicyclohexylcarbodiimide (275 mg, 1.35 mmol) was added slowly over a period of 3.5 hr. When the addition was completed, the solution was refluxed for 2 more hours after which tlc on silica gel (*n*-BuOH–MeOH–H_2O–NH_4OH, 40:30:20:10) indicated quantitative conversion to the morpholidate. The solution was cooled and the crystals which had formed after 16 hr were collected and washed with *t*-BuOH. The filtrate was evaporated to remove *t*-BuOH and the remaining aqueous phase extracted three times (with filtration after the first extraction) with ether. The aqueous layer was evaporated to dryness *in vacuo* (<40°) and the residue re-evaporated several times with ethanol to remove water. After the residue was taken up in methanol (1 ml), ether (60 ml) was added to precipitate the

[2] Communication with Dr. Waldo E. Cohn, Director, NAS-NRC Office of Biochemical Nomenclature, has led us to adopt the abbreviation poly(dUz) for poly(2′-azido-2′-deoxyuridylic acid) in harmony with the recommendations proposed for other 2′-modified polynucleotides (*e.g.*, see *J. Mol. Biol. 55*, 299 (1971)). In this fashion, the other unusual 2′-modified polynucleotides will be abbreviated as follows: poly(2′-fluoro-2′-deoxyuridylic acid), poly(dUf); poly(2′-chloro-2′-deoxyuridylic acid), poly(dUcl); poly(2′-amino-2′-deoxyuridylic acid), poly(dUa); poly(2′-*O*-methyluridylic acid), poly(Um).

morpholidate which was isolated after centrifugation and washing with ether.

The morpholidate was dried by four consecutive additions and evaporations of anhydrous pyridine and was dissolved in pyridine (dry, 4 ml). To this solution, a dried solution of mono(tri-*n*-butylamine) orthophosphate (prepared from 0.069 ml (1 mmol) of orthophosphoric acid and 0.24 ml of tri-*n*-butylamine) was added. The combined solutions were further dried by addition and evaporation of pyridine and finally dissolved in dry pyridine (5 ml). After 50 hr at room temperature, paper chromatography and tlc indicated complete disappearance of the morpholidate. The reaction mixture was evaporated to a viscous residue which was dissolved in water and the aqueous solution evaporated to remove residual pyridine. This residue was again dissolved in water (40 ml) and the pH of the solution adjusted to 8.1 with dilute alkali. The solution was then applied to a column of Dowex 1-X8 (Cl^-) (200–400 mesh). The column was washed with water (75 ml); then elution was commenced with 0.01 N HCl which eluted a small amount of uv-absorbing material. Some 5′-monophosphate and a considerable amount of orthophosphate were eluted with 0.01 N HCl–0.03 M LiCl. Finally the 5′-diphosphate was eluted with 0.01 N HCl–0.10 M LiCl. The diphosphate fractions were combined, the pH adjusted to 5.0 with fresh dilute lithium hydroxide, and the solution was evaporated *in vacuo* (<40°) to a colorless residue which was dried by several evaporations and addition of ethanol. The solid was suspended in methanol (5 ml) and then acetone (35 ml) was added. The resulting precipitate was centrifuged and washed with methanol–acetone (10:70) until no more chloride ions were detectable in the supernate ($AgNO_3$). The precipitate was washed with acetone and then ether to give after drying *in vacuo* the lithium salt of 2′-azido-2′-deoxyuridine 5′-diphosphate (62 mg, 41%). A second preparation gave the 5′-diphosphate in 60% yield (from monophosphate): uv $\lambda_{max}^{H_2O}$ 262 nm (ϵ 10,000); ν_{max} 2120 cm^{-1} (N_3); R_F(UDP) 1.75 (system A). *Anal.* Calcd for $C_9H_{16}N_5O_{14}P_2 \cdot HLi_2 \cdot 3H_2O$: N, 14.15; P, 12.51. Found: N, 14.53; P. 12.32.

The diphosphate contained no detectable inorganic phosphate and was hydrolyzed by bacterial alkaline phosphatase to give 2′-azido-2′-deoxyuridine as the only uv-absorbing product both by paper chromatography and silica gel tlc, each in two solvent systems.

Preparation of Poly(2′-azido-2′-deoxyuridylic acid). Conditions for polymerization. A typical incubation mixture contained per milliliter: 0.1 mmol of Tris (pH 8.6 for $MgCl_2$ or pH 8.0 for Mn^{2+}), 6–8 phosphorolysis units of *Micrococcus luteus* polynucleotide phosphorylase, 12–17 μmol of 2′-azido-2′-deoxyuridine 5′-diphosphate, and 6–8 μmol of $MgCl_2$ (or 1.5–2.0 μmol of $MnCl_2$). Incubation was at 37°. Progress of the reaction was followed by inorganic phosphate analyses on aliquots removed at various time intervals. While under these conditions, UDP or ADP is polymerized to the extent of 50–60%, dUzDP showed no phosphate release even up to 2–2.5 hr. At 6 hr phosphate release had usually reached a maximum and, at that point, the reaction was terminated by cooling in ice and proceeding with the purification procedure. Mn^{2+} increased the yield of polymer; thus, under conditions where Mg^{2+} as cofactor gave a 40% yield of polymer (based on phosphate analysis), Mn^{2+} raised the yield to 50%. All properties recorded in this paper were determined with polymer synthesized with Mg^{2+} as cofactor.

Purification of the polynucleotide. The reaction mixture was diluted to 0.5 ml with ammonium bicarbonate (0.04 M) and exhaustively extracted (six–eight times) either with Genetron 113 or with $CHCl_3$–isoamyl alcohol (5:3, v/v). The organic phases were combined and extracted with ammonium bicarbonate (0.04 M) (0.5 ml) and the resulting aqueous solution was repeatedly extracted (four–five times) with the above organic system. The resulting two aqueous solutions were combined and extracted once with ether, and nitrogen was blown over the surface of the aqueous layer to remove residual ether. This solution was then applied to either a Sephadex G-100 or G-200 column (1.5 × 25 cm) and eluted with ammonium bicarbonate (0.04 M). The polymer was always excluded in the void volume. The appropriate fractions containing polynucleotides were combined and lyophilized to dryness. The polymer was then taken up in water (10–15 ml) and dialyzed successively against 2-l. changes of 0.01 M EDTA–0.1 M NaCl (pH 7.0), 0.001 M EDTA–0.1 M NaCl (pH 7.0), and 1 M NaCl, and then exhaustively against distilled water. The resulting aqueous solution was then clarified and sterilized by filtration through a Millipore filter and lyophilized. Usually 5′-diphosphate (2 mg) was polymerized as described above. After purification, the yield of polymer was 5–8 OD_{260} (optical density) units (13–20%)

It should be pointed out that preparations of poly(dUz) which were not dialyzed and passed through Millipore filter often exhibited a significant extraneous absorption in the 300–290-nm region. This absorption was apparently not due to a precipitate, since no absorption was observed in the visible range.

Determination of the extinction coefficient of Poly(dUz). Determination of ϵ_{max} for poly(dUz) by hydrolysis with KOH or RNase was not possible because of the absence of a 2′-OH group; in addition, because of the small hyperchromicity (5–10%) associated with degradation to mononucleotide, digestion with other hydrolytic enzymes followed by determination of the concentration of mononucleotide was not a sufficiently accurate method. The method of choice was determination of inorganic phosphate after digestion with mixed acids as described by Howard *et al.* (1971). Three determinations gave values of ϵ_{max} of 9320, 9490, and 9490 at 24° in 0.01 M $MgCl_2$–0.001 M KH_2PO_4 (pH 7.6). The ϵ_{max} was unchanged in 0.2 M NaCl–0.01 M sodium cacodylate (pH 7.0). As a check on the accuracy of this method ϵ_{max} was also determined by acid digestion of poly(dUz) followed by determination of N by the diffusion method. This procedure gave ϵ_{max} 9100 in 0.01 M $MgCl_2$–0.001 M KH_2PO_4 (pH 7.6) at 24°. An average value for the three P determinations gave 9430 as ϵ_{max} for poly(dUz). The error in this value is probably less than ±2%. The ϵ at 260 nm for poly(U) in 0.01 M $MgCl_2$ was determined as 9400.

