THE EFFECT OF PHOSPHONYLMETHYL ANALOGS OF RIBONUCLEOSIDE 5'-DIPHOSPHATES ON (*E. coli*) POLYNUCLEOTIDE PHOSPHORYLASE CATALYZED REACTIONS

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This paper is dedicated to Prof. M. Ikehara, Faculty of Pharmaceutical Sciences, Osaka University, Osaka, Japan on the occasion of his retirement from Osaka University.

Ribonucleoside 5'-O-phosphorylphosphonylmethyl ethers (*I*) are not substrates of *Escherichia coli* polynucleoside phosphorylase in the presence of either Mg^{2+}- or Mn^{2+}-ions. Compounds *I* are efficient competitive inhibitors of the polymerization of naturally occurring ribonucleoside 5'-diphosphates (K_i/K_m $2 . 10^{-3} - 10^{-1}$). The analogs are not incorporated either into the polymer chain or into its termini during copolymerization with the natural substrate yet the homopolymer obtained shows a larger (by 20—85%) chain length. The presence of short oligonucleotides containing analogs *II* was not found after the polymerization reaction either. PolyA and polyC phosphorolyses are not affected by the presence of either naturally occurring 5'-diphosphates or of compounds *Ib—Id*. Adenine analog *Ia* as the only one significantly stimulates the phosphorolysis reaction catalyzed by *E. coli* polynucleotide phosphorylase.

The O-phosphonylmethyl derivatives of ribonucleosides are isopolar nucleotide analogs which differ from the latter by the CH_2-group inserted between the phosphorus and the oxygen atom of the sugar residue (*cf.* review[1]).** This structural alteration results in both chemical and enzymatical stability of the bond between the phosphorus residue and the nucleoside. According to theoretical assumptions, which have already[2] been confirmed by physicochemical studies, these analogs can adopt a conformation isosterical with that of the natural nucleotide during the interaction with the enzyme. As a result some analogs show an inhibitory effect on certain enzymes, such as 5'-nucleotidase[3], uridine kinase[4], or DNA-dependent RNA polymerase[5].

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** Abbreviations: NDP ribonucleoside 5'-diphosphate. The other symbols comply with the one-letter symbols suggested by the IUPAC-IUB Commission on Biochemical Nomenclature. 1 OD_{260} one optical density unit at 260 nm.

Our studies on these "phosphonate" nucleotide analogs have been focused mainly on the preparation and investigation of polymers derived from these compounds. Such polynucleotide analogs composed exclusively or partly of units of the above analog types (*A*, *B*) could possess parameters interesting physicochemically or from the viewpoint of molecular biology. Modified internucleotide bonds in such polymers should resist degradations by enzymes: Such an assumption[3] has recently been confirmed by a detailed examination of various types of diribonucleoside monophosphate analogs containing a phosphonate bond. These bonds are resistant to the attack of both ribonucleases and also of various types of exonucleases[6]. Polymers of this type can be synthesized either chemically or enzymatically. The enzymatic polymerization reactions require the transfer of the activated O-phosphonylmethyl nucleoside residue to the hydroxyl function of the subsequent molecule of the same substrate. The first type of these reactions, the so-called synthesis from the 5'-terminus, is represented in the ribonucleotide series by reactions of ribonucleoside 2',3'-cyclic phosphates catalyzed by ribonucleases (*cf.* review[7]). In the phosphonates series, however, such reactions, *i.e.* the homopolymerization of six-membered 2',3'--cyclic O-phosphonylmethyl ribonucleosides derived from uridine or guanosine or their reactions with ribonucleosides in the presence of ribonuclease A, T1 or T2, do not proceed[8].

The other type of polymerization reactions of naturally occurring nucleotides are reactions yielding polymers with the free 3'-terminus of the chain. These reactions usually make use of activation of the 5'-nucleotide by a mixed anhydride bond. Most enzymes catalyzing these reactions (DNA polymerases, RNA polymerases, DNA-dependent RNA polymerases) utilize 5'-triphosphates. Such enzymes could utilize also the phosphonate analogs of nucleoside 5'-triphosphates on condition that the phosphorus atom in the α-position only has been modified. The reaction could occur particularly in such cases where the catalysis of the transfer reaction does not depend on the activation of this atom but on the activation of, *e.g.*, the β-phosphorus atom. Indirect evidence in favor of such a possibility is the donor activity of 5'-O-diphosphorylphosphonylmethyl ribonucleosides (ATP and GTP analogs) during uridine phosphorylations catalyzed by uridine kinase from L-1210 cells[9]. A direct proof of the utilization of ribonucleoside 5'-triphosphate analogs with an α-phosphonate bond is the initiation of chain formation and the synthesis of phosphonate analogs of short oligoribonucleotides catalyzed by DNA-dependent RNA polymerase from *E. coli*[10].

Other enzymes capable of catalyzing the synthesis of both homo- and heteropolyribonucleotides are polynucleotide phosphorylases (EC 2.7.7.8) (polynucleotide: orthophosphate nucleotidyl transferase) (*cf.* reviews[11,12]). Unlike in the preceding reactions the substrates of these enzymes are ribonucleoside 5'-diphosphates; inorganic phosphate is liberated in the course of the reaction. The polynucleotide phosphorylases are moreover capable of catalyzing the reversed reaction, *i.e.* the

phosphorolysis of the polyribonucleotides by inorganic phosphate (this is obviously their physiological role[13]) or, eventually, the terminal exchange reaction of β-phosphates in ribonucleoside 5'-diphosphates[14]. The activity of these enzymes, especially the utilization of the structurally modified substrates considerably varies with the enzyme source or with the activator used (a bivalent metal ion, such as, *e.g.*, Mg^{2+} or Mn^{2+}) (refs[15,16]), and, in the case of highly purified enzymes, also with the presence of the primer (*e.g.*, a trinucleotide[17]). Nucleotide analogs with a modified phosphorus atom can also interact with polynucleotide phosphorylases: Adenosine 5'-hypophosphate, *e.g.*, inhibits both polymerization and phosphorolysis[18], and so does also adenosine 5'-methylenediphosphonate[19], whereas adenosine 5'-phosphorylphosphothioate is a substrate of the enzyme[20].

