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Stepwise Enzymatic Oligoribonucleotide Synthesis Including Modified Nucleotides†

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ABSTRACT: A method has been developed for the routine synthesis of 2′(3′)-*O*-monoacyl ribonucleoside 5′-diphosphates for stepwise synthesis of oligoribonucleotides with *Escherichia coli* polynucleotide phosphorylase. The use of triethyl orthoisovalerate allows the facile preparation of 2′(3′)-*O*-isovaleryl-UDP, -CDP, -ADP, -GDP, -IDP, -ϵ-ADP, -ϵ-CDP, and N^6-isopentenyl-ADP. The synthesis of N^6-isopentenyl-ADP from ADP by N^1-alkylation and the Dimroth rearrangement to N^6 is reported. The effects of several factors including the nature of the divalent cation, pH, salt concentration, and time on the efficiency of the polynucleotide phosphorylase catalyzed single additions of the 2′(3′)-*O*-isovaleryl ribonucleoside 5′-diphosphates to an oligoribonucleotide primer are reported. The syntheses of many tetranucleoside triphosphates and two pentanucleoside tetraphosphates in yields of 20–75% are reported. The 2′(3′)-*O*-isovaleryl derivatives of IDP, ϵ-ADP, ϵ-CDP, and N^6-isopentenyl-ADP were all accepted by polynucleotide phosphorylase as substrates for the monoaddition reaction. The extension of the method to include the syntheses of oligoribonucleotides containing modified nucleosides offers a means of studying the roles of these modifications by the use of relatively simple model compounds.

Oligoribonucleotides of defined sequence are useful model compounds for the study of the structure and function of RNA. Chemical synthesis of ribo oligomers has not developed as rapidly as their deoxyribo counterparts primarily due to the difficulty in ensuring the correct 3′ → 5′ internucleotide linkage. Although sequences as long as nine residues have been achieved chemically (Ohtsuka *et al.*, 1973), the syntheses are involved and time consuming. An alternate approach to oligoribonucleotide synthesis employs primer dependent polynucleotide phosphorylase from *Micrococcus luteus* (Thach and Doty, 1965). Although the enzymatic techniques are rapid and assure the correct inter-

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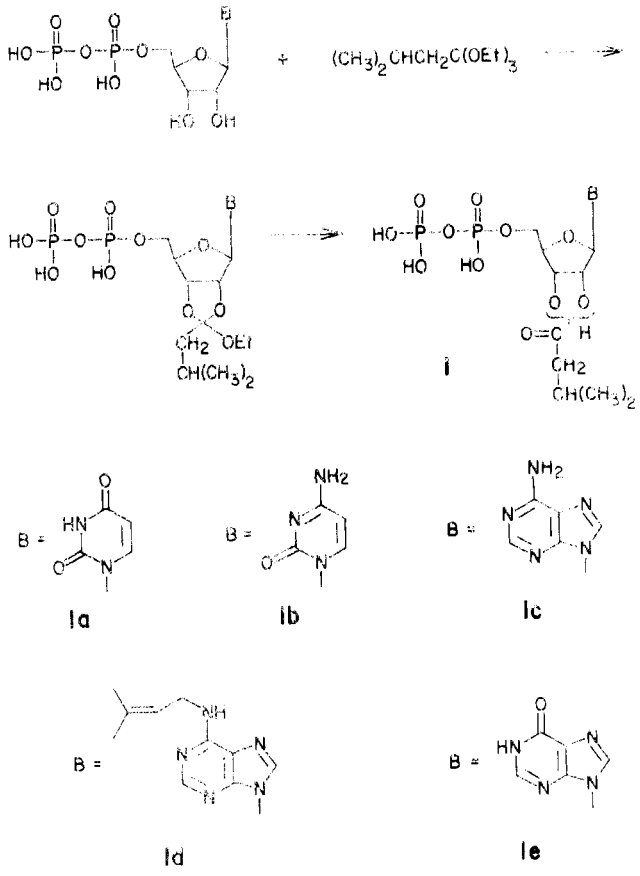

FIGURE 1: The synthesis of the 2′(3′)-*O*-isovaleryl derivatives of UDP, CDP, ADP, N^6-isopentenyl-ADP, and IDP using triethyl orthoisovalerate.

nucleotide linkage, yields are usually very low and the specificity of the enzyme places severe restrictions on the sequences which can be made. Recently a method has been described which combines much of the ease and specificity of an enzymatic reaction with the generality of a chemical synthesis. The enzyme polynucleotide phosphorylase from *Escherichia coli* is used to catalyze the addition of a chemically blocked ribonucleoside 5′-diphosphate to an oligonucleotide primer. An ideal blocking group would allow the addition of a single residue and would prevent subsequent additions. The most successful derivatives of the ribonucleoside 5′-diphosphates found thus far are the 2′-*O*-(α-methoxyethyl) (Mackey and Gilham, 1971; Bennett *et al.*, 1973) and 2′(3′)-*O*-isovaleryl (Kaufmann *et al.*, 1971).

One of the major limitations of the technique as described to date is the difficulty of preparing large quantities of blocked ribonucleoside 5′-diphosphates. The 2′(3′)-*O*-(α-methoxyethyl) and 2′(3′)-*O*-isovaleryl derivatives were obtained by partial reaction of the nucleotide with methyl vinyl ether and the imidazolide of isovaleric acid, respectively, to give mixtures of di-, mono-, and unsubstituted nucleoside 5′-diphosphates which were subsequently separated by paper chromatography to give the desired monosubstituted product. Only moderate yields are obtained and the paper chromatographic purifications are suitable for only relatively small quantities. For the technique to become a viable procedure for the routine synthesis of oligoribonucleotides, a facile method of efficiently preparing monosubstituted nucleotides was required. Such a technique is described in this paper for the synthesis of 2′(3′)-*O*-isovaleryl derivatives and the syntheses of a number of oligoribonucleotides prepared with them are reported.

Since polynucleotide phosphorylase will accept a variety of modified nucleoside 5′-diphosphates as substrates for polymerization, it seemed possible that the monoaddition reaction could be extended to allow the synthesis of hitherto unavailable oligonucleotides containing modified bases. Such compounds could then serve as useful models in the elucidation of the roles of minor bases found in naturally occurring RNA. In the present study we describe the synthesis of the 2′(3′)-*O*-isovaleryl derivatives of N^6-isopentenyl-ADP, -IDP, -ε-ADP,[1] and -ε-CDP and also their suitability as monoaddition substrates for oligomer synthesis.

