NUCLEIC ACID COMPONENTS AND THEIR ANALOGUES. CXLVII.*
PREPARATION OF 5-ETHOXYCARBONYLURIDINE, 5-CARBOXYURIDINE AND THEIR NUCLEOTIDIC DERIVATIVES

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Reaction of the urethane derivative *V* with ethyl orthoformate gave the ethoxymethylene derivative *VI*, the condensation of which with 2,3-O-isopropylidene-β-D-ribofuranosylamine (*VIII*) led to 2',3'-O-isopropylidene-5-ethoxycarbonyluridine (*XI*). Removal of protecting group from compound *XI* afforded 5-ethoxycarbonyluridine (*XII*), the alkaline hydrolysis of which led to 5-carboxyuridine (*II*). The isopropylidene derivative *XIII* was obtained by the alkaline hydrolysis of the isopropylidene derivative *XI*. 2',3'-Cyclic phosphates *XV* were prepared from the free nucleosides *II* and *XII* by successive reactions with triethyl phosphite and hexachloroacetone. Both phosphates are substrates for pancreatic ribonuclease and ribonuclease T 2. Phophorylation of compound *XII* with phosphorus oxychloride in triethyl phosphate afforded the 5'-phosphate *XIX* which was converted by alkaline hydrolysis to the 5-carboxyuridine derivative *XX*. The nucleotides *XIX* and *XX* represent substrates for nonspecific phosphomonoesterases; only compound *XIX* is dephosphorylated by the action of snake venom 5'-nucleotidase. Condensation of pyridinium salts of phosphates *XXI* and *XXVII* with 2',3'-O-ethoxymethylene derivative *XXII* and the subsequent weakly alkaline hydrolysis afforded the corresponding dinucleoside phosphates *XXIII* and *XXVIII*, the acidic hydrolysis of which led to uridylyl-(3'→5')-5-ethoxycarbonyluridine (*XXIV*) and guanylyl-(3'→5')-5-ethoxycarbonyluridine (*XXIX*). Alkaline hydrolysis of compounds *XXIII* and *XXVIII* led to the corresponding derivatives *XXV* and *XXX* which were treated with acetic acid to afford uridylyl-(3'→5')-5-carboxyuridine (*XXVI*) and guanylyl-(3'→5')-5-carboxyuridine (*XXXI*). The dinucleoside phosphates *XXIV* and *XXIX* are split by the action of snake venom phosphodiesterase while the 5-carboxyuridine derivatives *XXVI* and *XXXI* are quite resistant towards this enzyme.

In the course of investigations on the influence of various substituents at the uracil moiety on behaviour of nucleotidic derivatives towards nucleolytic enzymes, we have observed[1,2] some anomalous properties of nucleotides derived from orotidine (6-carboxyuridine; *I*). The nucleoside *I* occurs as metabolite in the synthesis of nucleic acids: by the action of orotidylate decarboxylase, orotidine 5'-phosphate is converted to uridine 5'-phosphate[3]. Orotidine (*I*) possesses an acidic character which might be the cause of a decreased affinity of orotidine nucleotides towards some enzymes

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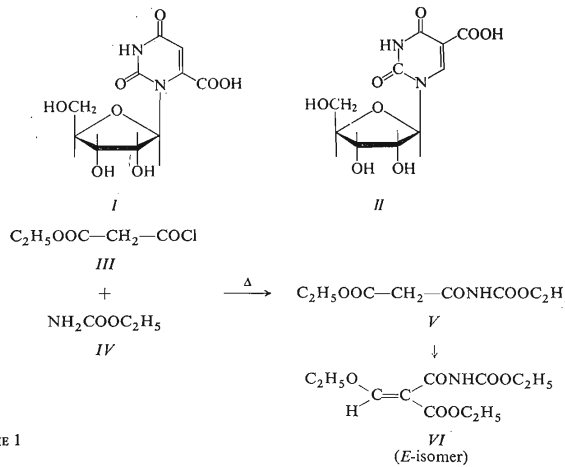
(pancreatic ribonuclease) or a complete enzymatic inactivity (snake venom phosphodiesterase[2], 5'-nucleotidase[4]). The nucleotide derivatives of orotidine may be bonded through the mediation of the carboxylic group to strongly basic centers of the enzyme molecule; this circumstance may lead to a decrease or complete loss of a suitable spatial orientation of the substrate to active centers of the enzyme. A similar situation may occur also with other nucleotide derivatives of acidic nucleosides. In the present paper, we wish to report the synthesis and properties of one orotidine isomer, namely, 5-carboxyuridine (*II*) and its nucleotides.