This study was undertaken in an effort to determine whether ribonucleoside 5'-diphosphate analogs, 5'-O-phosphorylphosphonylmethyl ribonucleosides (*I*) can serve as substrates or act as inhibitors of polynucleotide phosphorylases and also whether these enzymes can be employed for the synthesis of polymers containing either exclusively or partly analogous phosphonate internucleotide bonds.

EXPERIMENTAL

Materials. Adenosine 5'-diphosphate (sodium salt) was from Reanal (Hungary), uridine 5'-diphosphate (potassium salt) from Boehringer (F.R.G.) and cytidine 5'-diphosphate (sodium salt) from Pharma-Waldhof (F.R.G.). All 5'-diphosphates were purified by ion exchange chromatography before use. 5'-O-phosphonylmethyl ribonucleosides and 5'-O-phosphorylphosphonylmethyl ribonucleosides (sodium salts) used in this study were prepared according to ref.[21] and were pure as judged by HPLC. ApA, CpC, and ApApA were preparations of Pharma-Waldhof (F.R.G.), adenosine-3'-O-phosphonylmethyl-5'-adenosine and cytidine-3'-O-phosphonylmethyl--5'-cytidine were prepared as described in literature[6]. Sephadex A-25 and Sephadex G-50 (particle size 40—80 μ) were preparations of Pharmacia Fine Chemicals (Sweden), nitrocellulose membrane filters (0·45 μ) 25 mm in diameter, were purchased from Millipore (U.S.A.). [U-^{14}C]--Ribonucleoside 5'-diphosphates (spec. activity: UDP 15·76 MBq/μmol, CDP 16·65 MBq/μmol, ADP 17·76 MBq/μmol) were obtained from the Institute for Research, Production and Utilization of Radioisotopes, Prague (Czechoslovakia) and were 98·5—98·8% radiochemically pure according to TLC on PEI-cellulose (Polygram Cel 300 PEI/UV 254) in the system 1 mol l^{-1}. acetic acid and 4 mol l^{-1} lithium chloride, 4 : 1.

Enzymes. Polynucleotide phosphorylase *E. coli* B (10 units/mg protein) was purchased from P-L Biochemicals, Inc. (U.S.A.) or (5 units/mg protein) from Choay-Chimie (France). Polynucleotide phosphorylase *Micrococcus luteus* (purified, B grade) (0·033 units/mg protein) was a product of Calbiochem (U.S.A.). Snake venom (*Crotalus durissus*) exonuclease (2 units/mg protein, 2 mg/ml) and calf spleen exonuclease (1 mg/ml) were purchased from Boehringer (F.R.G.), alkaline phosphatase, type III-S from *E. coli* (10·5 units/mg protein, 10·3 mg/ml) was obtained from Sigma (U.S.A.), bovine pancreatic ribonuclease A from Lachema (Czechoslovakia) and ribonuclease T2 (*Asp. oryzae*) from Sankyo (Japan).

Methods. Paper chromatography was carried out on Whatman No. 3MM paper in system S1, 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2), paper electrophoresis (20 V/cm, 1 h) on the same paper in 0·1 mol l^{-1} triethylammonium hydrogen carbonate, pH 7·5, TLC was per-

formed on PEI-cellulose in system S2, 1 mol l^{-1} acetic acid–4 mol l^{-1} lithium chloride (4 : 1). The R_F values and electrophoretic mobilities are summarized in Table I. The ultraviolet absorption spectra were measured in aqueous solutions on Specord UV-VIS (C. Zeiss, Jena, G.D.R.) spectrophotometer and on 8451A Diode Array Spectrophotometer Hewlett–Packard. Analytical ultracentrifugation was performed in Model 6 Beckman–Spinco analytical ultracentrifuge (An-H Ti rotor) at 20°C in SSC buffer (4 800—56 000 rpm), for centrifugation Janetzki T 32c (G.D.R.) centrifuge was used. The radioactivity measurements were performed in Packard Tri-carb 300 liquid scintillation counter using a toluene scintillator. Phosphorus determinations were effected using the blue color method[22] or the yellow color method[23]. The deproteinization of enzyme assay mixtures was carried out according to ref.[24].

Purification of ribonucleoside 5'-diphosphates. The commercial ribonucleoside 5'-diphosphate sodium salt (200 mg) in 2 ml of 0·02 mol l^{-1} triethylammonium hydrogen carbonate was applied on a DEAE-Sephadex A-25 column (2·3 × 17 cm) equilibrated with the same buffer. Elution was effected by a linear gradient (0·02—0·5 mol l^{-1}, 500 + 500 ml) of the same buffer. The fraction containing the pure product was evaporated at 30°C/2 kPa, the dry residue was codistilled with methanol, the residue dissolved in 5 ml of water and applied on a Dowex 50 X8 column (2·3 × 12 cm) in Na^+-form. The column was eluted with water, the ultraviolet-absorbing fraction was evaporated at 30°C/2 kPa, the residue codistilled with ethanol and crystallized

