SUBSTRATE SPECIFICITY OF SPLEEN ACID EXONUCLEASE AND SPLEEN CYCLIC PHOSPHODIESTERASE*

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The spleen acid exonuclease degrades internucleotidic linkages in the order G > A > U ≫ C. With substances of the XpY type, the interaction with the enzyme is determined by the character of both bases; the -CpC- and -CpU- linkages as well as the (2′ → 5′) and (5′ → 5′) internucleotidic linkages are enzyme-resistant. A substituent at position 2′ of the 3′-nucleotide lowers the affinity towards the enzyme owing to sterical and conformational effects. The enzyme-affinity of alkyl esters of 3′-ribonucleotides is determined by the sterical effect of the substituent. The phosphomonoester group at the 5′-end of the molecule lowers the affinity but is of no influence when present at the 3′-end. The spleen cyclic phosphodiesterase degrades neither internucleotidic linkages nor linkages of the (2′ → 5′) or (5′ → 5′) type. The 2′,3′-cyclic phosphates of ribonucleosides are degraded to 2′-nucleotides regardless the character of the natural base. The alkyl esters of nucleoside 3′- or 2′-phosphates are enzyme-resistant. For the formation of a complex between the enzyme and the substrate, the linkage of the cyclic phosphodiester and the cooperative effect of the interaction with the heterocyclic base are necessary. Preparations have been reported of 2′-chloro-2′-deoxyuridylyl-(3′ → 5′)-uridine, 2′-O-methyluridylyl-(3′ → 5′)-uridine, 2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-uridine, 2′-deoxycytidylyl-(3′ → 5′)-guanosine, uridylyl-(2′→5′)-uridine, uridylyl-(2′ → 5′)-adenosine, and uridylyl-(5′ → 3′)-1-(β-D-arabinofuranosyl)cytosine by nucleotide condensations. Alkyl (methyl, ethyl, 1-propyl, 2-propyl, and 1-butyl) esters of 3′-ribonucleotides (Up, Cp, Ap) have been prepared from protected 3′-nucleotides by reaction with the corresponding alcohol in the presence of N,N′-dicyclohexylcarbodiimide.

The acid exonuclease of bovine spleen (spleen phosphodiesterase) constitutes one of the important enzymes widely used in sequentional investigations of oligo- and polynucleotides of the ribo- as well as deoxyribonucleic acid series. This enzyme (E.C.3.4.1), designated sometimes as phosphodiesterase II, has been reported for the first time by Heppel and Hilmoe[1] and its properties determined by Razzell and Khorana[2]. In contrast to the snake venom phosphodiesterase, the spleen acid exonuclease may be isolated free of endonucleolytic activities and is thus predetermined for the use in sequentional analyses. Although this enzyme belongs to the group of long-time discovered nucleotidic enzymes (for reviews see refs[3-5]), the knowledge on its properties, particularly on its structural requirements is very limited. The present knowledge on spleen acid exonuclease may be summarised as follows. (*a*) The enzyme degrades specifically the poly- and oligonucleotides of both the ribo and deoxyribo series to 3′-nucleotides regardless the character of

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the heterocyclic base[2]. (*b*) The enzyme possesses the typical character of an exonuclease and the degradation begins at the 5′-end of the oligonucleotide[2]. *c*) The enzyme is sensitive towards the secondary structure of the substrate (the native DNA is considerably resistant)[6]. *d*) Only the (3′ → 5′) internucleotidic linkages are degraded. The original report[2] that the spleen phosphodiesterase is also capable of degrading the 2′,3′-cyclic phosphates of ribonucleotides to 2′-nucleotides, which has been for a long time repeated in the literature, is obviously in error. Such an activity must be due to the presence of cyclic phosphodiesterase, the level of which in spleen is high and which is difficult to remove. The attempted isolation of exonuclease entirely free of cyclic phosphodiesterase has not so far met with success[5].

The mechanism of the action of the enzyme has not been hitherto studied in detail. According to some reports, the enzyme can also catalyze the reverse reaction *i.e.* formation of alkyl esters[8] from 3′-nucleotides and aliphatic primary alcohols. A complex of the enzyme with a 3′-nucleotide is obviously formed in the synthetic reaction while in the lytic reaction a complex is probably involved of the enzyme with the 3′-nucleotide bound in the (3′ → 5′)-phosphodiester molecule.

In connection with systematic investigations on the specificity of nucleolytic enzymes and enzymes of the nucleic acid metabolism, we have now also examined the spleen phosphodiesterase on synthetic substrates. The choice of substrates was performed with regard to the known effects accompanying modifications of the naturally occurring substrates and taking into account the hitherto known facts on the spleen exonuclease specificity[5]. In principle, two types of substrates have been now investigated: analogues of oligonucleotides and alkyl esters of 3′-nucleotides. Commercial preparation of spleen phosphodiesterase has been used in our experiments; of course, this preparation contained a high level of the above mentioned cyclic phosphodiesterase. Since the activities of the two enzymes may be readily differentiated on synthetic substrates, the substrate specificity of the spleen cyclic phosphodiesterase has been simultaneously investigated. In addition to the two substrate types mentioned, the conclusions on properties of spleen cyclic phosphodiesterase may be particularly inferred from behaviour of this enzyme towards the group of nucleoside 2′,3′-cyclic phosphates modified in the heterocyclic or sugar moiety. Because of the great number of test substances, the kinetic measurements have not been performed but our efforts were to gather qualitative and semiquantitative data on the extent of the degradation and on degradation products.

In addition to the two main activities, the commercial enzyme preparation contained only a small amount of adenylate-deaminase which did not interfere with our assays. Any additional nucleolytic activities have not been observed. According to the literature[5], the optimum pH value of exonuclease is 5·8, but a high activity may be observed at pH 7; the optimum pH value of cyclic phosphodiesterases also lies in the neutral region[9]. The whole study was therefore performed at pH 7 to ensure the minimum extent of nonenzymatical conversion of the substrates investigated.

Preparation of Substrates

Most compounds used in the present investigation on the substrate specificity of the two enzymes have been reported in earlier papers (see the references in Tables). Alkyl

(methyl, ethyl, 1-propyl, 2-propyl, 1-butyl) esters of 3′-nucleotides have been prepared by reaction of the pyridinium salt of 5′-O-acetyl-2′-O-tetrahydropyranyluridine 3′-phosphate[10], N^4,$O^{5'}$-diacetyl-2′-O-tetrahydropyranylcytidine 3′-phosphate or N^6, $O^{5'}$-diacetyl-2′-O-tetrahydropyranyladenosine 3′-phosphate[11] with the corresponding aliphatic alcohol by the action of N,N′-dicyclohexylcarbodiimide and the subsequent removal of protecting groups in alkaline and then acidic media:

$$\mathrm{AcNp^{THP}} + \mathrm{R{-}OH} \xrightarrow[2.\ OH]{1.\ DCC} \mathrm{Np^{THP}R} \xrightarrow{H^+} \mathrm{NpR}$$

(for abbreviations see ref.[11]; N, uridine, cytidine, adenosine; R, methyl, ethyl, 1-propyl, 2-propyl, 1-butyl).

Diribonucleoside phosphates of the (3′ → 5′)-type have been reported earlier[11]. The (2′ → 5′)-UpA and UpU have been prepared by condensation of 5′-O-acetyluridine 2′,3′-cyclic phosphate with uridine and adenosine resp. in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride[12] and the subsequent pancreatic ribonuclease degradation of the dinucleoside phosphate fraction. The resistant portion was isolated by paper chromatography and its structure was established by the snake venom exonuclease degradation to uridine and the corresponding 5′-nucleotide in equimolar ratios.