5-Carboxyuridine (*II*) has been reported[5] as a by-product of the oxidation of 5-hydroxymethyluridine. The corresponding 2'-deoxyribonucleoside has been obtained from 5-bromo-2'-deoxyuridine by reaction with alkyllithium and treatment of the lithium derivative with carbon dioxide[6]. Both procedures gave, however, very low yields. Consequently, we have attempted an independent synthesis which represents an analogy of the preparation of 5-cyanouridine[7]. The acyclic intermediate *VI* was prepared by the reaction sequence given in Scheme 1:



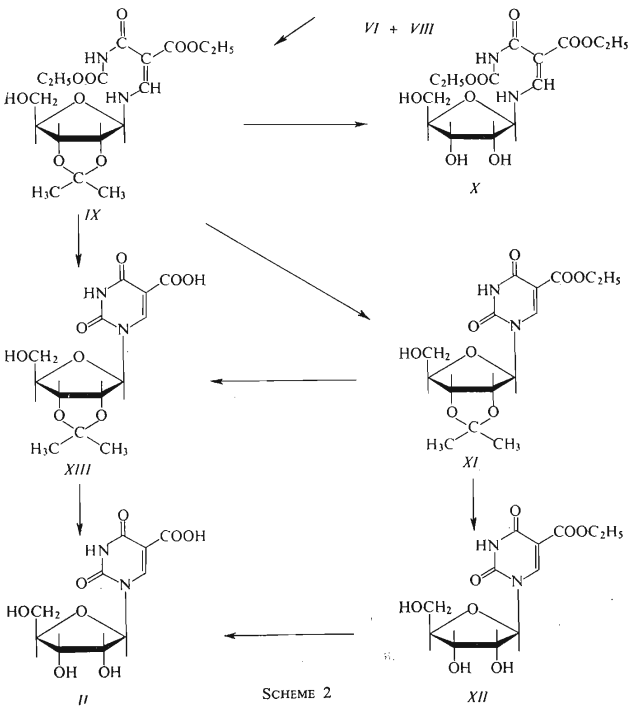
SCHEME 1

Thus, malonic ethyl ester chloride[8] (*III*) reacts in the melt with urethane (*IV*) at 100°C to give ethyl malonylurethane (*V*) which is then condensed[9] with triethyl orthoformate in refluxing acetic anhydride under the formation of the ethoxymethylene derivative *VI*. As shown by NMR spectrum, $H_{(2)}$ singlet at 8·23 p.p.m., and

infrared spectrum, $\nu(N—H)_{bonded}$ 3190, 3260 cm^{-1}, $\nu(N—H)_{free}$ absent, $\nu(C=O)_{bonded}$ (ester) 1706 cm^{-1}, $\nu(C=O)_{free}$ (ester absent), compound *VI* represents the pure isomer *E*.

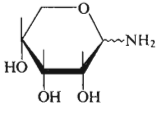
In the preparation of the sugar component *VIII* (ref.[7]), a mixture of acetone and triethyl orthoformate was used instead of 2,2-dimethoxypropane. Compound *VIII* is formed from the ribopyranosylamine *VII* probably *via* the cyclic orthoformate[10,11]. In comparison with the original method, the yield is somewhat lower, but the product is identical with the authentic specimen[7] and of the same anomeric purity.

Condensation of the amine *VIII* with compound *VI* (Scheme 2) is performed in the presence of methanolic sodium methoxide to afford the isopropylidene derivative *IX* which is converted by refluxing in acetic acid into the compound *X*. On the basis of NMR spectra, compounds *IX* and *X* were ascribed the β-anomeric structure.