Ultraviolet spectroscopic measurements. Ultraviolet spectra as well as mixing curves were determined on a Cary Model 15 spectrophotometer. Temperature–absorbance profiles were obtained by two different measurement systems referred to as methods 1 and 2 in the text.

Method 1. The profiles were obtained in a Beckman Kintrac VII spectrometer equipped with a thermostated cell housing. To minimize temperature gradients, the solutions were stirred continuously with a built-in magnetic stirrer. The temperature was monitored by a thermistor probe inserted in the cell compartment containing the polynucleotide solution. A water–methanol mixture from a Haake thermostat, coupled with a water bath cooler, was circulated through the thermostated cell holder. Dry nitrogen was flushed through the cell compartment to prevent fogging.

Method 2. Temperature–absorbance profiles were obtained on a Cary Model 14 spectrophotometer with a thermostated

sample compartment through which a water–ethylene glycol solution from a Forma or Haake water bath was circulated. The temperature of the sample was determined with a thermistor (Yellow Springs Instrument Co.) which was calibrated during each run with a mercury thermometer graduated in 0.1°. The probe of the thermistor was placed immediately adjacent to but not inside the sample cell. The reference solvent cell was kept at room temperature. Absorbances were determined 15–20 min *after* the thermistor indicated thermal equilibrium in the sample cell. The quartz cells were 10 mm in path length with ground Teflon stoppers. The entire spectrum at each temperature was recorded with an expanded chart scale (3 nm = 1 in.).

CIRCULAR DICHROISM MEASUREMENTS. Ultraviolet CD measurements were determined on a Cary Model 6002 CD attachment to a Cary 60 recording spectropolarimeter equipped with a thermostatable cylindrical cell holder. Results are reported in terms of the differential dichroism absorption ($\epsilon_L - \epsilon_R$) and are given on the basis of the molar concentration of nucleotide residues. Constant nitrogen flushing was employed over the wavelength of 320–200 nm. A water–methanol mixture from a Haake thermostat coupled with a water bath cooler was circulated through the thermostatable cylindrical cell holder. The temperature was measured with a sensitive thermistor which was inserted through a Teflon stopper directly into the top of the cell.

CONSTRUCTION OF MIXING CURVES. Mixing curves were constructed basically according to the methodology developed by Howard *et al.* (1971) and by Blake *et al.* (1967). Equimolar concentrations of polynucleotides were used to construct the curves. Spectra were determined on the Cary 15 at an expanded chart setting (3 nm = 1 in.). Appropriate quantities of polynucleotide and buffer were delivered from the micrometric syringe microburet. Mixtures were incubated at 24 ± 1°. Initially, solutions consisting of only 1:1 and 1:2 molar ratios of $(A)_n$ to $(dUz)_n$ were prepared. The complete spectrum (340–210 nm) of each mixture was determined immediately after mixing and for periods of 8–16 hr thereafter until the change in absorbance at several different wavelengths (280, 270, 260) was 0.002 or less. Solutions over the entire mixing curve were prepared and ultraviolet spectra determined as above after equilibrium had been obtained.

Isochromic wavelengths were determined by converting the absorbancy at 1-nm intervals to ϵ values for the 0, 50, 67, and 100 mol % poly(dUz) mixtures. Arbitrary wavelengths over the entire spectrum were selected and the ϵ values at each chosen wavelength were plotted *vs.* mole per cent poly(dUz). The isochromic wavelength was determined when the ϵ values for the 100, 50, and 0 or 100, 67, and 0 mol % poly(dUz) solutions were exactly colinear.

CHROMATOGRAPHIC METHODS. Paper chromatography was carried out by the descending technique either with Whatman No. 1 or 3 paper in the following solvent systems: (A) 95% ethanol–1.0 M ammonium acetate (pH 7.5, 7:3, v/v) or (B) isobutyric acid–1.0 M ammonium hydroxide–0.2 M EDTA (100:60:0.8, v/v). For thin-layer chromatography (tlc) silica gel GF plates (5 × 20 mm, Analtech) were used and the following solvent systems: (C) ethyl acetate–acetone (1:1, v/v); (D) benzene–acetone (1:4, v/v); (E) 1-butanol–methanol–water–concentrated ammonium hydroxide (60:20:20:1, v/v).

Enzymes. Polynucleotide phosphorylase (from *Micrococcus luteus*, ATCC 4698, type 15) was purchased from P-L Biochemicals (Milwaukee, Wis.). Pancreatic ribonuclease A (RNase A), bacterial alkaline phosphatase, snake venom phosphodiesterase, bovine spleen phosphodiesterase, deoxyribonuclease I (DNase I), and deoxyribonuclease II (DNase II) were all products of Worthington Biochemicals. Micrococcal nuclease was a gift from the laboratory of C. B. Anfinsen.

Conditions Employed to Compare the Degradation of Poly(U) and Poly(dUz) by Various Degradative Enzymes and KOH. For each experiment 0.5–1.0 OD_{260} unit of polynucleotides was dissolved in 50–100 μl of the indicated buffer and incubated with the indicated concentration of appropriate enzyme for 24 hr at 37°. After incubation, the solutions were spotted on Whatman No. 1 paper with appropriate reference compounds and the chromatograms developed in solvent system A: KOH (0.3 M); 5 μl of RNase A (1 mg/ml)–0.01 M Tris (pH 8); 5 μl of DNase I (2500 units/mg, 5 mg/ml)–0.01 M Tris (pH 7.5)–5 mM $MgCl_2$; 5 μl of DNase II (12,000 units/mg; 20,000 units/ml)–0.3 M acetate (pH 5.5)–1 mM $CaCl_2$; 5 μl of micrococcal nuclease (5 mg/ml)–0.01 M Tris (pH 7.5)–2 mM $CaCl_2$; 5 μl of snake venom phosphodiesterase (5 mg/ml)–0.01 M Tris (pH 7.5); 5 μl of bovine spleen phosphodiesterase (16 units/ml)–0.3 M acetate (pH 5.5).

Polynucleotides. Poly(A) and poly(U) were purchased from P-L Biochemicals (Milwaukee, Wis.). Poly(A) had $s_{20,w}$ = 9.8 S and poly(U) had $s_{20,w}$ = 8.0.

Other Methodology. Inorganic phosphate was assayed by a modification of the method of Fiske and Subbarow (1925). Infrared spectra were determined on a Perkin-Elmer 237B as Nujol mulls, and proton magnetic resonance (pmr) spectra were determined on a Varian A-60 with tetramethylsilane as the internal standard. Pmr are reported in parts per million with reference to the δ system.

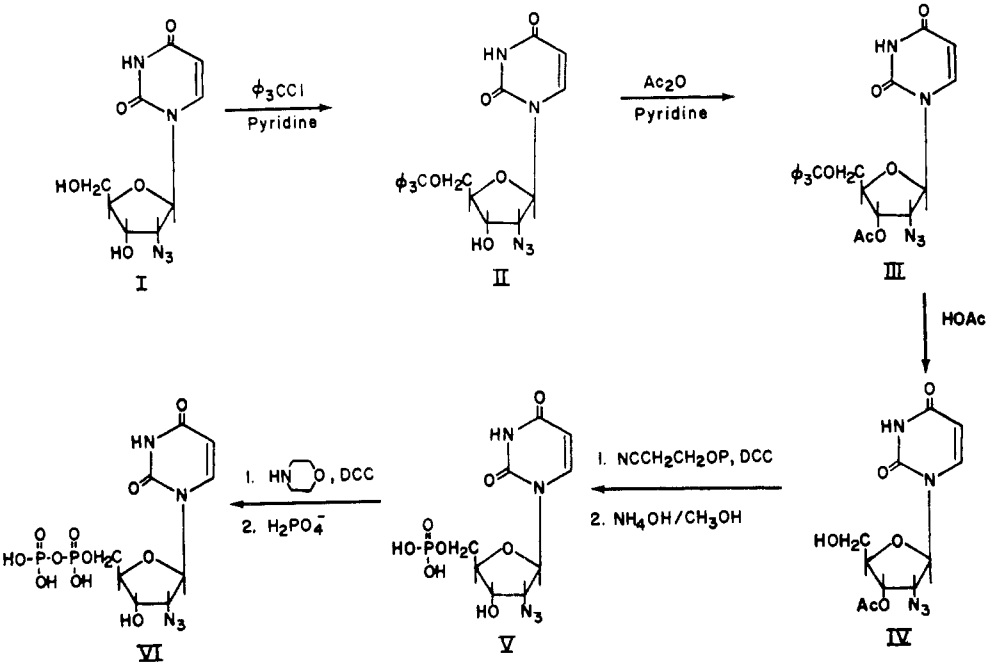
s_{20} values were determined with a Beckman Model E analytical ultracentrifuge using an AN-G titanium six-hole rotor and 0.1 M NaCl–0.001 M NaH_2PO_4–0.001 M EDTA (pH 6.5) buffer.

