Enzymatic Synthesis of Polynucleotides Containing 5,6-Methylene- and 5,6-Dihydropyrimidines†

Paul F. Torrence‡ and Bernhard Witkop*

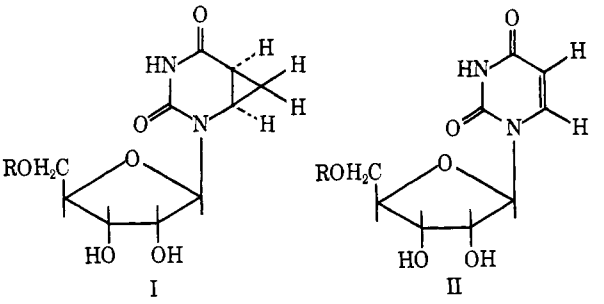
ABSTRACT: 1-(β-D-Ribofuranosyl)-(α+β)5,6-methyleneuracil 5′-diphosphate (Ib), a bicyclic isomer of 5-methyluridine, was synthesized and investigated as a substrate for polynucleotide phosphorylase from *Micrococcus luteus*. In the same way as dihydrouridine 5′-diphosphate (IIIb), Ib could not be converted directly into a homopolymer by the enzyme. However, both Ib and IIIb could be copolymerized with ADP, IDP, CDP, or UDP into polymers which contained 5–40 residues of Ib or IIIb per 100 [of the normal] nucleotide units and were shown to be distributed internally in the polynucleotide chain. The ratios obtained depended on the relative quantities of Ib or IIIb *vs.* the normal nucleotide in the incubation medium but, more significantly, on the use of magnesium or manganese ion as metal cofactor. In all cases, IIIb was more efficiently incorporated into the polymers than was Ib. These results are discussed in terms of known conformations of various nucleotides and in terms of the substrate specificity of polynucleotide phosphorylase. These experiments provide a useful synthetic access to the preparation of dihydrouridine copolymers as well as copolymers of other odd nucleotides.

In electronic and spectral terms the cyclopropane ring may be regarded as an intermediate between a carbon–carbon single and double bond; *e.g.*, the bond length of cyclopropane (Bastiansen *et al.*, 1964) bond angles of vicinal hydrogens (Bastiansen *et al.*, 1964) and CH hybridization (Wiberg, 1964) lie between the corresponding values of ethane and ethylene. Electronically, such an intermediacy is demonstrated by its ability to stabilize a photoexcited state (Yoshida and Ogoski, 1970) or an incipient carbonium ion (Deno *et al.*, 1965; Brown and Cleveland, 1966). In a purely steric sense this comparison fails, since the methylene group adds considerable bulk to the corresponding ethane or ethylene. Thus, investigation of the relative activities of substrates with ethane, ethylene, and cyclopropane unit variations in a dynamic enzymic system might permit an assessment of the relative importance of steric *vs.* electronic factors.

The recent synthesis (Kunieda and Witkop, 1971) of 1-(β-D-ribofuranosyl)-α(or β)-5,6-methyleneuracil ("cyclo-5-methyluridine," I) permits a test of this approach in the field of nucleic acids, since I can be considered an intermediate between uridine (II), 5-methyluridine, and their dihydro derivatives. The cyclopropane ring contributes to the aromaticity which shows up in the ultraviolet spectrum of I (λ_{max} 245 nm, shoulder), intermediate between II(λ_{max} 260 nm) and III (λ_{max} 206 nm). If electronic factors, *i.e.*, aromaticity, were overriding in determining the ability of I, II, and III to serve as substrates, the order should be II>I>III. If steric factors involving the 5,6 bond of uridine were important, then the order should be II>III>I.

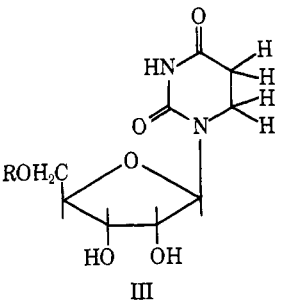
Initial results with compound I in biological systems have been discouraging. I does not arrest the growth of HeLa and L5178Y cells nor production of vaccinia virus in HeLa cells when tested at 10^{-4} M (C. Heidelberger, personal communication), is inactive against Vaccinia, Polio III, Herpes, Coe, Adeno, Rhino (two strains), Semlik Forest, and A-type influenza (K. Gerzon, personal communication), and is phosphorylated neither by rat liver uridine kinase (Torrence and Witkop[1]) nor by uridine kinase from Ehrlich ascites cells (G. A. LePage, personal communication). It is inactive in systems such as DNA polymerase and thymidylate synthetase (G. A. LePage, personal communication).

Because polynucleotide phosphorylase (nucleoside diphosphate:polynucleotide nucleotidyltransferase, EC 2.7.7.8)[2] has a relatively wide substrate specificity (Grunberg-Manago, 1963) and because of the interest in the mechanism



I

II



III

I, II, III, R = H
Ia, IIa, IIIa, R = P(O)(OH)$_2$
Ib, IIb, IIIb, R = P(O)(OH)OP(O)(OH)$_2$

† From the Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received January 24, 1972.*

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

[1] P. F. Torrence and B. Witkop, unpublished observations.

[2] Abbreviations used are those listed in *Biochemistry 5*, 1445 (1966), and *J. Mol. Biol. 55*, 299 (1971); D stands for dihydrouridine, I is represented as ΔU.

of action of this enzyme (Kapuler *et al.*, 1970) as well as in modified polynucleotides (Shen, 1970) the ability of the 5′-diphosphates of I and III to serve as substrates for this enzyme has been investigated in some detail.

Materials and Methods

Enzymes. Polynucleotide phosphorylase (PNPase, form I) was a gift of Dr. Maxine Singer and had been purified from *Micrococcus luteus* (formerly *lysodeikticus*, ATCC 4698) through the hydroxylapatite column step (Klee, 1969). Later experiments, in which large quantities of the polynucleotides were synthesized, made use of PNPase (type 15) purchased from P-L Biochemicals (Milwaukee, Wis.). Pancreatic ribonuclease, snake venom phosphodiesterase, and bacterial alkaline phosphatase were products of Worthington Biochemicals.

Other Compounds. Oligonucleotide primers, of the general formula $(Ap)_nA$, were purchased from Miles Laboratories (Elkhart, Ind.). The nucleoside diphosphates, ADP, CDP, IDP, and UDP, were purchased from P-L Biochemicals. [5-^{3}H]Cytidine diphosphate was purchased from Amersham-Searle (Chicago, Ill.) and sodium borotritide from New England Nuclear (Boston, Mass.).