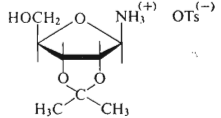


SCHEME 2

The NMR and infrared spectra of compound *IX* correspond unequivocally to the *E*-configuration of the glycosidically bonded aliphatic residue. On the other hand, compound *X* is partially isomerised (it contains about 30% of the *Z*-isomer. The absence of $\nu(NH)_{free}$ in infrared spectra of compound *IX* corresponds exclusively to the *E*-isomer, both NH groups of which are linked by hydrogen bonds to the carbonyl groups of ester functions while only the more acid amide NH group is linked in the *Z*-isomer. The chromophoric system of this grouping is characterised by absorption maxima at 233 and 285 nm in accordance with data for derivatives of β-aminoacrylic acid and at variance with spectra of the cyclic derivatives *XI*, *XII*, and *II* (*vide infra*) and 5-ethoxycarbonyluracil[12]. The same chromophoric system is present in compound *XIV* which was prepared by reaction of the derivative *VI* under the same conditions as compound *IX*.



VII

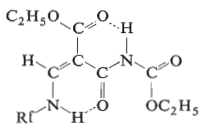


VIII

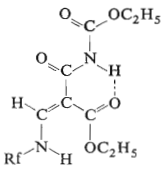
The *E*-configuration of compound *IX* is confirmed by cyclisation to the derivative *XI*. The reaction proceeds very slowly under usual condensation conditions but is quantitative when performed in refluxing 20% ethanolic triethylamine (30 min). As shown by NMR spectrum, the isopropylidene derivative *XI* represents a pure β-anomer. The isopropylidene group was removed by refluxing in 80% aqueous acetic acid under the formation of 5-ethoxycarbonyluridine (*XII*); the ester function at position 5 of the heterocyclic moiety did not suffer any hydrolytical change.

The ester function at position 5 of compounds *XI* and *XII* may be quantitatively hydrolysed with 2M-LiOH at room temperature. Thus, the hydrolysis of 5-ethoxycarbonyluridine (*XII*) affords 5-carboxyuridine (*II*) which was isolated as an amorphous ammonium salt or as a crystalline cyclohexylammonium salt. The analogous hydrolysis of the isopropylidene derivative *XI* leads to 2′,3′-O-isopropylidene-5-carboxyuridine (*XIII*) which may be also quantitatively obtained from compound *IX* by the action of aqueous alkali, obviously *via* cyclisation to the uridine derivative *XI* and the subsequent hydrolysis. Deblocking of compound *XIII* in acidic media leads to 5-carboxyuridine. In the synthesis of the derivative *XII*, it is not necessary to isolate the intermediate *IX*.

The structure of nucleosides *XII* and *II* was confirmed by elemental analysis as well as NMR and infrared spectra (Table I and II). Ultraviolet absorption spectra of compounds *II* and *XII* (Table III) are characterised by absorption maxima at 275 and 278 nm in acidic and alkaline pH range. The hypochromic effect accompanying the passage from the acidic region into the alkaline one confirms the presence of a free $N_{(3)}$-H group in the heterocyclic system. The bathochromic shift of absorption maximum from 260 nm (uridine) to the value of 275—278 nm with nucleosides *XII* and *II* might be explained by the presence of an electronegative substituent at position 5 of the

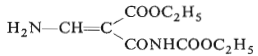


E-isomer

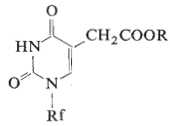


Z-isomer

IX, Rf = 2,3-O-isopropylidene-1-(β-D-ribofuranosyl)-
X, Rf = 1-(β-D-ribofuranosyl)



XIV



XV, Rf = 1-(β-D-ribofuranosyl)
a, R = H
b, R = CH_3

uracil chromophore and is roughly the same as the shift due to substitution by a bromo atom (λ_{max}^{pH2} 278 nm, ref.[13]).

CD-Spectra of nucleosides *II* and *XII* (Table IV) exhibit a positive Cotton effect B_{2u} (ref.[14]) at 284–285 nm, characteristic of β-anomers of uridine derivatives in the *anti*-conformation[14–16]. In accordance with the typical behaviour is also the negative Cotton effect B_{1u} at 253–254 nm (nucleosides *II* and *XII*) and the positive effect E_{1ub} (compound *II*, 202·5 nm; compound *XII*, 217 nm). The second negative effect (E_{1ua}) observed with uridine derivatives (Table V) substituted at position 5 as well as the homologous derivatives *XIVa,b* cannot be identified in the spectrum of compound *XII* probably because of coalescence with some of the vicinal bands. The existence of the E_{1ua} effect with compound *II* may be assumed from deformation of the negative part of spectrum at 230 nm. The CD-spectrum of 5-carboxyuridine differs considerably (Table IV) from that of orotidine (*I*) which exhibits two positive Cotton effects (B_{1u}, B_{2u}) at 274·5 nm and 236 nm and two negative effects (E_{1ua}, E_{1ub}) at 209 and 195 nm. In contrast to orotidine (*I*), the long-wavelength Cotton effect of 6-methyluridine is negative because of the *syn*-conformation of the base[14,15]; it may be consequently assumed that the isomeric carboxyuridines *I* and *II* possess the *anti*-conformation.