2′-Deoxycytidylyl- (3′ → 5′)-guanosine (dCpG) was obtained analogously to the preparation of dUpG (cf.[13]) by condensation of 2′,3′-O-ethoxymethylene-N^2-dimethylaminomethyleneguanosine 5′-phosphate with 5′-O-p-methoxytrityl-N^4-dimethylaminomethylene-2′-deoxycytidine[14] and the subsequent alkaline and then acidic hydrolysis of the reaction mixture:

$$\mathrm{MMTrd\overset{DMM}{C} + pG\overset{DMM}{>} EM} \xrightarrow[2.\ OH^-]{1.\ DCC} \mathrm{MMTrdCpG > EM} \xrightarrow{H^+} \mathrm{dCpG}$$

2′-O-Tetrahydropyranyluridylyl-(3′ → 5′)-uridine ($\mathrm{Up^{THP}U}$) was prepared from 5′-O-acetyl-2′-O-tetrahydropyranyluridine 3′-phosphate[10] and 2′,3′-di-O-benzoyluridine[15] by the action of N,N′-dicyclohexylcarbodiimide and the subsequent alkaline work-up of the reaction mixture:

$$\mathrm{AcUp^{THP}} + \mathrm{U^{Bz}_{Bz}} \xrightarrow[2.\ OH^-]{1.\ DCC} \mathrm{Up^{THP}U}$$

2′-Chloro-2′-deoxyuridylyl-(3′ → 5′)-uridine ($\mathrm{U^{Cl}pU}$) and 2′-O-methyluridylyl-(3′ → 5′)-uridine ($\mathrm{Up^{Me}U}$) were synthesized by reaction of 2′,3′-O-ethoxymethyleneuridine 5′-phosphate[16] with 5′-O-di-p-methoxytrityl-2′-chloro-2′-deoxyuridine and 5′-O-di-p-methoxytrityl-2′-O-methyluridine, resp., and the subsequent acidic removal of protecting groups:

$$DMTrU^{Cl} + pU > EM \xrightarrow[2.\ H^+]{1.\ DCC} Up^{Cl}U$$

$$DMTrU^{Me} + pU > EM \xrightarrow[2.\ H^+]{1.\ DCC} Up^{Me}U$$

(abbreviations: U^{Cl}, 2′-chloro2′-deoxyuridine; U^{Me}, 2′-O-methyluridine). The necessary trityl derivatives were prepared by reaction of di-*p*-methoxytrityl chloride with 2′-chloro-2′-deoxyuridine[17] and 2′-O-methyluridine, resp. The latter derivative was prepared from 2′-O-methylcytidine[18] by deamination with sodium hydrogen sulfite[19].

Uridylyl-(5′ ← 3′)-1-(β-D-arabinofuranosyl)cytosine (araCpU) was prepared from 1-(β-D-arabinofuranosyl)cytosine 3′-phosphate (araCp, *cf.*[20]) by benzoylation with benzoyl cyanide[21] and the subsequent reaction with 2′,3′-di-O-benzoyluridine[15]. The alkaline hydrolysis directly afforded the free diester:

$$araCp \rightarrow Bz\text{-}araCp^{Bz}; \quad Bz\text{-}araCp^{Bz} + U^{Bz}_{Bz} \xrightarrow[2.\ OH^-]{1.\ DCC} araCpU$$

Uridylyl-(3′ → 5′)-2′-deoxyuridine (UpdU) and uridylyl-(3′ → 5′)-2′-chloro-2′-deoxyuridine (UpU^{Cl}) were obtained by reaction of uridine 2′,3′-cyclic phosphate with 2′-deoxyuridine and 2′-chloro-2′-deoxyuridine, resp., in the presence of pancreatic ribonuclease:

$$U > p + dU \rightarrow UpdU; \quad U > p + U^{Cl} \rightarrow UpU^{Cl}$$

The remaining trinucleoside diphosphates[11], 3′-dinucleotides[22] XpYp and 5′-dinucleotides[11,23] pXpY were prepared according to the references given. Ribonucleoside 2′,3′-cyclic phosphates were obtained by the reported procedures; most of them were prepared according to a general method[24] consisting in reaction of the corresponding ribonucleoside with triethyl phosphite and the subsequent treatment of the resulting 2′(3′)-phosphite (Np_i) with hexachloroacetone:

$$N \xrightarrow{(C_2H_5O)_3P} Np_i \xrightarrow{Cl_3CCOCCl_3} N > p$$

The commercial (Calbiochem, U.S.A.) preparations of polyU, polyC, polyA and yeast tRNA were used directly.

Enzymatical Activity of Spleen Acid Exonuclease

The test substances include particularly all 16 combinations of (3′ → 5′)-diribonucleoside phosphates containing the naturally occurring nucleosides (Table I). From this group, the compounds of the UpN, ApN, and GpN type are good substrates for the exonuclease except for UpC which (in contrast to the other test substances mentioned) is not degraded quantitatively after 4 h; moreover, the extent

of degradation of UpC is low even after a longer incubation. In the dinucleoside phosphate series derived from cytidine 3′-phosphate, CpU and CpC are completely resistant, CpA is a very bad substrate, and only CpG is a good substrate for the exonuclease. Consequently, in the series of naturally occurring internucleotidic compounds, the enzyme affinity depends not only on the nature of the 3′-nucleotide (the 5′-end) but also on the ester-bound nucleoside. As indicated by the observed relations UpC < UpU, CpA < CpG, and CpA < UpA, the affinity order towards the enzyme is with both components G > A > U ≫ C. The affinity is therefore determined not only by the 5′-end nucleotide (the degradation begins at this nucleotide which obviously forms a complex with the enzyme, *cf.* catalysis of the synthetic reaction) but also by the character of the ester-bound nucleoside. The preference for the purine derivatives may be observed in both cases.

The degradation of polynucleotides also proceeds in accordance with the preceding observations on dinucleoside phosphates. Degradation of polyU and polyA is quantitative and leads to 3′-nucleotides (inosine 3′-phosphate is also formed in the case of polyA due to contamination with traces of adenylate deaminase) while polyC is completely enzyme-resistant, in accordance with the resistance of the -CpC- linkage. The stability of polyC towards exonuclease has been mentioned in the literature[6] and considered difficult to explain. On the basis of the above findings, this stability may be ascribed to the character of the linkage proper and not to the secondary structure of the polymer.

In order to estimate the influence of the growing chain of the oligonucleotide and the endonuclease activity if any (the low level of this activity appears as the intrinsic

TABLE I

Degradation of Oligo- and Polynucleotides with Spleen Acid Exonuclease after 4 and 24 Hours

Compound	4 h	24 h	Compound	4 h	24 h	Compound	4 h	24 h
poly U	+	+	CpA	−	+	pUpU	−	−
poly C	−	−	CpG	+	+	pCpU	−	−
poly A	+	+[a]	ApU	+	+	pdCpdC	−	−
poly G	+	+	ApC	+	+	UpUp	+	+
UpU	+	+	ApA	+	+	CpCp	−	−
UpC	+	+	ApG	+	+	UpUpU	+	+
UpA	+	+	GpU	+	+	ApApA	+	+
UpG	+	+	GpC	+	+	CpCpC	−	−
CpU	−	−	GpA	+[b]	+	CpUpA	−	+
CpC	−	−	GpG	+[b]	+			

[a] Partial deamination to inosine 3′-phosphate; [b] quantitative after 2 h.

property of the enzyme), the degradation of polyC was performed under extreme conditions and in the presence of alkaline phosphatase *E.coli* (free of diesterase activities). This technique makes possible to detect more readily the shorter oligonucleotides of the $Cp(Cp)_nC$ type. Under these extreme conditions neither cytidine nor a shorter oligoC is formed. The diffuse character of the spot in paper chromatography of the mixture indicates the presence of longer oligonucleotides (at least a pentanucleotide) containing mostly longer chains; this length has not been exactly determined. The endogeneous degradation thus appears to take place but it cannot be said whether a simultaneous multiple degradation is involved or if the degradation occurs successively and repeatedly. With trinucleotides (Table I), the situation is less complicated. Thus, UpUpU and ApApA are degraded quantitatively to a mixture of a 3'-nucleotide and nucleoside in the ratio of 2 : 1; on the other hand, CpCpC is enzyme-resistant as expected. The triplet CpUpA contains a combination of the resistant bond CpU- and of the -UpA bond which is capable of degradation. Under standard conditions, CpUpA is resistant, in accordance with the predominating exonuclease activity beginning from the 5'-end of the molecule; longer incubation and a higher concentration of the enzyme lead to a partial degradation under the formation of the 3'-terminal adenosine and a mixture of cytidine 3'-phosphate, uridine 3'-phosphate (ratio, 1 : 1). and CpUp. (The structure of the latter compound was proved by the alkaline phosphatase *E.coli* degradation to give CpU, the pancreatic ribonuclease degradation of which affords Cp and U in an equimolar ratio.) The mixture did not contain any UpA. The slow degradation of the first internucleotidic bond to Cp and UpA is followed by a rapid degradation of UpA to Up and adenosine. Of importance is the proof of the presence of CpUp which is formed by degradation of the other internucleotidic bond almost in the same proportion as the mixture of Up and Cp. Since CpUp (similarly to CpU) is practically enzyme-resistant, the enzyme preparation used must contain some endonucleotidic activity. Because of the extremum degradation conditions both with CpUpA and polyC, it is difficult to determine whether this activity is characteristic of the enzyme (as suggested in the literature) or if a contaminating activity is involved which may be noticed in analogous special cases only.