TABLE I
Chromatography and electrophoresis

Compound	R_F		E_{Up}[a]
	S1	S2	
Ado	0·59	—	−0·13
AMP	0·10	0·65	0·87
ADP	0·07	0·35	1·05
Ia	0·07	0·37	1·05
IIa	0·10	0·66	0·87
ApA	0·30	—	0·30
ApApA	0·05	—	0·90
Cyd	0·55	—	−0·12
CMP	0·08	0·64	1·00
CDP	0·06	0·33	1·19
Id	0·08	0·67	1·00
IId	0·06	0·35	1·18
CpC	0·26	—	0·35
Urd	0·50	—	0
UMP	—	0·66	1·00
UDP	0·05	0·32	1·17
Ib	0·05	0·34	1·16
Ino	0·46	—	0·12

[a] Electrophoretic mobility referred to uridine 3'-phosphate.

from ethanol–ether. The product was filtered off by suction, washed with ether and dried at 13 Pa over phosphorus pentoxide. The sodium salts of 5'-diphosphates thus obtained are more than 99% pure as judged by HPLC.

Preparative chromatography of the polymerization mixtures was carried out on a Sephadex G-50 column (1 × 95 cm) in water at 4°C. Flow rate 0·05 ml/min, fractions collected in 20 min intervals. The course of the chromatography was monitored by Uvicord (LKB, Sweden). Corresponding fractions were pooled and lyophilized, the products were stored at −20°C in a desiccator.

Enzyme degradation assays. a) Snake venom exonuclease assay: The incubation mixture (300 μl) contained 3·3 mmol l^{-1} ApA, CpC or their phosphonate analogs, 5 mmol l^{-1} $MgCl_2$ and 0·12 enzyme units in 0·1 mol l^{-1} Tris-HCl, pH 8·5. *b*) Pancreatic RNase assay: The incubation mixture (300 μl) contained 3·3 mmol l^{-1} CpC or analogs and 150 μg of enzyme protein in 0·1 mol l^{-1} Tris-HCl, pH 8·5. *c*) Ribonuclease T2 assay: The incubation mixture (100 μl) consisted of 3 mmol l^{-1} ApA (or analog) and 50 μg of enzyme protein in 0·05 mol l^{-1} ammonium acetate, pH 4·5. Incubation 20 h at 37°C. Blanks were assayed under identical conditions in the absence of the enzyme. The incubation mixtures were analyzed by paper electrophoresis. The UV-absorbing spots were cut out, eluted by 0·01 mol l^{-1} HCl and the product content determined spectrophotometrically at 260 nm.

Polymer digestion analysis. a) PolyA digestion: The incubation mixture (300 μl) contained 20 OD_{260} of polyA, 5 mmol l^{-1} $MgCl_2$, snake venom exonuclease (60 μg of protein) and alkaline phosphatase (100 μg) in 0·1 mol l^{-1} Tris-HCl, pH 8·5. *b*) PolyC digestion: The incubation mixture (150 μl) consisted of 4 OD_{260} polyC preparation, pancreatic RNase (30 μg) and alkaline phosphatase (50 μg) in 0·1 mol l^{-1} Tris-HCl, pH 7·5. Incubation 18 h at 37°C. The mixtures were applied on Whatman No. 3MM paper and developed in system S1. The spots of nucleoside, nucleoside 5'-phosphate and ApA or CpC were cut out, eluted by 0·01 mol l^{-1} HCl and the product content determined spectrophotometrically at 260 nm. With labeled polymers, the chromatograms were scanned and the spots corresponding to the above components were eluted with dilute ammonia (1 : 100), the solutions concentrated and the products separated by paper electrophoresis. The chromatograms were scanned and radioactivity determined in spots corresponding to the appropriate standard compounds. Low molecular weight fractions (NDP) from gel filtration of polymerization mixtures were analyzed as follows: *a*) The [^{14}C]ADP fraction (120 OD_{260}, 3 × 10^6 cpm) and alkaline phosphatase (200 μg) in 200 μl 0·1 mol l^{-1} Tris-HCl, pH 8·5 or *b*) [^{14}C]CDP fraction (28 OD_{260}, 3 × 10^6 cpm) and alkaline phosphatase (100 μg) in 150 μl 0·1 mol l^{-1} Tris-HCl, pH 8·5 were incubated 18 h at 37°C and the mixtures were separated by paper chromatography in system S1. The chromatograms were scanned and the radioactivity peaks were eluted and counted. The peaks corresponding to NMP or NpN were run on paper electrophoresis and the peaks corresponding to the above standards were counted.

Polymerization Experiments

Polymerization of ADP and of its analog *Ia* (phosphate release method): The incubation mixture (50 μl) contained 20 mmol l^{-1} ADP or *Ia*, 5 mmol l^{-1} $MgCl_2$ or $MnCl_2$, 0·2 mmol l^{-1} EDTA and 0·3 units of *E. coli* polynucleotide phosphorylase in 0·15 mmol l^{-1} Tris-HCl, pH 9·0. Incubation at 37°C, aliquots (10 μl) analyzed at intervals of 0·5—24 h by the blue color method. Under these conditions the reaction (release of P_i) proceeds with ADP to 60% after 2 h in the presence of $MgCl_2$ whereas no reaction can be observed with compound *Ia* after 24 h.

Polymerization of ADP, UDP, *Ia*, and *Ib* in the presence of primer: The composition of the

incubation mixture (50 μl) is identical to that described above; it contains 20 mmol l^{-1} ADP, UDP, *Ia* or *Ib* and 0—1 mmol l^{-1} ApApA. Incubation 30 min at 37°C, the phosphate liberated determined by the yellow color method. The reaction rate of ADP (0·8 mmol min^{-1}) is independent of ApApA concentration under these conditions, compounds *Ia* and *Ib* do not react at all.

