NUCLEIC ACID COMPONENTS AND THEIR ANALOGUES. CXL.*

PREPARATION OF 5′-L-RIBONUCLEOTIDES, SOME OF THEIR DERIVATIVES, AND 2′(3′) → 5′-HOMOOLIGO-L-RIBONUCLEOTIDES; CODING PROPERTIES OF L-RIBONUCLEOSIDE-CONTAINING OLIGONUCLEOTIDES**

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L-Uridine 5′-phosphate*** (*IVa*), L-cytidine 5′-phosphate (*IVb*), L-adenosine 5′-phosphate (*IVc*), and L-6-azauridine 5′-phosphate (*IVd*) were prepared (*a*) by phosphorylation of 2′,3′-O-ethoxymethylene derivatives *II* with β-cyanoethyl phosphate and the subsequent removal of protecting groups in acidic and then alkaline media, or (*b*) by phosphorylation of L-nucleosides *I* with phosphorus oxychloride in triethyl phosphate. L-Uridine 5′-diphosphate (*Va*), L-cytidine 5′-diphosphate (*Vb*), and L-adenosine 5′-diphosphate (*Vc*) were obtained from compounds *IV* by reaction with diphenylphosphoryl chloride and orthophosphate. L-Adenosine 5′-triphosphate (*IX*) was prepared from compound *IVc* by reaction with diphenylphosphoryl chloride and tri-n-butylammonium pyrophosphate. The behaviour of the above mono-, di-, and triphosphates towards some nucleolytic enzymes has been discussed.

Polymerisation of L-uridine 2′,3′-cyclic phosphate *Xa* or adenosine 2′,3′-cyclic phosphate *Xb* with bis(*p*-nitrophenyl)phosphoryl chloride led to oligohomo-L-ribonucleotides *XI* containing mixed 2′ → 5′ and 3′ → 5′ internucleotidic linkages. The corresponding D-ribonucleotides D-*XI* were prepared analogously. Compounds D-*XIa* and D-*XIb* stimulate the binding of Phe-tRNA and Lys-tRNA, resp., to ribosomes. The corresponding L-ribonucleotides *XIa, b* are completely inactive. Uridylyl-(3′ → 5′)-L-uridine (*XIV*) was prepared by condensation of the uridine 3′-phosphate derivative *XII* with compound *IIa*, followed by removal of protecting groups. Compound *XIV* was converted to GpUpL-U by reaction with guanosine 2′,3′-cyclic phosphate in the presence of ribonuclease T1. In contrast to GpUpU, compound *XIV* does not exhibit any activity for stimulation of the Val-tRNA binding to ribosomes. pGpL-U obtained by reaction of L-uridine (*Ia*) with 5′-O-phosphorylguanosine 2′,3′-cyclic phosphate in the presence of ribonuclease T1, is also inactive in this respect.

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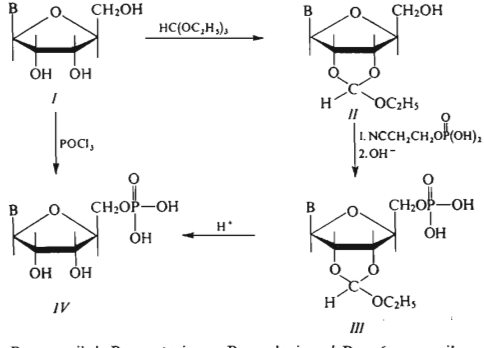
*** For oligonucleotides, the one-letter symbols are used, *cf.* Tentative Rules, IUPAC-IUB Commission on Biochemical Nomenclature, Biochim. Biophys. Acta *108*, 1 (1965). In the case of L-nucleosides and L-nucleotides, the prefix L is attached to the corresponding name or abbreviation. All phosphodiester linkages are of the 3′ → 5′ type unless stated otherwise.

In an earlier paper[1] of this Series, we have reported the synthesis of enantiomeric β-ribonucleosides derived from L-ribofuranose as well as the preparation of their 2′(3′)-nucleotides and 2′,3′-cyclic phosphates. The recent investigations of these compounds with a view to the specificity of nucleolytic enzymes have furnished interesting results and particularly new information on the interaction of enzymes with the heterocyclic base of the substrate. It was therefore felt desirable to extend the investigations on further nucleotide derivatives of β-L-ribonucleosides. In the present paper, we wish to report the preparation of β-L-ribonucleoside 5′-phosphates and some related derivatives as well as their biochemical properties.

The 5′-nucleotides *IV* were prepared by two independent routes, both starting from the L-ribonucleoside *I*, namely, *a*) specific protection of the 2′, 3′-*cis*-diol system by reaction with ethyl orthoformate[2], phosphorylation with β-cyanoethyl phosphate according to Tener[3] (in the case of the preparation of compound *IVb*, the amino group of the adenine ring had to be protected by reaction with a dimethylformamide acetal[4], and finally, successive removal of protecting groups in acidic and alkaline media; *b*) direct phosphorylation of the hydroxylic function at position 5′ of compounds *I* by reaction with phosphorus oxychloride in triethyl phosphate[5] (see Scheme 1). Compounds *IVa*–*IVc*, prepared by the above procedures, were free of 2′(3′)-isomers and, in respect to their chromatographic, electrophoretic and spectral characteristics, identical with authentic 5′-nucleotides derived from β-D-ribonucleosides.

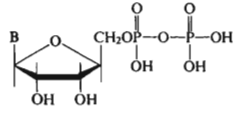
The β-L-ribonucleoside 5′-phosphates *IV* are entirely resistant to the action of *Crotalus adamanteus* snake venom 5′-nucleotidase (*cf.* ref.[6]), even with the use of excess enzyme and prolonged reaction periods. On the other hand, compounds *IV* are

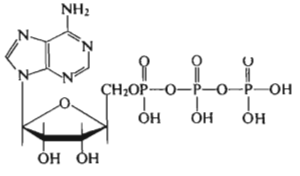


In formulae *I*—*IV a*: B = uracil; *b*: B = cytosine; *c*: B = adenine; *d*: B = 6-azauracil.