The above observations on the resistance of -CpC- and -CpU- bonds towards the spleen exonuclease are also confirmed by composition of the incubation mixture after the yeast tRNA degradation. Thus, the mononucleotidic fraction mainly consists of Up, Ap, and Gp. The mixture of dinucleotides was dephosphorylated with alkaline phosphatase *E.coli* to afford particularly CpC and CpU along with a lesser amount of CpA. The higher oligonucleotides of the incubation mixture were not characterised *per se* but their UV spectrum indicates a high content of cytidine.

The different affinity of spleen exonuclease towards oligonucleotides bearing the phosphomonoester group at the 3'- or 5'-end of the molecule has been several times mentioned in the literature. While the group at the 3'-end of the molecule is obviously

without any influence[2], the data on the changed affinity due to the presence of the 5'-terminal phosphate group appear somewhat confused. Thus, the original observations on the resistance of such compounds[2,25,26] have been corrected[27]. As it may be inferred from Table I, the stability of compounds pCpU and pdCpdC as well as CpUp and CpCp is ascribable to the general resistance of the CpPy (Py = U, C) bond. While UpUp is a good substrate, pUpU is entirely resistant under standard conditions. These findings are thus in accordance with the original observation[2] on the marked inhibition due to the presence of the 5'-terminal phosphate group. A similar but reverse inhibitory effect of the 3'-terminal phosphate has been reported with the snake venom exonuclease; in this case, the degradation begins at the 3'-end of the molecule[28]. The strictly sterically located effect of the phosphate group with the spleen exonuclease might be due to the destabilizing interaction between the phosphate group and the acidic amino acids in the proximity of the active enzyme center, similar to the hypothesis on the action of the snake venom exonuclease[29].

As shown by the affinity comparison of dinucleoside phosphates with modified internucleotidic bonds (see Table II), both the (2' → 5')-bonds[7] and the (5' → 5')-bonds are stable towards the action of exonuclease; only the naturally occurring (3' → 5')-internucleotidic bond may be degraded. It is also possible to use the spleen exonuclease in determinations of the degree of isomerisation, particularly in those cases when the corresponding ribonucleases are accessible only with difficulty or are poorly active (esters of adenosine 3'-phosphate).

The exonuclease is known[2] to degrade the bonds not only of 3'-ribo- but also of 2'-deoxyribonucleoside 3'-phosphates and thus not to require the presence of the 2'-hydroxylic function adjacent to the internucleotidic bond. Another group of sub-

TABLE II

Degradation of Dinucleoside Phosphate Analogues with Spleen Acid Exonuclease after 4 and 24 Hours

Compound	4 h	24 h	Compound	4 h	24 h	Compound	4 h	24 h
(2'—5')-UpU	—	—	$Up^{Cl}U$	+	+[a]	UpI	+	+
(2'—5')-UpA	—	—	$Up^{Me}U$	+	+[b]	$UpAzU^{f}$	+	+
(5'—5')-UpU	—	—	$Up^{THP}U$	—	—	$Up6\text{-}MeU^{g}$	+	+
dUpG	+	+	$Up^{THP}U > EM$	—	—	$Up\text{-}L\text{-}U^{h}$	—	—[e]
dCpG	—	—	UpdU	+[c]	+	$Up\alpha\text{-}U^{i}$	+	+
araUpU	+	+	UpU^{Cl}	+[d]	+	Gpα-U	+	+
araCpU	—	—						

[a] 38%; [b] 22%; [c] quantitative; [d] 50%; [e] 15% with 100 μg enzyme; [f] AzU, 6-azauridine, [g] 6-MeU, 6-methyluridine, [h] L-U, L-uridine, [i] α-U, α-uridine.

strates examined includes compounds modified both in the sugar moiety of the 3'-nucleoside molecule and in the molecule of the ester-bound nucleoside (Table II). It may be seen from comparison of members of the pair dUpG and dCpG that the rule on the low affinity of esters of the cytosine nucleosides is also valid in the 2'-deoxy series; dCpG is a poor substrate for the enzyme. The enzyme affinity is qualitatively not influenced by the *ribo-arabino* configurational change; while araUpU is degraded, araCpU is as a derivative of a cytosine 3'-nucleotide resistant (*cf.*[30]). The substitution at position 2' by the chloro atom in the *ribo* series with $Up^{Cl}U$ lowers the affinity (*cf.*[31]) as does the O-methylation of the 2'-hydroxylic function with $Up^{Me}U$. Substitution of the 2'-hydroxylic function by the bulkier tetrahydropyranyl group (with $Up^{THP}U$ and $Up^{THP}U > EM$) leads, however, to a complete resistance of the molecule to the exonuclease. Introduction of a bulky substitutent into the position adjacent to the internucleotidic bond to be degraded obviously interferes with the formation of a complex of the enzyme with the substrate since this position is not important at the hydrolytical stage of the reaction. The reason of this behaviour does not consist in the change of conformation of the nucleoside moiety of the 3'-nucleotide molecule (*cf.* the non-resistance of 3'-nucleotide esters of the *arabino* series), but in the conformational change of the phosphodiester molecule as the whole. The latter change makes impossible the participation of the ester-bound nucleoside on the complex formation. The observed resistance of 2'-O-acetylated polynucleotides[32] and the inhibitory influence of 2'-O-methylation in tRNA on the affinity towards exonuclease[5] are also in accordance with data obtained with model compounds.

Replacement of the ester-bound uridine residue with 2'-deoxyuridine (*cf.* UpdU) or with 2'-chloro-2'-deoxyuridine (cf. UpU^{Cl}) does not markedly influence the enzyme affinity. On the other hand, a marked decrease in the affinity towards the enzyme is observed when the above replacement is performed with the use of the enantiomeric L-uridine (*cf.* Up-L-U) or with the anomeric α-uridine (*cf.* Up-α-U or Gp-α-U). In the series of UpU analogues, it is hardly possible to expect stabilisation of the molecule conformation due to base-stacking; in other combinations, such a stabilisation may play an important role (*cf.* the highest enzyme affinity of those purine doublets of the highest base-stacking). The lowered affinity of UpU derivatives and analogues which cannot assume conformation similar to that of the naturally occurring UpU because of a bulky substituent at position 2' ($Up^{THP}U$) or for structural reasons (Up-L-U, Up-α-U) might be explained by the assumption that in addition to the interaction of the active center of the enzyme with the phosphorus atom of the phosphodiester bond, the enzyme-substrate complex is stabilised by a cooperative interaction with the heterocyclic base of 3'-nucleotide (most probably of the $\pi-\pi$ character since the aromatic purine systems appear to be bound more firmly than the pyrimidine ones) as well as with the heterocyclic base of the ester-bound nucleoside. To the present knowledge, however, the role of the 3'-nucleotide appears as the most important.

In connection with earlier observations, the group of 3′-nucleotide alkyl esters has been now investigated from the standpoint of the varying sterical effect of the alkyl substituent. It may be seen from Table III that the affinity order of all the three series of nucleotide esters is the same, namely, 1-butyl = 1-propyl > methyl > ethyl ≫ ≫ 2-propyl. The 2-propyl esters are in all the three cases resistant towards the action of the exonuclease (a similar resistance of 2-propyl esters has been also encountered with the pancreatic ribonuclease). The above affinity order might be explained by the growing sterical requirements of the alkyl group (2-propyl > ethyl > methyl). With 1-propyl and 1-butyl esters, the sterical effect of the 1-alkyl chain appears to be lowered by its hydrophobic character and thus the lesser affinity to the hydrophilic moiety of the sugar phosphate. The influence of the base is not as pronounced as with dinucleoside phosphates (the cytosine derivatives do not considerably differ from the uracil and adenine ones). The destabilisation of the enzyme-substrate complex with 3′-nucleotide alkyl esters is mainly due to the sterical effect which is practically the same in all cases. Similarly to internucleotidic compounds, the action of exonuclease on the alkyl esters is limited to 3′-nucleotide esters (uridine 2′-phosphate methyl ester as well as cytidine 2′-phosphate methyl ester are quite exonuclease-resistant).