Results

Synthesis of 2′-Azido-2′-deoxyuridine 5′-Diphosphate (VI). 2′-Azido-2′-deoxyuridine (I) (Wagner *et al.*, 1971) was phosphorylated with β-cyanoethyl phosphate–dicyclohexylcarbodiimide after protection of the 3′-OH function in a manner similar to that employed by Tener (1961) for the synthesis of thymidine 5′-monophosphate (Scheme I). The β-cyanoethyl group was removed by warming the intermediate with NH_4OH in CH_3OH, since control experiments had indicated that dUz underwent limited decomposition under normal conditions for elimination of the cyanoethyl function (0.1–1.0 N NaOH, 90–100°), whereas there was no detectable decomposition when I was warmed (60–70°) with NH_4OH–CH_3OH (1:1). The morpholidate method (Moffatt and Khorana, 1961) was used to convert the monophosphate to the diphosphate. Recently an independent synthesis of the 5′-monophosphate was reported (Wagner *et al.*, 1972) by the above route which gave a product which corresponded well to that reported herein.

Synthesis of Poly(dUz). Polynucleotide phosphorylase (*M. luteus*) polymerized the diphosphate VI with either magnesium or manganese as metal ion cofactor, although use of Mn^{2+} seemed to result in a somewhat higher yield of polymer (40–50%) than that obtained with Mg^{2+} (30–40%). Poly(dUz) synthesized in this manner was of high molecular weight as indicated by an *s* value of 8.0 S. Degradation of the polymer by a mixture of bacterial alkaline phosphatase and snake venom phosphodiesterase gave dUz as the only detectable product by paper chromatography in several different sys-

SCHEME I



tems. Although this system may not be as sensitive to impurities as other methods of analysis (Hobbs *et al.*, 1972b), the diphosphate VI, in contrast to the less stable dUcl nucleotides, showed no evidence of any transformation (other than limited conversion to the monophosphate) in prolonged (24–48 hr) treatment at 37° in the buffer used for polymerization.

Degradation Experiments. Under conditions that lead to nearly instantaneous degradation of poly(U) to mononucleotide, poly(dUz) showed complete stability to both pancreatic ribonuclease A and 0.3 N KOH for periods of at least 24 hr. Under conditions which led to degradation of poly(U) to nucleosides or nucleotide, poly(dUz) was also degraded by snake venom phosphodiesterase, bovine spleen phosphodiesterase, and micrococcal nuclease. Neither poly(dUz) nor poly(U) was detectably degraded by deoxyribonuclease I. Deoxyribonuclease II acted upon poly(U) to give oligonucleotide fragments, but was without effect on poly(dUz).

Ultraviolet Spectral Characteristics of Poly(dUz). Poly(dUz) (Figure 1) showed λ_{max} 260 nm (ϵ 9430) compared with λ_{max} 262 nm (ϵ 10,000) for dUzMP and dUzDP. The blue shift (2 nm) and the polymerization hypochromicity (6%) are, therefore, about the same as that observed for poly(U). The extinction coefficient for poly(dUz) was not altered by changing the solvent from 0.01 M Mg^{2+} to 0.21 M Na^+. Poly(dUz) also showed a slight blue shift (<0.5 nm) of λ_{max} compared to poly(U) (not shown).

Ultraviolet Circular Dichroism Spectra. Figure 2A records

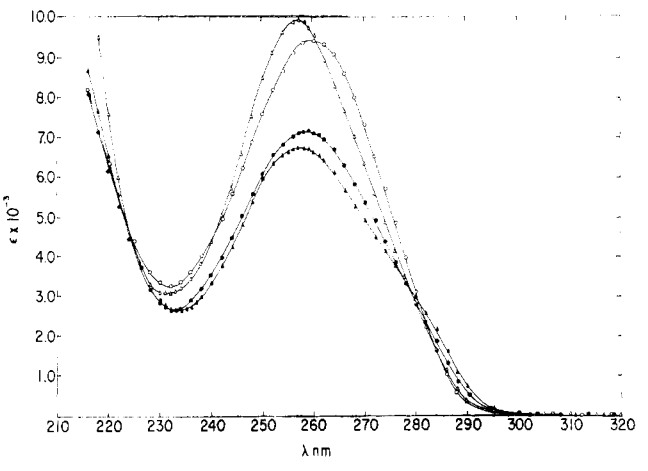


FIGURE 1: Ultraviolet spectra of the two-stranded and three-stranded helices at 25° in 0.2 M NaCl–0.01 M sodium cacodylate (pH 7.0) and of the homopolynucleotides from which they are formed: poly(A) (△—△), poly(dUz) (○—○), poly(A)·2poly(dUz) (●—●), and poly(dUz)·poly(A) (▲—▲).

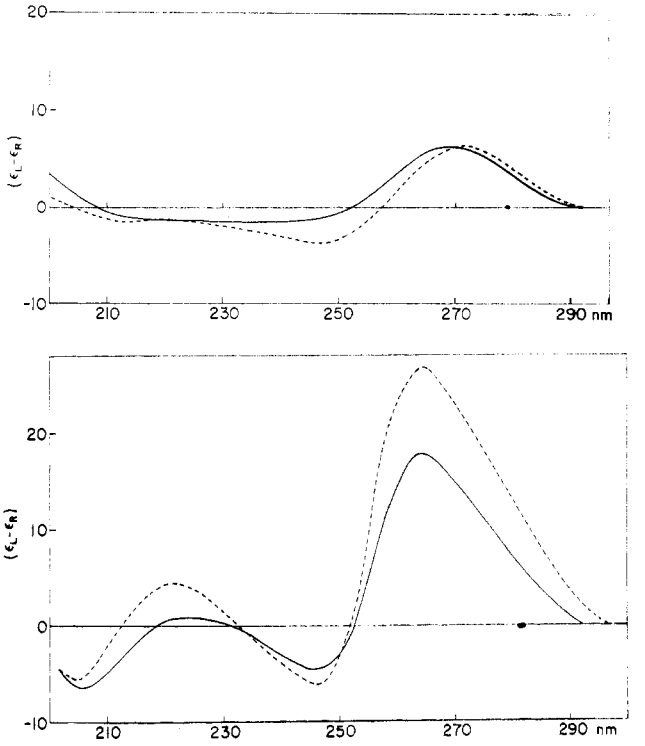


FIGURE 2: Ultraviolet circular dichroism spectra of poly(U) (- - -) and poly(dUz) (—) in 0.01 M $MgCl_2$–0.01 M KH_2PO_4 (pH 7.5). (A, top) T = 30°; (B, bottom) T = 2.5°.

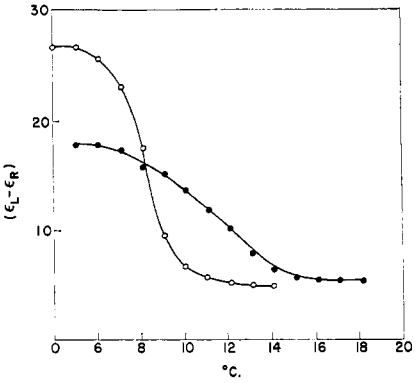


FIGURE 3: Melting profiles of poly(U) (○) and poly(dUz) (●) as monitored by ultraviolet circular dichroism: buffer, 0.01 M KH_2PO_4–0.01 M $MgCl_2$ (pH 7.5); wavelength, 264.5 nm.

the CD spectra of poly(U) and poly(dUz) at 30° in 0.01 M $MgCl_2$–0.01 M KH_2PO_4 (pH 7.5). No change in the spectra is observed if they are recorded at 23° (not shown). The long-wavelength CD band of poly(dUz) is shifted slightly to the blue as compared to poly(U), and its negative circular dichroism at the shorter wavelength is less intense. In Figure 2B, the CD of poly(U) and poly(dUz) is shown at 2.5° in the same buffer as above. Under these conditions the transition from the disordered to the ordered state is essentially complete as can be seen from the CD melting profiles in Figure 3. Although the line shape of the CD spectra is similar for both polymers, the ordered form of poly(dUz) exhibits less circular dichroism than poly(U). In both cases, however, the transition to the ordered forms is followed by a blue shift of the strong positive CD bands.