SCHEME 1

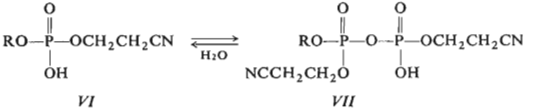
quantitatively dephosphorylated by alkaline phosphatase *E. coli*, intestinal alkaline phosphatase, and acidic wheat germ phosphatase under the formation of nucleosides *I*. The three last mentioned enzymes (splitting also the 2'(3')-isomeric L-nucleotides[1]) are non-specific, qualitatively independent of the nature of the alcoholic group of the phosphomonoester. The snake venom 5'-nucleotidase obviously belongs to the latter enzyme group where the orientation of the substrate to the enzyme depends on the interaction in at least three centers; at least one of these centers corresponds to the nucleoside moiety of the 5'-nucleotide molecule. Since this enzyme

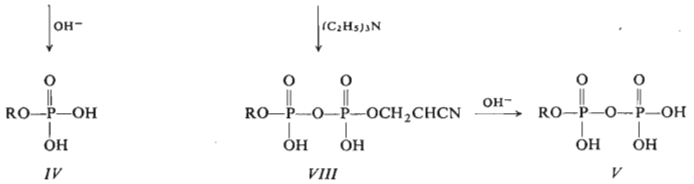




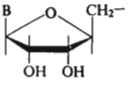
IX

Va, B = uracil
Vb, B = cytosine
Vc, B = adenine





In formulae *IV*–*VIII* R =



SCHEME 2

hydrolyses exclusively the 5'-D-nucleotides and not the corresponding phosphates of 1-O-alkyl-β-D-ribofuranosides, it may be assumed that an interaction is required between the enzyme and the heterocyclic basic moiety which is not suitably sterically situated with compounds *IV* (*cf.* ref.[1]).

The β-L-ribonucleoside 5'-diphosphates *V* were prepared from compounds *IV* by reaction with diphenylphosphoryl chloride and an orthophosphate according to Michelson[7], and identified on comparison with authentic D-enantiomeric derivatives by means of paper chromatography and electrophoresis.

Worthy of mention is the fact that the phosphorylation of compounds *II* with β-cyanoethyl phosphate (Scheme 1) results in the formation of a considerable amount of the 5'-diphosphate when the reaction mixture is processed first under anhydrous conditions with triethylamine and then in aqueous ammonia. This fact might be explained by reaction of the intermediate *VI* with a further molecule of the reagent under the formation of a triester of diphosphoric acid *VII*; in aqueous media, the latter substance must undergo a retrohydrolysis of the anhydride bond and afford the normal reaction product *IV*. The action of a strong base in anhydrous media might lead, however, to a β-elimination of the β-cyanoethyl group at the α-phosphate residue. The subsequent alkaline work-up of the intermediate *VIII* should afford the 5'-diphosphate *V*. This observation was made also in the preparation of other 5'-nucleotides by the Tener method when the reaction mixture was processed as above. Since the proportion of 5'-diphosphates may be as high as 30% in respect to the 5'-monophosphate, the above modification may be used preparatively in all cases when both 5'-mono- and 5'-diphosphates have to be synthesized. The structure of 5'-diphosphates resulting in phosphorylations with β-cyanoethyl phosphate was unequivocally established both on comparison with authentic specimens and by analysis of the *cis*-diol system present.

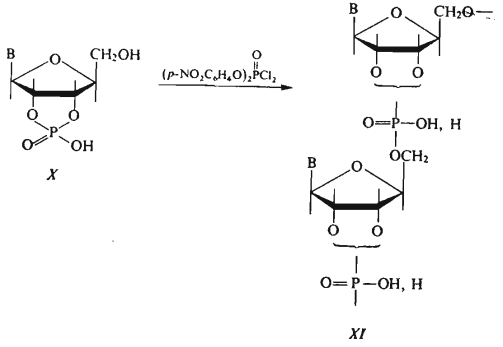
L-Uridine 5'-diphosphate (*Va*) and L-adenosine 5'-diphosphate (*Vc*) are weak but unequivocally positive substrates for polynucleotide phosphorylase *E. coli* (the detailed results of this investigation will be published elsewhere[8]).

Adenosine 5'-triphosphate represents another nucleotide derivative of a wide importance in metabolic processes. As reported in the literature[7], the enantiomeric derivative *IX* (L-ATP) was prepared from compound *IVc* by reaction with diphenylphosphoryl chloride and inorganic pyrophosphate. In respect to chromatography and electrophoresis, compound *IX* is identical with ATP. L-ATP does not represent substrate for RNA polymerase but inhibits competitively the incorporation of adenosine from ATP to RNA by this enzyme in the presence of native DNA as template[9].

As mentioned above, the L-5'-diphosphates *V* are relatively poor substrates for polynucleotide phosphorylase[8]. To obtain at least a remote idea on properties of L-oligonucleotides, we have attempted preparation of oligoribonucleotide derivatives composed of L-ribonucleotide units, by chemical polymerisation. In this connection, the preparation of oligo-L-ribonucleotides containing mixed 2' → 5' and

3′ → 5′ internucleotide linkages was used as the most accessible. In the presence of a diphenylphosphochloridate type reagent, the L-ribonucleoside 2′,3′-cyclic phosphates *X* (*cf.* ref.[1]) afford activated mixed anhydrides of the triester type; the latter anhydrides undergo autocondensation with the free hydroxylic function at position 5′ of compounds *X*, accompanied by a simultaneous opening of the triester ring under the formation of the oligonucleotides *XI*, see Scheme 3 (*cf.* ref.[10]). In contrast to the original procedure of Michelson[10], bis(*p*-nitrophenyl)phosphoryl chloride[11] was used as the condensation agent. This agent is more active and makes possible an easy control of the course of dialysis and gel filtration during the work-up of the reaction mixture because of the intensive colour of the released *p*-nitrophenol as well as the sensibility of both bis- and mono-*p*-nitrophenyl phosphate to detection in ultraviolet light. After an exhaustive dialysis against water and gel filtration, the oligonucleotides were isolated on a column of Sephadex G-200 in 0·02M triethylammonium hydrogen carbonate (pH 7·5); the fractions of the main ultraviolet-absorbing band with identical A_{260}^{250} and A_{260}^{280} values were pooled.

In connection with the above special type of homooligonucleotides with mixed internucleotide linkages, the corresponding derivatives of the D-ribonucleotide D-*XI* series were prepared under identical conditions of polymerisation and isolation of polymers, and used as comparison material in our investigations. Sedimentation constants of our homooligonucleotides (Table I) indicate that the isolated material is composed predominantly from shorter chains the average length of which is about



In formulae *X*, *XI a*: B = uracil; *b*: B = adenine; D-*X*, D-*XI* are enantiomeric compounds, represented by mirror-image formulae.

SCHEME 3

15—20 monomer units. Furthermore, the data of Table I indicate a high similarity between the properties of the corresponding enantiomeric pairs L-*XIa*, *b* and D-*XIa*, *b*.