Two additional types of compounds should be mentioned in connection with the above discussion. One of them, the five-membered cyclic phosphodiesters, namely,

TABLE III

Degradation of 3′-Ribonucleotide Alkyl Esters (NpR) with Spleen Acid Exonuclease

N / UCA	U	C	A	Incubation time, h
	4	+	−	−
CH_3	24	+72%[a]	+27%[a]	−
	4	−	−	+23%
C_2H_5	24	+	+15%	+
	4	+	+N	+30%
n-C_3H_7	24	+quant.	+64%	+
	2	−	−	−
i-C_3H_7	24	−	−	−
	4	−	+	+4%
n-C_4H_9	24	+quant.	+63%	+

[a] 2′-Nucleotide methyl ester is resistant after 24 hours.

ribonucleoside 2',3'-cyclic phosphates were claimed substrates of exonuclease in an early paper[2]. As mentioned above, however, this activity must be ascribed to the accompanying cyclic phosphodiesterase since the thoroughly purified spleen exonuclease is practically free of such an activity[7]. The principal argument against the potential degradation of 2',3'-cyclic nucleotides with exonuclease consists in the formation of 2'-nucleotides as degradation products (*vide infra*). Such a formation is at variance with the present knowledge on the specificity of exonuclease. The stability of these compounds towards the pure spleen exonuclease might be due to the stereochemistry of the molecule with a fixed five-membered cyclic system of the phosphodiester since the mutual distance of the phosphorus atom (the center at which the linkage with the active site takes place) and the heterocyclic base of the nucleotide (the center of the cooperative interaction) might not correspond to the requirements of the enzyme. A similar explanation can be applied to the other type of cyclic 3'-nucleotide esters, *i.e.*, ribonucleoside 3',5'-cyclic phosphates that also are stable towards the exonuclease. The stereochemistry of the molecule is completely changed because of the mutual annelation of the five-membered and six-membered ring. The secondary-alcoholic character of the 2'-hydroxylic function may be excluded as the potential source of the resistance with 2',3'-cyclic phosphates (in 3',5'-cyclic phosphates, the 3'-nucleotide bond is resistant in spite of the intramolecular esterification with a primary alcoholic function).

Enzymatical Activity of the Spleen Cyclic Phosphodiesterase

As mentioned in the introduction, the enzymatical preparations of the spleen exonuclease contain practically always an additional activity of the cyclic phosphodiesterase (cPDase); this activity was originally assumed to be an intrinsic property of the spleen exonuclease[2]. The same activity is also present in the preparation used in the present investigations. The spleen cyclic phosphodiesterase has not been so far characterised in detail; in view of its properties, especially the formation of 2'-ribonucleotides as degradation products, it resembles the 2',3'-cyclic phosphodiesterases isolated from bacteria or nerve tissues[9,33]. As shown by investigations on the affinity to the above types of compounds with the use of the above exonuclease preparation contaminated with the cyclic phosphodiesterase, the two enzymes do not interfere with each other. It is therefore possible to use the mixture for the study of both the enzymes. Thus, CpC, CpMe, and C-2'-pMe were resistant towards exonuclease as well as cyclic phosphodiesterase while cytidine 2',3'-cyclic phosphate was degraded. Also the (2' → 5')-internucleotidic compounds are resistant to the mixture of enzymes. Consequently. ribonucleoside 2',3'-cyclic phosphate constitutes the only type of substrate for the 2',3'-cyclic phosphodiesterase. The reasons which exclude the activity of exonuclease in degradations of 2',3'-cyclic phosphates have been mentioned above. The investigations on the cPDase activity have been effected simultaneously

TABLE IV

Degradation of Nucleoside 2′,3′-Cyclic Phosphates with Spleen Cyclic Phosphodiesterase

Formula	Nucleoside	4h[a]	24h[b]	Formula	Nucleoside	4h[a]	24h[b]
I	uridine	+	63%	*XXV*	4-pyrimidinone 3-riboside[44]	+	40%
II	5-methyluridine[37]	+	100%	*XXVI*	6-methyluracil 3-riboside[45]	+	52%
III	5-chlorouridine[24]	+	69%	*XXVII*	1,6-dimethyluracil 3-riboside[45]	—	0
IV	5-bromouridine[38]	—	0[c]	*XXVIII*	L-uridine[41]	—	0
V	5-aminouridine[24]	+	63%	*XXIX*	α-uridine[40]	—	0
VI	5-dimethylaminouridine[24]	+	50%	*XXX*	thymine 1-β-D-ribopyranoside[46]	—	0
VII	5-ethoxycarbonyluridine[39]	—	29%	*XXXI*	adenosine	+	40%[d]
VIII	5-diethylaminomethyluridine	—	20%	*XXXII*	L-adenosine	+	50%[d]
IX	6-methyluridine[24]	—	0	*XXXIII*	adenine 9-α-L-lyxofuranoside[47]	+	12%
X	5,6-dimethyluridine[24]	—	0	*XXXIV*	adenine 9-α-D-lyxofuranoside[47]	—	5[d]
XI	5-carboxyuridine[39]	—	0	*XXXV*	6-dimethyladenosine	—	+
XII	6-carboxyuridine[24]	—	0	*XXXVI*	tubercidine	+	100%
XIII	3-methyluridine[24]	—	24%	*XXXVII*	inosine[48]	+	100%
XIV	3-hydroxyethyluridine[42]	—	0	*XXXVIII*	6-thioinosine[48]	+	+
XV	cytidine	±	30%	*XXXIX*	xanthosine[48]	+	+
XVI	5-methylcytidine[24]	+	25%	*XL*	2-aminopurine riboside	+	44%
XVII	4-dimethylcytidine[24]	+	36%	*XLI*	2,6-diaminopurine riboside	+	42%
XVIII	isocytidine[24]	+	20%	*XLII*	isoguanosine	+	100%
XIX	6-azauridine[43]	—	0	*XLIII*	guanosine	+	100%
XX	5-methyl-6-azauridine[24]	+	44%	*XLIV*	L-guanosine[41]	±	11%
XXI	3-methyl-6-azauridine[24]	—	0	*XLV*	8-bromoguanosine	—	±
XXII	6-azacytidine[43]	+	42%	*XLVI*	8-aminoguanosine	+	15%
XXIII	2-pyrimidone-1-riboside[44]	+	100%	*XLVII*	8-hydroxyguanosine	+	100%
XXIV	4-methyl-2-pyrimidinone 1-riboside	+	100%	*XLVIII*	8-mercaptoguanosine	—	±

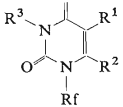
[a] Qualitative evaluation of degradation; [b] percentage of degradation; [c] 20% degradation with 20 μg enzyme; [d] 2′-nucleotide formation.

with the study on the closely related exonuclease specificity. In the present work, the attention has been focussed to a series of nucleoside 2',3'-cyclic phosphates modified both in the heterocyclic and the sugar moiety, also with regard to the structure of the resulting products. Along with the preceding results (Tables I–III), the following conclusions can be drawn from investigations on 2',3'-cyclic phosphates (Table IV).

a) The cPDase degrades 2',3'-cyclic phosphates of both pyrimidine and purine nucleosides. The (2' → 5')-, (3' → 5')-, and (5' → 5')- phosphodiesters of the dinucleoside phosphate type, the alkyl esters of 2'- and 3'-ribonucleotides as well as the diesters of 2'-deoxy-3'-ribonucleotides are stable towards the action of cPDase. None of the characteristic functional groups of the naturally occurring pyrimidine and purine bases does exert any appreciable influence on the affinity; the functional groups do not therefore play any role in the catalysis of the hydrolytical stage of the reaction. The enzyme does not consequently posses any specific recognition site for the heterocyclic base; the interaction with this base only enables the formation of the enzyme-substrate complex.

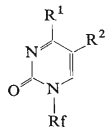
b) In the series of pyrimidine derivatives, there is only a small qualitative influence of the substitution at position 4 of the substrate (*I*, R = OH; *XV*, R = NH_2; *XVII*, R = $N(CH_3)_2$; *XXIII*, R = H; *XXIV*, R = CH_3) on the affinity towards the enzyme. Substitution that increase the acidic character of the base (*IV*, *XI*, *XII*, *XIX*), decrease the affinity towards the enzyme. Replacement of $C_{(6)}$—H by —N= (compound *XXII*) is without any influence unless it leads to an increased acidity (*cf.* the 6-azauridine derivative *XIX*).