Inhibition Experiments

Effect of GMP, compounds *Ic* and *IIc* on ADP polymerization: The incubation mixture (50 μl) contained 20 mmol l^{-1} ADP, 5 mmol l^{-1} $MgCl_2$, compounds *Ic*, *IIc* or GMP in concentrations of 0·25, 0·5, 1·0, 2·0, 3·0, and 10 mmol l^{-1} and 0·075 units of *E. coli* polynucleotide phosphorylase. Incubation 30 min at 37°C. The phosphate liberated was determined by the yellow color method. In a control experiment in the absence of the effector v_0 = 0·70 mmol P_i min^{-1}, GMP and *IIc* are without effect, in the presence of 0·5 mmol l^{-1} *Ic*, v_0 = 0·1 mmol P_i min^{-1}.

Mg^{2+}-Dependence of NDP polymerization: The standard assay mixture (50 μl) consisted of 40—80 μmol . l^{-1} [^{14}C]NDP (4·44 kBq), $MgCl_2$ (0·01—10 mmol l^{-1}) and of 0·3 units of *E. coli* polynucleotide phosphorylase in 0·15 mmol l^{-1} Tris-HCl, pH 9. Incubation 10 min at 37°C. Yeast RNA solution (0·5 ml, 0·5 mg/ml) and 10% trichloroacetic acid solution (0·5 ml) were added, the mixture was set aside at 0°C for 10 min, filtered through a Millipore filter, the filter was washed three times with 3 ml of 5% trichloroacetic acid, then dried and radioactivity counted in a scintillation counter. The results are shown in Fig. 1. The optimum Mg^{2+} concentrations estimated from the slopes of the Mg^{2+} dependence (1·5 mmol l^{-1} for UDP and ADP, 5 mmol l^{-1} for CDP) were used in all subsequent kinetic experiments.

Determination of K_m-values of NDP polymerization: The experiments were performed in standard mixtures at optimum Mg^{2+} concentrations and varying NDP concentrations, and processed as above. The K_m-values are summarized in Table II.

Determination of K_i-values for compounds *I* in NDP polymerization: The reactions were performed as above in standard mixtures at optimum Mg^{2+} concentrations and different concentrations (0—20 mmol l^{-1}) of compounds *I*. The mixtures were processed under standard conditions. The K_i and K_i/K_m values are summarized in Table II, a typical Dixon-plot of the inhibition is shown in Fig. 2.

Preparation of PolyA and PolyC

A) *In the absence of inhibitors*: The incubation mixture (3 ml) contained 15 mmol l^{-1} CDP or 20 mmol l^{-1} ADP, $MgCl_2$ (2 mmol l^{-1} for CDP, 1 mmol l^{-1} for ADP polymerization) and *E. coli* polynucleotide phosphorylase (10 units) in 0·15 mmol l^{-1} Tris-HCl, pH 9. Incubation 18 h at 37°C. After the addition of chloroform–3-methylbutanol (5 ml, 3 : 1), the mixture was centrifuged 10 min at 3 000 rpm and the organic layer was discarded. The aqueous layer was treated twice more in the same manner, the organic layers discarded and the combined interphase layers were washed with water (1 ml). The combined aqueous solutions were freeze-dried, dissolved in water (2 ml) and applied on a Sephadex G-50 column. The elution patterns are shown in Fig. 3, the physical characteristics of the polymer fractions are summarized in Table III. The polymer and low molecular weight fractions were freeze-dried.

B) *In the presence of inhibitors*: The polymerizations were performed under the conditions given under *A*) except that the CDP polymerization mixture contained 0·15 mmol l^{-1} *Id* and the ADP mixture 2 mmol l^{-1} *Ia*. The elution pattern and the characteristics of the polymers are given in Fig. 3 and Table III.

C) *Labeled polymers*: The polymerizations were performed as described under *A*) and *B*)

with *E. coli* or *Micrococcus luteus* polynucleotide phosphorylase except that the ADP polymerization mixture contained 370 kBq [U-^{14}C]ADP and the CDP mixture 550 kBq [U-^{14}C]CDP. The yields and the specific radioactivity values of the polymers are given in Table IV.

TABLE II

NDP polymerization by polynucleotide phosphorylase: kinetic parameters of inhibition by compounds *I* (K_i, μmol l^{-1}), K_i/K_m values in parentheses

Inhibitor	Substrate		
	UDP	CDP	ADP
None (K_m)	2 976 ± 69	217 ± 1	70 ± 4
Ia	4·8 ± 3·1 (0·002)	9·6 ± 0·07 (0·044)	2·8 ± 0·7 (0·040)
Ib	11·3 ± 1·6 (0·004)	14·6 ± 0·4 (0·087)	3·8 ± 0·6 (0·054)
Ic	4·6 ± 0·8 (0·002)	5·2 ± 1·5 (0·024)	1·0 ± 0·2 (0·014)
Id	5·8 ± 11·6 (0·002)	12·6 ± 4·1 (0·058)	8·1 ± 2·9 (0·116)

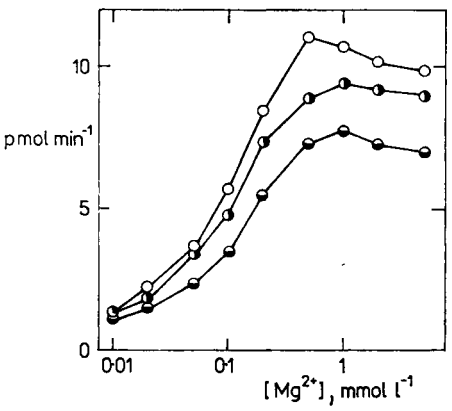


FIG. 1

Dependence of ADP polymerization on Mg^{2+}-ions at different substrate concentrations. [ADP] (μmol l^{-1}) ◒ 40, ◑ 60, ○ 80