Chromatographic Methods. Paper chromatography was carried out by the descending method using either Whatman No. 1 or 3 or DE-81 paper in the following solvent systems: (A) 1.0 M ammonium bicarbonate; (B) 95% ethanol–1.0 M ammonium acetate (pH 7.5, 7:3, v/v); (C) isobutyric acid–1.0 M ammonium hydroxide–0.2 M EDTA (100:60:0.8, v/v); (D) 1-butanol saturated with water; (E) *n*-butyl alcohol–methanol–water–ammonia (60:20:20:1, v/v); (F) *tert*-butyl alcohol–methyl ethyl ketone–water–ammonium hydroxide (concentrated) (40:30:20:10).

Thin-layer chromatography was carried out either on MN-300F cellulose plates (Analtech) or on silica gel GF plates (Analtech) with systems B, D, E, and F above.

Other Methods. Thin-layer plates were scanned for radioactivity using a Vanguard Autoscanner 880. Aqueous samples were counted in a Packard Tri-Carb (Model 3375) instrument using Aquasol (New England Nuclear) as fluor, whereas a toluene-based fluor (from Liquifluor, New England Nuclear) was used to count cut-up, dried filter paper from chromatograms. Appropriate quenching corrections were made when necessary. Inorganic phosphate was determined by a modification of the method of Fiske and Subbarow (1925). Ultraviolet spectra were determined on a Cary-15 instrument, infrared spectra on a Perkin-Elmer 237B, and nuclear magnetic resonance (nmr) spectra on a Varian A-60. Nmr spectra are reported in parts per million using the τ system.

Determination of Polymer Synthesis. Polymerization of nucleoside diphosphates was followed either by measurement of orthophosphate release and/or by incorporation of radioactivity as determined by chromatography on Whatman DE-81 in solvent system A. In this system, the nucleoside diphosphates migrate near the solvent front whereas small oligonucleotides have R_F values intermediate between the diphosphate and higher molecular weight polymers which remain at the origin.

Determination of Base Ratios in Polymers. Polymer base ratios were determined by one or more of the following methods: (1) determination of per cent incorporation of (radioactive) unusual nucleotides Ib or IIb into polymer coupled with total phosphate release, (2) determination of per cent incorporation of (radioactive) normal nucleotide (CDP) into polymer coupled with total phosphate release, (3) degradation of the (radioactive) polymer with HCl followed by determination of the specific activity per absorbance unit. When all three methods were applied to several samples, the results agreed to within 10%.

Conditions for Polymerization. The incubation mixture contained per milliliter: 0.1 mmole of Tris (pH 9.0 for Mg^{2+} and pH 8.0 for Mn^{2+}), 0.5–2.5 phosphorolysis units of enzyme, 5–30 μmoles of $MgCl_2$ or 5–15 μmoles of $MnCl_2$, and 5–30 μmoles of nucleoside diphosphates. In initial studies, the $(Ap)_3A$ concentration was 0.25–1.0 μg/ml, the bovine serum albumin concentration was 0.20 mg/ml, and the EDTA concentration was 0.5 μmole/ml. Incubation was carried out at 37° for period ranging from 1 to 12 hr. For extended incubation, a trace of sodium azide was added to prevent bacterial contamination. When necessary, aliquots were removed and cooled in an ice bath when required for chromatographic analysis, or, alternatively for determination of phosphate, the aliquot was quenched with a cold solution of uranyl acetate in perchloric acid.

Synthesis of 1-(β-D-Ribofuranosyl)-5,6-methyleneuracil 5′-Diphosphate. 2′,3′-ISOPROPYLIDENE-3-DIPHENYLMETHYLURIDINE (V). 2′,3′-Isopropylideneuridine (Tipson, 1968) (IV) (5.2 g, 19.2 mmoles) was dissolved in 100 ml of warm methanol and diphenyldiazomethane (Fieser and Fieser, 1967) (53 mmoles, from 10.4 g of benzophenone hydrazone) added.[3] The resulting deep violet solution was gently refluxed and stirred for 16 hr until the solution became yellow. When this solution was kept at 0° for several hours, benzophenoneazine precipitated; it was then collected and washed with a small amount of cold methanol. The filtrate was evaporated *in vacuo* to give a viscous dark residue which was chromatographed on a silica gel column. Elution with benzene (500 ml) removed several impurities. Benzene–acetone (1:1, v/v) was used to elute the products (5.5 g). Further elution with the same solvent system gave 800 mg of unreacted isopropylideneuridine. The corrected yield of V was 84%; R_F (silica gel tlc) 0.6 in benzene–acetone (1:1); ir 3300 (m), OH; 1720 (m), 1670 (vs), 1640 (s) sh, all C=O, 1105 (s), 1075 cm^{-1} (s), both C=O; nmr ($CDCl_3$): 8.68 (s, 3, CH_3), 8.45 (s, 3, CH_3), 2.50 (d, 1, 6-H), 4.25 (d, 1, 5-H), 7.10 (t, 1, 5′-O*H*), 6.25 (m, 2, 5′ CH_2), 2.65 (m, 11, $(C_6H_5)_2$–CH–N), 5.75 (m, 1, 4′H), 5.10 (m, 2, 2′ and 3′ H's), 4.40 (m, 1, 1′H). *Anal.* Calcd for $C_{25}H_{26}N_2O_6$: C, 66.67; H, 5.78; N, 6.62. Found: C, 66.74; H, 5.70; N, 6.24.

2′,3′-Isopropylidene-3-diphenylmethyl-5′-trityluridine (VI). 2′,3′-Isopropylidene-3-diphenylmethyluridine (9.5 g, 21 mmoles) was dissolved in dry pyridine (50 ml) and triphenylmethyl chloride (8.0 g, 29 mmoles) was added. The mixture was gently refluxed for 4 hr, cooled, and evaporated *in vacuo* to leave a viscous residue which was taken up in acetone (25 ml) and the acetone solution was dropped slowly into a vigorously stirred ice–water mixture. The gummy precipitate which formed was extracted into ether and the ether extract dried over anhydrous sodium sulfate and, after filtration, evaporated to dryness *in vacuo*. The resulting semisolid residue was taken up in a small amount of benzene and applied to a column of silica gel. Elution with benzene removed several

[3] The quantity of methanol used as solvent in this reaction seems somewhat critical: when smaller quantities are used (*e.g.*, 10 ml per 2 g of IV) considerable amounts of a second product with an R_F just slightly faster than V on tlc (chloroform–methanol, 9:1) is formed. This product probably results from *O*-diphenylmethylation of IV as a result of the higher enolic content of IV in more nonpolar systems, *i.e.*, the large excess of diphenyldiazomethane decreases the polarity of the methanol solvent system.

impurities including trityl alcohol. Elution with benzene–acetone (24:1) gave VI and subsequent elution with benzene–acetone (1:1) gave a small amount of V (400 mg). The yield of amorphous VI was 12.0 g or 87% based on recovery of starting material. VI had an R_F of 0.6 in benzene–acetone (24:1) on silica gel tlc. This compound was identical with that prepared by diphenylmethylation of 5′-trityl-2′,3′-isopropylideneuridine; ir 1770 (m), 1675 (vs), 1660 (s)sh, all C=O; 1105 (s), 1075 cm^{-1} (s), both C=O; nmr 8.68 (s, 3, CH_3), 8.47 (s, 3, CH_3), 2.40 (d, 1, 6H), 4.55 (d, 1, 5H), 2.70 (m, 26, (C_6H_5)-H's and C_6H_5-C*H*-N), 6.55 (d, 2, 5CH_2), 5.70 (m, 1, 4′H), 5.20 (m, 2, 2′3′H's), 4.10 (m, 1, 1′H), 4.40 (m, 1, 2′H). *Anal.* Calcd for $C_{44}H_{40}N_2O_6$: C, 76.30; H, 5.78; N, 4.05. Found: C, 76.36; H, 5.60; N, 3.96.