Experimental Procedure

Proton nuclear magnetic resonance (nmr) spectra were recorded on Varian Associates A-60 and HA-100 spectrometers using TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-d_4) or TMS (tetramethylsilane) as an internal standard. The ultraviolet spectra were determined on a Cary Model 15 spectrophometer or on a Perkin-Elmer Coleman 124D double beam spectrophotometer. Infrared spectra were determined on a Perkin-Elmer 337 grating infrared spectrophotometer. All scintillation counting was performed using a Beckman LS-230 liquid scintillation system. Microanalyses were performed by Mr. Josef Nameth and his associates.

Polynucleotide phosphorylase, free of nuclease and phosphatase activity, was isolated from *E. coli* B by gradient sievorptive chromatography (Kirkegaard, 1973) and passage over Sephadex G-200 (manuscript in preparation). One unit of polynucleotide phosphorylase activity is defined as that amount which catalyzes the incorporation of 1 μmol of ADP into acid precipitable polymer in 15 min at 37° in a reaction mixture containing 0.1 M Tris buffer (pH 8.5), 17 mM ADP, 10 mM $MgCl_2$, and 0.4 M NaCl. Ribonucleases A (Grade RAF) and T_1 and bacterial alkaline phosphatase (Grade BAPC) were purchased from Worthington Biochemical Corp., Freehold, N.J.

Triethyl Orthoisovalerate. Isovaleronitrile was synthesized in 64% yield by the reaction of 1-bromo-2-methylpropane with NaCN in dimethyl sulfoxide (Friedman and Shechter, 1960). The corresponding iminoester hydrochloride was prepared in 48% yield and then was converted to triethyl orthoisovalerate in 47% yield (McElvain and Nelson, 1942): ir no absorption between 2000 and 1500 cm^{-1}; nmr neat δ 1.00 (6 H, d, $C(CH_3)_2$), 1.20 (9 H, t, $OCCH_3$), 1.63 (2 H, d, CCH_2C), 1.61–2.08 (1 H, m, CCHC), 3.59 (6 H, q, OCH_2C).

Anal. Calcd for $C_{11}H_{24}O_3$: C, 64.67; H, 11.84. Found: C, 64.69; H, 11.78.

Triethyl Orthoisobutyrate. Isobutyronitrile was converted to triethyl orthoisobutyrate by a similar procedure to that used for triethyl orthoisovalerate: ir no absorption between 2000 and 1500 cm^{-1}; nmr neat δ 0.90 (6 H, d, $C(CH_3)_2$), 1.08 (9 H, t, $OCCH_3$), 1.93 (1 H, m, CH), 3.40 (6 H, q, OCH_2).

2′(3′)-O-Isovaleryl Esters of CDP and UDP. Trifluoroacetic acid (0.4 ml) was added to a stirring mixture of pyrimidine nucleoside 5′-diphosphate trilithium salt (1 mmol)

[1] Abbreviations used are: ε-ADP, 3-β-D-ribofuranosylimidazo[2,1-*i*]purine 5′-diphosphate; ε-CDP, 5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidine 5′-diphosphate; iA, N^6-isopentenyladenosine.

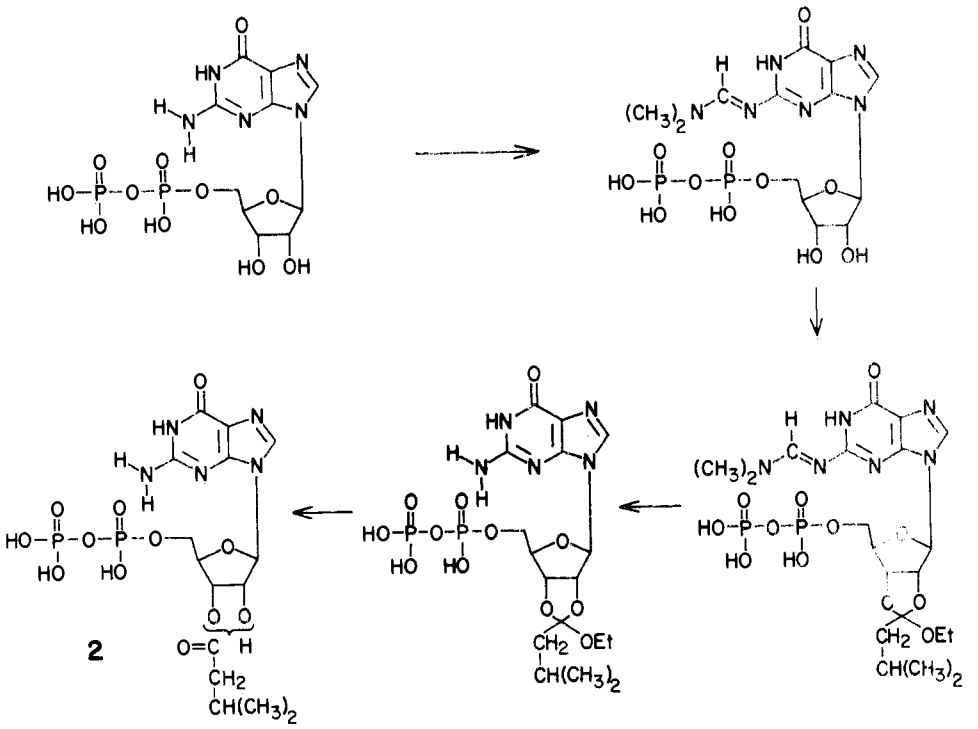

FIGURE 2: The synthesis of 2′(3′)-*O*-isovalerylguanosine 5′-diphosphate. The amino group of GDP is protected as the dimethylaminomethylene derivative prior to the reaction with triethyl orthoisovalerate and then is removed with aqueous methanolic ammonia prior to the opening of the cyclic orthoester with aqueous acid.

and 1.25 ml of triethyl orthoisovalerate in 12–20 ml of *N,N*-dimethylformamide. After 40–60 min at 25° 2 ml of H_2O was added and the reaction was stirred for 2 hr at room temperature. After removal of the solvent *in vacuo* the 2′(3′)-*O*-isovaleryl nucleoside 5′-diphosphate was separated from a small amount of the unreacted nucleoside diphosphate by chromatography on Sephadex LH-20 (670 g) in 95% ethanol–distilled water (33% v/v).