In accordance with the influence of the electronegative substituent at position 5 on the electron density of the uracil system, the observed pK_a value 8·64 of the nucleo-

TABLE I

Chemical Shifts [p.p.m.] and Interaction Constants [Hz]
Measured in hexadeuteriodimethyl sulfoxide; tetramethylsilane as internal standard.

Compound	$H_{1'}$, $H_{2'}$	$H_{3'}$	$H_{4'}$	2 $H_{5'}$	H_6	N_3H	$(CH_3)_2C<$	OH	CH_3CH_2	CH_3CH_2	$J_{1',2'}$
II[a]	5·87 d 4·10—4·20 m	4·10—4·20 m	3·95—4·10 m	3·70—3·80 m	8·60 s	—	—	6·80 (6 H)	—	—	3·5
IX[b]	5·13 dd 4·75 dd	4·93 [d]	4·38 m	3·75 m	8·11[d] [d]	11·22 br s	1·34 s 1·52 s	5·05 br s	1·25 t 1·30 t	4·17 q 4·21 q	1·2[f]
X[b]	[c]	[c]	[c]	[c]	8·24 d[d,e] (8·16 d)	11·12 br s	—	—	1·24 t 1·27 t	4·12 q 4·17 q	[g]
XI	5·90 brs 4·86 m	4·86 m	4·35 m	3·75 m	8·73 s	11·50 br s	1·33 s 1·54 s	4·89 t	1·30 t	4·25 q	1·0
XII	5·84 d 4·0 —3·15 m	4·0 —4·15 m	3·90—4·0 m	3·60—3·75 m	8·94	11·49 br s	—	5·49 bs 5·08 t 5·0 bs	1·26 t	4·18 q	3·0
XIII	5·88 d 4·78—4·85 m	4·78—4·85 m	4·05—3·20 m	3·60—3·70 m	—	—	1·32 s 1·53 s	—	—	—	2·0

[a] 3·0 p.p.m. (1 H), 1·10—2·10 p.p.m. (10 H, cyclohexylamine); [b] acyclic substituent: *IX* 10·98 p.p.m. (q), *X* 10·45 p.p.m. (m); [c] unsufficiently resolved; [d] CH=N; [e] mixture of stereoisomers (2 : 1); [f] $J_{2',3'} = 6{\cdot}0$, $J_{3',4'} = 0$, $J_{4',5'} = 2{\cdot}0$, $J_{1',NH} = 9{\cdot}5$, $J_{NH,CH} = 14{\cdot}0$; [g] $J_{NH,CH} = 13{\cdot}5$.

side *XII* is somewhat shifted when compared with the pK_a value 9·17 of uridine. The same effect may be observed with 5-bromouridine (Table IV). The carboxylic group at position 5 of compound *II* is of the same strongly acidic character (pK_{a1} 4·20) as with orotidine (pK_{a1} 2·68) and compound *XVa* (pK_{a1} 4·8, ref.[17]). On the other hand, the acidity of the $N_{(3)}$—H function of compounds *I* and *II* is decreased (*I*, pK_{a2} 9·45; *II*, pK_{a2} 9·87), obviously due to the presence of a carboxylate group with an electronegative charge. An analogous observation has been made[17,18] with compound *XVa* (pK_{a2} 9·83).