Ultraviolet CD–Temparature Profile for Poly(dUz). CD melting profiles monitored at 264.5 nm are presented for poly(U) and poly(dUz) in Figure 3. The buffer employed was the same as that used in determination of the CD spectra (0.01 M MgCl–0.01 M KH_2PO_4 (pH 7.5)). The T_m value of 8.2° for poly(U) is in good agreement with that of 8.5° obtained by uv absorbance–melting curves in the presence of Mg^{2+} (Swierkowski *et al.*, 1965; Szer and Shugar, 1962). The ordered form of poly(dUz) shows, however, a significantly higher T_m of about 11° in excellent agreement with uv absorbance–temperature profiles in 0.01 M Mg^{2+} (*vide infra*). In addition to the higher T_m, poly(dUz) also exhibits a less steep (or less cooperative) melting profile than poly(U) in the same solvent system.

Ultraviolet Absorbance–Temperature Profiles for Poly(dUz). Lipsett (1960) first observed the *helicogenic* property of low concentrations of Mg^{2+} on poly(U). When a solution of poly(dUz) in 0.01 M $MgCl_2$–0.001 M KH_2PO_4 (pH 7.6) was cooled at 0°, a significant decrease in the uv absorbance at 260 nm was observed. Figure 4A records the variance (at 260 nm) of the absorbance with temperature in the same buffer. Included for comparison is the melting profile for poly(U) under the same conditions. The transitions observed were completely reversible (not shown). The T_m (11.6°) for poly(dUz) is significantly elevated (3.6°) compared to the T_m (8.0°) for poly(U). The melting profile for poly(dUz) is somewhat less cooperative and its hyperchromicity on melting is smaller than that for poly(U). These T_m values agree well with those obtained by CD.

In order to reduce the possibility of changes in aggregation during the thermal transitions, the T_m values of poly(dUz) and poly(U) were determined in solvents in which no change in

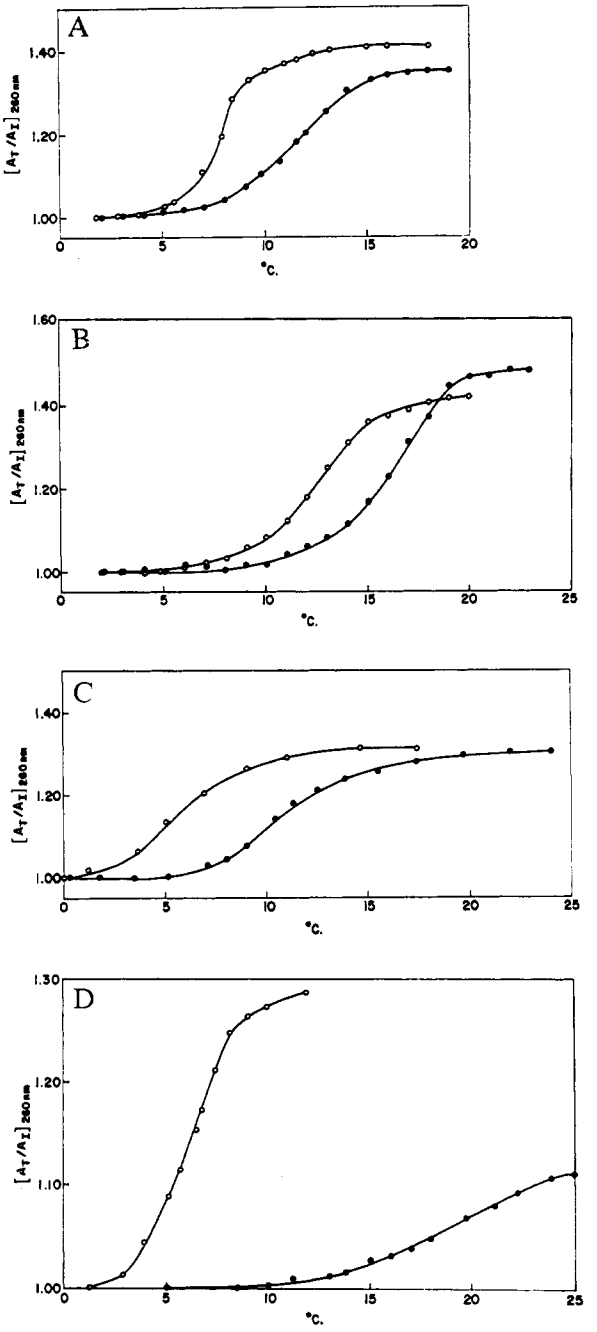


FIGURE 4: Ultraviolet absorbance–temperature profiles of poly(dUz) (○) and poly(U) (●) in four different solvents: (A) 0.01 M KH_2PO_4–0.01 M $MgCl_2$ (pH 7.5); poly(U), c 4.57 × 10^{-5} M; poly(dUz), c 4.77 × 10^{-5} M; (B) 0.5 M CsCl–0.01 sodium cacodylate–10^{-4} M EDTA (pH 6.6); poly(U), c 4.46 × 10^{-5} M; poly(dUz), c 4.03 × 10^{-5} M; (C) 1.0 M KCl; poly(U), c 7.6 × 10^{-5} M; poly(dUz), c 7.1 × 10^{-5} M; (D) 0.11 M $MgCl_2$ (pH 7.6); poly(U), c 7.0 × 10^{-5} M; poly(dUz), c 6.6 × 10^{-5} M. A_t/A_i is the ratio of absorbance at temperature T over the absorbance at the initial temperature. Method 1 was used for the curves in A and B, whereas method 2 was used for C and D.

apparent molecular weight occurs during the melting of poly(U). Figure 4B shows the melting profiles recorded in 0.5 M CsCl–0.01 M sodium cacodylate–10^{-4} M EDTA (pH 6.6). The value for poly(U), 12.5°, is of the same order as obtained by CD melting studies in this buffer (Thrierr and Leng, 1969; Thrierr *et al.*, 1971). Poly(dUz) has a T_m of 16.2° and a slightly higher melting hyperchromicity is observed compared with poly(U). In addition, the steepness of the melting profile is

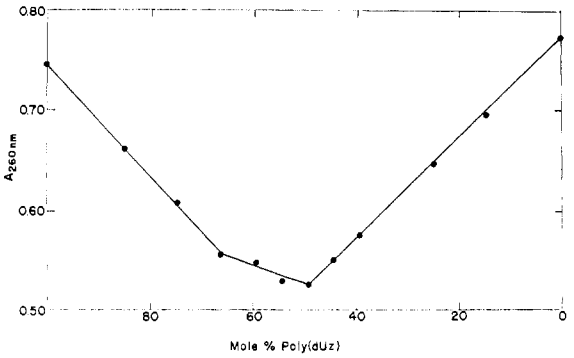


FIGURE 5: Ultraviolet mixing curve as determined at 260 nm for the interaction of poly(A) with poly(dUz) in 0.20 M NaCl–0.01 M sodium cacodylate (pH 7.0) at 24°.