2-(2′,3′-Isopropylidene-5′-tritylribofuranosyl)-4-diphenylmethyl-2,4-diazabicyclo[4.1.0]heptane-3,5-dione (VII). 2′,3′-Isopropylidene-5′-trityl-3-diphenylmethyluridine (12.0 g, 17.3 mmoles) in dry tetrahydrofuran (20 ml) was added in one portion to a cool solution of dimethyloxosulfonium methylide (Corey and Chaykovsky, 1965) (42 mmoles prepared from 42 mmoles of sodium hydride and 60 mmoles of trimethyloxosulfonium chloride). The solution was gently refluxed under nitrogen for 6 hr until the ultraviolet absorption shoulder at 260 nm had disappeared. The reaction mixture was cooled, filtered, and evaporated *in vacuo* at <30° to a gum. This residue was taken up in ether, the ether layer washed with water, dried, filtered, and then evaporated to give an amorphous solid which was then chromatographed on a silica gel column. Elution with benzene–acetone (24:1) gave 6.1 g (50%) of chromatographically pure product which had an R_F of 0.6 in benzene–acetone (24:1) on silica gel tlc. Nmr showed the disappearance of the 5 and 6 hydrogens of the uracil ring and the appearance of 4 protons characteristic of the cyclopropane ring; ir 1725 (m), C=O; 1680 (s), C=O; 1660 cm^{-1} (m)sh, C=O. *Anal.* Calcd for $C_{45}H_{42}N_2O_6$: C, 76.45; H, 5.97; N, 3.98; Found: C, 76.00; H, 5.86; N, 4.02.

2-(β-D-Ribofuranosyl)-4-diphenylmethyl-2,4-diazabicyclo-[4.1.0]heptane-3,5-dione (VIII). To a solution of VIII (6.1 g, 8.6 mmoles) in warm methanol (500 ml) water (50 ml) was added slowly with stirring. To this clear solution was added Dowex-50 (H^+)-X4 (500–100 mesh, 50 ml) and the mixture was stirred at room temperature for 48 hr after which time tlc (benzene–acetone) (24:1) showed that no starting material remained. When Dowex 50 (H^+)-X8 is used in this hydrolytic step, at least a week is required for completion of the reaction. The solution was filtered from the resin, which was washed with 100 ml of methanol, and the filtrate evaporated *in vacuo* at a temperature <35° to a small volume containing a large quantity of precipitate (trityl alcohol). The residue was taken up in dioxane, treated briefly with activated charcoal (2 g), filtered, and then used directly for the next step.

1-(β-D-Ribofuranosyl)-5,6-methyleneuracil (I). To the above aqueous dioxane–methanol solution of VIII was added 10% palladium on charcoal (1.0 g) catalyst and the mixture was hydrogenated at 1 atm hydrogen pressure for 1.5 hr until hydrogen uptake was complete and tlc (silica gel, benzene–acetone, 1:1) showed complete conversion to the desired product. The hydrogenation mixture was filtered carefully over Celite and the filtrate evaporated *in vacuo* at <30° to a small volume. A small amount of dioxane and water was added and the resultant solution filtered once more to remove insoluble matter. The filtrate was evaporated almost to dryness and thoroughly mixed with water and the entire aqueous mixture was extracted first with ether and then ethyl acetate until no trityl alcohol or diphenylmethane could be detected by tlc. The aqueous layer was evaporated to a small volume *in vacuo* at <30° and this evaporation repeated several times with absolute ethanol to remove water. The residue was taken up in absolute methanol and absolute ether was added with stirring until no additional precipitate formed. The precipitate was centrifuged, washed with ether, and then taken up again in methanol. Evaporation of this methanolic solution gave, after drying of the glassy solid, 1-(β-D-ribofuranosyl)-5,6-methyleneuracil (1600 mg, 73% based on VII, 27% based on IV). The hygroscopic solid was homogeneous by the criteria of tlc in several different solvent systems including one which was capable of separating I and II (system F). It had identical chromatographic and spectral properties with those of authentic I (Kunieda and Witkop, 1971).

1-(2′,3′-Isopropylidene-β-D-ribofuranosyl)-5,6-methyleneuracil (IX). 1-(β-D-Ribofuranosyl)-5,6-methyluracil (I, 1.0 g, 4.0 mmoles) was suspended in anhydrous (magnesium sulfate) acetone (50 ml); 2,2-dimethoxypropane (Hampton, 1961) (3 ml), anhydrous copper sulfate (0.50 g), and concentrated sulfuric acid (0.02 ml) were added (Tipson, 1968). The solution was stirred at room temperature for 24 hr with protection from light. The mixture was then evaporated to about 20 ml *in vacuo* at <30° and poured into cold water, and the pH quickly adjusted to 7.0 with Dowex-1(OH^-). The solution was filtered to remove resin, and the filtrate evaporated to leave a small volume of aqueous solution which was extracted once with ether. The aqueous layer was then taken to dryness *in vacuo* at <30°. This residue was taken up in a small volume of methanol and applied to a silica gel column. Elution with benzene–acetone gave IX (950 mg, 80%) as an amorphous solid with chromatographic properties identical with those of 2′,3′-isopropylideneuridine. Hydrolysis of IX with Dowex-50 (H^+) in methanol gave back I quantitatively. Compound IX had λ_{max} 245 (sh), with ϵ 1000. This absorption was quickly lost upon addition of hydroxide.

1-(β-D-Ribofuranosyl)-5,6-methyleneuracil 5′-Monophosphate (Ia). Compound IX (950 mg, 3.2 mmoles) was dissolved in dry (calcium hydride) pyridine (10 ml) and β-cyanoethyl phosphate (Tener, 1961) (12 ml of pyridine solution, 1 mmole/ml) was added. The solution was evaporated *in vacuo* at <30°; the residue taken up in dry pyridine and evaporated again. This process was repeated three more times. Then dicyclohexylcarbodiimide (4.0 g, 19.5 mmoles) was added in dry pyridine (30 ml). The flask was well stoppered and protected from light and the mixture was stirred at room temperature. Almost immediately crystals of dicyclohexylurea began to form.