TABLE II

Infrared Spectra (wavenumbers in cm^{-1})

Compound	ν(C=C)	ν(C=O)	ν(NH)bonded	ν(OH)free	ν(OH)bonded
IX[a]	1 599	1 752, 1 677 (1 659 sh)	3 235, 3 155 sh	3 610	3 445
X[b]	1 599	1 676, 1 761	3 220	—	—
XI[a]	1 623	1 693 sh[c], 1 720, 1 747 sh	3 210[d]	3 615	3 480
XII[b]	1 620	1 725[e], (1 711 sh), (1 696 sh)	—	—	—
XIII[b]	1 628	1 710, 1 690	3 180—3 190	—	—

[a] In tetrachloromethane; [b] in potassium bromide, [c] ester group; [d] ν(NH)free 3 375 cm^{-1}; [e] ν(C—O) 1 573—1 584 cm^{-1}, 1 375—1 384 cm^{-1} (carboxylate); ν(NH) 1 404 cm^{-1} (NH_4^+).

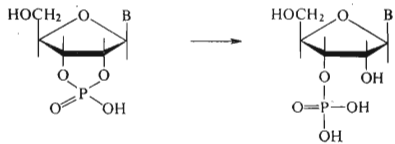
TABLE III

Ultraviolet Absorption Spectra (pH 2) (wavelengths in nm)

Compound	$\lambda_{max}(\varepsilon)$	$\lambda_{min}(\varepsilon)$	ε_{260}	$A_{250/260}$	$A_{280/260}$	$A_{290/260}$
II[a]	278 (14 200)	240 —	5 900	0·42	2·42	1·17
IX	233 (14 600)	252 (8 500)	5 400	0·56	4·6	4·6
	285 (27 000)					
X	233 (14 300)	252 (8 700)	5 500	0·55	4·4	4·7
	285 (26 600)					
XI	275 (15 100)	240 (2 100)	8 500	0·47	1·58	0·82
XII	276 (15 200)	240 (1 800)	8 000	0·45	1·62	0·86
XIII	286 (12 400)	240 (1 300)	7 000	0·44	1·69	0·93

[a] pH 12: λ_{max} 271 nm (ε_{max} 9 600), λ_{min} 250 nm, ε_{260} 4 400, $A_{250/260}$ 0·77, $A_{280/260}$ 0·92, $A_{290/260}$ 0·37.

In a mineral medium with glucose, the nucleosides *II* and *XII* do not exhibit any inhibitory effect on the growth of *Escherichia coli* (strain B) at concentrations up to 10 μg/ml. A strong growth inhibition may be observed at the concentration of 100 μg/ml while at 1000 μg/ml, a complete inhibition occurs.* No toxic changes have been observed with AKR mice when the ammonium salt of compound *II* has been applied in the dosis of 450 mg/kg.



XVI *XVII*

Phosphorylation of the 2′,3′-*cis*-diol system of compounds *II* and *XII* was performed on treatment of unprotected ribonucleosides with triethyl phosphite and hexachloroacetone[1]. The structure of resulting 2′,3′-cyclic phosphates *XVI* was established by acidic hydrolysis to 2′(3′)-monophosphates *XVII* and enzymatic dephosphorylation of the latter compounds to the starting nucleosides *II* and *XII*. Phosphates *XVII* are resistant towards the snake venom 5′-nucleotidase and differ by their chromatographic behaviour from the 5′-isomers *XIX* (*vide infra*). These findings simultaneously prove the isomeric purity of phosphates *XVII*.

5-Ethoxycarbonyluridine 2′,3′-cyclic phosphate (*XVIa*) is a good substrate for pancreatic ribonuclease and ribonuclease T 2. With both enzymes, the degradation leads to the 3′-isomer *XVIIa*, identical on chromatography and electrophoresis with a specimen (mixture of isomers) obtained by acidic hydrolysis of the cyclic phosphate *XVIa*; enzymatic dephosphorylation of compound *XVIIa* leads to the nucleoside *XII*.

5-Carboxyuridine 2′,3′-cyclic phosphate *XVIb* is also split by both ribonucleases to the corresponding 3′-isomer *XVIIb*, identical with a mixture of isomers obtained by acidic hydrolysis of compound *XVIb* (*vide supra*). In this case, the degradation is more difficult than with the 5-ethoxycarbonyl derivative *XVIa*. Thus, roughly 50% of compound *XVIb* is split under conditions which lead to a quantitative degradation of compound *XVIa* within 2 hours. These observations are in accordance with our earlier findings[1] on the behaviour of 5- and 6-substituted uridine 2′,3′-cyclic phosphate derivatives towards the two above mentioned ribonucleases. The electroneutral substituent at position 5 of the uracil moiety has no qualitative influence on the affinity of the substrate to the enzyme. On the other hand, the orotidine

* These experiments were performed by Dr I. Votruba, Department of Molecular Biology of this Institute.