Substitution by the methyl group at position 5 enhances the affinity towards cPDase; this effect is particularly striking with the 6-azauracil derivatives *XIX* and *XX*. On the other hand, introduction of the methyl group into the position 3 results in a considerable decrease of the affinity (compounds *XIII* and *XXI*). This decrease



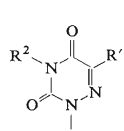
I, $R^1 = R^2 = R^3 = H$
II, $R^1 = CH_3$, $R^2 = R^3 = H$
III, $R^1 = Cl$, $R^2 = R^3 = H$
IV, $R^1 = Br$, $R^2 = R^3 = H$
V, $R^1 = NH_2$, $R^2 = R^3 = H$
VI, $R^1 = N(CH_3)_2$, $R^2 = R^3 = H$
VII, $R^1 = COOC_2H_5$, $R^2 = R^3 = H$
VIII, $R^1 = CH_2N(C_2H_5)_2$, $R^2 = R^3 = H$
IX, $R^1 = R^3 = H$, $R^2 = CH_3$
X, $R^1 = R^2 = CH_3$, $R^3 = H$
XI, $R^1 = COOH$, $R^2 = R^3 = H$
XII, $R^1 = R^3 = H$, $R^2 = COOH$
XIII, $R^1 = R^2 = H$, $R^3 = CH_3$
XIV, $R^1 = R^2 = H$, $R^3 = CH_2CH_2OH$

is not due to any hydrophobic interactions since compound *XIV* is also resistant in spite of the substitution by the hydrophilic 2-hydroxyethyl group. A similar decrease of the affinity may be observed with the 6-methyl group as substituent (compounds *IX* and *X*); since this substitution leads to the *syn*-conformation of the nucleoside moiety, the methyl group assumes the same orientation to the sugar moiety as with the N^3-methyl derivatives (compounds *XIII*, *XIV*, and *XXI*).

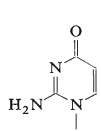


XV, $R' = NH_2$, $R^2 = H$
XVI, $R' = NH_2$, $R^2 = CH_3$
XVII, $R' = N(CH_3)_2$, $R^2 = H$
XXIII, $R' = R^2 = H$
XXIV, $R' = CH_3$, $R^2 = H$

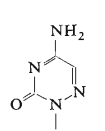
c) With the purine derivatives, substitution at position 2 and 6 by a hydroxy, amino, dialkylamino, or mercapto group as well as replacement of N^7 by —CH= (compound *XXXVI*) do not exert any qualitative effect on the enzyme affinity; compounds *XXXI* and *XXXV* to *XLIII* are substrates for cPDase. In all these purine derivatives, the pyrimidine ring is orientated above the plane of the sugar moiety; whenever the conformation is changed due to substitution at 8 (compounds *XLI*, *XLVI*, and *XLVIII*), the enzyme affinity decreases. This effect is similar to that caused by substitution on N^3 or $C_{(6)}$ in the case of the pyrimidine nucleosides (*vide supra*) where the bulky substituent occurs at that side of the heterocyclic base which is averted from the plane of the sugar ring (similar to the bulky pyrimidine ring of the 8-substituted purine derivatives of the *anti* conformation, *cf.*[34]). The 8-hydroxy derivative *XLVIII* is not



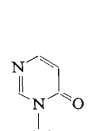
XIX, $R' = R^2 = H$
XX, $R' = CH_3$, $R^2 = H$
XXI, $R' = H$, $R^2 = CH_3$



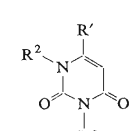
XVIII



XXII



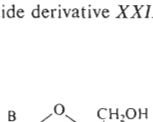
XXV



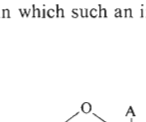
XXVI, $R' = CH_3$, $R^2 = H$
XXVII, $R' = R^2 = CH_3$

exception to this rule since it has been shown in earlier papers[35] that the energy barrier of the rotation about the nucleosidic bond is low and that there is roughly an equal probability of the occurence of both conformations.

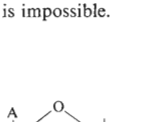
d) Noteworthy is the difference in affinity of the enantiomeric L-derivatives of 2',3'-cyclic nucleotides in the pyrimidine and purine series. Thus, the L-uridine derivative *XXVIII* is enzyme-resistant while the purine L-derivatives *XXXII* and *XLIV* are degraded. Conformation of the nucleoside remains intact in the L-enantiomers. With compound *XXVIII*, the pyrimidine moiety is out of the enzyme region responsible for the interaction between the enzyme and the heterocyclic base of the substrate. With the purine derivatives *XXXII* and *XLIV*, however, the pyrimidine ring is situated owing to their *syn*-conformation above the plane of the sugar cycle and interferes at least partly with the region mentioned. The importance of the cooperative interaction between the heterocyclic base and the enzyme during the stabilisation of the enzyme-substrate complex is supported *inter alia* by the resistance of the α-nucleotide derivative *XXIX* in which such an interaction is impossible.



XXVIII, B = uracil
XXXII, B = adenine
XLIV, B = guanine



XXXIII, A = adenine

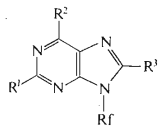


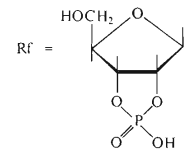
XXXIV, A = adenine

e) The change of configuration at position 4' of the sugar ring leads to a decreased affinity in both enantiomeric series (compounds *XXXIII* and *XXXIV*) in the same sense as in the D- and L-*ribo* series (compounds *XXXI* and *XXXII*). The cause of the decreased affinity with lyxofuranosides is not quite clear; a sterical effect might be involved or a hydrophilic effect of the hydroxymethyl group may play an important role; the latter group gets into the vicinity of the phosphorus atom and could interfere with the active center bond or with the hydrolytical process. In this connection, the cPDase-resistance of the ribopyranosyl derivative *XXX* is of interest (the vicinal 4'-hydroxylic function is also *cis*-orientated in respect to the phosphate).

f) Degradation of 2',3'-cyclic phosphates both in the pyrimidine[2,7] and the purine series affords always nucleoside 2'-phosphates as products. This fact has been now

confirmed on numerous purine derivatives. The 2'-nucleotides are also formed in the case of the lyxofuranosyl derivative *XXXIII* and the two enantiomeric derivatives *XXXII* and *XXXIV*. The latter observation indicates that the stereospecific degradation of the cyclic phosphodiester obviously depends on the electron density distribution over the sugar ring (the distribution is equal with both the enantiomers) and

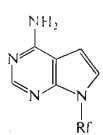




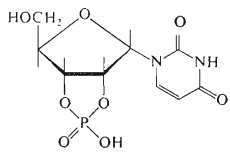
XXXI, $R^1 = R^3 = H$, $R^2 = NH_2$
XXXV, $R^1 = R^3 = H$, $R^2 = N(CH_3)_2$
XXXVII, $R^1 = R^3 = H$, $R^2 = OH$
XXXVIII, $R^1 = R^3 = H$, $R^2 = SH$
XXXIX, $R^1 = R^2 = OH$, $R^3 = H$
XL, $R^1 = NH_2$, $R^2 = R^3 = H$
XLI, $R^1 = R^2 = NH_2$, $R^3 = H$

XLII, $R^1 = OH$, $R^2 = NH_2$, $R^3 = H$
XLIII, $R^1 = NH_2$, $R^2 = OH$, $R^3 = H$
XLV, $R^1 = NH_2$, $R^2 = OH$, $R^3 = Br$
XLVI, $R^1 = R^3 = NH_2$, $R^2 = OH$
XLVII, $R^1 = NH_2$, $R^2 = R^3 = OH$
XLVIII, $R^1 = NH_2$, $R^2 = OH$, $R^3 = SH$

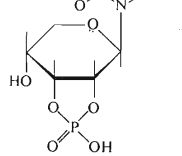
that the activation of the phosphorus atom in the enzyme–substrate complex is symmetrical to the plane of symmetry of the sugar ring (its carbon atoms 1' and 4'); it would be otherwise difficult to explain simultaneously the observations of paragraph *d* and the formation of 2'-isomers from both enantiomers *XXXI* and *XXXII* Concerning the mechanism of the 2'-nucleotide formation, a more detailed study will be necessary. By this formation, the cyclic phosphodiesterases differ from ribonucleases which catalyse the hydrolysis of 2',3'-cyclic phosphates to 3'-ribonucleotides.