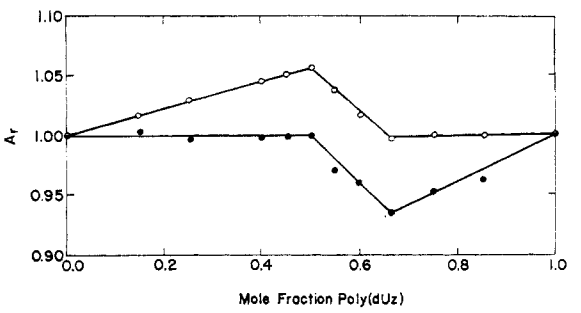


FIGURE 6: Ultraviolet mixing curve plotted in terms of relative molar absorbance ($A_r = \epsilon_{obsd}/(X_a\epsilon_a + X_z\epsilon_z)$, where ϵ_{obsd} is the experimentally determined extinction coefficient for the mixture, X_a and X_z are the mole fractions of the two components, and ϵ_a and ϵ_z are the extinction coefficients of the two components at the isochromic wavelength. The wavelength 281.3 nm (○) is selective for the two-stranded helix and 280.6 nm (●) is selective for the three-stranded helix.

now of the same order for both polymers. Figure 4C gives the melting profiles in 1.0 M KCl (Millar and Mackenzie, 1970). In this system, poly(U) has T_m of ~6.0° and poly(dUz) has a T_m of 10.6°. The T_m for poly(U) is ~1.0° lower than that found previously (Millar and Mackenzie, 1970), but this is likely due to differences in methodology. The melting hyperchromicity and the steepness of the curves in this buffer are nearly identical for both poly(U) and poly(dUz). A separate determination (not shown) of the T_m of poly(dUz) in 1.0 M NaCl gave a similar T_m (11°), but showed a reduced melting hyperchromicity (20–25%). What is most significant is the consistently higher T_m for poly(dUz) compared with poly(U) in the above three buffers. The difference in T_m values is the same (3.6°) in both Mg^{2+} and Cs^+ buffers. The difference increases somewhat (4–4.6°) in KCl, but this may be due to the fact that poly(U) has not reached a constant absorbance at 0° under these conditions.

The T_m values for poly(dUz) and poly(U) were also determined in 0.11 M $MgCl_2$ (Figure 4D) so that comparison could be made with poly(Um) (Zmudzka and Shugar, 1970). A striking change in the T_m (16–17°) of poly(dUz) was observed in this system, whereas poly(U) showed little difference from the T_m recorded in 0.01 M Mg^{2+}. In addition to a significantly elevated T_m, the poly(dUz) transition became less cooperative and considerably less hyperchromicity was apparent on melting. The absorbance of poly(dUz) did not change significantly above 25°.

Interaction of Poly(dUz) with Poly(A). Preliminary crude mixing curves of poly(dUz) with poly(A) in 0.1 M NaCl–0.01 M KH_2PO_4 (pH 7.0) indicated that the homopolymers could form a 1:1 complex, but these unrefined curves did not indicate the formation of any triple-stranded complex at several different wavelengths. In order to define this system quantitatively, a mixing curve was constructed in 0.2 M NaCl–0.01 M sodium cacodylate (pH 7.0), since the poly(A)·poly(U) system has been well characterized under these conditions and since the triply stranded complex, if it did exist, would be more stable under these conditions of higher ionic strength. Equimolar solutions of poly(dUz) and poly(A) were mixed in varying proportions in the above buffer and the spectra of the 0.50 and 0.67 mol fraction poly(dUz) solutions were determined at 30 min, 24 hr, and 64 hr after mixing. The absorbance (at 10-nm intervals) of the 67 mol % poly(dUz) mixture underwent little change from 30 min to 64 hr indicative of quick attainment of equilibrium. The 50 mol % mixture showed very small (<0.01 absorbance unit) changes at various wavelengths between 30 min and 24 hr, but reached equilibrium between 24 and 64 hr (<0.002 unit changes). Spectra for the entire mixing curve were recorded at 66 hr. The results (Figure 5) are given in the form of a classical mixing curve at λ 260 nm. The reasonably well-defined breaks at 0.67 and 0.50 mol fraction poly(dUz) indicated formation of poly(A)·2 poly(dUz) and poly(A)·poly(dUz) complexes, respectively.

A still more refined resolution of the composition of the above mixtures was obtained by determining the isochromic wavelengths (Blake *et al.*, 1967) for the double- and triple-stranded complexes in this buffer system. On the basis of the methodolody of Howard *et al.* (1971), 281.3 nm was determined as the isochromic wavelength for the triple-stranded complex and 280.6 nm as the isochromic wavelength for the double-stranded complex. In Figure 6, the same mixing curves presented in Figure 5 are given, except that the results are plotted in terms of relative molar absorbance at the isochromic wavelengths for both complexes. The end points are well defined and occur at ratios which are stoichiometric for each complex. The results are internally consistent: where poly(A)·poly(dUz) reaches a maximum, there is no poly(A)·2-poly(dUz), and *vice versa*. Furthermore, as the complex of poly(A)·poly(dUz) decreases, there is a parallel increase in the complex of poly(A)·2poly(dUz). The formation of both complexes at their stoichiometric end points may be virtually quantitative.

The ultraviolet spectra of the poly(A)·2poly(dUz) and the poly(A)·poly(dUz) complexes as well as the constituent homopolymers are recorded in Figure 1.

Course of the Thermal Dissociation of the Complexes. Uv absorbance–temperature profiles were determined for equilibrium mixtures of 0.05 mol fraction poly(dUz) and 0.67 mol fraction poly(dUz) (Figures 7A and B). The 1:1 complex showed but one transition at all wavelengths (290–255 nm at 1-nm intervals) examined and had a T_m of 59°. The presence of but one transition in the 0.5 mol fraction mixture must represent the 2 → 1 transition; in contrast to the poly(A)·poly(U) (Blake *et al.*, 1967; Stevens and Felsenfeld, 1964) and poly(A)·poly(rT) (Howard *et al.*, 1971), no 2 → 3 transition occurs at [Na^+] of 0.21 M. The double-helical structure appears to melt out directly to constituent homopolymers rather than *via* rearrangement to the triple-stranded complex.

The 2:1 complex also showed a monophasic transition with T_m = 59°. No other transitions were observable at all wavelengths examined (290–255 nm at 1-nm intervals). The triply stranded poly(A)·2poly(dUz) complex apparently melts out directly to constituent homopolymers, *i.e.*, a 3 → 1 transition, with no prior rearrangement of a 3 → 2 transition. Poly(A)·