(*I*) derivative carrying at position 6 of the uracil moiety the carboxylate grouping is a poor substrate for pancreatic ribonuclease and a very poor substrate for ribonuclease T 2, similarly to compound *XVIb* substituted by the carboxylate function at position 5 of the uracil moiety.

In the preparation of 5'-nucleotides derived from compounds *II* and *XII*, we have used the direct phosphorylation of the free nucleoside *XII* or its 2',3'-isopropylidene derivative *XI* with phosphorus oxychloride in triethyl phosphate[20]. This is the only possible method with compound *XII* in view of the sensibility of the ester group at the heterocyclic moiety to alkaline hydrolysis (*vide supra*). Both derivatives thus afforded the 5'-phosphate *XIX*, the degradation of which with bacterial alkaline

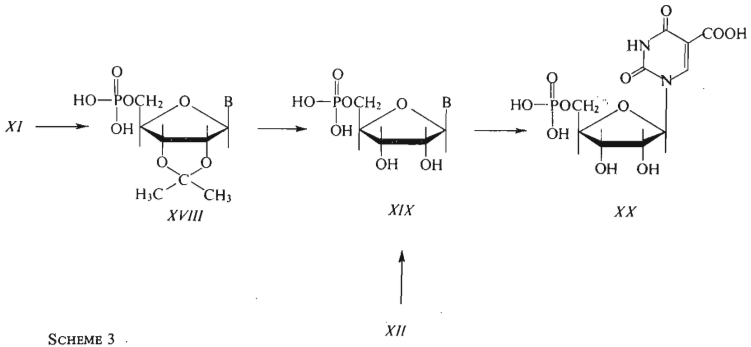
TABLE IV

Circular Dichroism Spectra (molar ellipticities Θ are in parentheses) and p*K*-Values of Some Uridine Derivatives

Compound	Circular dichroism[a,b]				pK_a[b]
	B_{2u}	B_{1u}	E_{1ua}	E_{1ub}	
Uridine	267 (+8 500)	240 (−3 700)	215 (− 4 400)	196 (+ 7 600)	9·17[c]
5-Methyluridine[d]	272 (+5 500)	242 (−4 000)	217 (− 4 400)	196 (+11 000)	—
5-Bromouridine	284 (+4 160)	245 (−3 710)	222 (− 5 500)	197·5 (+19 300)	8·12
II[e]	285 (+4 390)	254 (−5 710)	230 sh (− 2 140)	202·5 (+11 700)	4·20 9·87
XII	284 (+9 560)	253 (−9 300)	—	217 (+16 300)	8·64
I[e]	274·5 (+7 240)	236 (+6 060)	209 (−10 450)	195 sh (− 7 100)	2·68 9·45
XVa[f]	270 (+7 980)	240 (−5 070)	219 (− 7 250)	197 (+16 200)	4·8[f] 9·83
XVb[f]	271 (+8 470)	240 (−4 750)	219 (− 6 300)	197 (+17 100)	—

[a] The terminology of transitions is that adopted in literature[14,15]; [b] in aqueous solutions at 20°C; [c] ref.[28]; [d] ref.[14]; [e] cyclohexylammonium salt; [f] ref.[17].

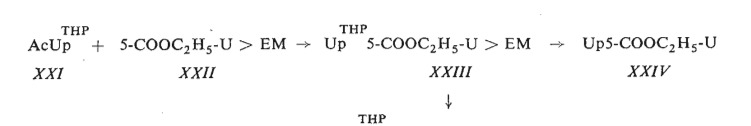
phosphatase or intestinal alkaline phosphatase led to the nucleoside *XII* as the sole product (Scheme 3). Alkaline hydrolysis of compound *XIX* afforded 5-carboxyuridine 5′-phosphate (*XX*). This procedure in the preparation of compound *XX* was used for that reason that the direct phosphorylation of compound *II* could be complicated (analogously to that of orotidine, ref.[2]) by the presence of the carboxylate function. The structure of compound *XX* was also confirmed by degradation to 5-carboxyuridine (*II*) with bacterial alkaline phosphatase and by comparison of the properties with those of the 3′-isomer *XVIIb* (*vide supra*).