The purified snake venom (*Crotalus terr. terr.*) phosphodiesterase degradation of D-oligonucleotides D-*XI* is quantitative (the 2′,3′-cyclic phosphate group which is attached to the 3′-end of the chain in latter compounds does not inhibit the enzymatic reaction in contrast to the free 3′-phosphate group[12]). On the other hand, the L-oligonucleotides *XI* are not cleaved by the above enzyme both under standard conditions and with the use of excess enzyme and prolonged incubation period. This observation is in accordance with the considerably decreased enzyme activity towards the internucleotide linkage occurring in the L-ribonucleoside 5′-phosphate *IV* (*vide infra* and ref.[1]). Nevertheless, a partial degradation to shorter oligonucleotides cannot be excluded although their chromatographic separation failed under the conditions applied.

The ability of L-oligonucleotides *XI* to serve as messenger-RNA-type molecules in the proteosynthesis was studied *in vitro* in the binding-assay system[13] in respect to the potential stimulation of the aminoacyl-tRNA binding to ribosomes in the presence of compounds *XI*. The activity of compound *XI* was compared with that of the corresponding triplet codons (UpUpU with phenylalanine; ApApA with lysine) and D-oligonucleotides D-*XI*. For the results see Figs 1 and 2. It may be seen that the stimulating activity of compounds D-*XI* is somewhat lower than that of the triplet codon but, nevertheless, the activity is quite significant (in the concentration dependence on Figs 1 and 2, the hyperchromicity of compounds *XI* and D-*XI* is not taken into account and the values are expressed in optical density units). As indicated by physicochemical properties of the above oligonucleotides[14], their chains do not exhibit any significant organized secondary structure and their ability to form complexes with complementary biosynthetic polynucleotides as well as with each other

TABLE I

Yields and Characterisation of Homooligo-L-nucleotides *XI* and their D-Enantiomers D-*XI*

Sedimentation constants were determined on Spinco Model E, AnD-Rotor, 59780 r.p.m. in 0·1M sodium chloride at 20°C. UV spectra at pH 7; for degradation, snake venom phosphodiesterase was used.

Compound	λ_{max}, nm	$A\frac{250}{260}$	$A\frac{280}{260}$	Yield A_{260}	$s_{20,w}$	Degradation
XIa	262	0·82	0·67	800	1·3	none
D-*XIa*	263	0·82	0·56	1·5	1·5	complete
XIb	258	0·83	0·34	2 400	1·6	none
D-*XIb*	258	0·83	0·35	1 700	1·45	complete

(L-*XIa*–L-*XIb*, D-*XIa*–D-*XIb*) is considerably lower. The data suggest a disordered coil structure exhibiting a great probability of the occurrence of trinucleotide units with a 3′ → 5′ internucleotide linkage separated by regions of 2′ → 5′ internucleotide linkages. Since the occurrence of a 2′ → 5′ internucleotide linkage in an oligonucleotide leads to a considerable decrease of the template activity[15,16], the dependence in Figs 1 and 2 reflects in the case of D-*XI* the amount of fragments with 3′ → 5′ internucleotide linkages in the polymer. The decreased stimulating activity of compounds D-*XI* in comparison with that of the trinucleoside phosphates UpUpU or ApApA may be thus interpreted in these terms.

In contrast to the significant stimulating activity of compounds D-*XI* in the binding-assay system, the L-derivatives *XI* do not exhibit any template activity. It may be concluded that the L-polyribonucleotide chains cannot form sufficiently stable ternary complex with ribosomes and aminoacyl-tRNA presumably because of the impossibility of an interaction between the right-handed chain of the messenger-like L-oligonucleotide *XI*, based on base-pairing of complementary bases of the codon and anticodon.

By means of the binding-assay system, we have also investigated the template activity of codons containing both D- an L-nucleosides. As a model system, we have used the codon for valine, namely, GpUpX (X represents any from the four naturally

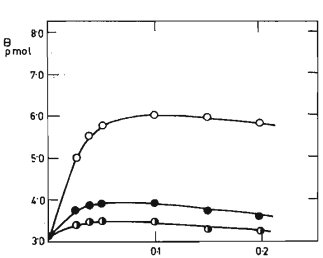


FIG. 1

The Effect of Oligonucleotides on the Binding of [^{14}C]-Phe-tRNA to Ribosomes

Conditions as described in Experimental part. The incubation mixture contained 0·50A_{260} of tRNA acylated with 25·2 pmol [^{14}C]- phenylalanine. A_{260} template concentrations; B [^{14}C]-Phe-tRNA bound; ○ UpUpU, ● D-*XIa* ◑ *XIa*.

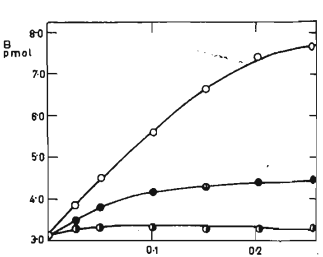


FIG. 2

The Effect of Oligonucleotides on the Binding of [^{14}C]-Lys-tRNA to Ribosomes

Conditions as given in the Experimental part. The incubation mixture contained 0·3A_{260} of tRNA acylated with 22·5 pmol [^{14}C]-lysine. A_{260} template concentrations; [^{14}C]-Lys-tRNA bound. ○ ApApA, ● D-*XIb*, ◑ *XIb*.

occurring nucleosides). As shown earlier[17,18], the dinucleoside phosphate 5'-phosphate pGpU stimulates the Val-tRNA binding to ribosomes. The corresponding L-uridine-containing derivative, namely, pGpL-U was prepared by an enzymatically catalysed reaction of 5'-O-phosphorylguanosine 2',3'-cyclic phosphate[19] with L-uridine in the presence of ribonuclease T1 analogously to the preparation of pGpU (*cf.* ref.[17]). As shown by data of Table II, pGpL-U does not exhibit any template activity for Val-tRNA. It may be consequently concluded that replacement of the naturally occurring D-ribonucleoside by a L-enantiomer at the second position of triplet codon leads to a loss of the coding response of such a fragment. A similar effect may be expected in the case of an analogous replacement at the first position of the triplet codon.