After 80 hr, water (10 ml) was added to the reaction mixture which was stirred for an additional 2 hr. The mixture was filtered and the precipitate of dicyclohexylurea was washed with water. The filtrate was evaporated *in vacuo* (room temperature) to a semisolid residue which was taken up in water and again filtered. The filtrate was evaporated, the residue taken up in methanol–water (1:1, 100 ml), and Dowex-50 (H^+) resin (30 ml) was added. The solution was then stirred for 24 hr in the dark.

After filtration of the solution and washing of the resin with water, the combined filtrate and washings were evaporated to remove methanol at 30° *in vacuo*. The aqueous solution was slowly and carefully neutralized to pH 7.0 at 5–10° with saturated barium hydroxide solution. The resultant solution was concentrated to 10 ml *in vacuo* and ethanol (20 ml) added. The colorless precipitate which formed was centrifuged and washed once with ethanol–water (1:1). The precipitate was practically free of ultraviolet-absorbing materials. The com-

bined supernatants were evaporated *in vacuo* to remove ethanol and then the aqueous residue deionized with Dowex-50 (H^+) in a batch process. After the resin was filtered off and washed with water, the filtrate was carefully adjusted to pH 7.0 with neat pyrrolidine. This solution was then evaporated to a viscous residue which was taken up in methanol and reevaporated. This process was repeated several times to insure complete removal of water. The residue was next dissolved in methanol–pyrrolidine (9:1, 20 ml) and refluxed gently.

After 4 hr of refluxing, no starting material was detected by tlc. The cooled solution was evaporated to a viscous residue and taken up in methanol (5 ml). Addition of ether (50 ml) produced an oil which was centrifuged and washed with ether. The oil was taken up in water and deionized with Dowex-50 (H^+). The resin was filtered off and the filtrate adjusted to a pH of 7.2 with saturated barium hydroxide solution. The small amount of precipitate which formed was centrifuged and the supernatant was concentrated *in vacuo* at room temperature to 10 ml. Slow addition of ethanol (20 ml) produced a copious precipitate which was centrifuged, washed with ethanol, acetone, and finally ether, and dried *in vacuo*.

The yield of barium salt was 710 mg (47%). It had an ultraviolet spectrum identical with that of I and its behavior on cellulose tlc in a variety of solvent systems was the same as that of uridine 5′-monophosphate. Treatment with bacterial alkaline phosphatase gave I. The nuclear magnetic resonance spectrum of the sodium salt of Ia in D_2O (obtained through Dowex-50 (H^+) treatment and subsequent neutralization with sodium hydroxide) was practically superimposable on that of I. This sodium salt was chromatographically identical with the 5′-monophosphate of I from *p*-nitrophenyl phosphate (Janion *et al.*, 1970).

1-(β-D-Ribofuranosyl)-5,6-methyleneuracil 5′-Diphosphate. The barium salt of Ia (650 mg, 1.38 mmoles) was suspended in water and treated with enough Dowex-50 (H^+) to bring about complete solution of the salt. This solution was then passed through a column of Dowex-50 (H^+) and the column was thoroughly washed with water. The eluent was taken to near dryness *in vacuo* at room temperature and then evaporated several times with ethanol to remove water.

This residue was suspended in methanol (10 ml) and tri-*n*-octylamine (490 mg, 1.4 mmoles) added. The mixture was refluxed gently until all the octylamine had dissolved and the solution was clear (about 10 min). The methanol was removed *in vacuo*, the residue taken up in dimethylformamide, and the solution evaporated *in vacuo* to remove traces of moisture.

To the solution of the resulting residue in dioxane (10 ml) were added diphenyl phosphorochloridate (0.42 ml, 2.8 mmoles) and tri-*n*-butylamine (0.62 ml, 2.6 mmoles). The solution was left at room temperature for 4 hr and then refrigerated overnight. After concentration to a thick syrup *in vacuo*, anhydrous ether (35 ml) was added, after which on vigorous shaking a precipitate formed. After several hours at 0°, the ether was decanted and the residue was taken up in dry pyridine. Several evaporations with dry pyridine removed traces of moisture.

Separately, tri-*n*-butylamine (0.66 ml, 2.8 mmoles) and orthophosphoric acid (85%, 0.19 ml, 2.8 mmoles) were dissolved in dry pyridine, and the solution was evaporated several times with dry pyridine and then mixed in the presence of dry pyridine with the above residue containing the nucleoside diphenyl pyrophosphate. The mixture was protected from moisture and light and stirred at room temperature overnight. After removal of solvent *in vacuo*, the residue was shaken with dry ether and the precipitated pyridinium salt was dissolved in water. The pH of this solution was adjusted to 2 with Dowex-50 (H^+) and, after filtration, the solution adjusted to a pH of 7.0 with saturated barium hydroxide. The solution was concentrated to a volume of 10 ml *in vacuo*, ethanol (20 ml) was added to give a precipitate which was centrifuged, washed with ethanol, acetone, and ether, and dried. The yield of crude barium salt of diphosphate was 740 mg.

The barium salt was dissolved in water with the aid of Dowex-50-(H^+) and then deionized by passage through a Dowex-50(H^+) column. The eluent was neutralized to pH 7.0 with dilute sodium hydroxide and, after concentration *in vacuo* at room temperature, applied to a column of Dowex-1-(Cl^-)-X8. Elution with water removed some ultraviolet-absorbing impurities and elution with 0.01 N HCl–0.03 M LiCl removed a small quantity of Ia. Finally, the diphosphate (Ib) was removed by elution with 0.01 N HCl–0.10 M LiCl. The eluent containing Ib was adjusted to pH 6.0 with lithium hydroxide and then evaporated to near dryness *in vacuo* at room temperature. After several additional evaporations with absolute ethanol, the residue was taken up in methanol and acetone added until no more precipitate formed. The solid precipitate was centrifuged and washed with acetone–methanol until the supernatant no longer gave a precipitate with silver ion. The precipitate was then washed with acetone and ether and dried.

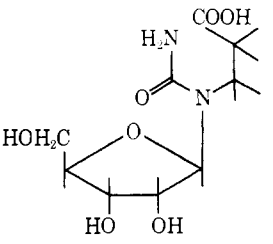
The yield was 310 mg (46% based on monophosphate, 16% based on I) of pure colorless lithium salt of Ib; inorganic phosphate content, 0.18%; $\lambda_{max}^{H_2O}$ 245 nm (sh), ϵ 1000. *Anal.* Calcd for $C_{10}H_{13}N_2O_{12}P_2 \cdot 3Li \cdot 3H_2O$: N, 5.72; P, 12.65. Found: N, 5.64; P, 12.32. Found: ratio of acid labile phosphate of total phosphate (1.0/2.1); Calcd: 1.0/2.0. The R_F of the salt was identical with that of uridine diphosphate in a number of different systems. Treatment with bacterial alkaline phosphatase gave I.

[α-^{32}P]Ib was prepared from [^{32}P]cyanoethyl phosphate (Amersham-Searle, Chicago, Ill.) by the above series of reactions. It was chromatographically homogeneous on Whatman No. 1 in solvent systems B, C, and D and on Whatman DE-81 in solvent system A.