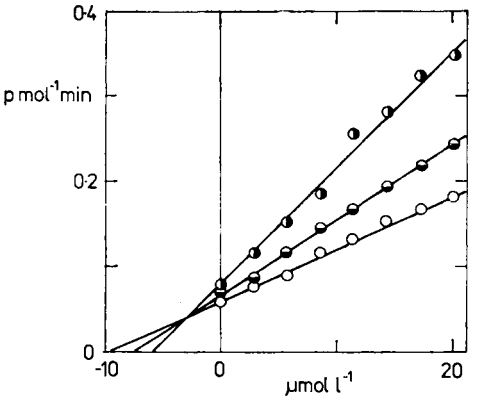


FIG. 2

Dixon plot of inhibition of ADP polymerization by compound *Ia*. [ADP] (μmol l^{-1}) ◑ 40, ◒ 60, ○ 80

Phosphorolysis Experiments

Effect of NDP or *I* on polyA and polyC phosphorolysis: The standard incubation mixture (100 µl) consisted of 10 mmol l^{-1} disodium hydrogen phosphate, 1 mmol l^{-1} $MgCl_2$, 1 OD_{260} [^{14}C]polyA (*cf.* Table IV, 650 µmol l^{-1} monomer, 682 Bq/OD_{260}) or 0·59 OD_{260} [^{14}C]polyC (*cf.* Table IV, 1 377 Bq/OD_{260}, 650 µmol l^{-1} monomer), 0—10 µmol l^{-1} NDP or compound *Ia*, *Id* and 0·03 units of *E. coli* polynucleotide phosphorylase in 0·15 mol l^{-1} Tris-HCl, pH 9.

TABLE III

Effect of compounds *I* on properties of homopolymers prepared by *E. coli* polynucleotide phosphorylase

Polymer	*I*/NDP	Yield %	S^{20}	Mol. weight	Polymer length (units)
PolyC	0	17	4·77	$7 . 10^4$	217
	0·01 (*Id*)	5	5·6	$13 . 10^4$	402
Poly A					
Fraction I[a]	0	11	6·9	$3·1 . 10^5$	893
	0·1 (*Ia*)	11	7·3	$3·8 . 10^5$	1 095
Fraction II	0	17	6·1	$1·9 . 10^5$	548
	0·1 (*Ia*)	8	6·6	$2·6 . 10^5$	749

[a] *Cf.* Fig. 3.

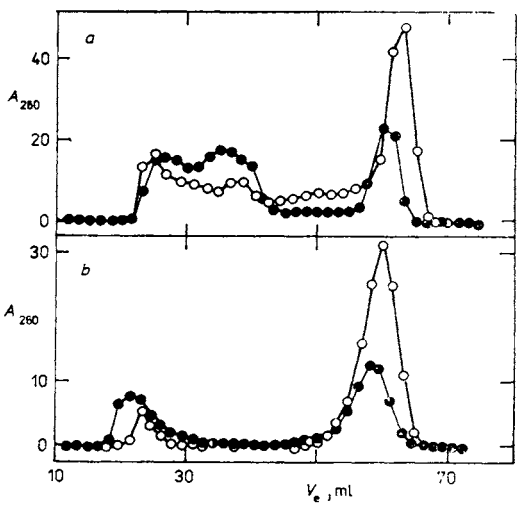


FIG. 3

Gel filtration (G-50) elution profile of *a* ADP and *b* CDP polymerization mixture prepared in absence ○ or presence ● of inhibitors (*Ia* in *a*, *Id* in *b*)

Incubation 10 min at 37°C. The mixture was processed as described under kinetic measurements of polymerization reactions. The data are summarized in Fig. 4.

Effect on Mg^{2+} on polyA phosphorolysis in the presence or absence of *Ia*: The standard phosphorolysis incubation mixture was the same as above, without or with (1 μmol l^{-1}) *Ia* and varying concentrations (0·2—5·0 mmol l^{-1}) of $MgCl_2$. The mixture was processed as described above. The data are shown in Fig. 5.

TABLE IV

[^{14}C]-Labeled polyribonucleotides prepared by *Micrococcus luteus* polynucleotide phosphorylase

Polymer	*I*/NDP	Yield %	Polymer length (units)	Specific radioactivity KBq/OD_{260}
PolyC	0	14	196	2·894
	0·01 (*Id*)	3	—	3·100
PolyA				
Fraction *I*[a]	0	14	222	0·682
	0·1 (*Ia*)	10	—	0·684
Fraction II[a]	0·1 (*Ia*)	8	—	0·610

[a] *Cf.* Fig. 3.