XXXI



XXIX



XXX

Observations obtained in the present work on the specificity of two related enzymes occurring in the same tissue have revealed some interesting rules concerning the structural effects with the two non-specific enzymes which catalyse the hydrolysis of various types of phosphodiesters. A comparison appears desirable between the present observations and the effects of other related enzymes, *e.g.*, the snake venom exonuclease and the nerve tissue cyclic phosphodiesterase.

EXPERIMENTAL

Solutions were taken down on a rotatory evaporator at 40°C/15 Torr unless stated otherwise. Substances were dried over phosphorus pentoxide at 0·1 Torr. Descending paper chromatography was performed on paper Whatman No 1 (preparative runs on paper Whatman No 3 MM) in the solvent system 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2). Paper electrophoresis was carried out by the technique of Markham and Smith[36] on paper Whatman No 3 MM in 0·1M triethylammonium hydrogen carbonate (pH 7·5; 20 V/cm; 1 h). Ultraviolet absorption spectra were measured in aqueous solutions on a Zeiss Specord apparatus.

Ribonucleoside 3′-Phosphate Alkyl Esters

The calcium salt (5 mmol) of 5′-O-acetyl-2′-O-tetrahydropyranyluridine 3′-phosphate[10], of N^4,$O^{5'}$-diacetyl-2′-O-tetrahydropyranylcytidine 3′-phosphate[11] or of N^6,$O^{5'}$-diacetyl-2′-O-tetrahydropyranyladenosine 3′-phosphate[11] in 20% aqueous pyridine (10 ml) was applied to a column of pyridinium Dowex 50 X 8 ion exchange resin (50 ml). The column was eluted with 20% aqueous pyridine (150 ml), the eluate evaporated under diminished pressure almost to dryness, and the residue coevaporated with five 20 ml portions of pyridine at 30°C/0·1 Torr. The final residue was made up to the volume of 25 ml with pyridine and the solution was divided into five 5 ml portions. Each portion (1 mmol of the pyridinium salt of the corresponding nucleotide) was treated with 2 ml of the appropriate alcohol (methanol, ethanol, 1-propanol, 2-propanol, 1-butanol) and 0·6 g of N,N′-dicyclohexylcarbodiimide, and the mixture was kept under exclusion of atmospheric moisture for 5 days at room temperature. Water (2 ml) was then added, the mixture kept for additional 1 h, diluted with water (100 ml), and washed with two 25 ml portions of ether. The aqueous phase was concentrated under diminished pressure to the volume of about 20 ml and the concentrate was kept with an equal volume of conc. aqueous ammonia at room temperature overnight. The mixture was evaporated under diminished pressure to dryness and the residue heated at 50°C in 50% aqueous acetic acid (5 ml) 30 min. The resulting mixture was chromatographed (*vide supra*) on 3 sheets of paper Whatman No 3 MM. Bands of the corresponding products were eluted with 1 : 100 dilute aqueous ammonia (50 ml), the eluates evaporated under diminished pressure, the residue coevaporated with ethanol (50 ml), and reprecipitated from methanol (5 ml) with ether (100 ml). The precipitate of the ammonium salt of the 3′-nucleotide alkyl ester was collected by centrifugation, washed, and dried under diminished pressure to afford the following products (% yield, R_F, and E_{Up} given): UpMe, 28, 0·45, 0·50; UpEt, 35, 0·50, 0·50; Upn-Pr, 37, 0·60, 0·50; UpiPr, 24, 0·65, 0·50; Upn-Bu, 27, 0·63, 0·50; CpMe, 42, 0·45, 0·45; CpEt, 39, 0·47, 0·45; Cpn-Pr, 25, 0·60, 0·45; CpiPr, 20, 0·64, 0·45; Cpn-Bu, 34, 0·62, 0·45; ApMe, 36, 0·58, 0·32; ApEt, 38, 0·60, 0·32; Apn-Pr, 42, 0·66, 0·32; ApiPr, 25, 0·66, 0·32; Apn-Bu, 35, 0·70, 0·30.

Uridylyl-(2′ → 5′)-uridine (*cf.*[12])

To a solution of the tri-n-butylammonium salt of 5′-O-acetyluridine 2′,3′-cyclic phosphate (1 mmol) in dimethylformamide (4 ml) there was added 2,4,6-triisopropylbenzenesulfonyl chloride (0·9 g)

and then (after 10 min at room temperature) uridine (0·73 g; 3 mmol). The mixture was stirred at room temperature under exclusion of atmospheric moisture for 2 h, treated with water (5 ml) and tri-n-butylamine (2 ml), kept at room temperature overnight, diluted with water (50 ml), and washed with two 25 ml portions of ether. The aqueous phase was evaporated under diminished pressure, the residue dissolved in 10% aqueous pyridine (50 ml) and the solution incubated with pancreatic ribonuclease (20 mg) at 37°C for 16 h. The mixture was applied to a column of pyridinium Dowex 50 ion exchange resin (100 ml) and the column eluted with 20% aqueous pyridine (250 ml). The eluate was adjusted to pH 8 by the addition of aqueous ammonia, and the whole evaporated under diminished pressure to dryness. The residue was dissolved in water (20 ml) the solution filtered through a layer of Hyflo Super Cell, and the filtrate applied to a column (80 × 4 cm) of DEAE-cellulose (Cellex D, standard capacity, HCO_3^- form). The column was then washed with water (3 ml per min) to the drop of the initial UV-absorption and eluted with a linear gradient of triethylammonium hydrogen carbonate pH 7·5 (2 l of water in the mixing chamber and 2 l of a 0·3M buffer solution in the reservoir; rate, 3 ml per min; the fractions were taken in 10 min intervals). The product fraction (0·08—0·10M) was evaporated under diminished pressu and the residue chromatographed (*vide supra*) on 2 sheets of paper Whatman No 3 MM. The band of the product was eluted with dilute (1 : 100) aqueous ammonia (50 ml) and the eluate freeze-dried to afford 50 μmol of the product, chromatographically and electrophoretically identical with UpU but resistant towards the action of pancreatic ribonuclease. The snake venom phosphodiesterase degradation (standard conditions) afforded uridine 5′-phosphate and uridine in the ratio of 1 : 0·97, R_F, 0·27; E_{Up}, 0·30.

Uridylyl-(2′ → 5′)-adenosine (*cf.*[12])

The title compound was prepared analogously to (2′ → 5′)-UpA (*vide supra*) with the use of adenosine (3 mmol) instead of uridine. Yield, 100 μmol; R_F, 0·33; E_{Up}, 0·23 (both the latter values are identical with those of UpA). The substance is resistant towards pancreatic ribonuclease. The snake venom phosphodiesterase degradation affords adenosine 5′-phosphate and uridine in the ratio of 1 : 1·05.

2′-Deoxycytidylyl-(3′ → 5′)-guanosine

The title compound was prepared by condensation of the pyridinium salt of 2′,3′-O-ethoxymethylene-N^2-dimethylaminomethyleneguanosine 5′-phosphate[13] (1 mmol) with 5′-O-p-methoxytrityl-N^4-dimethylaminomethylene-2′-deoxycytidine[14] (2·5 mmol). The mixture of reactants was dried together by codistillation with five 20 ml portions of pyridine at 30°C/0·1 Torr. The residue was dissolved in pyridine (10 ml) and the solution was shaken with 2,4,6-triisopropylbenzenesulfonyl chloride (1·5 g) at room temperature overnight. The mixture was then evaporated under diminished pressure to dryness, the residue heated in 50% aqueous acetic acid (50 ml) for 1 h at 50°C, the whole evaporated, the residue coevaporated with three 20 ml portions of water, dissolved in water (50 ml). and the aqueous solution washed with three 20 ml portions of ether. The aqueous phase was neutralised with aqueous ammonia and chromatographed on a column of DEAE-cellulose analogously to (2′ → 5′)-UpU. The 0·05—0·08M fraction was evaporated under diminished pressure and the residue rechromatographed on 4 sheets of paper Whatman No 3 MM. Bands of the product were eluted with dilute (1 : 100) aqueous ammonia and the eluate freeze-dried to afford 0·38 mmol (38%) of the ammonium salt of dCpG, homogeneous on chromatography (R_F, 0·20) and electrophoresis (E_{Up}, 0·25). The snake venom phosphodiesterase degradation affords guanosine 5′-phosphate and 2′-deoxycytidine in the ratio 1 : 0·92.