Dihydrouridine 5′-Diphosphate (IIIb). IIIb was synthesized by rhodium-on-alumina hydrogenation of IIb (Roy-Burman, 1968; Janion and Shugar, 1960) and converted to the colorless lithium salt; inorganic phosphate, 0.23%. *Anal.* Calcd: for $C_9H_{13}N_2O_{12}P_2 \cdot 3Li \cdot 1.5H_2O$: P, 13.90. Found: P, 13.85. Found: ratio of acid labile phosphate to total phosphate: 1.0/2.2; Calcd: 1.0/2.0.

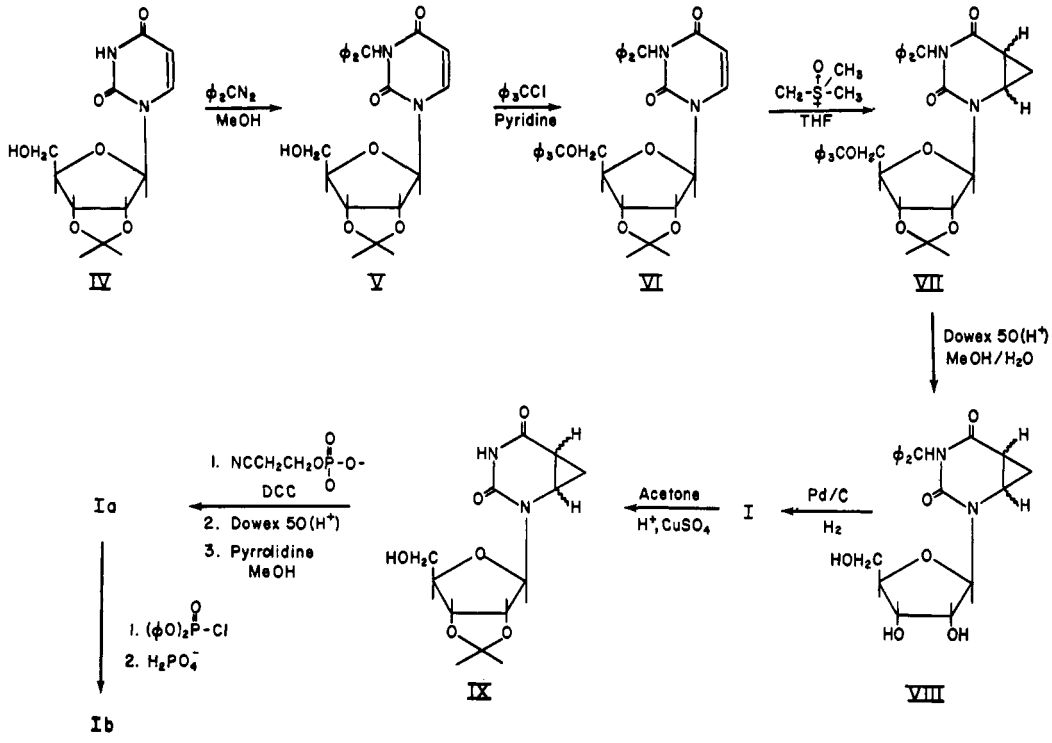
In order to ascertain that the [2-^{14}C]dihydrouridine diphosphate was completely free of radioactive UDP, the following degradation was performed.

[2-^{14}C]Dihydrouridine diphosphate (30 μl, 0.5 μCi) was mixed with Tris buffer (pH 8.5, 30 μl) and 5 units of bacterial alkaline phosphatase and the mixture incubated overnight at 37°. Chromatography of an aliquot on silica gel GF in solvent system F showed that neither diphosphate nor monophosphate



X

SCHEME I



were present, but failed to effect a distinct radiochemical separation of uridine from dihydrouridine (III). Treatment of this solution with 50 μl of 5.0 N potassium hydroxide overnight converted III to the ring-opened γ-ureidopropionic acid (X) (Cerutti *et al.*, 1968b) whose R_F of 0 was substantially different in chloroform–methanol (6:4) from that of uridine (R_F 0.5) which was stable to alkaline ring opening under the same reaction conditions. All the radioactivity was associated with X on cellulose tlc; no activity could be found corresponding to uridine even at the highest possible GM amplification.

Results

Synthesis of the Diphosphate of I. While the synthesis of I had been previously reported (Kunieda and Witkop, 1971), low yields and difficulty in reproducibility led us to attempt to find a new route to I. The key was the use of a different protecting group for N-3, namely, diphenylmethyl (Prystas and Sorm, 1962; Pliml *et al.*, 1963). In contrast to the pivaloyloxymethyl group used previously (Kunieda and Witkop, 1971), the diphenylmethyl group is stable under conditions of the ylide reaction and can be easily removed by hydrogenation at one atmosphere over a palladium-on-charcoal catalyst. Use of Scheme I routinely resulted in yields of 20–40% of I from 2′,3′-isopropylideneuridine.

While I could be phosphorylated with wheat shoot phosphotransferase and *p*-nitrophenyl phosphate (Janion *et al.*, 1970), for large-scale preparations it was more convenient to synthesize Ia using the method of Tener (Tener, 1961). It was however, necessary to modify the method of elimination of the cyanoethyl group from the nucleoside phosphate ester, since the cyclothymine moiety of I undergoes ring opening at room temperature in 0.1 N sodium hydroxide ($t_{1/2}$ = 2 min). It was found that pyrrolidine in methanol at reflux temperatures effected elimination of the cyanoethyl group without any detectable ring opening. The phosphorochloridate method (Michelson, 1964) proved somewhat superior to the morpholidate procedure (Moffatt and Khorana, 1961) in converting Ia to the diphosphate Ib.

Attempted Polymerization of Ib and IIIb. Both by the criteria of phosphate release and incorporation of radioactivity, Ib failed to polymerize even with variations of pH, concentration of enzyme, substrate, or magnesium ion, either with or without primer. Substitution of manganese for magnesium ion also did not bring about polymerization (Janion *et al.*, 1970; Babinet *et al.*, 1965; Rottman and Heinlein, 1968; Chou and Singer, 1971; Zmudzka *et al.*, 1969). The same results were obtained with IIIb, in confirmation of earlier reports (Szer and Shugar, 1961; Ochoa and Heppel, 1957).

Inhibition of Polymerization by Ib and IIIb. While Ib and IIIb are not converted to homopolymers by polynucleotide phosphorylase, they do inhibit the polymerization of UDP and ADP. The inhibition of the polymerization of ADP by varying amounts of Ib and IIIb is represented in Figure 1.