TABLE II

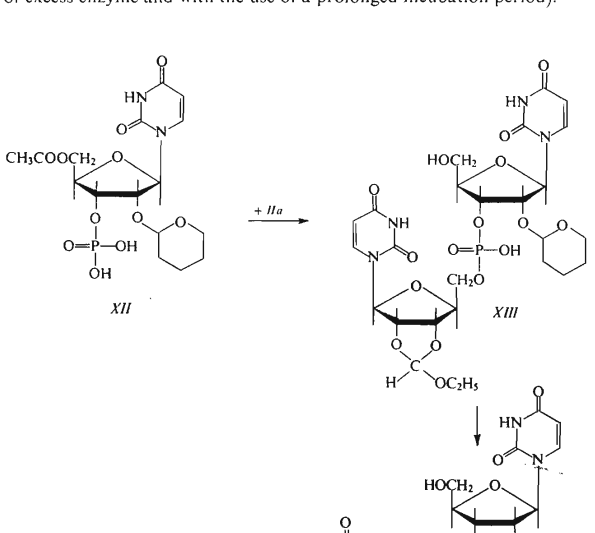
Effect of L-Uridine Containing Oligoribonucleotides upon [^{14}C]-Val-tRNA Binding to Ribosomes

Conditions as described in the Experimental part. The incubation mixture contained 0·55A_{260} of tRNA acylated with 22 pmol of [^{14}C]-valine.

Oligonucleotide (0·10A_{260})	[^{14}C]-Val-tRNA bound	
	pmol	Δpmol
—	0·41	—
GpUpU	2·38	1·97
GpUpL-U	0·55	0·14
pGpUpU	4·21	3·80
pGpUpL-U	0·78	0·37
pGpU	1·20	0·79
pGpL-U	0·43	0·02

The replacement at the third position of the codon was studied with the use of the valine codon GpUpL-U. The key compound, uridylyl-(3' → 5')-L-uridine (*XIV*) was prepared by reaction sequence given in Scheme 4. Thus, condensation of 5'-O-acetyl-2'-O-tetrahydropyranyluridine 3'-phosphate[20] (*XII*) with 2',3'-O-ethoxymethylene-L-uridine (*IIa*) in the presence of N,N'-dicyclohexylcarbodiimide led to the protected derivative *XIII*, identical on chromatography and electrophoresis with the analogous UpU derivative (*cf.* ref.[21]). Removal of protecting groups with acetic acid afforded UpL-U (*XIV*), identical with an authentic specimen of UpU in respect to ultraviolet spectra as well as chromatographic and electrophoretic characteristics and containing

less than 3% of the uncleavable 2′ → 5′ isomer, as shown by pancreatic ribonuclease degradation. In contrast to the quantitative cleavage of UpU to uridine 5′-phosphate and uridine, compound *XIV* is completely resistant under standard conditions to the snake venom phosphodiesterase (less than 1% of the material is cleaved with the use of excess enzyme and with the use of a prolonged incubation period).



XII *XIII* *XIV*

SCHEME 4

The final step in the synthesis of the trinucleoside diphosphate GpUpL-U was effected by the enzymatically (ribonuclease T1) catalysed reaction of UpL-U with guanosine 2′,3′-cyclic phosphate[22]. After purification by paper chromatography and electrophoresis, the required product was obtained in 3·5% yield. Its properties corresponded to those of GpUpU and its T2 ribonuclease degradation afforded

Gp, Up and L-uridine in an equimolecular ratio. The oligonucleotide pGpUpL-U was prepared analogously from compound *XIV* and 5'-O-phosphorylguanosine 2',3'-cyclic phosphate[19]. For the stimulating activity data of these oligonucleotides upon the Val-tRNA binding to ribosomes see Table II.

Replacement of uridine at the third position of the GpUpU valine codon by L-uridine leads to the loss of the oligonucleotide template activity. It may be assumed on the basis of earlier investigations[18] that the nucleoside base at the 3'-terminus (*i.e.*, at the third position of the triplet codon) contributes cooperatively by the base-pair formation with the complementary anticodon base (or, by pairing with inosine according to the wobble hypothesis[23]) to the stability of the ternary complex of aminoacyl-tRNA, the oligonucleotide, and ribosomes. The formation of this complex depends *inter alia* on the base-pairs between the first codon bases and the other two anticodon bases. When this cooperative effect is not possible (due to the methylation of the uridine $N_{(3)}$—H group[24] and the like), the triplet is inactive In the case of GpUpL-U, the stereochemistry of the whole molecule and especially of the 3'-portion is obviously quite different. In spite of a weak base-stacking between two vicinal uracil nuclei and, consequently, a weak contribution of this part of molecule to the conformation of the whole molecule, it cannot be expected that the uracil residue of L-uridine in GpUpL-U could assume an analogous position in respect to the anticodon as in the case of GpUpU. Such a conformation would cause a considerable change in the sugar-phosphate backbone of the -UpL-U molecule fragment which represents a further stabilising factor (complexes with magnesium ions) in the formation of the ternary complex with ribosomes and aminoacyl-tRNA. On the other hand, when the sugar-phosphate backbone stereochemistry of this fragment is analogous to that of GpUpU, then the uracil portion of L-uridine is too remote from the anticodon region. Moreover, because of the *anti* conformation of L-uridine, the $N_{(3)}$—H grouping of the uracil portion is not situated favourably enough for the formation of hydrogen bonding with anticodon base.

Notwithstanding, pGpUpL-U exhibited some template activity for valine (Table II) in accordance with results concerning substitutions of the 5'-end of valine codon triplets by a phosphate grouping. Stabilisation of the ternary complex or an increased probability of its formation caused by an interaction of the phosphate group with ribosomes[18] can compensate the loss of the cooperative effect in the interaction between the third codon base and the anticodon base. This loss, however, is apparent from the decreased template activity of pGpUpL-U in comparison with that of pGpUpU. Since this effect is not general for all codons, it may be assumed that the introduction of a L-ribonucleoside to any codon position of the messenger-RNA chain must result in a considerable decrease or loss of coding activity, and consequently, in inhibition of the proteosynthesis *in vitro*.

EXPERIMENTAL

Methods

Descending chromatography was performed on paper Whatman No 1 (preparative runs on paper Whatman No 3 MM) in the solvent systems S_1, 7 : 1 : 2 2-propanol–concentrated aqueous ammonia–water; S_2, 5 : 2 ethanol–1M ammonium acetate; and S_3, 7 : 1 : 2 2-propanol–concentrated aqueous ammonia–0·1M triethylammonium borate (pH 8).

Paper electrophoresis was performed in an apparatus according to Markham and Smith[25] on paper Whatman No 3 MM in the buffer solution E_1, 0·1M triethylammonium hydrogen carbonate (pH 7·5) at 20 volt per cm (1 hour).