2′-Deoxy-2′-chlorouridylyl-(3′ → 5′)-uridine

A solution of uridine 5′-phosphate sodium salt (2 mmol) in water (5 ml) was applied to a column of Dowex 50 (H^+ form) ion exchange resin (20 ml). The elution was performed with water using the Uvicord apparatus. The UV-absorbing eluate was evaporated to dryness, the residue coevaporated with four 25 ml portions of ethanol and one 25 ml portion of toluene, and the final foamy residue dissolved in a mixture of dimethylformamide (10 ml) and triethyl orthoformate (5 ml). As shown by paper chromatography (R_F, 0·28; R_F of UMP, 0·13) the reaction mixture was homogeneous after 3 days at room temperature. The mixture was evaporated at 40°C/0·1 Torr and the residual 2′,3′-O-ethoxymethyleneuridine 5′-phosphate dried by coevaporation with four 20 ml portions of pyridine at the same temperature and pressure.

A solution of 2′-deoxy-2′-chlorouridine[17] (1·6 g; 6 mmol) and di-*p*-methoxytrityl chloride (3·0 g; 9 mmol) in pyridine (20 ml) was kept at room temperature for 2 days, poured into 200 ml of ice-cold water, and extracted with three 25 ml portions of ethyl acetate. The extract was washed with two 20 ml portions of water, dried over anhydrous magnesium sulfate, and evaporated under diminished pressure. The residue (homogeneous on thin-layer chromatography on Silufol UV_{254} ready-for-use silica gel plates, developed in $CHCl_3$; R_F, 0·24) was dissolved in pyridine (20 ml) and the solution added to the residue of pU > EM of the preceding paragraph. The whole mixture was dried by coevaporation with four 25 ml portions of pyridine at 35°C/0·1 Torr, and the final residue dissolved in pyridine (25 ml). 2,4,6-Triisopropylbenzenesulfonyl chloride (1·5 g; 5 mmol) was added to the solution and the mixture kept at room temperature under exclusion of atmospheric moisture overnight. Water (5 ml) was then added, the mixture kept at room temperature for additional 1 h, evaporated under diminished pressure to dryness, the residue coevaporated with toluene, and the residual foam dissolved in a mixture of acetic acid (50 ml) and 50% aqueous methanol (50 ml). The resulting solution was heated at 50°C for 2 h, evaporated under diminished pressure, the residue diluted with water (100 ml), and washed with two 50 ml portions of chloroform and one 50 ml portion of ether. The aqueous phase was evaporated under diminished pressure to dryness, the residue coevaporated with two 50 ml portions of water, and finally dissolved in water (20 ml). The solution was neutralised with aqueous ammonia, filtered through a layer of Hyflo Super Cel, and the filtrate chromatographed on a column of DEAE-cellulose analogously to (2′ → 5′)-UpA. The fraction of the product (0·05—0·12M) was evaporated and the residue rechromatographed on 6 sheets of paper Whatman No 3 MM. Bands of the product were eluted with water (50 ml) and the eluates freeze-dried to afford 0·90 mmol (45%) of the ammonium salt of $Up^{Cl}U$, homogeneous on chromatography (R_F, 0·28) and electrophoresis (E_{Up}, 0·37). The product is resistant towards pancreatic ribonuclease; the snake venom phosphodiesterase degradation affords uridine 5′-phosphate and 2′-deoxy-2′-chlorouridine in the ratio 1 : 1·02.

2′-O-Methyluridylyl-(3′ → 5′)-uridine

A solution of 2′-O-methylcytidine[18] (0·8 g; 3·1 mmol), sodium hydrogen sulfite (5 g), and water (20 ml) was kept at room temperature overnight, diluted with water (50 ml), and precipited with the required amount of hot saturated aqueous barium hydroxide (100 ml). The mixture was filtered while hot through a layer of Hyflo Super Cel and the material on the filter washed with water (50 ml). The filtrate and washings were combined, neutralised by the addition of Dowex 50 (H^+ form) ion exchange resin, filtered, and the resin washed with water (100 ml). The combined filtrates were evaporated to dryness under diminished pressure, the residue coevaporated with two 20 ml portions of ethanol, and dried under diminished pressure until a solid foam resulted. Yield, 0·72 g (2·85 mmol) of 2′-O-methyluridine, homogeneous on thin-layer chromatography

on Silufol UV_{235} ready-for-use silica gel plates, developed in 75 : 25 chloroform–ethanol, R_F 0·52; 2′-O-methylcytidine, R_F 0·08). To a solution of this residue in pyridine (10 ml), there was added di-*p*-methoxytrityl chloride (1·7 g; 5 mmol) and the whole stirred until a solution was obtained. The solution was kept at room temperature for 3 days, poured into ice-cold water (200), ml, and proceessed analogously to $Up^{Cl}U$ (*vide supra*). To the residue of the thus-prepared trityl derivative there was added a solution of 2 mmol of the pyridinium salt of 2′,3′-O-ethoxymethyleneuridine 5′-phosphate (for its preparation see $Up^{Cl}U$) in pyridine (20 ml); the condensation, work-up, chromatography on a column of DEAE-cellulose, and the final purification by paper chromatography was effected analogously to the above $Up^{Cl}U$. The product was obtained by freeze-drying as the ammonium salt (yield, 46%), homogeneous on chromatography (R_F, 0·25) and electrophoresis (E_{Up}, 0·44). The product is resistant towards pancreatic ribonuclease; the snake venom phosphodiesterase degradation affords uridine 5′-phosphate and 2′-O-methyluridine. UV spectrum: λ_{max} 262 nm.

2′-O-Tetrahydropyranyluridylyl-(3′ → 5′)-uridine

The pyridinium salt of 5′-O-acetyl-2′-O-tetrahydropyranyluridine 3′-phosphate[10] (2·5 mmol) was prepared as given under the heading of 3′-nucleotide alkyl esters. To a solution of this salt in pyridine (25 ml) there was added 2′,3′-di-O-benzoyluridine (3·2 g; 7 mmol; *cf.*[15]) and the whole mixture dried by coevaporation with five 20 ml portions of pyridine at 30°C/0·1 Torr. The residue was then taken up into pyridine (10 ml) and N,N′-dicyclohexylcarbodiimide (4 g) was added. The reaction mixture was kept at room temperature under exclusion of atmospheric moisture for 7 days. Water (5 ml) and triethylamine (0·5 ml) were added, the mixture kept at room temperature for 1 h, diluted with conc. aqueous ammonia (30 ml) and methanol (30 ml), heated at 50°C for 2 h, and evaporated under diminished pressure. The residue was diluted with water and washed with two 25 ml portions of ether. The aqueous phase was filtered through a layer of Hyflo Super Cel, the filtrate concentrated under diminished pressure to the volume of about 20 ml, and the concentrate chromatographed on a column of DEAE-cellulose as above. The elution was performed analogously to (2′ → 5′)-UpU. The fraction of the product (0·12—0·18M) was evaporated under diminished pressure, the residue coevaporated with two 25 ml portions of ethanol, the final residue dissolved in ethanol, and the solution filtered through a layer of Hyflo Super Cel. The filtrate was added dropwise into ether (500 ml), the precipitate collected with suction, washed with ether, and dried under diminished pressure to afford 1·03 g (56%) of the triethylammonium salt of $Up^{THP}U$, homogeneous on chromatography (R_F, 0·32) and electrophoresis (E_{Up}, 0·47). Content as determined spectrophotometrically, 98%. The product is resistant towards pancreatic ribonuclease; heating in 50% aqueous acetic acid for 30 min at 50°C affords quantitatively UpU, identical with an authentic specimen.

Uridylyl-(5′ → 3′)-1-(β-D-arabinofuranosyl)cytosine

To a suspension of the ammonium salt of 1-(β-D-arabinofuranosyl)cytosine 3′-phosphate[20] (1·7 g; 5 mmol) and benzoyl cyanide (2·6 g; 20 mmol) in acetonitrile (25 ml) there was added with stirring triethylamine (3 ml), the whole stirred at room emperature for 4 h, and poured into ether (300 ml). The precipitate was collected with suction, washed with ether, and dried under diminished pressure to afford 2·6 g (71%) of the triethylammonium salt of 1-(2,5-di-O-benzoyl-1-β-D-arabinofuranosyl)cytosine 3′-phosphate, homogeneous on chromatography in 5 : 2 ethanol–1M ammonium acetate; R_F, 0·80).