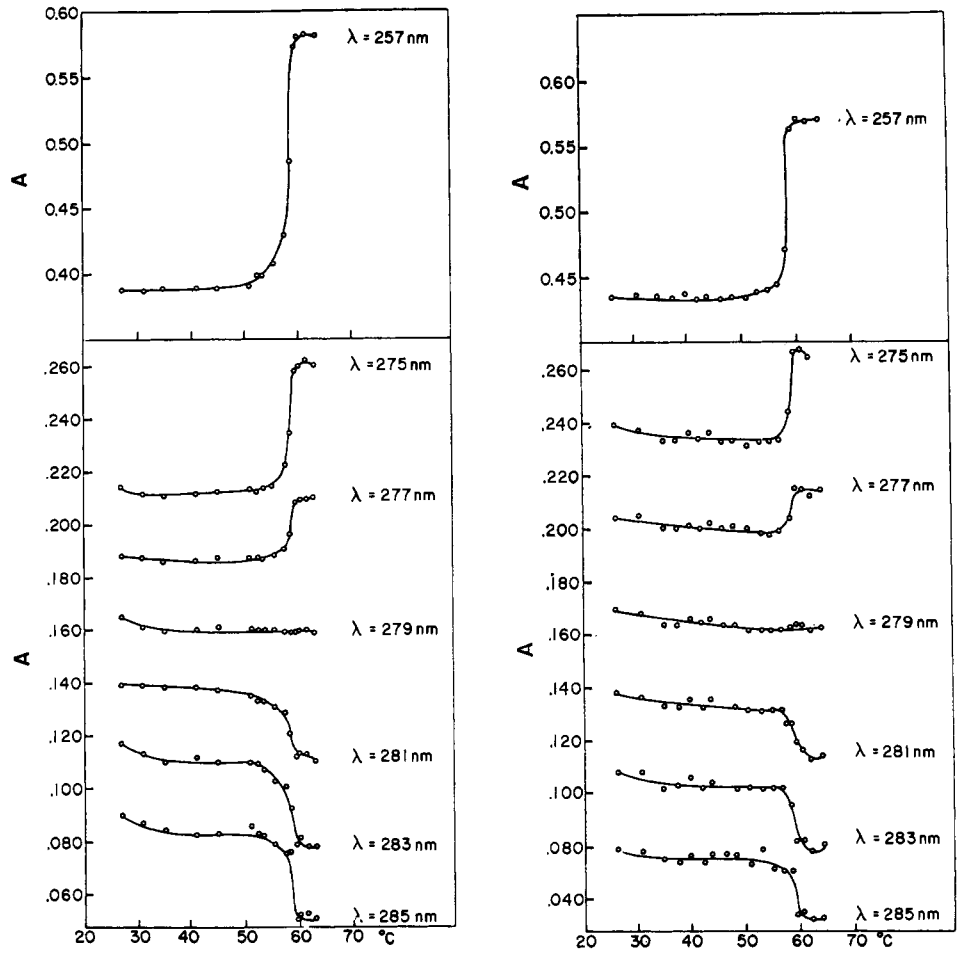


FIGURE 7: Ultraviolet melting curves of (A, left) 1:1 molar mixture and (B, right) 1:2 molar mixture of poly(A) and poly(dUz), respectively. The solvent in both cases was 0.2 M NaCl–0.01 M sodium cacodylate (pH 7.0).

poly(rT) (Howard *et al.*, 1971) also undergoes the 3 → 1 transition with no strandwise (3 → 2) dissociation evident for a [Na^+] of 0.03–0.7 M. Poly(A)·2poly(U) (Blake *et al.*, 1967; Stevens and Felsenfeld, 1964) does undergo strandwise dissociation (3 → 2 → 1) but only at low ionic strength (0.1 M Na^+ and below). At 0.20 M Na^+ and above only the 3 → 1 transition is observed.

The apparent coincidence of the T_m values (59°) for both the 2 → 1 and 3 → 1 transitions need not in itself be considered surprising in that in a given range of salt concentrations, the T_m values of the double- and triple-stranded forms in the A-U system (Blake *et al.*, 1967; Stevens and Felsenfeld, 1964) and the A-rT system (Howard *et al.*, 1971) are also nearly the same. Nonetheless, other avenues have been explored in order to verify the above findings. One possibility was that the system was not at equilibrium when the mixing curve was determined. This seems unlikely because the isochromic wavelength mixing curves show the sharp breaks and are internally consistent. This would not be the case if equilibrium had not been attained. Since the T_m values were determined in solutions (stored in buffer at 0°) approximately 6 weeks after mixing, there is little chance that they do not represent true equilibrium situations. Finally, 3 months after the original mixing curve had been obtained, the spectra of the various mixtures were again determined. While slight evaporation had apparently occurred (as indicated by the slight increase in absorbance of the 100 mol % solutions), the basic shape of the mixing curve at 260 nm was unchanged indicative of equilibrium of the system at the time of collection of the original data.

After it was ascertained that the transitions for the poly(A)·poly(dUz) and poly(A)·2poly(dUz) complexes were 2 → 1 and 3 → 1, respectively, the variation with salt of the T_m of the 1:1 complex was studied in the expectation that at higher salt concentration, the 2 → 3 transition might be observed. While a positive dependence on salt concentration was observed for the T_m of the 1:1 complex, no evidence for the 2 → 3 transition could be found up to 0.7 M NaCl.

In order to compare the T_m of poly(dUz)·poly(A) with poly(A)·poly(U), the T_m values of 0.50 mol fraction mixtures for both complexes were determined in 0.11 M NaCl. Under these conditions, poly(dUz)·poly(A) showed a monophasic transition with T_m = 58° and poly(A)·poly(U) had T_m = 56° (Stevens and Felsenfeld, 1964).

Discussion

Physicochemical Properties of Poly(dUz). It has become abundantly evident that the presence, absence, or modification of the 2'-hydroxyl function of poly- and oligoribonucleotides results in significant differences in the conformation and relative stabilities of ordered structures of such nucleic acids; *e.g.*, oligo- and polydeoxyribonucleotides exhibit greatly reduced rotatory powers compared to their ribo counterparts (Ts'o *et al.*, 1966; Adler *et al.*, 1967; Vournakis *et al.*, 1967; Brahms and Sadron, 1966); 2',5'-phosphodiester linkages in

ribonucleotides bring about a decrease in secondary structure (Adler *et al.*, 1968; Brahms *et al.*, 1967a,b; Maurizot *et al.*, 1969); oligoarabinonucleotides show less ordered structure than oligo-, ribo-, or even oligodeoxyribonucleotides (Adler *et al.*, 1968; Maurizot *et al.*, 1968). Generally, double-stranded homopolymer pairs seem to follow a trend that the all-ribose duplexes have a higher T_m than the all-deoxyribose duplexes as well as the hybrid duplexes (Chamberlin, 1965; Chamberlin and Patterson, 1965; Riley *et al.*, 1966; Barszcz and Shugar, 1968); replacement of the 2′-OH of poly(uridylic acid) by hydrogen (Zmudzka and Shugar, 1970), fluorine (Janik *et al.*, 1972), chlorine (Hobbs *et al.*, 1971), or amino (Hobbs *et al.*, 1972a) leads to polynucleotides which are incapable of forming ordered secondary structures down to 0°, whereas replacement of the 2′-OH by methoxy (Zmudzka and Shugar, 1970) leads to a polynucleotide with a T_m equal to or surpassing (in certain buffers) that of poly(U).

The possibility that the 2′-OH contributes structure and stability to ordered conformations of nucleic acids by means of several different types of hydrogen bonding (Ts'o *et al.*, 1966; Brahms *et al.*, 1967b; Maurizot *et al.*, 1969; Rabczenko and Shugar, 1972) has been largely excluded by the results of studies on modified oligo- and polynucleotides (Adler *et al.*, 1968; Zmudzka and Shugar, 1970; Bobst *et al.*, 1969a–c) and by the finding that the unperturbed coil dimensions of apurinic acid at high temperature are the same as the dimensions for poly(U) and poly(A) (Achter and Felsenfeld, 1971). More recently, the hypothesis has been advanced that steric factors involving the 2′-OH group may account for some of the observed differences between ribo- and deoxyribonucleotide polymers (Khurshid *et al.*, 1972; Cross and Crothers, 1971; Kondo *et al.*, 1972).

It is generally agreed that poly(U) is essentially a random coil at room temperature (Inners and Felsenfeld, 1970, and references cited therein). Simpkins and Richards (1967) reached this conclusion on the basis of the Cotton effect in mono-, oligo-, and poly(uridylic acids) at intermediate ionic strength. At high ionic strength, some base stacking in poly(U) may occur even at room temperature (Michelson and Monny, 1966). CD studies on the dinucleotide UpU in 4.7 M KF appear to show appreciable stacking even above room temperature (Brahms *et al.*, 1967b). It is possible that such observations may rather be due to restrictions in backbone rotations (Achter and Felsenfeld, 1971). For the comparative CD study on poly(U) and poly(dUz), however, the ionic strength conditions are such that poly(U) at least should behave like a random coil at room temperature. The close resemblance of the CD of poly(U) and poly(dUz) (Figure 2A) makes it reasonable to assume that poly(dUz) shows a similar degree of disorder as poly(U) at or above room temperature. The small differences in the CD spectra might be caused by small differences in the puckering of the ribose ring (C-3 endo, C-2 endo, and intermediate conformation), and/or the geometry of the base relative to the sugar (syn, anti, or intermediate rotamers) due to the presence of the 2′-azido group.