2′(3′)-*O*-Isovaleryluridine 5′-diphosphate (**1a**) was obtained in 80% yield (assuming ϵ_{262} (pH 7.0) 10,000): λ_{max}(H_2O) 262 nm, λ_{min}(H_2O) 231; λ_{max}(H_2O) (acid) 262; λ_{min}(H_2O) 230, λ_{max}(H_2O) (base) 262, λ_{min}(H_2O) 243; nmr (D_2O) δ 0.97 and 1.00 (6 H, d, d, $C(CH_3)_2$), 2.10 (1 H, m, CH), 2.40 and 2.43 (2 H, d, d, $COCH_2$), 4.00–4.97 (sugar H's), 5.37 (1 H, m, 2′(3′)-H's of esterified hydroxyls), 6.07 (2 H, m, 1′-H and 5-H), 7.92 and 7.95 (1 H, d, d, 6-H).

2′(3′)-*O*-Isovalerylcytidine 5′-diphosphate (**1b**) was obtained in 82% yield (assuming ϵ_{272} (pH 7.0) 9,100): λ_{max}(H_2O) 272 nm, λ_{min}(H_2O) 250; λ_{max}(H_2O) (acid) 280, λ_{min}(H_2O) 241; λ_{max}(H_2O) (base) 273, λ_{min}(H_2O) 250; nmr (D_2O) δ 0.97 and 1.00 (6 H, d, d, $C(CH_3)_2$), 2.07 (1 H, m, CH), 2.38 and 2.41 (2 H, d, d, $COCH_2$), 4.00–4.08 (sugar H's), 5.33 (1 H, m, 2′(3′)-H's of the esterified hydroxyls), 6.10 (2 H, m, 1′-H and 5-H), 7.94 (1 H, d, 6-H).

2′(3′)-O-Isovaleryl Esters of ADP and IDP. Trifluoroacetic acid (0.4 ml) was added to a stirring mixture of nucleoside 5′-diphosphate trilithium salt (1 mmol) and 1 ml of triethyl orthoisovalerate in 10 ml of $(CH_3)_2SO$. After 30 min at 25° 2 ml of H_2O was added and the mixture was stirred for 2.5 hr at room temperature. After precipitation by dropwise addition to 150 ml of stirring acetone, the product was collected by centrifugation and was washed with acetone. Chromatography on Sephadex LH-20 (670 g) in 95% ethanol–distilled water (33% v/v) yielded the monoacylated nucleoside 5′-diphosphate.

2′(3′)-*O*-Isovaleryladenosine 5′-diphosphate (**1c**) was obtained in 68% yield (assuming ϵ_{259} (pH 7.0) 15,400): λ_{max}(H_2O) 259 nm, λ_{min}(H_2O) 227; λ_{max}(H_2O) (acid) 257, λ_{min}(H_2O) 230; λ_{max}(H_2O) (base) 259, λ_{min}(H_2O) 227; nmr (D_2O) δ 0.86 and 0.93 (6 H, d, d, $C(CH_3)_2$), 2.16 (1 H, m, CH), 2.34 and 2.38 (2 H, d, d, $COCH_2$), 4.08–5.20 (sugar H's), 5.60 (1 H, m, 2′(3′)-H's of esterified hydroxyls), 6.16 and 6.22 (1 H, d, d, 1′-H), 8.20 and 8.52 (1 H, 1 H, s, broad s, purine H's).

2′(3′)-*O*-Isovalerylinosine 5′-diphosphate (**1d**) was obtained in 80% yield (assuming ϵ_{249} (pH 6.0) 12,200): λ_{max}(H_2O) 249 nm, λ_{min}(H_2O) 223; λ_{max}(H_2O) (acid) 251, λ_{min}(H_2O) 223; λ_{max}(H_2O) (base) 253, λ_{min}(H_2O) 228.

2′(3′)-O-Isovalerylguanosine 5′-Diphosphate (**2**). The synthetic scheme is outlined in Figure 2. Dimethyl sulfoxide (2.5 ml) and 0.25 ml of *N,N*-dimethylformamide dimethyl acetal was added to the triethylammonium salt of guanosine 5′-diphosphate. At the end of 20 hr at room temperature no GDP could be detected by thin-layer chromatography and the ultraviolet spectrum had shifted from that of guanosine with its maximum absorbance at 252 nm to that reported for N^2-dimethylaminomethyleneguanosine (Zemlicka and Holy, 1967); λ_{max}(H_2O) 295, λ_{min}(H_2O) 252. Then 0.5 ml of H_2O was added to hydrolyze any groups which had reacted with the sugar hydroxyls (Zemlicka and Holy, 1967). The N^2-dimethylaminomethyleneguanosine 5′-diphosphate was then collected by dropwise addition to 50 ml of acetone. The product was dried by three evaporations with absolute ethanol followed by drying *in vacuo* at 50° for a few minutes. Then 2.5 ml of dimethyl sulfoxide, 0.25 ml of triethyl orthoisovalerate, and 0.1 ml of trifluoroacetic acid were added and the reaction was allowed to proceed for 40 min. Then 10 ml of methanolic ammonia was added followed by 50 ml of concentrated aqueous ammonia and the solution was stirred for 3.5 hr while slowly bubbling in NH_3. This removal of the N^2-dimethylami-

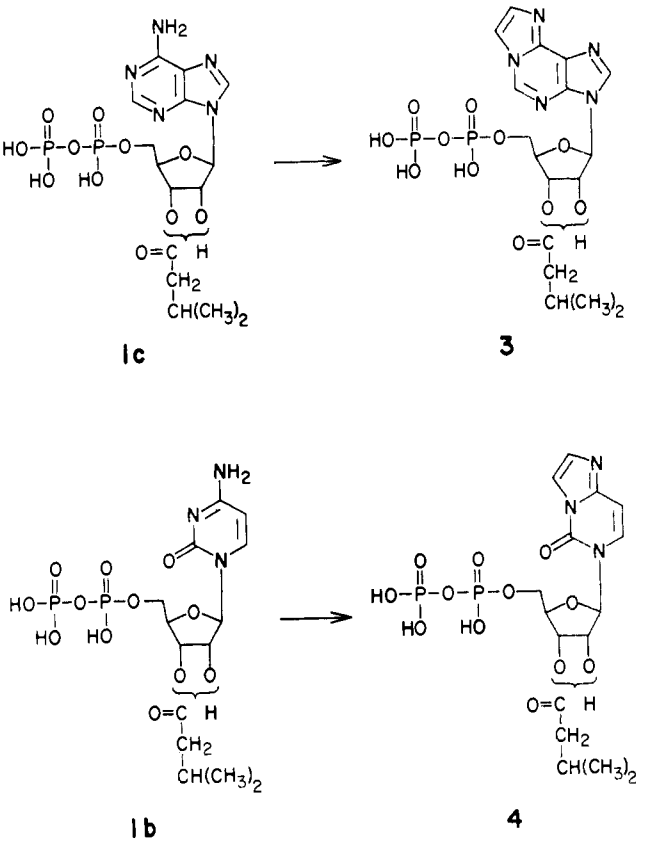

FIGURE 3: The synthesis of 2′(3′)-*O*-isovaleryl-ϵ-ADP and -ϵ-CDP by treatment of the isovaleryl derivatives with chloroacetaldehyde.