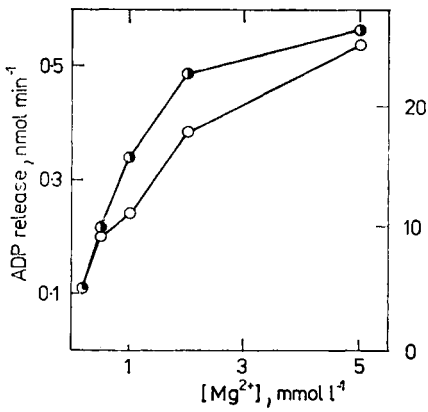


FIG. 4

Effect of ADP and compound *Ia* on phosphorolysis of polyA at different Mg^{2+} concentrations. ○ ADP, ◑ *Ia*

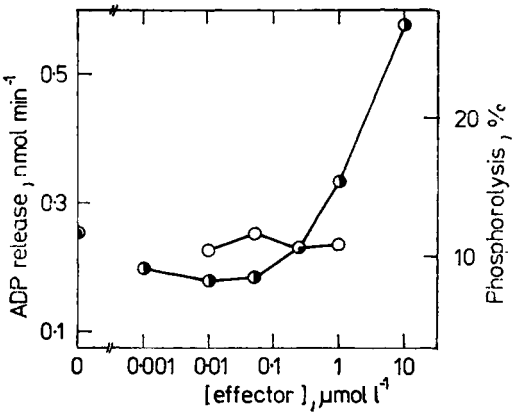


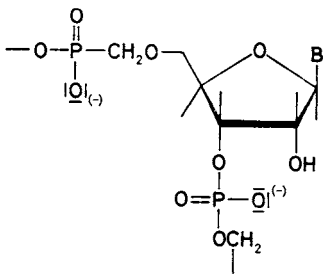
FIG. 5

Mg^{2+} Effect on polyA phosphorolysis in presence ◑ or absence ○ of compound *Ia*

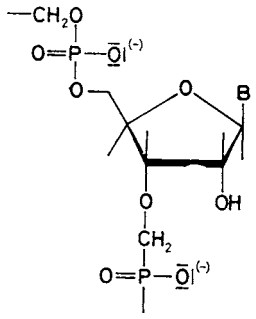
RESULTS AND DISCUSSION

During the first stage of this study the ability of naturally occurring ribonucleoside 5'-diphosphate analogs *I* to serve as *E. coli* polynucleotide phosphorylase substrates was examined. The phosphate release method was used as a tool of this investigation. Under the experimental conditions described, ADP is polymerized with the liberation of inorganic phosphate; this reaction arrives at an equilibrium after 2 h, the conversion being 60%. By contrast, no measurable reaction could be observed with compound *Ia* even after 24 h incubation. The analogs of other NDP's, namely compounds *Ib*, *Ic*, and *Id*, are also inert under these conditions (data not shown). To eliminate the possible effect of Mg^{2+}-ions on the polymerization reaction we repeated the same experiments with Mn^{2+}-ions in the concentration range 2·5 to 10 mmol l^{-1}. However, no measurable liberation of inorganic phosphate was observed even under these conditions with analog *Ia*. We observed though that even the degree of ADP polymerization is approximately three times lower in the presence of Mn^{2+}-ions than in the presence of Mg^{2+}-ions.

The polymerization of ADP, UDP and of their analogs *Ia* and *Ib* by the *E. coli* enzyme was also performed in the presence of various concentrations of ApApA



A



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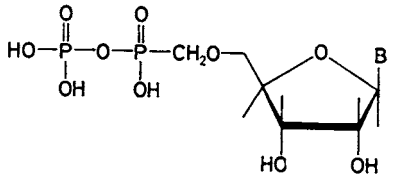
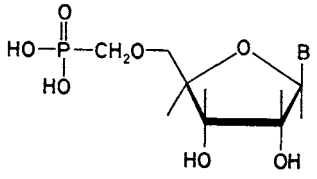


I



II

In formulae *I*, *II*: *a*, B = adenin-9-yl, *b*, B = uracil-1-yl, *c*, B = guanin-9-yl, *d*, B = cytosin-1-yl.

primer. The polymerization of ADP was independent of the primer in the range 1 μmol l^{-1} – 1 mmol l^{-1} ApApA at 10 mmol l^{-1} concentration of substrate whereas the UDP polymerization was weakly primer-dependent: The reaction rate increased twice at 1 mmol l^{-1} ApApA concentration. No polymerization of compounds *Ia* and *Ib* was observed even in the presence of the primer.

These experiments with compounds *I* as the only substrates of the reaction demonstrated that the analogs cannot be utilized by the enzyme for the synthesis of a polymer. In an effort to determine whether the failure of compounds *I* to react is not caused by the mere presence of the —P—CH_2—O— group in the molecule of compounds *I* we studied the effect of an analog of GMP, *i.e.* of compound *IIc* on ADP polymerization. Both GMP and its analog *IIc* are entirely without any effect on the rate of ADP polymerization. By contrast, the 5'-diphosphate analog *Ic* markedly decreases the rate of ADP polymerization; a 60% decrease of the latter can be observed even at a concentration ratio of ADP to *Ic* equal to 100 : 1. Consequently, the 5'-diphosphate analogs *I* are not substrates but inhibitors of polynucleotide phosphorylase. This feature results from the characteristic structure of the integral molecule of compound *I* and does not reflect the presence of the group in the neighborhood of the phosphorus atom in position α itself.