Figure 2B gives the CD spectra of the two polymers in 0.01 M $MgCl_2$ at 2.5° where the transition to the ordered form is nearly complete as the uv and CD melting profiles indicate. The transition to the ordered form is in both cases accompanied by a blue shift of the strong positive CD bands, an indication of the formation of base pairs. It thus seems likely that, as for poly(U), the formation of the ordered structure of poly(dUz) consists of double strands paired by hydrogen bonding. At least two explanations for the difference in intensity of the CD bands can be considered. It is possible that poly(dUz) binds Mg^{2+} less strongly than poly(U). It was recently shown that the CD spectrum of poly(U) is strongly dependent on Mg^{2+} concentration (Carroll, 1972). At a Mg^{2+} concentration equal to or greater than 10^{-2} M no significant changes in $\epsilon_L - \epsilon_R$ at 265 nm occurred, whereas at lower $[Mg^{2+}]$ the intensity of the CD bands became smaller. The decrease in intensity was interpreted as an incompleteness of the conversion of the disordered poly(U) to the ordered form. Should poly(dUz) bind Mg^{2+} less strongly than poly(U), its smaller CD at 2.5° reflects possibly less conversion to the ordered form in 0.01 M Mg^{2+}. Experiments to clarify this point have not been possible with the limited amounts of poly(dUz) presently available. Another possible explanation for the difference in intensity of the CD bands of the two polymers might be found in the existence of subtle changes with respect to the geometries of the ordered structures. It was, for instance, shown that the long-wavelength CD band of uridine can change its sign from positive to negative, if the base–sugar conformation changes from anti to syn (Teng *et al.*, 1971).

The T_m of the transition (observed by uv or CD) from the ordered to the disordered structure of poly(dUz) is 3–4° higher than the corresponding T_m for poly(U) (Figures 3 and 4). This relationship is valid in at least four different buffer systems, *i.e.*, 0.01 M Mg^{2+}, 1 M NaCl, 1 M KCl, and 0.5 M CsCl. In the latter two systems in which poly(U) is observed not to undergo any change in molecular weight (Thrierr and Leng, 1969; Millar and Mackenzie, 1970), the steepness (or cooperativity) and the hyperchromicity changes are approximately the same for both polymers. In 0.1 M Mg^{2+}, a significant increase in the T_m of poly(dUz) occurs, but it is difficult to draw conclusions from this effect, since the cooperativity and melting hyperchromicity have changed considerably. This high concentration of Mg^{2+} may lead to aggregation and thus confuse the monomolecular transition.

That poly(dUz), in analogy to poly(U), forms an ordered structure with intrastrand base pairing (indicated by the blue shift of the strong positive CD band during the thermal transition) may be a valid conclusion; however, the nature of this structure is not known although it probably resembles the proposed structure of poly(U), a single hairpin (antiparallel helix) (Thrierr *et al.*, 1971). Although hydrogen bonds are almost certainly formed in both ordered structures, the question of which atoms are involved and in what manner is still unanswered. What seems to be clear is that neither the 2′-OH function nor the 2′-oxygen atom are necessary for the formation of ordered structure, at least in the case of poly(U).

Interaction of Poly(dUz) with Poly(A). The usefulness of any modified polynucleotide duplex in determining the effect of that same specific structural feature in any physicochemical or biological system requires first the determination of the stoichiometry of the interaction and secondly the conditions under which the possible stoichiometric complexes exist and interconvert. The use of isochromic wavelengths in the construction of mixing curves leads to the conclusion that poly(dUz) can form both double- and triple-stranded complexes with poly(A). It appears that in the case of both complexes, melting proceeds directly to the constituent homopolymers ($3 \rightarrow 1$ and $2 \rightarrow 1$) with no evidence for $3 \rightarrow 2$ or $2 \rightarrow 3$ interconversions. On the assumption that the transition observed for the 50 mol % poly(dUz) mixture is $2 \rightarrow 1$, then the conclusion can be drawn that the replacement of the 2′-OH by 2′-azido in the uridylic acid strand of poly(U)·poly(A) does not significantly affect the thermal stability of the double-stranded complex, whereas the stability of the triple-stranded complex

(poly(A)·2poly(dUz), T_m = 59°) is slightly less compared to poly(A)·2poly(U) (T_m = 62° (0.2 M NaCl)).

Conclusions

Poly(dUz) can be effectively used as a probe for polynucleotide structure in biological systems. For instance, we have recently found that poly(dUz)·poly(A) is a much less effective interferon inducer than poly(A)·poly(U) or poly(I)·poly(C), indicative of the possible importance of the intact 2′-OH group in the induction process. Since the azido group possesses an infrared absorption at around 2120 cm^{-1}, it may prove possible to use it as a probe for monitoring a helix–coil transition at a site remote from the bases involved in hydrogen bonding. Introduction of the azido group into other polynucleotide systems (*e.g.*, poly(A)) is of interest with respect to the general role of the 2′-OH group in polynucleotide secondary structure and also because the azido group can be regarded as a pseudohalogen possessing many properties similar to bromine (Treinen, 1971). The ability to introduce a large polarizable group, such as azido, becomes especially important when it is realized that the synthesis of 2′-bromo- or 2′-iodo-substituted polynucleotides may be a difficult task because of the strong tendency toward intramolecular reaction (*e.g.*, arabinoside formation (Codington *et al.*, 1964)).

Acknowledgments

The authors are grateful to Dr. J. G. Moffatt for an authentic sample of I, to Dr. C. B. Klee for her assistance in determining sedimentation constants, and to Drs. H. T. Miles and F. B. Howard for their constructive criticisms and helpful suggestions.

References

Achter, E. K., and Felsenfeld, G. (1971), *Biopolymers 10*, 1625.

Adler, A., Grossman, L., and Fasman, G. D. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 423.

Adler, A., Grossman, L., and Fasman, G. D. (1968), *Biochemistry 7*, 3836.

Babinet, C., Roller, A., Duberg, J. M., Thang, M. N., and Grunberg-Manago, M. (1965), *Biochem. Biophys. Res. Commun. 19*, 95.

Barszcz, D., and Shugar, D. (1968), *Eur. J. Biochem. 5*, 91.

Blake, R. D., Massoulie, J., and Fresco, J. R. (1967), *J. Mol. Biol. 30*, 291.

Bobst, A. M., Cerutti, P. A., and Rottman, F. (1969a), *J. Amer. Chem. Soc. 91*, 1246.

Bobst, A. M., Rottman, F., and Cerutti, P. A. (1969b), *J. Amer. Chem. Soc. 91*, 4603.

Bobst, A. M., Rottman, F., and Cerutti, P. A. (1969c), *J. Mol. Biol. 46*, 221.

Brahms, J., Maurizot, J. C., and Michelson, A. M. (1967a), *J. Mol. Biol. 25*, 465.

Brahms, J., Maurizot, J. C., and Michelson, A. M. (1967b), *J. Mol. Biol. 25*, 481.

Brahms, J., and Sadron, C. (1966), *Nature (London) 212*, 1309.

Brentnall, H. J., and Hutchinson, D. W. (1972), *Tetrahedron Lett.*, 2595.

Carroll, D. (1972), *Biochemistry 11*, 426.

Chamberlin, M. J. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24*, 1446.

Chamberlin, M. J., and Patterson, D. (1965), *J. Mol. Biol. 12*, 410.

Chou, J. Y., and Singer, M. F. (1971), *Biochem. Biophys. Res. Commun. 42*, 306.

Codington, J. F., Doerr, I. L., and Fox, J. J. (1964), *J. Org. Chem. 29*, 558.

Colby, C. (1971), *Progr. Nucl. Acid Res. Mol. Biol. 11*, 1,

Cross, A. D., and Crothers, D. M. (1971), *Biochemistry 10*, 4015.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Grunberg-Manago, M. (1963), *Progr. Nucl. Acid Res. Mol. Biol. 1*, 93.

Hendler, S. S., Shannahoff, D. H., and Sanchez, R. A. (1971), Abstracts, 162nd National Meeting of the American Chemical Society, Washington, D. C., Sept 1971, No. BIOL-20.

Hobbs, J., Sternbach, H., and Eckstein, F. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 15*, 345.

Hobbs, J., Sternbach, H., and Eckstein, F. (1972a), *Biochem. Biophys. Res. Commun. 46*, 1509.

Hobbs, J., Sternbach, H., Sprinzl, M., and Eckstein, F. (1972b), *Biochemistry 11*, 4336.

Howard, F. B., Frazier, J., and Miles, H. T. (1971), *J. Biol. Chem. 246*, 7073.

Inners, L. D., and Felsenfeld, G. (1970), *J. Mol. Biol. 50*, 373.

Janik, B., Kotick, M. P., Kreiser, T. H., Reverman, L. F., Sommer, R. G., and Wilson, D. P. (1972), *Biochem. Biophys. Res. Commun. 46*, 1153.