nomethylene group was followed by monitoring the shift of the absorption maximum from 295 back to 252 nm. After removal of the ammonia and solvent *in vacuo* and then treatment with 2 ml of 5% trifluoroacetic acid for 2 hr at room temperature the product was precipitated by dropwise addition to stirring acetone and was collected by centrifugation. Chromatography on Sephadex LH-20 in 95% ethanol–distilled H_2O (33% v/v) gave a 45% yield (assuming ϵ_{252} (pH 7.0) 13,700) of 2′(3′)-*O*-isovalerylguanosine 5′-diphosphate: $\lambda_{max}(H_2O)$ 252 nm, $\lambda_{min}(H_2O)$ 225; $\lambda_{max}(H_2O)$ (acid) 255, $\lambda_{min}(H_2O)$ 230; $\lambda_{max}(H_2O)$ (base) 262, $\lambda_{min}(H_2O)$ 232.

N^6-Isopentenyladenosine 5′-Diphosphate. A mixture of 1.86 mmol of adenosine 5′-diphosphate trilithium salt and 1.0 ml (0.77 g, 5.3 mmol) of 1-bromo-3-methyl-2-butene in 10 ml of dimethyl sulfoxide was stirred at room temperature in a stoppered flask. The course of the reaction was followed by cellulose thin-layer chromatography in 1-propanol–concentrated aqueous ammonia–H_2O (55:10:35). After 24 hr the product was precipitated by dropwise addition to stirring acetone and the acetone was decanted. The ultraviolet spectrum in base of the product at this stage showed the long wavelength tail which is characteristic of N^1-substituted adenosines (Grimm and Leonard, 1967). The sticky residue was dissolved directly in 200 ml of dilute aqueous ammonia and was heated on a steam bath for 3 hr. The progress of the rearrangement to the N^6-substituted compound was followed by monitoring the disappearance of the long wavelength tail (in base) and the shifting of the $\lambda_{max}(H_2O)$ to 267 nm. Chromatography on DEAE-Sephadex A-25 (500 ml) with a 4-l. linear gradient of triethylammonium bicarbonate buffer (pH 7.5) (0.3–1.0 M) gave a 43% yield (assuming ϵ_{267} (pH 7.0) 19,200) of *N^6*-isopentenyladenosine 5′-diphosphate. A trace of ADP remaining after the chromatography on DEAE-Sephadex A-25 was removed by chromatography on Sephadex LH-20 in 95% aqueous ethanol–distilled H_2O (33% v/v): $\lambda_{max}(H_2O)$ 267 nm, $\lambda_{min}(H_2O)$ 231; $\lambda_{max}(H_2O)$ (acid) 264, $\lambda_{min}(H_2O)$ 231; $\lambda_{max}(H_2O)$ (base) 267, $\lambda_{min}(H_2O)$ 231; nmr (D_2O) δ (shifts are reported from TMS in a concentric capillary) 1.47 (6 H, s, CH_3), 5.09 (1 H, m, vinylic proton), 7.89 (1 H, s, 2-H), 8.15 (1 H, s, 8-H).

N^6-Isopentenyl-2′(3′)-O-isovaleryladenosine 5′-Diphosphate (**1d**). The 2′(3′)-*O*-isovaleryl derivative of N^6-isopentenyladenosine 5′-diphosphate was prepared using similar conditions to those used for UDP and CDP and after purification by chromatography on Sephadex LH-20 in 95% ethanol–distilled H_2O (33% v/v) it was obtained in 82% yield (assuming ϵ_{267} (pH 7.0) 19,200): $\lambda_{max}(H_2O)$ 267 nm, $\lambda_{min}(H_2O)$ 232; $\lambda_{max}(H_2O)$ (acid) 265, $\lambda_{min}(H_2O)$ 232; $\lambda_{max}(H_2O)$ (base) 267, $\lambda_{min}(H_2O)$ 234; nmr (D_2O) δ (downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS)) 0.76 and 0.99 (d, d, $C(CH_3)_2$), 1.75 (broad s, $C(CH_3)_2$), 6.16 and 6.27 (d, d, 1′-H's), 8.21 and 8.25 (s, s, 2-H's), 8.46 and 8.50 (s, s, 8-H's).

2′(3′)-O-Isovaleryl-ϵ-adenosine 5′-Diphosphate (**3**). A solution of 0.095 mmol of 2′(3′)-*O*-isovaleryladenosine 5′-diphosphate (**1c**) in 10 ml of 2 M aqueous chloroacetaldehyde was stirred at 37° for 3 days maintaining the pH at 4.0–4.5 with aqueous $NaHCO_3$, and then was evaporated to dryness *in vacuo*. Chromatography on Sephadex LH-20 (200 ml) in 31% ethanol in distilled H_2O gave 2′(3′)-*O*-isovaleryl-ϵ-adenosine 5′-diphosphate in 85% yield (assuming ϵ_{275} (pH 7.0) 6200). The uv spectrum was identical with ϵ-adenosine (Barrio *et al.*, 1972): $\lambda_{max}(H_2O)$ 275, 265, 258 (sh), 300 (sh), $\lambda_{min}(H_2O)$ 247; $\lambda_{max}(H_2O)$ (acid) 273, $\lambda_{min}(H_2O)$ 244 nm.

2′(3′)-O-Isovaleryl-ϵ-cytidine 5′-Diphosphate (**4**). A solution of 0.11 mmol of 2′(3′)-*O*-isovalerylcytidine 5′-diphosphate (**1b**) in 8 ml of 2 M aqueous chloroacetaldehyde was stirred at 37° for 5 days, maintaining the pH at 3.5 with aqueous $NaHCO_3$, and then was evaporated to dryness *in vacuo*. Chromatography on Sephadex LH-20 (200 ml) in 95% ethanol–distilled water (33% v/v) gave 2′(3′)-*O*-isovaleryl-ϵ-cytidine 5′-diphosphate in a yield of 58% (assuming ϵ_{271} (pH 7.0) 11,700). The uv spectrum was identical with ϵ-cytidine (Barrio *et al.*, 1972): $\lambda_{max}(H_2O)$ 271, 280 (sh), 292 (sh) nm, $\lambda_{min}(H_2O)$ 230; $\lambda_{max}(H_2O)$ (acid) 285, 302 (sh), 248 (sh), $\lambda_{min}(H_2O)$ 230; $\lambda_{max}(H_2O)$ (base) 271, 280 (sh), 292, (sh), $\lambda_{min}(H_2O)$ 235.