When determining the kinetic parameters of inhibition by compound *I*, the complexity of the reaction kinetics of polymerizations catalyzed by polynucleotide phosphorylase should be considered. The most important role in these reactions plays the activating effect of magnesium ions which form relatively rigid complexes[25] with the substrates of the reaction. It is likely that the diphosphate analogs *I* also form analogous complexes with Mg^{2+}-ions even though their stability will be probably lower[9]. The inhibitory effect of compounds *I* could thus be caused, to a certain degree, by competition for Mg^{2+}-ions with the natural substrates of the reaction. Since the plot of the polymerization rate *versus* the concentration of Mg^{2+}-ions depends on the substrate concentration we carried out our kinetic studies at the optimal concentration of Mg^{2+}-ions. The latter was determined from plots obtained with the individual substrates and chosen to correspond to such a concentration range of Mg^{2+}-ions over which the plot is linear and parallel over the entire range of substrate concentrations used. This optimalization procedure is shown in Fig. 1 demonstrating the Mg^{2+}-dependence of the rate of ADP polymerizations. The range of substrate concentrations for the kinetic measurements was established from the preliminary K_m-values of all three substrates (UDP, CDP, ADP) used, determined for 40–160 mmol l^{-1} NDP. The estimation of the initial reaction rate was carried out with [^{14}C]-labeled substrates, *i.e.* by measuring the synthesis of the [^{14}C]-labeled polymer by the membrane filter technique. The optimal values of Mg^{2+}-concentrations found are 1·5 mmol l^{-1} and 5 mmol l^{-1} for UDP and ADP and for CDP, respectively. The corrected K_m-values of all three substrates were then obtained from the Lineweaver-Burke plots under the conditions found in experi-

ments with 5–8 different substrate concentrations. These K_m-values are listed in Table II.

The determination of the inhibition constants K_i of analogs *Ia–Id* for the polymerization of natural substrates UDP, CDP, and ADP (GDP is a very poor substrate of the enzyme) was carried out by the same technique using the Dixon plot. The values of K_i and K_i/K_m obtained by the method of least squares are listed in Table II together with the standard error values. A typical plot of ADP polymerization is given in Fig. 2. All these measurements show that the diphosphate analogs *I* are competitive inhibitors of NDP polymerizations. Since the ratio of inhibitor concentration to the total concentration of Mg^{2+}-ions is 1 : 75 or 1 : 250 resp. at the most, the effect of compounds *I* on the decrease of the actual concentration of the metal ion activator can be eliminated. It is obvious from the data given in Table II (K_i/K_m) that all the analogs are very efficient inhibitors of the enzyme. This inhibition is not absolutely base specific (each compound *I* inhibits the reactions of any NDP); it differs thus from, *e.g.*, the absolute base specificity of the inhibition of DNA-dependent RNA polymerase by 5'-triphosphates analogous to compounds *I* (*cf.*[3]). In spite of that it is obvious that purine derivatives *Ia* and *Ic* are more efficient inhibitors than pyrimidine compounds *Ib* and *Id*. The K_m-values of naturally occurring NDP's differ over a wide concentration range thus documenting their different affinity for the enzyme. By contrast, the affinity of inhibitors of type *I* is obviously not affected too much by the character of the heterocyclic base.

Hence, the substitution at the α-phosphorus atom does not eliminate the ability of analog *I* to interact with polynucleotide phosphorylase; it interferes, however, with the course of the reaction catalyzed. We cannot exclude the possibility that compound *I* could play a role in the initiation period of the reaction (*i.e.* a situation analogous to that observed with DNA-dependent RNA polymerase[10]) or, *vice versa*, in the chain termination, even more so because the polymerization reaction involves two subsites of the enzyme which need not necessarily be simultaneously occupied by the analog[26]. A direct proof of this statement in an experiment with the labeled analog is troublesome because of the difficult accessibility of [^{14}C]- or[^{32}P]-labeled analogs *I* and because of the insufficient reliability of experiments with [^{3}H]-labeled compounds in the reactions catalyzed by the enzyme. We have therefore proceeded *via* the preparation and isolation of polynucleotides prepared from [^{14}C]-labeled NDP's in the presence or absence of inhibitors *I* and *via* the analysis of both high molecular weight and low molecular weight products of the polymerization reaction. A reliable analytical method for this purpose are enzymatic degradations: We have shown recently that internucleotide links involving the phosphonates (type *A*) are resistant to the action of most nucleolytic enzymes (ribonucleases, exonucleases[6]). The presence of a resistant oligonucleotide, which in the sequence of type *A* or *B* must contain one labeled nucleoside at least, after exhaustive digestion of the polynucleotide with a nucleolytic enzyme would represent unambiguous evidence of

incorporation of the analog into the terminus or into the middle portion of the chain.

We prepared polyA, polyC, and [^{14}C]-labeled polymers using the *E. coli* and *Micrococcus luteus* enzymes, in parallel experiments in the absence and in the presence of inhibitor *I* containing a heterocyclic base identical with the substrate. Because of the high inhibitory effect of compounds *I* we had to choose, however, such ratios which would still permit a preparative isolation of the polymer (100 : 1 for CDP : *Id*, 10 : 1 for ADP : *Ia*). The polymerization mixtures were treated under standard conditions (see a typical elution pattern in Fig. 3) and both the polymer fractions and the low molecular weight portion were isolated. The yields and the characteristics of the polynucleotides prepared are listed in Table III and IV. Pancreatic ribonuclease was used in the degradation experiments with polyC. We have proved in an independent experiment that phosphonate analog CpC is entirely resistant toward the action of the enzyme (*cf.* also[6]) even under conditions of prolonged incubation. The polymer fraction was digested with a mixture of ribonuclease A and alkaline phosphomonoesterase which can dephosphorylate cytidylic acids yet not 5'-O-phosphonylmethyl cytidine (*IId*). According to our expectation, cytidine was the main product of the digestion; no significant radioactivity was detected in the region of compound *IId*. Thus the incorporation of the analog into the 5'-terminus of the chain can be excluded. By contrast, a significant radioactivity peak was observed in the region of dinucleoside phosphate, a peak which also according to its electrophoretic behavior corresponds to this type of compound. Its quantity was 0·07% of input radioactivity. An equally significant quantity (with respect to the background which was below 30–40 cpm) of an analogous compound was detected, however, also when the polymer prepared by polymerization in the absence of compound *Id* was degraded. Obviously, contamination with the resistant isomer (2'–5')--CpC arising either during the polymerization itself or rather during the isolation of the polymer, its cleavage and separation can be suspected. We may thus conclude that the preparation of polyC obtained in the presence of *Id* does not contain any analog incorporated either into the terminus or inside the chain. The accuracy of the determination with respect to the chain length is higher than 1 monomer unit.