Reaction of Ib and IIIb with (Ap)$_3$A. It was found that when increasing amounts of the primer, (Ap)$_3$A, were used in attempted polymerization of Ib, rising increments of ortho-

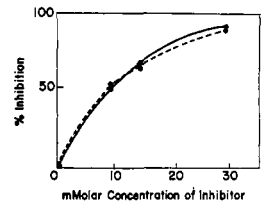


FIGURE 1: Inhibition of ADP polymerization by Ib (—) and by IIIb (——). Data were obtained at a time when, in the uninhibited reaction, 15% of the ADP was utilized. ADP concentration was 13 mM. The Mg concentration was increased in proportion to increasing inhibitor concentration.

TABLE I: Effect of Concentration of $(Ap)_3A$ on Polymerization of Ib.

Molar ratio[a]	60	22	6.9	2.2	1.8	1.2
% polymerization[b]	4	10	15	33	40	60

[a] Micromoles of Ib/micromoles of $(Ap)_3A$. [b] Micromoles of orthophosphate released/micromoles of Ib × 100%.

phosphate were released (Table I). Paper chromatographic examination of these incubation mixtures, on Whatman No. 1 filter paper in solvent system C, revealed a variety of products, whose R_F indicated that they were composed of primer molecules with the addition of one or more residues derived from Ib. Similar results were obtained with IIIb. There was no detectable formation of phosphate when the enzyme was incubated with $(Ap)_3A$ alone. There was also no detectable reaction in the absence of enzyme. No attempt was made to characterize further these oligonucleotide products, since they appeared quite similar to the products encountered previously in the addition of GDP (Singer *et al.*, 1960) or ADP (Kaufmann and Littauer, 1969) to oligonucleotides.

Copolymerization of Ib and IIIb with Nucleoside Diphosphates. Since both the inhibition studies and limited oligonucleotide formation indicated that Ib and IIIb could interact with the enzyme's active site and could undergo limited reaction, we attempted the copolymerization of Ib and IIIb with various "normal" diphosphates which were thought to "dilute out the mistake" (Cramer, 1969) caused by the unusual analog. Indeed, as Table II demonstrates, Ib or IIIb copolymerized with various diphosphates.

In order to confirm and extend these observations, a more detailed study of the copolymerization was carried out with Ib or IIIb and UDP (Table III). The results clearly demonstrate that in the polymerization of Ib or IIIb with UDP over a wide range of substrate ratios, IIIb is more efficiently incorporated into the polymeric product. This was further confirmed by a double label experiment in which [^{32}P]Ib and [^{14}C]IIIb were allowed to compete, in the same incubation mixture, for a growing copolymer chain based on CDP (Table IV). Again, IIIb is several more effective as a substrate for copolymerization than is Ib.

Finally, Ib and IIIb were copolymerized with nucleoside diphosphates in the presence of manganese ion (Babinet *et al.*, 1965) which seems to decrease the substrate specificity of polynucleotide phosphorylase (Janion *et al.*, 1970; Rottman and Heinlein, 1968; Chou and Singer, 1971; Zmudzka *et al.*, 1969) (Table V). Use of manganese ion increased the relative amount of odd nucleotide incorporated, but IIIb was still several times more effectively incorporated than Ib.

TABLE II: Copolymerization of Ib and IIIb with Nucleoside Diphosphates.

NDP[a]	Ib Substrate Ratio[b]	Ib Polymer Ratio[b]	% Yield[c]
	1-(β-D-Ribofuranosyl)-5,6-methyleneuracil 5′-Diphosphate (Ib)		
UDP	2.5	0.08	24
ADP	2.5	0.06	26
CDP	2.5	0.10	25
IDP	2.5	0.10	27
	IIIb Substrate Ratio[b]	IIIb Polymer Ratio[b]	
	Dihydrouridine 5′-Diphosphate (IIIb)		
UDP	1.1	0.14	40
ADP	1.1	0.15	49
CDP	0.9	0.14	42
IDP	0.9	0.08	46

[a] Nucleoside diphosphate. [b] Micromoles of Ib or IIIb/micromoles of NDP. [c] Micromoles of product/micromoles of NDP + Ib or IIIb × 100%.

TABLE III: Effect of Input Substrate Ratio on Resulting Polymer Base Ratio.

IIIb Input[a]	IIIb Polymer[a]	IIIb % Yield[b]	Ib Input[a]	Ib Polymer[a]	Ib % Yield[b]
0.10	0.05	34	0.35	0.05	31
0.64	0.11	33	0.78	0.06	30
1.1	0.14	15	1.3	0.08	24
1.25	0.25		2.5	0.03	24
1.7	0.30	15			

[a] Micromoles of Ib or IIIb/micromoles of UDP. [b] Micromoles of product/micromoles of UDP + Ib or IIIb × 100%.

TABLE IV: Competition between Ib and IIIb in Copolymerization with CDP.[a]

Substrate Input Ratio (Ib/IIIb)	Substrate $^{32}P/^{14}C$	Polymer Product $^{32}P/^{14}C$	Polymer Product Polymer Ratio (Ib/IIIb)
1.7	0.445	0.117	0.45

[a] The reaction mixture consisted of: 60 μl of 0.2 M Tris buffer (pH 9.0, 20 mM Mg^{2+}), 20 μl of bovine serum albumin (1 mg/ml), 10 μl of enzyme preparation (50 units/ml), 0.4 mg of CDP, 0.5 mg of [2-^{14}C]IIIb, 0.85 mg of [α-^{32}P]Ib, and 75 μl of water. A control reaction contained the same constituents in the same concentration except that 10 μl of water was added instead of the enzyme. After incubation at 37°, aliquots (40 μl) of reaction and control solutions were chromatographed on DE-81 in solvent system A. The chromatograms were cut into 1-cm strips and counted in Liquifluor. The ratio of $^{32}P/^{14}C$ in the nucleoside diphosphates of the control and the ratio of $^{32}P/^{14}C$ in the uv-absorbing origin (polymer) of the reaction were then determined. A similar experiment in which Sephadex G-75 was used to separate polymer from diphosphates gave the same results within 15%.

TABLE V: Copolymerization in the Presence of Manganese Ion.

NDP[a]	Ib Substrate Ratio[b]	Ib Polymer Ratio[b]	% Yield[c]
1-(β-D-Ribofuranosyl)-5,6-methyleneuracil 5′-Diphosphate (Ib)			
ADP	1.5	0.10	35
UDP	1.5	0.15	33
CDP	1.5	0.13	37
IDP	1.5	0.13	40
	IIIb Substrate Ratio[b]	IIIb Polymer Ratio[b]	
Dihydrouridine 5′-Diphosphate (IIIb)			
ADP	1.1	0.42	49
UDP	1.1	0.41	50
CDP	1.1	0.40	52
IDP	1.1	0.35	50

[a] Nucleoside diphosphate. [b] Micromoles of Ib or IIIb/micromoles of NDP. [c] Micromoles of product/micromoles of NDP + Ib or IIIb × 100%.