Khurshid, M., Khan, A., and Rottman, F. M. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 28*, 25.

Kleinschmidt, W. J. (1972), *Annu. Rev. Biochem.*, 517.

Kondo, N. S., Fang, F. N., Miller, P. S., and Ts'o, P. O. P. (1972), *Biochemistry 11*, 1991.

Lipsett, M. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 455.

Mackey, J. K., and Gilham, P. T. (1971), *Nature (London) 233*, 551.

Maurizot, J. C., Brahms, J., and Eckstein, F. (1969), *Nature (London) 222*, 559.

Maurizot, J. C., Wechter, W. J., Brahms, J., and Sadron, C. (1968), *Nature (London) 219*, 377.

Michelson, A. M., and Monny, C. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1528.

Millar, D. B., and Mackenzie, M. (1970), *Biochim. Biophys. Acta 204*, 82.

Moffatt, J. G., and Khorana, H. G. (1961), *J. Amer. Chem. Soc. 83*, 649.

Rabczenko, A., and Shugar, D. (1972), *Acta Biochim. Pol. 19*, 89.

Riley, M., Maling, B., and Chamberlin, M. J. (1966), *J. Mol. Biol. 20*, 359.

Rottman, F., and Heinlein, K. (1968), *Biochemistry 7*, 2634.

Simpkins, H., and Richards, E. G. (1967), *J. Mol. Biol. 29*, 349.

Stevens, G., and Felsenfeld, G. (1964), *Biopolymers 2*, 293.

Swierkowski, M., Szer, W., and Shugar, D. (1965), *Biochim. Z. 342*, 429.

Szer, W., and Shugar, D. (1962), *J. Mol. Biol. 5*, 580.

Tazawa, I., Tazawa, S., Alderfer, J. L., and Ts'o, P. O. P. (1972), *Biochemistry 11*, 4931.

Tener, G. M. (1961), *J. Amer. Chem. Soc. 83*, 159.

Teng, N. N. H., Itzkowitz, M. S., and Tinico, I., Jr. (1971), *J. Amer. Chem. Soc. 93*, 6258.

Thang, M. N., Graffe, M., and Grunberg-Manago, M. (1965), *Biochim. Biophys. Acta 108*, 125.

Thrierr, J. C., Dourlent, M., and Leng, M. (1971), *J. Mol. Biol. 58*, 815.

Thrierr, J. C., and Leng, M. (1969), *Biochim. Biophys. Acta*

182, 577.
Torrence, P. F., Waters, J. A., and Witkop, B. (1972), *J. Amer. Chem. Soc. 94*, 3638.
Torrence, P. F., and Witkop, B. (1972), *Biochemistry 11*, 1737.
Treinen, A. (1971), *in* The Chemistry of the Azido Group, Patai, S., Ed., New York, N. Y., Interscience, pp 1–55.
Ts'o, P. O. P., Rapaport, S. A., and Bollum, F. J. (1966), *Biochemistry 5*, 4153.
Vournakis, J. N., Poland, D., and Scheraga, H. A. (1967), *Biopolymers 5*, 403.
Wagner, D., Verheyden, J. P. H., and Moffatt, J. G. (1971), *J. Org. Chem. 36*, 250.
Wagner, D., Verheyden, J. P. H., and Moffatt, J. G. (1972), *J. Org. Chem. 37*, 1876.
Zmudzka, B., Bollum, F. J., and Shugar, D. (1969a), *J. Mol. Biol. 46*, 169.
Zmudzka, B., Janion, C., and Shugar, D. (1969b), *Biochem. Biophys. Res. Commun. 6*, 895.
Zmudzka, B., and Shugar, D. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 8*, 52.

Fragments Produced by Cleavage of λ Deoxyribonucleic Acid with the *Hemophilus parainfluenzae* Restriction Enzyme Hpa II†

Bernard Allet

ABSTRACT: One of the restricting enzymes extracted from *Hemophilus parainfluenzae*, Hpa II, is shown to cleave λ DNA at more than 50 sites. The resulting DNA fragments have been prepared from a variety of deletion or deletion–substitution mutants of λ, and analyzed by polyacrylamide gel electrophoresis. The major DNA segments (larger than 0.3 Mdalton) produced by digestion of λ DNA have been mapped and many of the cleavage sites in the immunity region, in the *b*2 region, and to the right of the immunity region have been identified.

Endonucleases are known that cleave DNA at specific sites, chiefly the so-called restriction enzymes from bacteria (Arber, 1971). Since it was realized that they can be used *in vitro* to prepare specific DNA fragments (Danna and Nathans, 1971), many investigators have devoted much work to characterizing these enzymes and to discovering new ones. When cleaved in favorable substrates, the fragments can be identified genetically with considerable precision (Danna *et al.*, 1973) and should therefore be useful for analysis of DNA functions.

In a previous report we described the cleavage of linear λ DNA with the endonuclease R·RI and the isolation and characterization of the six DNA fragments that were produced (Allet *et al.*, 1973a). Because one of these DNA fragments contained the early promoters, p_R and p_L, as well as the binding sites for repressor, the operators o_R and o_L, it seems that endonuclease R·RI does not cut the immunity region. In this paper I report the analysis of fragments produced by cleavage of λ DNA with one of the restriction enzymes isolated from *Hemophilus parainfluenzae* (Hpa II, which introduces one cut into SV40 DNA) (Sharp *et al.*, 1973). The *Hemophilus* enzyme cuts the immunity region in at least two places, permitting separation of p_L and o_L from p_R and o_R.

To map the DNA fragments that were produced, I compared by gel electrophoresis the pattern of the pieces cleaved from λ (λ*c*I857, S7) with those from a variety of deletion and substitution derivatives of λ. It is assumed throughout this work that all phage strains used were isogenic except for the indicated mutations. This implies that if a DNA fragment from a mutant has the same electrophoretic mobility as a fragment from the control λ, then both fragments are derived from an identical region of the λ genome. In the case of deletion–substitution mutants, it is further assumed that electrophoretic mobility alone is sufficient to identify a fragment. It is clear that if either assumption is invalid, then analysis of a number of mutants should quickly lead to an inconsistent interpretation. In fact, the internal consistency of the data presented in this paper entirely justifies both assumptions. Although the technique and the mutants used permit one to analyze many different regions of λ DNA, in this paper I have focused attention mainly on the immunity region, the *b*2 region, and the late promoter (p'_R) region.

Materials and Methods

Propagation of Phages and Extraction of DNA. The phage strains λ*b*511, λ*b*519, λ*bio*16A, λ*bio*7-20, λ*bio*69, λ*bio*10, λ*bio*256, λ*bio*3h-1 *nin*5, λ*bio*24-5 *nin*5, λ*bio*30-7 *nin*5, λ*bio*t124 *nin*5, λ*bio dc*30-7 *nin*5, λ*b*2 P4, φ80-λi^{λ}, φ80λi^{434}, and φ80λi^{21} were a kind gift from F. Blattner and W. Szybalski. The phages were propagated and the DNA was extracted by standard methods (Bovre and Szybalski, 1971) with minor modifications described previously (Allet *et al.*, 1973b).

Digestion of DNA with Hpa II (and R·RI) Endonucleases. The endonuclease Hpa II was prepared from *H. parainfluenzae* by Joe Sambrook (Sharp *et al.*, 1973). Samples of DNA (12–15 μg) were digested with the enzyme (50 μl) in a 400-μl reaction mixture containing 6.6 mM Tris (pH 7.5), 6.6 mM $MgCl_2$, and 6.6 mM β-mercaptoethanol (Calbiochem) for 10–15 hr at 37°. Addition of more enzyme or extension of the incubation time did not alter the pattern of cleavage, an indication that the reaction goes to completion. The DNA fragments were precipitated with 4–5 ml of ethanol (−20° for 3–4 hr) and collected by centrifugation for 20 min at 45,000 rpm in a Spinco SW50.1 rotor. The pellet was dried under vacuum and

† From the Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724. *Received May 29, 1973.* Supported by Grant CA 13106 from the National Cancer Institute, U. S. Public Health Service, and a grant to R. Gesteland from the National Science Foundation.