Detection was performed under ultraviolet light. The nucleotide derivatives were detected by reaction with ammonium molybdate according to Isherwood[26]. For chromatographic and electrophoretic data see Table III.

Preparative column chromatography was performed on a 100 × 4 cm DEAE-cellulose column (Cellex D, standard capacity, purchased from Calbiochem Ltd., Los Angeles, U.S.A.) in HCO_3^- cycle. Standard conditions: elution rate 3 ml per min; frations were taken in 10 min intervals. The ultraviolet absorption of eluates was measured continuously on a Uvicord apparatus (LKB, Uppsala, Sweden). Elution was performed with the use of a linear gradient of triethylammonium hydrogen carbonate, pH 7·5 (2 l of water in the mixing chamber, 2 l of the buffer solution in the reservoir). Chromatographic fractions were pooled, evaporated to dryness at 35°C/15 Torr, and the residue coevaporated twice with methanol under the same conditions. The content was determined spectrophotometrically after dilution of an aliquot with 0·01M-HCl.

Enzymatic degradations were performed with 10—15 A_{260} units of the substrate in 100 µl of the buffer solution at 37°C for 6 hours. The following enzymes were used: *a*) pancreatic ribonuclease (Calbiochem Ltd., A grade, 5 fold recrystd.), 10 µg, 0·05M Tris-HCl (pH 8·0); *b*) *Crotalus terr. terr.* snake venom phosphodiesterase (Boehringer, Mannheim, Germany), 20 µl of a solution of the enzyme in 50% aqueous glycerol, 0·05M Tris-HCl (pH 9·0); *c*) alkaline phosphatase *E. coli* (Worthington, A grade), 20 µl of an enzyme suspension in saturated ammonium sulfate, 0·05M Tris-HCl (pH 9·0); *d*) calf intestinal alkaline phosphatase (Boehringer), 50 µg of the enzyme, 0·05M Tris-HCl (pH 9·0); *e*) *Crotalus adamanteus* snake venom 5′-nucleotidase (Worthington), 50 µg of the enzyme, 0·05M Tris-HCl (pH 9·0), 0·005M magnesium sulfate; and *f*) ribonuclease T2 (prepared by Professor Dr Dr H. Witzel, Marburg, Germany), 20 µg of the enzyme, 0·1M sodium γ,γ-dimethylglutarate (pH 6·2), 0·1M sodium chloride. When no degradation was observed under the above conditions, a five fold excess of the enzyme was used and the incubation period was prolonged to 16 hours (at 37°C). Blank tests were performed under analogous conditions in the absence of the corresponding enzyme.

Ultraviolet spectral measurements were performed in an 1 cm cell on a Beckmann DU apparatus. Quantitative determinations and qualitative comparisons were performed with the use of values tabelated for D-ribonucleosides and their derivatives[27] at pH 2 (0·01M-HCl). The optical density unit ($1A_{260}^{pH2}$) is that amount of the test substance in 1 ml of a solution (pH 2) which exhibits in 1 cm cell at 260 nm the absorbancy equal to one.

Starting Materials and Reagents

For the preparation of L-ribonucleosides (*I*) and 2′ 3′-cyclic phosphates *X* see an earlier paper[1]. The pyridinium salt of β-cyanoethyl phosphate was prepared from the barium salt (Calbiochem Ltd.) on a column of pyridinium Dowex 50 ion exchange resin; the solution in aqueous pyridine

was evaporated, dried by repeated coevaporations with pyridine, and the 1M stock solution of the reagent stored under exclusion of atmospheric moisture at 4°C. The bis(tri-n-butyl)ammonium salt of phosphoric acid was prepared by neutralisation of phosphoric acid with tri-n-butylamine, drying by repeated coevaporations with pyridine, and stored in the form of a 1M stock solution in pyridine at 4°C. The 1M pyridine solution of the pyridinium salt of pyrophosphoric acid was

TABLE III

Paper Chromatography and Electrophoresis

Compound	R_F			E_{Up}[a] in E_1
	S_1	S_2	S_3	
Uridine	0·48	0·74	0·42	--
Ia	0·48	0·74	0·42	--
Ib	0·50	0·68	0·42	--
Ic	0·56	0·70	0·46	--
Id	0·48	—	0·40	0·47
IIa	0·78		--	--
IIb	0·76		--	--
IIc	0·80	--	--	--
IIIa	0·28	0·54	--	0·90
IIIb	0·38	0·57	--	0·80
IIIc	0·35	0·46	--	0·83
Uridine 3′-phosphate	0·10	0·40	0·15	1·00
IVa	0·10	0·28	0·05	1·00
IVb	0·12	0·32	0·07	0·80
IVc	0·14	0·24	0·06	0·85
IVd	0·08	0·23	0·04	1·24
Cytidine 3′-phosphate	0·12	0·22	0·17	0·80
Adenosine 3′-phosphate	0·14	0·23	0·24	0·85
6-Azauridine 3′-phosphate	0·08	0·23	0·22	1·24
Va	0·05	0·14	—	1·10
Vb	0·06	0·15	—	0·95
Vc	0·07	0·20	—	0·97
IX	0·03	0·08	—	1·10
Xa	0·43	0·60	—	0·65
Xb	0·45	0·57	—	0·45
Xc	0·48	0·58	—	0·45
XIII	0·52	--	--	0·40
XIV	0·23	—	—	0·40
GpUpL-U	—	--	—	0·52
pGpUpL-U	—	—	—	0·79
pGpL-U	—	--	—	0·87

[a] Electrophoretical mobility referred to uridine 3′-phosphate.

prepared from the sodium salt (analogously to the preparation of pyridinium β-cyanoethyl phosphate) and stored under exclusion of atmospheric moisture at 4°C. Guanosine 2',3'-cyclic phosphate[28] and 5'-O-phosphorylguanosine 2',3'-cyclic phosphate[19] were prepared according to the references given. The preparation of ribosomes, [^{14}C]-aminoacyl-tRNA from *E. coli* was performed according to ref.[29] The [^{14}C]-amino acids were obtained from ÚVVVR Prague; specific activity, Phe 113 mCi/mmol, Lys 82 mCi/mmol, and Val 146 mCi/mmol. Specific activities of aminoacyl-tRNA Phe 50 pCi/1A_{260}, Lys 75 pCi/1A_{260}, Val 40 pCi/1A_{260}.

The 6M solution of hydrogen chloride in dimethylformamide was prepared by saturation of dimethylformamide with dry hydrogen chloride at 0°C and dilution with additional dimethylformamide. Phosphorus oxychloride was freshly distilled before use. Bis(*p*-nitrophenyl)phosphoryl chloride was prepared according to the ref.[12] and then crystallised from light petroleum.