Synthesis of Oligoribonucleotide Primers. Primers dependent polynucleotide phosphorylase was prepared by the method of Klee (1969). Diribonucleoside monophosphates were either purchased commercially or were obtained by digestion of particular polymers and were converted to triribonucleoside diphosphates with primer dependent polynucleotide phosphorylase (Thach and Doty, 1965a,b) often with the use of a specific nuclease (Thach *et al.*, 1966; Stanley *et al.*, 1966; Uhlenbeck *et al.*, 1971). Radioactively labeled oligonucleotides were prepared by the primer dependent polynucleotide phosphorylase catalyzed addition to the required oligomer of a terminal nucleotide, tritium labeled to high specific activity (*ca.* 10 Ci/mmol) (Uhlenbeck *et al.*, 1970). Preparation of ApApC with ^3H in the H-8 of the internal A residue was accomplished by the synthesis of GpApA from GpA and [^{3}H]ADP, treatment with ribonuclease T_1, and the addition of C as indicated above.

Monoaddition Reaction. A reaction mixture containing

0.1 M Tris buffer (pH 8.5), *ca.* 0.7 mM oligoribonucleotide primer, 10 mM $MnCl_2$, 0.8 M NaCl (only for pyrimidine nucleotide additions), and the concentrations of 2′(3′)-*O*-isovaleryl ribonucleoside 5′-diphosphate and enzyme indicated in Table I was incubated at 37° for the time shown. At the end of the incubation period a 200-fold volume of 50% aqueous methanol saturated with NH_3 at 0° was added and the solution was allowed to stand at room temperature for 1 hr. After removal of the solvent *in vacuo* the mixture was separated by paper chromatography in ethanol–1 M ammonium acetate (6:4). The extent of reaction was determined by liquid scintillation counting of segments of the chromatogram in 2,5-diphenyloxazole–toluene (4 g/l.). The oligonucleotide products were usually characterized by digestion with specific nucleases and then comparison of the R_F's of the uv absorbing spots and the peak of radioactivity with the R_F's of the authentic compounds.

Results and Discussion

Synthesis of 2′(3′)-O-Acyl Ribonucleoside 5′-Diphosphates. The most convenient method of achieving selective 2′(3′)-O-monoacylation of nucleosides or nucleotides is by the use of an orthoester intermediate. Zemlicka (1964) and Jarman and Reese (1964) protected the 2′,3′-*cis*-hydroxyl groups of nucleosides by treatment with trimethyl orthoformate in the presence of an acid catalyst to give the 2′,3′-*O*-methoxymethylidene derivatives. These cyclic orthoesters could be converted in high yield to the corresponding 2′(3′)-*O*-formyl nucleosides by treatment with aqueous acid. This approach has also been used to synthesize 2′(3′)-*O*-glycyladenosine and the corresponding 5′-mono-, di-, and triphosphates by reaction with *N*-carbobenzoxyglycine triethyl orthoester (Zemlicka and Chladek, 1966, 1968). Thus it seemed probable that 2′(3′)-*O*-isovaleryl nucleoside 5′-diphosphates could also be efficiently synthesized through an orthoester intermediate.

Isovaleronitrile was conveniently prepared from isobutyryl bromide (Friedman and Shechter, 1960) and then was converted to triethyl orthoisovalerate (McElvain and Nelson, 1942). Triethyl orthoisobutyrate was also prepared in a similar fashion.

The trilithium salts of UDP and CDP reacted readily with triethyl orthoisovalerate in *N,N*-dimethylformamide in the presence of trifluoroacetic acid. Addition of water to the reaction mixture hydrolyzed the orthoesters to the 2′(3′)-*O*-isovaleryl nucleoside 5′-diphosphates (Figure 1). ADP reacted smoothly when dimethyl sulfoxide was used as the solvent instead of *N,N*-dimethylformamide and the product could be recovered by precipitation in acetone, avoiding the troublesome removal of dimethyl sulfoxide by evaporation. After the treatment with water the reaction mixture contained the 2′(3′)-*O*-isovaleryl ester of the nucleotide and small amounts of the original nucleoside 5′-diphosphate as well as a trace of a compound with a higher R_F on cellulose thin-layer chromatography in 1-butanol–acetic acid–water (5:2:3) than the desired compound. Since the fast moving compound had the same uv spectrum as the nucleoside 5′-diphosphate, it presumably was the 2′,3′-diisovaleryl nucleoside 5′-diphosphate. Because the enzyme utilizes unmodified nucleoside 5′-diphosphates at a faster rate than the 2′(3′)-*O*-isovaleryl derivatives it was necessary to purify the compounds to remove all traces of unmodified nucleoside 5′-diphosphates. This was achieved by chromatographing the reaction mixture on Sephadex LH-20 in 95% ethanol–distilled water (33% v/v). The gel is being used in an absorptive mode rather than a sieving mode as the unreacted diphosphate comes off the column first. The above synthesis and purification allowed the preparation of 2′(3′)-*O*-isovaleryl-UDP (**1a**), -CDP (**1b**), and -ADP (**1c**) in yields of 80, 82, and 63%, respectively.

In order to utilize the orthoester method to synthesize 2′(3′)-*O*-isovaleryl-GDP it was necessary to protect the amino group of the base from reaction with triethyl orthoisovalerate. Zemlicka and Holy (1967) had shown that the amino group of guanosine could be protected by treatment with *N,N*-dimethylformamide dimethyl acetal to form the N^2-dimethylaminomethylene derivative. The procedure had to be modified for GDP by using the triethylammonium salt to increase the solubility and employing dimethyl sulfoxide as the solvent instead of *N,N*-dimethylformamide. The reaction appeared to be complete in 12 hr on the basis of the ultraviolet spectral change. After the addition of water to hydrolyze any groups that had reacted with the sugar, the N^2-dimethylaminomethylene-GDP was isolated by precipitation in acetone. The reaction with triethyl orthoisovalerate then proceeded smoothly in a few minutes. The dimethylaminomethylene group was removed with aqueous methanolic ammonia prior to the opening of the cyclic orthoester with aqueous acid. The 2′(3′)-*O*-isovaleryl-GDP (**2**) was obtained with 45% yield after chromatography on Sephadex LH-20 in 95% ethanol–distilled water (33% v/v). The synthetic scheme is outlined in Figure 2.