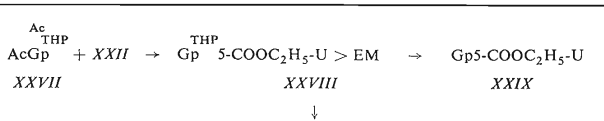


SCHEME 3

Compounds *XIX* and *XX* differ distinctly in their affinity towards the snake venom 5′-nucleotidase. Thus, compound *XIX* is quantitatively degraded to the nucleoside *XII* while only 5–6% of the phosphomonoester linkage of compound *XX* is split under the same conditions. Since orotidine 5′-phosphate is also practically resistant to the snake venom 5′-nucleotidase, it may be assumed that the lack of activity must be due to the acidic character of the substituent and not to its location on the heterocyclic nucleus.

The sensibility of the snake venom phosphodiesterase on substitution of the substrate by an acidic function was studied on the dinucleoside phosphates *XXIV*, *XXVI*, *XXIX* and *XXXI* which were prepared as follows (ref.[21,22]):





(Abbreviations: 5-EtOOC-U, *XII*; 5-HOOC-U, *II*; for other abbreviations see ref.[21])

The reaction of the nucleoside *XII* and triethyl orthoformate[10] afforded the 2',3'-O-ethoxymethylene derivative *XXII*. Condensation of *XII* with the pyridinium salt of 5'-O-acetyl-2'-O-tetrahydropyranyluridine 3'-phosphate[21,23] (*XXI*) or N^2,$O^{5'}$-diacetyl-2'-O-tetrahydropyranylguanosine 3'-phosphate[22] (*XXVII*) by the action of N,N'-dicyclohexylcarbodiimide and the subsequent hydrolysis at pH 9 (the strongly alkaline medium would affect the ester function located on the heterocyclic moiety of compound *XXII*) led to the corresponding partially blocked dinucleoside phosphates *XXIV* and *XXIX*. Treatment of compounds *XXIII* and *XXVIII* with aqueous sodium hydroxide resulted in a selective hydrolysis of the ester function on the heterocyclic moiety under the formation of the protected dinucleoside phosphates *XXV* and *XXX*. The ester function was hydrolysed in the stage of protected dinucleoside phosphates in order to exclude the cleavage of the internucleotidic bond in the presence of a free 2'-hydroxylic group. Deblocking of compounds *XXV* and *XXX* in acidic media led to the free dinucleoside phosphates *XXVI* and *XXXI*.

The dinucleoside phosphates *XXIV*, *XXVI*, *XXIX*, and *XXXI* were isolated by freeze-drying as chromatographically and electrophoretically homogeneous ammonium salts. Pancreatic ribonuclease degradation of uridine 3'-phosphate derivatives *XXIV* and *XXVI* and ribonuclease T1 degradation of guanosine 3'-phosphate derivatives *XXIX* and *XXXI* afforded equimolar ratios

TABLE V

Affinity of Substituted Uridine Nucleotides Towards Nucleotidic Enzymes[a]

Enzyme	5-$COOC_2H_5$	5-COOH	6-COOH	L-Nucleotide
Alkaline phosphatase *E. coli*	+	+	+	+
Alkaline phosphatase (calf intestine)	+	+	+	+
5'-Nucleotidase (snake venom)	+	(+)	—	—
Pancreatic ribonuclease	+	(+)	(+)	—
Ribonuclease T 2	+	(+)	(+)	—
Phosphodiesterase (snake venom)	+	—	—	—

[a] +, good substrate; (+), poor substrate; —, a resistant compound.

of uridine 3′-phosphate or guanosine 3′-phosphate and the nucleoside *II* or *XII*. The quantitative course of these degradations simultaneously confirms the isomeric purity of the (3′→5′)-internucleotidic linkage.