To a mixture of the latter salt (1·5 g; 2 mmol), 2′,3′-di-O-benzoyluridine[15] (2·3 g; 5 mmol), and pyridine (30 ml) there was added 2,4,6-triisopropylbenzenesulfonyl chloride (3 g; 10 mmol)

and the whole stirred at room temperature under exclusion of atmospheric moisture overnight. Water (10 ml) was then added, the mixture kept at room temperature for additional 1 h, evaporated under diminished pressure, and the residue coevaporated with three 25 ml portions of toluene. Water (40 ml), methanol (40 ml), and triethylamine (20 ml) were added, the solution heated at 60°C for 3 h, evaporated under diminished pressure, the residue diluted with water (100 ml), and washed with two 25 ml portions of ether. The aqueous phase was filtered through Celite, the filtrate concentrated under diminished pressure to the volume of about 20 ml, and the concentrate chromatographed on a column of DEAE-cellulose, the elution being performed analogously to the preparation of (2′→ 5′)-UpU as described above. The product was isolated from the 0·10—0·12M buffer fraction, rechromatographed on 6 sheets of paper Whatman No 3 MM, eluted with dilute (1 : 100) aqueous ammonia (20 ml), and freeze-dried. Yield, 0·48 mmol (24%) of the ammonium salt, homogeneous on chromatography (R_F, 0·18) and electrophoresis (E_{Up}, 0·32), and resistant towards pancreatic ribonuclease.

Preparation of Ribonucleoside 2′,3′-Cyclic Phosphates

This preparation was performed by a reported method[24] from reported ribonucleosides. The products were isolated by chromatography on DEAE-cellulose, rechromatographed on paper in the solvent system 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2), and freeze-dried as ammonium salts. In addition to paper chromatography and electrophoresis, the unreported derivatives were characterised by degradation to 2′(3′)-nucleotides on heating in 50% aqueous acetic acid at 50°C. The UV spectra of the present ribonucleoside 2′, 3′-cyclic phosphates were in accordance with those of the corresponding ribonucleosides.

Uridylyl-(3′→ 5′)-2′-chloro-2′-deoxyuridine and Uridylyl-(3′→ 5′)-2′deoxyuridine

A solution of uridine 2′,3′-cyclic phosphate lithium salt (0·1 mmol) and 2′-deoxyuridine (Calbiochem) or 2′-chloro-2′-deoxyuridine[17] (0·5 mmol) each in 50% aqueous pyridine (250 µl) containing 20 µg of pancreatic ribonuclease (Lachema, Czechoslovakia) was incubated at 0°C for 24 h and applied to one sheet of paper Whatman No 3 MM. The chromatographic band of the product was eluted with dilute aqueous ammonia (10 ml) and the eluate freeze-dried to afford uridylyl-(3′→ 5′)-2′-deoxyuridine (12%; R_F, 0·26; E_{Up}, 0·40) and uridylyl-(3′→ 5′)--2′-chloro-2′-deoxyuridine (18%; R_F, 0·28; E_{Up}, 0·37), resp. Both these compounds are quantitatively degraded with pancreatic ribonuclease to equimolar mixtures of uridine 3′-phosphate and 2′-deoxyuridine or uridine 3′-phosphate and 2′-chloro-2′deoxyuridine.

Enzymatical Degradations

A. Analytical degradations. The test substance (2 µmol) in 0·05M-Tris-HCl buffer solution pH 8·5 (100 µl) containing 20 µg of pancreatic ribonuclease (Worthington) or the snake venom (*Crotalus adamanteus*) phosphodiesterase (Boehringer) or the bacterial alkaline phosphatase (Worthington) was incubated at 37°C for 4 h and the mixture analysed by paper chromatography or electrophoresis (*vide supra*).

B. Spleen acid exonuclease assay. The test substance (2 µmol) in 0·05M-Tris-HCl buffer solution pH 7·0 (100 µl) containing 10 µg of the enzyme protein (Boehringer) was incubated at 37°C. After 4 and 24 h, samples were withdrawn and analysed by paper chromatography. The blank was performed under identical conditions but in the absence of the enzyme. Spots of the starting substance and the degradation products were eluted with 0·01M-HCl (10 ml each); the

ratio of components was determined spectrophotometrically at 260 nm. The difference exceeding 10% in respect to the nonenzymatic blank degradation was taken as significant.

C. Spleen cyclic phosphodiesterase assay. This assay was performed analogously to that of paragraph *B*. In the case of negative results, the assay was repeated with the use of 25 μg of the enzyme protein (24 h at 37°C).

Degradation of Poly C with Spleen Acid Exonuclease

The control assay was performed under the above conditions (paragraph *B*); as shown by paper chromatography (3 days), no products were present except for the spot of the polymer at the start line. Degradations in the presence of alkaline phosphatase were performed analogously to paragraph *B* except for the addition of 10 μg of the bacterial alkaline phosphatase (Worthington) to the incubation mixture. After 24 h of incubation, the mixture was separated by paper chromatography (3 days) using CpC and CpCpC as standards. By this technique, the mixture was shown to contain mainly the resistant polymer, *cf.* the spot at the start line and small amounts (not exceeding 10% of the total density) of diffused spots of the following R_{Cp} values (referred to cytidine 3′-phosphate): 0·07, 0·09, 0·15 (CpC, 2·20; CpCpC, 0·80). The spot of cytidine was not detected.

Degradation of Yeast tRNA with Spleen Acid Exonuclease

The incubation mixture contained 25 mg of yeast tRNA (Calbiochem) in 1 ml of 0·2M-TRIS-HCl buffer solution pH 7·0 and 100 μg of the enzyme protein. After 48 h at 37°C, the mixture was treated with additional 100 μg of the enzyme and incubated for additional 24 h at 37°C. Paper chromatography was then performed on one sheet of paper Whatman No 3 MM (3 days). The four UV-absorbing bands were eluted with dilute (1 : 100) aqueous ammonia, the eluate freeze-dried, and the content determined spectrophotometrically. Fraction 1 (R_F, 0; 200 A_{260}) represents a mixture of oligonucleotides of longer chains; the alkaline phosphatase degradation of this mixture and paper electrophoresis lead to a diffused spot (pH 7·5, E_{Up} 0·90; pH 3·5, E_{Up} 0·74). Fraction 2 (R_{Up}, 0·50) contains 80 A_{260} of guanosine 3′-phosphate identical with an authentic specimen on paper chromatography, paper electrophoresis at the two pH values, and UV-spectrum, and affording guanosine by the action of alkaline phosphatase. Fraction 3 (E_{Up}, 1) contains 170 A_{260} of a mixture of Up and Cp and dinucleotides; this mixture was separated by electrophoresis at pH 3·5 (the ratio of Up to Cp was 7 : 3). Dephosphorylation of fraction 3 with alkaline phosphatase *E. coli* afforded a mixture of nucleosides (Urd + Cyd) and dinucleoside phosphates in the ratio 4 : 1. The fraction of dinucleoside phosphates was isolated by paper electrophoresis at pH 7·5 and analysed by electrophoresis at pH 3·5. By this technique, the mixture was shown to contain an approximately equimolar ratio of CpU (λ_{max} 271 nm at pH 2) and CpC (λ_{max} 278 nm at pH 2) along with CpA (judged from the spot mobility) as the minor component. Fraction 4 contained 120 A_{260} of adenosine 3′-phosphate, identical with an authentic specimen on paper chromatography and paper electrophoresis at the two pH values, and affording adenosine by the action of alkaline phosphatase.

Identification of Purine 2′- and 3′-Ribonucleotides After the Spleen Cyclic Phosphodiesterase Degradation

The degradation was performed under standard conditions (*vide supra*). The spots of 2′ and 3′-nucleotides were eluted with water and the eluates chromatographed on paper Whatman No 1 in the solvent system saturated aqueous ammonium sulfate–1M ammonium acetate–2-propanol

(79 : 19 : 2). The blank test (without the enzyme) was processed analogously. As standards served either the pure 3'-nucleotides (prepared by the ribonuclease T 2 degradation) or a mixture of a 2'- and 3'-nucleotide (prepared by hydrolysis of the 2',3'-cyclic phosphate with 50% aqueous acetic acid at 50°C/5 h). When the direct allotment was not possible, the spot of the higher R_F value was empirically ascribed to the 2'-isomer.

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