Characterization of the New Polynucleotides. The data in Tables II, III, and V indicate that the ratio of Ia to normal nucleotide in the polymer product ranges from 5 to 15 per 100, whereas with IIIb the corresponding ratios range from 5 to 42 per 100. Both the results of DE-81 paper and Sephadex G-75 column chromatography showed the absence of any significant amounts of smaller oligonucleotides in the preparations. Chromatographic evidence as well as end group analysis with pancreatic ribonuclease indicated a minimum average chain length of 30–60 and perhaps as high as 100 nucleotides. It was nonetheless possible that IIIb and especially Ib were acting as chain terminators for the polymerization reaction, thereby being incorporated only at the 3′-OH end of the polymer. It was, therefore, necessary to degrade these products to determine that the odd nucleotide residues were mostly inside the copolymer chain. The extreme sensitivity of I and III ruled out acid and base hydrolysis, in which rather complicated mixtures were obtained. The copolymer poly(D,U) was degraded by pancreatic ribonuclease (Klee, 1966). In this way poly([^{14}C]D,U) gave 3′-UMP, U, 3′-[^{14}C]DMP, and [^{14}C]D, demonstrating the occurrence of the dihydrouridine residues in internal nucleotide bonds. The 3′-[^{14}C]-DMP was always found in quantities at least 15-fold greater than that of the [^{14}C]D; in addition, the results were not dependent on whether the polymer had been synthesized in the presence of magnesium or manganese.

Degradation of poly([^{32}P]ΔU,U) to establish the distribution of Ia units was more difficult since digestion with standard enzymes (such as pancreatic ribonuclease, spleen phosphodiesterase, snake venom phosphodiesterase) would not serve to identify the [^{32}P]phosphate with I in any way to permit differentiation of internal *vs.* external residues. Degradation of poly([^{3}H]C,ΔU) with pancreatic ribonuclease demonstrated the presence of terminal 3′-OH cytidine residues, thus disproving the exclusive presence of I residues in the terminal positions by establishing the presence of cytidine residues there. This degradation was equivocal because it assumes the identity of all molecules of polynucleotide and relies heavily on the purity of the ribonuclease.

The sodium borohydride dark reduction of dihydrouridine residues provided an alternate means of qualitatively determining the distribution of residues formed from Ib. This reduction of dihydrouridine to a ureidopropanol (Cerruti *et al.*, 1968a,b; Cerutti and Miller, 1967) has been applied to the determination of dihydrouridine residues in tRNA (Cerutti *et al.*, 1968a). The heterocyclic ring of I undergoes an analogous ring opening with borohydride (Kunieda and Witkop, 1971). Thus, reduction of poly(U,ΔU) with sodium [^{3}H]borohydride ought to give a labeled polymer which, after ribonuclease digestion, would allow a distinction of internal *vs.* terminal Ia residues. While it was not possible to predict the pattern which would be obtained, concurrent reduction of poly(U,D) would give some means of comparison, since the distribution of D residues in this polymer had already been determined.

Samples of poly(U), poly(U,D), and poly(U,ΔU) were synthesized and purified on Sephadex G-75. These samples were then treated with sodium [^{3}H]borohydride and rechromatographed on Sephadex. The labeled polynucleotides were digested by pancreatic ribonuclease and the digests chromatographed on Whatman No. 1 in solvent system B (Figure 2).

Perhaps the most surprising result is the significant labeling of poly(U) by [^{3}H]borohydride. This incorporation is real because activity persists in the products of ribonuclease hydrolysis. This activity might arise from minor impurities in the commercial samples of UDP or quite possibly, from the reduction of photoexcited uridine by sodium [^{3}H]borohydride (Cerutti *et al.*, 1968a,b). In this case light does not act as the source of excitation, but the radioactive decay of tritium might provide the energy. Poly(U) shows significantly less tritium incorporation than poly(U,ΔU) which in turn shows less incorporation than poly(D,U), confirming the previous comparisons using ^{14}C and ^{32}P substrates. The ribonuclease digests demonstrate the internal occurrence of Ia units in the polynucleotide chain because of the analogy with poly(D,U). If only terminal residues of I existed, all the activity should be associated with the free nucleoside.

Discussion

The suitability of Ib, IIb, and IIIb as substrates for polynucleotide phosphorylase can be discussed in terms of several structural parameters: (a) steric bulk about the 5,6 double bond, (b) aromaticity, and (c) planarity of the modified pyrimidine ring, (d) conformation of the base with respect to the sugar, (e) puckering of the ribofuranose ring, (f) conformation of the 5′-hydroxyl with respect to the ribofuranose ring.

The results presented above indicate that the steric influence of a methylene group and/or two hydrogens at the 5,6 bond of uridine is more important than electronic considerations (aromaticity) in determining substrate preference for polynucleotide phosphorylase. This is somewhat surprising, since uridine diphosphates with diverse substituents, such as 5-methyl (Griffin *et al.*, 1958), 5-chloro (Michelson *et al.*, 1962), 5-bromo (Michelson *et al.*, 1962), 5-iodo (Michelson *et al.*, 1962), 5-ethyl (Swierkowski and Shugar, 1970), and 5-sulfonylmethyl (Carpenter and Shaw, 1970), can be polymerized into homopolymers. The puckering of the dihydrouracil ring (Suck *et al.*, 1971; Sundaralingam *et al.*, 1971) in IIIb