Assay of Aminoacyl-tRNA Binding to Ribosomes

The general procedure of Nirenberg and Leder[13] was applied. The incubation mixtures contained (0·05 ml, total volume) 0·05M Tris acetate (pH 7·2), 0·1M ammonium chloride, 0·03M magnesium acetate, 2·5A_{260} units of ribosomes and those amounts of oligonucleotides and labelled aminoacyl-tRNA as shown in Table II and Figs 1 and 2. After incubation (20 min at 24°C) the mixtures were diluted with 3 ml of the same buffer, filtered on Milipore filters, and washed with four 5 ml portions of cold buffer. The filters were mounted on planchets, dried, and the radioactivity was determined in a proportional methane-flow counter (Frieseke and Hoepfner).

L-Ribonucleoside 5'-Phosphates *IV*

A. *By reaction of the* 2' 3'-O-*ethoxymethylene derivatives* II *with* β-*cyanoethyl phosphate.* L-Uridine (*Ia*; 5 mmol) was converted to the 2',3'-O-ethoxymethylene derivative *IIa* according to the ref.[2] and L-adenosine (*Ib*; 5 mmol) was transformed to N^6-dimethylaminomethylene-2',3'-O-ethoxymethylene-L-adenosine analogously to the preparation of the corresponding adenosine derivative[4]. A mixture of the protected nucleoside *II* (5 mmol) pyridinium β-cyanoethyl phosphate (15 mmol), and pyridine (20 ml) was evaporated to dryness at 30°C/0·1 Torr and the residue coevaporated with five 20 ml portions of pyridine under the same conditions. The final residue was dissolved in pyridine (40 ml), the solution treated with N,N'-dicyclohexylcarbodiimide (15 g), the whole mixture allowed to stand at room temperature for 6 days under exclusion of atmospheric moisture, and then treated with triethylamine (5 ml). After 15 min, water (20 ml) was added, the whole evaporated to dryness at 35°C/15 Torr, the residue diluted with water (100 ml), washed with two 50 ml portions of ether, the aqueous phase filtered, the filtrate evaporated to dryness under the above mentioned conditions and the residue heated in dilute (1 : 1) aqueous ammonia (50 ml) for 2 hours at 50°C. The reaction mixture was evaporated to dryness as above, the residue heated in 50% aqueous acetic acid (20 ml) for 30 min at 50°C, the solution evaporated to dryness, and the residue coevaporated with three 20 ml portions of water. The final residue was dissolved in water (50 ml), adjusted with aqueous ammonia to pH 8, and applied to a column of DEAE-cellulose. The column was eluted with water to remove the nucleoside and then with the use of a linear gradient of triethylammonium hydrogen carbonate (pH 7·5; final concentration, 0·4M). The nucleotide fraction was processed as usual. As shown by chromatography in the solvent system S_1, the eluate contained the 5'-diphosphate *V* in addition to compound *IV*. The eluate was evaporated and the residue chromatographed for 3 days on 6 sheets of paper Whatman No 3 MM in the solvent system S_1. The bands of compounds *IV* and *V* were separately eluted with 1 : 100 dilute aqueous ammonia (50 ml), the eluate evaporated to dryness, and the residue freeze-dried (its content was determined spectrophotometrically at pH 2).

Compounds *IV* and *V* thus obtained were homogeneous on chromatography and electrophoresis and their properties were identical with those of the authentic derivatives of the D-*ribo* series. Yields, 45% (*IVa*), 13% (*Va*), 36% (*IVb*), 12% (*Vb*), 39% (*IVc*), and 12% (*Vc*).

B. *By phosphorylation of* L-*ribonucleosides* I *with phosphorus oxychloride* (*cf.* ref.[5]). Phosphorus oxychloride (1 ml; 1·67 g; 10·9 mmol) was added under ice-cooling to a suspension of the nucleoside *I* (5 mmol) in triethyl phosphate (10 ml), the whole was stirred at 0°C for 4 hours, and then added dropwise under stirring into 100 ml of ether. The precipitate was collected by centrifugation, washed with ether (100 ml), and dissolved without delay in 1% aqueous lithium hydroxide (50 ml). The solution was allowed to stand at room temperature overnight and then applied to a column (50 ml) of pyridinium Dowex 50 ion exchange resin. The column was eluted with 10% aqueous pyridine (200 ml), the eluate made alkaline with aqueous ammonia, and concentrated at 35°C/15 Torr to the volume of about 50 ml. The concentrate was applied to a column of DEAE-cellulose (*vide supra*) and the unreacted nucleoside *I* cluted with water. Further work-up was analogous to that of procedure *A*. Bands of compounds *IV* were separated from a small amount of 2′(3′),5′-diphosphates by chromatography in the solvent system S_1 (for 2 days). The product was eluted with 1 : 100 dilute aqueous ammonia (50 ml) and freeze-dried in the form of the ammonium salt. The yields were determined spectrophotometrically. In this manner, compounds *IVa* (56%) and *IVc* (62%) were prepared.

L-6-Azauridine 5′-Phosphate (*IVd*)

The title compound was prepared by a modification of procedure *B*. Phosphorus oxychloride (0·15 ml; 1·64 mmol) was added at 0°C to a suspension of L-6-azauridine[1] (*Id*; 0·4 mmol) in triethyl phosphate (1 ml), the whole stirred at 0°C for 4 hours, and treated then with 0·4M triethylammonium hydrogen carbonate, pH 7·5 (20 ml) and triethylamine (2 ml). The mixture was heated at 50°C for 20 min and evaporated to dryness at 35°C/15 Torr. The residue was chromatographed on 2 sheets of paper Whatman No 3 MM in the solvent system S_1 for 3 days. Bands of compound *IVd* were eluted with 1 : 100 dilute aqueous ammonia (50 ml) and the eluate freeze-dried. Yield (as determined spectrophotometrically), 0·32 mmol (80%) of the ammonium salt *IVd*, homogeneous on chromatography as well as electrophoresis and identical in this respect with a specimen of the authentic D-derivative.

The 5′-nucleotidase degradation of 5′-nucleotides *IV* does not occur either under standard conditions or at higher concentrations of the enzyme. On the other hand, alkaline phosphatase *E. coli* degradation and intestinal alkaline phosphatase degradation afford quantitatively under standard conditions (*vide supra*) the starting L-nucleoside *I*, identified on comparison with an authentic specimen in the solvent systems S_1 and S_2 as well as in the buffer solution E_1. The isomer purity of compounds *IV* was also proved by chromatography in the solvent system S_3. In this system, the 5′-isomers are separated from 2′(3′)-isomers on the basis of complex formation with boric acid.