The orthoester method for synthesizing 2′(3′)-*O*-isovaleryl derivatives was extended to include certain minor nucleoside 5′-diphosphates. N^6-Isopentenyl-ADP was synthesized in 40% yield by alkylation of ADP at N^1 followed by the Dimroth rearrangement to the N^6-substituted compound. The conditions were modified from those reported for the synthesis of N^6-isopentenyl-AMP (Grimm and Leonard, 1967) and may also be used successfully for the synthesis of N^6-isopentenyl- and N^6-benzyl-cAMP (A. Vincze, G. C. Walker, and N. J. Leonard, unpublished results). The 2′(3′)-*O*-isovaleryl derivative of N^6-isopentenyl-ADP (**1d**) was prepared in 85% yield using the same conditions employed for the pyrimidine nucleoside 5′-diphosphates. Apparently the presence of the N^6-isopentenyl group made the nucleotide sufficiently soluble in *N,N*-dimethylformamide that it was not necessary to use dimethyl sulfoxide as a solvent. The trilithium salt of inosine 5′-diphosphate reacted smoothly with triethyl orthoisovalerate under the same conditions employed for ADP to give an 80% yield of the 2′(3′)-*O*-isovaleryl derivative (**1e**) after purification on Sephadex LH-20 in 95% ethanol–distilled water (33% v/v). Monosubstituted derivatives of the fluorescent ϵ-nucleoside 5′-diphosphates (Secrist *et al.*, 1972) were prepared by acylating the sugar *via* an orthoester intermediate prior to modification. The 2′(3′)-*O*-isovaleryl derivatives of ADP and CDP were then treated with chloroacetaldehyde and purified by chromatography on Sephadex LH-20 to yield 2′(3′)-*O*-isovaleryl-ϵ-ADP (**3**) and 2′(3′)-*O*-isovaleryl-ϵ-CDP (**4**) in yields of 85 and 58%, respectively.

Stepwise Synthesis of Oligoribonucleotides. Figure 4a shows a chromatogram analyzing the radioactive products of an ApApC and 2′(3′)-*O*-isovaleryl-ADP reaction after removal of the blocking group. The profile is identical if the reaction is treated with alkaline phosphatase prior to chromatography. This demonstrates that no phosphorolysis of the $(Ap)_2C$ to [^{3}H]CDP has occurred. Since the radioactive label is in the terminal C residue of the primer, the reaction yield may be determined directly. Under the conditions of

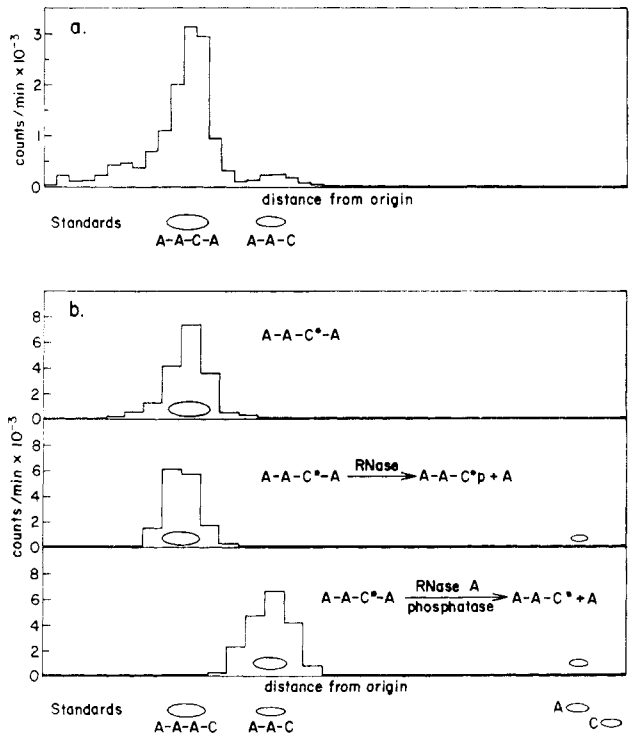

FIGURE 4: (a) Products of the polynucleotide phosphorylase catalyzed reaction of 2′(3′)-*O*-isovaleryl-ADP with ApApC. A reaction mixture (25 μl) containing 0.1 M Tris buffer (pH 8.5), 0.7 mM ApApC, 16 μM ApApC (with the terminal C ^{3}H-labeled, 10 Ci/mmol), 10 mM $MnCl_2$, 6.3 mM 2′(3′)-*O*-isovaleryl-ADP, and 0.1 unit of *E. coli* polynucleotide phosphorylase was incubated for 20 min at 37°. After treatment with 50% aqueous methanolic ammonia for 1 hr at room temperature the reaction mixture was chromatographed on paper in ethanol–1 M ammonium acetate (6:4). The chromatogram was examined under ultraviolet light and oligonucleotides which quenched the fluorescence of the paper were located (ovals). The chromatogram was then cut into 1-cm strips which were counted with a liquid scintillation spectrophotometer. (b) Characterization of the monoaddition product by treatment with RNase A and phosphatase. Paper chromatography was carried out in ethanol–1 M ammonium acetate (6:4). The positions of the uv absorbing spots were recorded and the distribution of radioactivity was determined by liquid scintillation counting.

the reaction, 7% of the label remains as ApApC, 76% of the label has the mobility expected for $(Ap)_2CpA$, and 12% has a mobility expected for $(Ap)_2CpApA$.