Ribonuclease T2 would seem the enzyme of choice for a similar degradation of polyA. The cleavage, however, proceeds very imperfectly in accordance with the known[27] resistance of this polymer toward ribonuclease T2. We used therefore for the digestion a mixture of snake venom exonuclease, yielding AMP as the main degradation product, and alkaline phosphomonoesterase. In model experiments the cleavage of ApA phosphonate analog under extreme conditions is below 8%. The analysis of the incubation mixture showed that radioactivity was contained, in addition to adenosine, also in the region of inosine, a deamination product (minor amount). As little as 0·2% of input radioactivity was found in the region of polar compounds; this region does not correspond, however, to compounds of either type

ApA or ApApA according to electrophoresis. Even in the latter case, however, the control polymer prepared in the absence of inhibitor *Ia* shows a completely identical digestion pattern. Hence, incorporation of the analog into the chain was not demonstrated in this case either, even though the substrate to inhibitor ratio is more favorable in the case of polyA synthesis.

The low molecular weight fraction from gel filtration of the polymerization mixture, containing the unreacted substrate and the inhibitor as the main components, can comprise also low molecular weight oligonucleotides, *e.g.* doublets and triplets. To analyze this fraction it is sufficient to digest it with alkaline phosphomonoesterase yielding the nucleoside and (unlabeled) compound *II*. We did not find any polar radioactive material indicating the presence of analogous short-chain oligonucleotides. The small amount of residual radioactivity in the nucleotide region (ca 0·2% of total input) is identical for the polymer prepared both in the presence and in the absence of the inhibitor. This pattern is identical in fractions obtained by the polymerization of ADP and CDP. This radioactivity most likely originates from an artifact present in the starting labeled substrate. Hence, no short oligonucleotides are formed during the polymerization of the four variants studied.

Comparing the parameters of control polymers with those of polynucleotides prepared in the presence of inhibitors *I* we observed that the presence of the inhibitor decreases the yield of the polymer yet significantly increases the polymer chain length. This increase is as high as 85% in the case of polyC (Table III). When the polyA reaction mixture is subject to gel filtration, two separate high molecular weight portions are obtained (Fig. 3) the first of which has its chain lenghtened by 20% whereas the second one (whose quantity is decreased in the presence of the inhibitor) has a chain length increased by 40%. Such an effect of the inhibitor could be interpreted in terms of suppression of phosphorolysis of the arising polymer, *i.e.* of the reversed enzymatic reaction. We studied therefore kinetically also the effect of compounds *I* on the phosphorolysis of polyA and polyC in buffers containing inorganic phosphate under the conditions analogous to those which have been recorded for phosphorolysis in the literature[28]. We employed various [^{14}C]-labeled polynucleotides as substrates of the reaction using the technique of membrane filters. The phosphorolysis was carried out in the presence of magnesium ions; the concentration used (1 mmol l^{-1} $MgCl_2$) roughly corresponds to the K_m-value for Mg^{2+}-ions in this reaction, derived from an independent experiment (data not shown) and is identical to reported data[28]. The effect of analog *Ia* on the phosphorolysis of polyA as a function of the concentration of magnesium ions is shown in Fig. 4. It is evident that the analog does not inhibit but on the contrary stimulates phosphorolysis, the difference from the control experiment being the greatest and constant over the range of 1–2 mmol l^{-1} Mg^{2+}. A detailed investigation of the effect of UDP, CDP, ADP, GDP and of their phosphonate counterparts *Ib–Id* on the phosphorolysis of both polymers has shown that these compounds have no significant effect on

the phosphorolysis over the concentration range of $0{\cdot}01-10\ \mu mol\ l^{-1}$ (data not shown). Adenine derivative *Ia* is the only one which significantly stimulates the phosphorolysis reaction. This increase may even double the initial reaction rate at a higher effector concentration (10 μmol) (Fig. 5). The stimulating effect on the phosphorolytic reaction has not been described so far for any analog. Most of the compounds studied as yet are either without any effect on the reaction or act as phosphorolysis inhibitors (adenosine 5'-phosphohypophosphate[18], 5'-methylenediphosphonate[19] or 2'-deoxyadenosine 5'-diphosphate[25]). All those compounds inhibit both reactions – polymerization and phosphorolysis – whereas the effect of compound *Ia* in both reactions is adversed. This unexpected behavior is difficult to interpret. These experiments, however, provide conclusive evidence that the increase in the length of the polymer chain during polymerization in the presence of inhibitor *I* is not caused by the suppression of the phosphorolytic reaction. It appears therefore that an explanation for the chain lengthening should be sought rather in an allosteric effect of analog *I* on the two-subsite enzyme and/or on the formation of active complexes of the enzyme, respectively.

We may conclude that ribonucleoside 5'-diphosphate analogs *I* are not substrates but efficient inhibitors of the enzyme. They are obviously not capable of incorporation into mixed polymers (a study at an increased inhibitor to substrate ratio is practically excluded). By contrast, these compounds, even when present in low quantities in the polymerization mixture, possess an ability to markedly increase the length of the arising polymer chain; this effect may result from an allosteric activation of the enzyme. This activity could receive practical application in preparative synthesis of polynucleotides by polynucleotide phosphorylase.

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