L-Ribonucleoside 5′-Diphosphates *V* (*cf.* ref.[7])

A mixture of the ammonium salt of the 5′-nucleotide *IV* (1 mmol), tri-n-octylamine (0·7 g), and methanol (50 ml) was refluxed until the solid dissolved. The solution was evaporated to dryness at 35°C/15 Torr, the residue coevaporated with two 25 ml portions of ethanol, and the tri-n-octylammonium salt of compound *IV* dried over phosphorus pentoxide at 0·1 Torr overnight. This salt was shaken with a mixture of dioxane (7 ml), tri-n-butylamine (0·50 ml), and diphenylphosphoryl chloride until the solid dissolved. The solution was allowed to stand at room tem-

perature for 3 hours and evaporated at 30°C/0·1 Torr to dryness. The residue was decanted with two 50 ml portions of ether and coevaporated with dioxane (5 ml) at 30°C/0·1 Torr. Pyridine (2 ml) and an 1M solution (3 ml) of the bis-tri-n-butylammonium salt of phosphoric acid in pyridine were then added to the residue and the whole allowed to stand at room temperature overnight under exclusion of atmospheric moisture. Water (2 ml) was then added and the mixture evaporated to dryness at 35°C/15 Torr. The residue was dissolved in water (25 ml), the solution adjusted with 1 : 1 dilute hydrochloric acid to pH 3, allowed to stand at room temperature for 2 hours, made alkaline with aqueous ammonia, and evaporated. The residue was chromatographed for 3—4 days on 6 sheets of paper Whatman No 3 MM in the solvent system S_1. Bands of compound *V* were separated from a small portion of the unreacted compound *IV* and eluted with 1 : 100 dilute aqueous ammonia (50 ml). The content was determined spectrophotometrically. The ammonium salt of compound *B* obtained on lyophilisation was homogeneous on chromatography in the solvent systems S_1 and S_2 as well as on electrophoresis in the buffer solution E_1 and identical in this respect with the corresponding authentic D-derivatives. Yields, 63% (*Va*), 54% (*Vb*), and 69% (*Vc*). Compounds *V* prepared by this procedure were identical with those resulting as by-products in the synthesis of compounds *IV* according to the procedure *A*.

L-Adenosine 5′-Triphosphate (*IX*) (*cf.* ref.[7])

The title compound was prepared from 1 mmol of compound *IVc* (ammonium salt) according to the procedure applied in the preparation of compounds *V* except for the use of an 1M solution of a tri-n-butylammonium salt of pyrophosphoric acid (3 ml) instead of the salt of phosphoric acid. After the work-up at pH 3, the resulting mixture was made alkaline with aqueous ammonia, washed with two 10 ml portions of ether, the aqueous phase evaporated to dryness at 35°C/15 Torr, the residue dissolved in water (20 ml), and the solution treated with calcium chloride (1·5 g) in water (2 ml) and with ethanol (300 ml). The precipitate of the calcium salt was collected by centrifugation, washed with two 50 ml portions of ethanol, the ether, air-dried, redissolved in water (20 ml), and centrifuged. The supernatant was applied to a column (50 ml) of ammonium Dowex 50 ion exchange resin and the column eluted with water. The ultraviolet-absorbing fraction was concentrated to a small volume and chromatographed for four days on paper Whatman No 3 MM in the solvent system S_1. Band of the product *IX* was eluted with 1 : 100 dilute aqueous ammonia (50 ml) and the content of the eluate determined spectrophotometrically. The eluate was freeze-dried. Yield, 0·45 mmol (45%) of the ammonium salt of compound *IX*, identical with ATP on chromatography on the solvent systems S_1 and S_2 as well as on electrophoresis in the buffer solution E_1.

Preparation of 2′(3′)→5′-Homooligo-L-nucleotides *XI* and the Corresponding D-Enantiomers D-*XI*

The ammonium salt (1 mmol) of L-uridine 2′,3′-cyclic phosphate[1] *Xa* or of the D-enantiomer D-*Xa* in 10 ml of water was applied to a column (10 ml) of pyridinium Dowex 50 ion exchange resin. The column was eluted with 20% aqueous pyridine (50 ml) and the eluate treated with tri-n-butylamine (2 ml) and such an amount of ethanol to obtain solution. The solution was evaporated to dryness at 30°C/15 Torr, the residue coevaporated with two 50 ml portions of ethanol and dried over phosphorus pentoxide at 0·1 Torr overnight.

The tri-n-butylammonium salt of adenosine 2′,3′-cyclic phosphate (D-*Xb*) and of the L-enantiomer *Xb* were prepared analogously from ammonium salts. Prior to the polymerisation, the salt was dissolved in dimethylformamide (10 ml) and dimethylformamide dimethylacetal (5 ml)

was added. After 16 hours at room temperature, the mixture was evaporated at 40°C/0·1 Torr, coevaporated with 50% aqueous pyridine (50 ml) under the same conditions, then with two 20 ml portions of pyridine, and dried again over phosphorus pentoxide at 0·1 Torr.

A mixture of the above tri-n-butylammonium salt *X* or D-*X*, dioxane (10 ml), dimethylformamide (5 ml), tri-n-butylamine (0·8 ml), and bis(*p*-nitrophenyl)phosphoryl chloride was shaken until the material dissolved and the solution was allowed to stand at room temperature for 3 days under exclusion of atmospheric moisture. The reaction mixture was evaporated to dryness at 35°C/0·1 Torr, the residue treated with 50 ml of 0·4M triethylammonium hydrogen carbonate (pH 7·5), and the solution adjusted to pH 9–9·5 with triethylamine. The solution was allowed to stand at room temperature overnight, diluted with water (50 ml), washed with three 25 ml portions of ether, the aqueous phase concentrated to the volume of about 25 ml, and the concentrate dialysed against water. The dialysate was centrifuged at 3000 r.p.m. (the precipitate does not contain any nucleotides) and the supernatant (about 3000 A_{260} units) evaporated at 25°C/0·1 Torr almost to dryness. The residue was applied to a column (40 × 4 cm) of Sephadex G-200 equilibrated with 0·02M triethylammonium hydrogen carbonate (pH 7·5). The column was eluted with the same buffer solution at the rate of 0·2 ml per min, the fractions being taken in 15 min intervals. Each five fractions of the ultraviolet-absorbing eluate were combined, evaporated at 30°C/15Torr, and subjected to measurements of the optical density and $A\frac{250}{260}$ and $A\frac{280}{260}$ values Except for the material corresponding to the descending part of the elution peak, the values were constant in the range of 0·02. The eluates possessing constant spectral ratios were pooled and freeze-dried. For yields, spectral data, and sedimentation constant of compounds *XI* and D-*XI* see Table I.