In Figure 4b the major peak is identified as the product of a single addition of an A residue to $(Ap)_2C$. Treatment of the peak with RNase A alone gave a nonradioactive product migrating with a mobility identical with adenosine and a single radioactive product which comigrates with an $(Ap)_2Cp$ marker. If the major peak is treated with both RNase A and alkaline phosphatase, nonradioactive adenosine and radioactive $(Ap)_2C$ are the only products. It is important to note that the $(Ap)_2CpA$ product peak is not contaminated by the tetranucleotides $(Ap)_2CpC$ or $(Ap)_3A$ which might arise from phosphorolysis of $(Ap)_2C$ and subsequent repolymerization or from transnucleotidation (Kaufmann *et al.*, 1971). Since the C residue is labeled, radioactive cytidine would be released if $(Ap)_2CpC$ were treated with RNase A. Since greater than 99% of the radioactivity remains as $(Ap)_2Cp$, the product must contain less than 1% $(Ap)_2CpC$. The absence of $(Ap)_2CpC$ in the product peak is consistant with absence of detectable [^{3}H]CDP in Figure 4a since both would indicate phosphorolysis of the $(Ap)_2C$ primer. In order to confirm that the product peak did not contain $(Ap)_3A$, $(Ap)_2C$ primer labeled with a 8-^{3}H in the internal A was used to synthesize $(Ap)_2CpA$ as before. The products of this reaction appeared identical with Figure 4a and treatment of the tetramer product peak with RNase A and alkaline phosphatase resulted in greater than 99% conversion of the radioactive tetramer to radioactive $(Ap)_2C$ and nonradioactive adenosine. If any of the $(Ap)_2C$ primer was converted to $(Ap)_3A$, some of the product radioactivity would not have been converted to $(Ap)_2C$ upon treatment with RNase A and alkaline phosphatase. Thus, contamination of the tetramer product peak with $(Ap)_3A$ derived from the primer is excluded. Finally, if the reaction in Figure 4a is run under identical conditions in the absence of $(Ap)_2C$ primer with [^{3}H]isovaleryl-ADP and subsequently treated with alkaline phosphatase, no radioactivity can be detected at the tetramer position, thereby eliminating the possibility that any contaminatory $(Ap)_3A$ can arise from the isovaleryl ADP. We therefore conclude that the major product peak in Figure 4a is $(Ap)_2CpA$.

Since the reaction conditions described for Figure 4a were adusted to maximize the yield of tetramer, not enough of the slower moving pentamer product could be obtained to allow analysis. When an identical reaction mixture was incubated six times longer (2 hr), a larger amount of pentamer was produced. Treatment of this compound with ribonuclease and alkaline phosphatase gave 89% of the radioactivity in $(Ap)_2C$, 6% in ApC, and 5% in C thus indicating that while the major product was $(Ap)_2C(pA)_2$, contaminating amounts of other pentamers such as $(Ap)_2CpApC$, $(Ap)_2(Cp)_2A$, and $(Ap)_2(Cp)_2C$ were present. Thus appreciable amounts of phosphorolysis do take place at reaction times which greatly exceed those required for the desired single addition synthesis. The incubation of oligomers with polynucleotide phosphorylase in the presence of the released phosphate leads to scrambled sequences. Indeed, under the extended reaction times used to prepare diaddition product, the $(Ap)_2CpA$ was contaminated by 3% $(Ap)_2CpC$. However, in a normal reaction, the small amount of pentamer is presumably the result of adding two A residues to the primer molecule. It appears unlikely that much of this diaddition arises from hydrolysis of the blocking group from the 3′-terminal nucleotide of the monoaddition product because the amount of diaddition does not show the expected dependence on the duration of the reaction; increasing the amount of diaddition does not show the expected dependence on the duration of the reaction; increasing the amount of polynucleotide phosphorylase and proportionately shortening the reaction time give the same amount of diaddition product. It is also unlikely that the diaddition product is a result of the transfer of a 3′-terminal nucleotide from one oligomer to another (Kaufman *et al.*, 1971) since only one [^{3}H]cytidine residue is detected in the product and diaddition can be observed even when the primer concentration is 10^{-3}-fold lower. A bimolecular reaction would have shown a strong dependence on oligomer concentration.

A variety of reaction conditions for the synthesis of $(Ap)_2CpA$ have been examined and several general characteristics of the monoaddition reaction have emerged. The most striking observation is the critical importance of the nature of the divalent metal ion in the reaction. When Mg^{2+} was used, the reaction rate was much slower and the monoaddition of a blocked diphosphate was not favored sufficiently over multiple additions to obtain useful yields. However, the substitution of Mn^{2+} for Mg^{2+} increased the rate of reaction and the yield of monoaddition product. This observation is consistent with numerous reports of polynu-

Table I: Oligoribonucleotides Synthesized Using 2′(3′)-*O*-Isovaleryl Ribonucleoside 5′-Diphosphates.

Monoaddition Product	Moles of Blocked Diphosphate/ mole of Primer	Units of Polynucleotide Phosphorylase/ μmole of Primer	Reaction Duration (min)	Yield (%)
A-A-C-A	9	6	20	76, 60[a]
U-U-C-A	9	6	120	64
A-A-C-C	60	12	60	44, 32[a]
G-A-U-C	20	4	100	35
G-A-C-C	17	3	45	36
A-G-C-C	8	3	120	26
A-A-C-U	60	12	60	39
A-G-C-U	6	3	100	39
A-A-C-G	11	6	70	71
C-C-G-G	12	6	90	60
A-A-A-U-A	9	6	30	75
A-G-C-C-A	11	0.7	50	36
A-A-A-iA	23	13	85	70
A-A-C-iA	17	12	60	65
A-A-C-I	12	6	60	74
A-A-C-ϵA	24	6	60	55
A-A-C-ϵC	21	6	60	24

[a] Synthesized using 2′(3′)-*O*-isobutyryl ribonucleoside 5′-diphosphates.

cleotide phosphorylase accepting a modified nucleoside 5′-diphosphate as a substrate more easily when Mn^{2+} was used as the divalent ion (Thang and Grunberg-Manago, 1968; Chou and Singer, 1971). Mn^{2+} had previously been employed in oligomer syntheses using blocked nucleoside diphosphates (Mackey and Gilham, 1971; Kaufman *et al.*, 1971). The optimal Mn^{2+} concentration seems to be 10 mM although Mn^{2+} concentrations down to 2 mM work quite well. At higher Mn^{2+} concentrations more multiple addition products are observed.

The pH optimum of the monoaddition reaction was found to be 8.5. Although the pH optimum of polynucleotide phosphorylase is slightly higher, the rate of hydrolysis of the isovaleryl group increases rapidly above pH 8.5 leading to multiple addition of unblocked diphosphates. At pH 8.5 at 37° the half-life of 2′(3′)-*O*-isovaleryl-ADP is 27 hr. Lower pH values decrease the rate of ester hydrolysis but also severely decrease the rate of the enzyme reaction. Since the pH of the reaction is so critical, 0.1 M Tris-HCl buffer is used in all reactions.