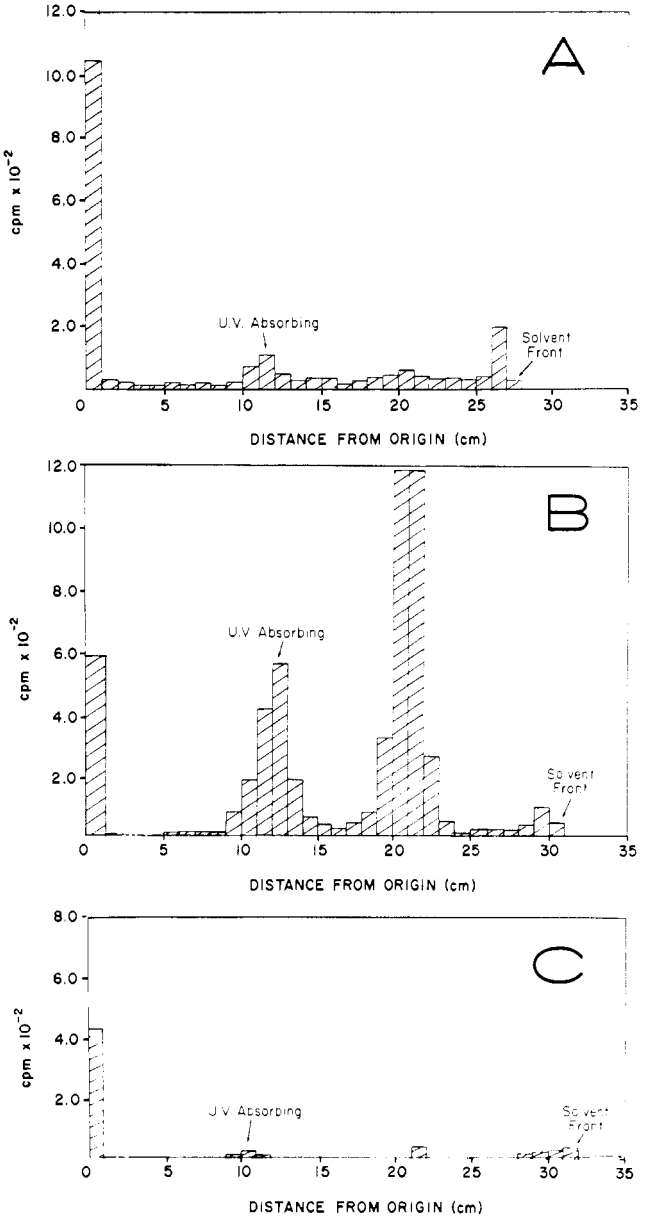


FIGURE 2: Sodium [^{3}H]borohydride reduction and ribonuclease digestion of poly(U), poly(U,ΔU), and poly(U,D). A. Pancreatic ribonuclease degradation of poly(U,ΔU) after borotritide reduction. Digestion carried out on 7 O.D. units of polymer. Specific activity of polymer after reduction was 870 cpm/O.D. unit. B. Pancreatic ribonuclease digestion of poly(U,D) after borotritide reduction. Digestion was carried out on 16 O.D. units. Specific activity of polymer after reduction was 3000 cpm/O.D. unit. C. Pancreatic ribonuclease digestion of poly(U) after borotritide reduction. Digestion was carried out on 8 O.D. units. Specific activity of polymer after reduction was 330 cpm/O.D. unit. Polymers were synthesized in the usual manner and purified on Sephadex G-75 with 0.1 M ammonium bicarbonate as the eluting agent. The poly(U,ΔU) and poly(U,D) polymers were synthesized with substrate ratios which led to 5 and 30 residues of odd nucleotide per 100 uridine residues, respectively. Each of the polynucleotides was dissolved in 200 μl of pH 9.8 borate buffer to which sodium [^{3}H]borohydride (5 mg, 10 mCi/mmole) was added. The solutions were kept at room temperature in the dark for 2 hr after which time an additional quantity (1.0 mg) of sodium [^{3}H]borohydride (10 mCi/mmole) was added. After standing an additional hour, 1.0 ml of 1.0 N acetic acid was added to each sample, and after another hour the solutions were lyophilized to dryness. Each sample was taken up in water, and chromatographed on Sephadex G-75 with 0.1 M ammonium bicarbonate as eluting buffer. After the appropriate fractions were lyophilized to dryness the residues were taken up in 100 μl of pH 8 Tris buffer and 5 units of pancreatic ribonuclease added. After incubation overnight at 37°, each sample was chromatographed on Whatman No. 2 paper in solvent system B. The chromatograms were visualized under uv light and then cut into 1-cm strips and counted in a toluene fluor. The material at 10–12 cm on the above chromatograms corresponded to 3′-UMP and the material at 22–23 cm corresponded to free nucleoside.

would also seem to be ruled out as an important factor, since the partial rigidity of the pyrimidine ring of Ib does not lead to more efficient incorporation than IIIb. In addition, molecular models of I and III do not indicate any difficulty in the interconversion of the syn and anti conformations of the nucleosides, a factor to be considered (Kapuler *et al.*, 1970). According to X-ray analysis III exists in the anti conformation (Suck *et al.*, 1971; Sundaralingam *et al.*, 1971) as does dihydrothymidine (Konnert *et al.*, 1970) and the common nucleotides.

The possibility also exists that differences in the conformation of the furanoside ring of I, II, and III, induced by saturation of the 5,6 double bond, could explain their relative behavior toward polynucleotide phosphorylase. Two major differences between II and III exist: (1) the furanoside ring of III has the rare conformation $^2T_1$ or C(2′)endo–C(1′)exo (Sundaralingam *et al.*, 1971) and (2) perhaps more significantly in III, as well as in dihydrothymidine (Konnert *et al.*, 1970), the conformation of the 5′-hydroxyl with respect to the ribose is predominantly gauche–trans (Sundaralingam *et al.*, 1971), whereas in the known nucleosides and polynucleotides the conformation is gauche–gauche (Sundaralingam, 1969). That the second point may be much more important is indicated by detailed nmr studies which demonstrated rapid interconversion between the conventional puckered ring formations of the ribose. In solution as well as in the solid state a definite preference for a particular rotamer of the exocyclic CH_2OH is shown (Hruska *et al.*, 1970, 1971; Blackburn *et al.*, 1970). β-Cyanuric acid riboside (Dugas *et al.*, 1971) in which the oxo function may interact with the 5′-CH_2OH exhibits a definite preference for the gauche–trans or trans–gauche rotamer. Such interaction, although not as pronounced in I and III, may be responsible for the proven gauche–trans conformation in III and the corresponding likely conformation in I. Verification of this hypothesis will require determination of the X-ray structure of I.

Such considerations may be important in the binding of the nucleoside diphosphate to the enzyme or in the conformation of the resultant polymer and its binding to the enzyme, since the interaction of both is apparently of importance (Godefroy, 1970; Chou and Singer, 1970). The importance of the binding of the polynucleotide product is indicated by the apparent equal inhibitory activity of both Ib and IIIb toward the enzyme. While the conformation of a nucleoside (nucleotide) in solution probably depends more on intramolecular forces, the overall conformation in the solid state or in a polynucleotide should depend on the sum of intra- and intermolecular interactions (Saenger, 1971).

I can possess two isomers, one with the methylene carbon of the cyclopropane ring on the same side of the pyrimidine as the ribofuranose oxygen, the other with the 5 and 6 hydrogen atoms on the same side of the pyrimidine as the ribofuranose oxygen. It is possible, although unlikely, that one of these isomers could be taken up selectively by the enzyme, a possibility which we are attempting to investigate. Optical rotatory dispersion data have shown that synthetic I contains both isomers in nearly equal amounts (Torrence and Witkop[3]); even allowing for complete selectivity of the enzyme for one isomer, IIIb is still a more effective substrate than Ib according to the data presented.

Copolymerization of IIIb with polynucleotide phosphorylase either in the presence of magnesium or manganese makes possible the preparation of various copolymers containing dihydrouridine residues. Copolymers, such as poly(D,C), have

been inaccessible by catalytic hydrogenation procedures, although they can be obtained to some degree by photoreduction with sodium borohydride (Cerutti, 1968). To illustrate the utility of this enzymic approach, we synthesized two samples of poly(C,D), one containing 5 D per 100 C, the other containing 50 D per 100 C and tested these polynucleotides for their ability to induce interferon, either as single strands or with poly(I). Neither polymer showed significant activity (Baron *et al.*[4]). The synthesis of other unusual copolymers is under investigation.

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