The snake venom phosphodiesterase degradation of compounds D-*XI* was quantitative under standard (*vide supra*) conditions (the ratio nucleoside to 5′-phosphate being 1 : 12 to 1 : 15). As shown by chromatography in the solvent system S_2, the compounds *XI* were resistant to the enzyme mentioned, even at higher concentrations of the enzyme (*vide supra*).

Uridylyl-(3′ → 5′)-L-uridine (*XIV*)

A mixture of L-uridine (*Ia*; 366 mg; 1·5 mmol), dimethylformamide (7 ml), ethyl orthoformate (3 ml), and 6M hydrogen chloride in dimethylformamide (0·2 ml) was stirred until the material dissolved and the resulting solution (acidic reaction) allowed to stand at room temperature overnight. As shown by thin-layer chromatography on silica (Silufol plates, Kavalier Glassworks, Votice, Czechoslovakia) in chloroform containing 10% of ethanol, the conversion of *Ia* to *IIa* (R_F value 0·60) was quantitative. Triethylamine (1 ml) was then added to the mixture, the whole evaporated to dryness at 40°C/0·1 Torr, and the residue dissolved in 30% aqueous pyridine (20 ml).

The calcium salt[20] of compound *XII* (0·5 mmol) in 30% aqueous pyridine (10 ml) was applied to a column (10 ml) of pyridinium Dowex 50 ion exchange resin, the column eluted with 30% aqueous pyridine (60 ml), and the eluate concentrated to about 20 ml at 30°C/0·1 Torr. The concentrate was added to the solution of compound *IIa* (*vide supra*), the mixture evaporated to dryness at 30°C/0·1 Torr, and the residue coevaporated with five 20 ml portions of pyridine under the same conditions. The residue was dissolved in pyridine (10 ml) and N,N′-dicyclohexylcarbodiimide (2·5 g) was added. The resulting solution was allowed to stand at room temperature for 5 days under exclusion of atmospheric moisture and then treated with water (2 ml) and triethylamine (1 ml). After additional 1 hour at room temperature, the mixture was diluted with water (100 ml), washed with two 25 ml portions of ether, the aqueous phase filtered, and the filtrate concentrated at 35°C/15 Torr to the volume of about 20 ml. The concentrate was heated at 50°C for 2 hours with concentrated aqueous ammonia (20 ml), the mixture evaporated to the

volume of about 20 ml at 35°C/15 Torr, the concentrate filtered through Celite, and the filtrate applied to a column of DEAE-cellulose. The elution was performed under standard conditions (*vide supra*) with the use of 2 l of 0·2M triethylammonium hydrogen carbonate (pH 7·5) in the reservoir. From the first fraction, there was recovered 0·75 mmol (50%) of compound *IIa*. The second fraction (0·08—0·10M) was processed as usual and then chromatographed on 3 sheets of paper Whatman No 3 MM in the solvent system S_1 overnight. Bands of the product *XIII* were eluted with 1 : 100 dilute aqueous ammonia (50 ml), the eluate evaporated to dryness at 30°C/15 Torr, and the yield determined spectrophotometrically. Content, 0·2 mmol (40%, based on compound *XII*).

The residue of compound *XIII* (0·2 mmol) was heated at 50°C for 30 min in 50% aqueous acetic acid (10 ml), the mixture evaporated at 30°C/15 Torr, and the whole chromatographed for 2 days on 2 sheets of paper Whatman No 3 MM. Bands of the product *XIV* were eluted with 1 : 100 dilute aqueous ammonia (50 ml), the eluate concentrated at 30°C/15 Torr to the volume of about 5 ml, and the concentrate freeze-dried. Yield, 85 mg (0·15 mmol) of the ammonium salt of compound *XIV*, identical with UpU on chromatography in the solvent systems S_1 and S_2 and on electrophoresis in the buffer solution E_1. Pancreatic ribonuclease degradation (standard conditions) led to uridine 3′-phosphate and L-uridine in the ratio 1 : 1·02 (97%). In contrast to the quantitative degradation of UpU, the snake venom phosphodiesterase degradation of compound *XIV* does not occur under standard conditions; with the use of excess enzyme (*vide supra*), 7—8% of compound *XIV* was split to uridine and L-uridine 5′-phosphate (*IVa*).

Preparation of GpUpL-U and pGpUpL-U (*cf.* ref.[17,18])

A mixture of guanosine 2′,2′-cyclic phosphate or 5′-O-phosphorylguanosine 2′,3′-cyclic phosphate (20 μmol of the ammonium salt, compound *XIV* (50 μmol of the ammonium salt), 0·05M-Tris-HCl (0·4 ml; pH 7·0) containing 0·01M ethylenediamine tetraacetic acid, and 4 e.u. of ribonuclease T1 (Sankyo Ltd., Japan) was incubated at 0°C for 6 hours. The mixture was chromatographed on paper Whatman No 3 MM in the solvent system 5 : 2 : 3 1-butanol–acetic acid–water overnight. The slowest ultraviolet-absorbing band was eluted with water and subjected to electrophoresis on paper Whatman No 3 MM in the buffer solution E_1. The band of product was eluted with water and the yield determined spectrophotometrically. Yield, 3·5% of GpUpL-U and 1·5% of pGpUpL-U. Ribonuclease T2 degradation of GpUpL-U afforded guanosine 3′-phosphate, uridine 3′-phosphate, and L-uridine in an almost equimolar ratio. Alkaline phosphatase *E. coli* degradation of pGpUpL-U afforded quantitatively GpUpL-U.

Preparation of pGpL-U (*cf.* ref.[17])

The title compound was prepared analogously to the synthesis of pGpUpL-U with the use of 5′-O-phosphorylguanosine 2′,3′-cyclic phosphate (20 μmol) and L-uridine (*Ia*; 50 μmol). After paper chromatography and electrophoresis, the yield was 2·5%. Electrophoretical characteristics (in the buffer solution E_1) of pGpL-U were identical with those of pGpU. Alkaline phosphatase *E. coli* degradation of pGpL-U afforded GpL-U, identical on electrophoresis in the buffer solution E_1 with GpU.

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