Table I summarizes oligoribonucleotide synthesis reactions between a variety of primers and 2′(3′)-*O*-isovaleryl nucleoside diphosphates. In each case, the conditions given have been adjusted to optimize the yield of the single addition product and the product was identified enzymatically in a manner similar to $(Ap)_2CpA$. Considerable variation in the rate and extent of reaction is observed with the different diphosphates. Thus, when 2′(3′)-*O*-isovaleryl-GDP is added to $(Ap)_2C$ under the same conditions as Figure 4, much less monoaddition product is obtained unless the incubation time is increased from 20 to 70 min. In the case of the pyrimidine diphosphates the addition reactions to $(Ap)_2C$ not only proceed more slowly, but show a greater tendency to give products with more than one nucleotide added. Thus, under the conditions given in Table I, the yield of $(Ap)_2CpU$ was 39% while 50% remained as unreacted $(Ap)_2C$ and 11% migrated as $(Ap)_2C(pU)_2$. If a longer incubation time or more enzyme is used, the amount of $(Ap)_2C$ decreases and the amount of $(Ap)_2C(pU)_2$ increases but the yield of $(Ap)_2CpU$ remains the same. Thus, the reaction conditions and incubation times given in Table I are adjusted to maximize the amount of single addition product and minimize the amount of multiple addition product. It was found that the use of 0.8 M NaCl in the reaction mixture favored the monoaddition of pyrimidine nucleotides somewhat by partially suppressing the tendency toward multiple addition to the primer. High salt concentrations did not increase the monoaddition of the purine nucleotides. Other attempts to increase the yield of pyrimidine nucleotide addition have not been successful. Indeed, the apparent inability of the pyrimidine nucleotides to give monoaddition products in high yield is one of the most important factors limiting the number of consecutive steps which can be carried out using the technique.

The 2′(3′)-*O*-isobutyryl derivatives of ADP and CDP were prepared using triethyl orthoisobutyrate and were tested as substrates but seemed to give lower yield of monoaddition products and showed a greater tendency toward multiple addition than did the corresponding isovaleryl nucleotides. The 2′(3′)-*O*-isovaleryl derivatives of inosine, N^6-isopentenyl-ADP, -ϵ-ADP, and -ϵ-CDP were tested as substrates and all gave monoaddition products as described in Table I. The N^6-isopentenyl and ϵ-modifications considerably increased the mobility of oligomers on the paper chromatograms, but the monoaddition products could still be characterized as described earlier.

Data in Table I show that although different primers give different optimal yields of monoaddition product, the effect is not as large as the dependence on which ribonucleotide is being added. Thus, while the rate of addition of a blocked diphosphate to an oligomer with a high pyrimidine content is considerably slower than to an oligomer with a high purine content, the yields of monoaddition product are comparable. This observation means that the method shows considerable generality with respect to the sequences which may be synthesized. The yield of monoaddition product is not strongly dependent on the primer concentration in the reaction mixture so that it is possible to use primer concentrations as low as 0.7 μM which is useful in monoaddition reactions to radioactive oligomers of high specific activity. The rate and extent of the monoaddition product show marked dependence on the chain length of the primer. The shortest oligonucleotides which are effective as primers for monoaddition are triribonucleoside diphosphates. The rate of addition to diribonucleoside monophosphates is very slow and multiple addition products predominate. Limited experience with tetranucleoside triphosphate primers indicates that while the rate of reaction increases considerably, the longer primers are also more susceptible to phosphorolysis. Although this difficulty can be overcome to some degree by lowering the enzyme concentration (as in the case of ApGpCpCpA synthesis in Table I) or by raising the diphosphate concentration, the synthesis of long sequences may not be possible. This important point is currently under investigation.

Evaluation. Due to the complexity of chemical techniques, enzymatic methods are more convenient for prepar-

ing short oligoribonucleotides of defined sequence to be used in other experiments. Primer dependent polynucleotide phosphorylase from *M. luteus* has been used to synthesize model oligoribonucleotides for thermodynamic studies (Uhlenbeck *et al.*, 1973; Martin *et al.*, 1971), circular dichroism studies (Borer *et al.*, 1973), oligomer binding studies (Uhlenbeck, 1972), and for use in the preparation of synthetic messenger RNAs (Stanley *et al.*, 1966; Thach *et al.*, 1966). The major weakness of these syntheses is that yields are low at each step and only certain sequences can be made with any efficiency. The development of a stepwise enzymatic oligomer synthesis technique should increase the variety of oligomers which can be synthesized and also hopefully improve yields. In this paper methods are described which allow synthesis of large quantities of a variety of 2′(3′)-*O*-isovaleryl nucleoside 5′-diphosphates in high yields. These diphosphates are tested as monoaddition substrates in a variety of reactions using *E. coli* polynucleotide phosphorylase. It was found that even though pyrimidine nucleotides do not add as efficiently as do purine nucleotides the yields are still high enough to make blocked diphosphate synthesis the method of choice for the preparations of short oligomers.

The method complements primer dependent techniques in many respects. A wide variety of new sequences can now be made. For example, the oligomer UpUpCpA is virtually impossible to make by a primer dependent polynucleotide phosphorylase reaction but can be synthesized in 64% yield by the technique described here. On the other hand, if a block of four or five residues had to be added to an oligomer, the primer dependent enzyme would be more suitable than sequential stepwise additions of the same nucleotide.

Two major factors limit the use of blocked diphosphates for the synthesis of extended sequences of RNA. First, multiple addition of pyrimidine diphosphates to oligomers prevents high yields of single addition products. Besides searching for a better blocking group, temporary modification of the pyrimidine bases in order to make the diphosphates better substrates might possibly increase the yield of monoaddition product. The N^4-dimethylaminomethylene derivative of 2′(3′)-*O*-isovaleryl-CDP did not prove to be sufficiently stable for this purpose. The second factor limiting the synthesis of extended sequences is the relative increase in the rate of phosphorolysis with respect to the rate of polymerization when longer oligomers are used as primers. If the yields decrease and the range of acceptable reaction times narrow greatly, the monoaddition reaction with blocked diphosphate and primer independent polynucleotide phosphorylase converges to the same reaction as unblocked diphosphate and primer dependent polynucleotide phosphorylase and the advantage disappears. This difficulty might be circumvented by coupling the reaction with an enzyme which utilizes phosphate in order to shift the equilibrium in a synthetic direction.

The ability of polynucleotide phosphorylase to accept 2′(3′)-*O*-isovalerylinosine 5-diphosphate and N^6-isopentenyl-ADP allows the ready synthesis of defined oligoribonucleotides longer than dinucleoside monophosphates which contain these modified bases. The synthesis of model oligomers containing minor bases will be a valuable tool in the elucidation of the role of these modifications in naturally occurring RNA (Nishimura, 1973). In addition, the ease of preparing and monoadding ϵ-ADP and ϵ-CDP allows specific fluorescent probes to be inserted into oligoribonucleotides at a desired position in the sequence.

It is anticipated that the blocked diphosphate techniques developed in this paper coupled with the established techniques of enzymatic oligomer synthesis will allow the production of sequences useful in a variety of applications.

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