The snake (*Crotalus terr. terr.*) venom phosphodiesterase degradation occurs only with 5-ethoxycarbonyluridine derivatives *XXIV* and *XXIX* under the formation of uridine or guanosine and the 5′-nucleotide *XIX*. On the other hand, the 5-carboxyuridine-containing dinucleoside phosphates *XXVI* and *XXXI* are completely resistant in this respect both under standard conditions and with the use of excess enzyme. These findings might be explained similarly to the behaviour of 5′-nucleotides *XIX* and *XX* towards the snake venom 5′-nucleotidase. Substitution by an electroneutral group at position 5 of the uracil moiety exerts no influence on the affinity of the substrate to the snake venom phosphodiesterase. With an acidic substituent at position 5 or 6 (orotidine derivatives, cf.[2]), the enzymatic reaction does not take place with dinucleoside phosphates containing orotidine (*I*) and 5-carboxyuridine (*II*) at the 3′-end.

The presence of a strongly acidic substituent at the heterocyclic moiety of uridine nucleotides influences the affinity of these compounds to various nucleolytic enzymes. Table V represents a comparison of the 5-ethoxycarbonyluridine and 5-carboxyuridine derivatives on the one hand and the orotidine derivatives on the other. It may be seen that substitution of the uracil nucleus at position 5 by an ethoxycarbonyl group does not exert any qualitative effect on the substrate activity. With the nucleotide derivatives of 5- and 6-carboxyuridine, the investigated enzymes may be divided into two groups. One group comprises nonspecific phosphomonoesterases (alkaline bacterial and intestinal phosphatase) which split well the nucleotide derivatives of both the above mentioned acidic nucleosides. The other group is formed by enzymes, the affinity of which to the substrate is lowered by introduction of a strongly acidic substituent. This effect differs with different enzymes and is the strongest with both snake venom enzymes (5′-nucleotidase and phosphodiesterase).

Comparison of the affinity of the same enzymes to L-nucleotide derivatives[24,25] leads to interesting conclusions. Enzymes sensitive to substitution of the uracil nucleus by a carboxylic group do not split the L-ribonucleotide derivatives while the nonspecific enzymes of the first group dephosphorylate even the L-nucleotides. The resistance of L-nucleotide derivatives to enzymes might be explained by the assumption that the appropriate enzyme binds the substrate (or interacts with the substrate) in at least three different regions, one of which probably corresponds to the interaction with the heterocyclic base[24–26]. In such a case, any change in the heterocyclic moiety may result in a changed interaction of this moiety with the enzyme. Substitution by an acidic function introduces into the very vicinity of the heterocyclic base an electrostatic charge since the substituent is deprotonated under the test conditions. This circumstance obviously influences orientation of the heterocyclic moiety in respect to the active center of the enzyme, especially to that region which

TABLE VI

Paper Chromatography and Electrophoresis

Compound	R_F			$E^{a,b}$	
	S_1	S_2	S_3	E_1	E_2
Uridine	0·50	0·76	0·30	—	—
Uridine 2′(3′)-phosphate	0·12	0·30	0·10	1·00	1·00
Guanosine 2′(3′)-phosphate	0·06	0·20	0·05	0·86	0·80
Uridine 2′,3′-cyclic phosphate	0·37	0·65	0·12	0·67	1·05
Guanosine 2′,3′-cyclic phosphate	0·27	—	0·15	0·52	0·82
Uridylyl-(3′ → 5′)-uridine	0·21	0·50	—	0·40	—
I	0·37	0·59	0·17	0·65	1·11
II	0·25	0·40	0·20	0·56	0·50
IX	0·82	—	—	0	0
X	0·49	0·70	—	0	0
XI	0·75	—	—	0·24	0
XII	0·52	0·80	0·40	0·22	0
XIII	0·52	—	—	0·57	0·50
XVIa	0·40	0·66	0·18	0·60	0·82
XVIb	0·20	0·32	0·08	0·85	1·35
XVIIa	0·14	0·40	0·17	0·96	0·85
XVIIb	0·05	0·10	0·06	1·20	1·30
XVIII	0·29	0·68	—	0·92	0·90
XIX	0·16	0·40	0·16	0·94	0·90
XX	0·03	0·08	0·04	1·18	1·30
XXII	0·77	—	—	0·24	—
XXIII	0·60	0·85	—	0·47	—
XXIV	0·21	0·60	—	0·47	—
XXV	0·39	0·70	—	0·60	—
XXVI	0·13	0·28	—	0·60	—
XXVIII	0·56	0·74	—	0·32	—
XXIX	0·17	0·54	—	0·31	—
XXX	0·27	0·50	—	0·43	—
XXXI	0·06	0·37	—	0·43	—

[a] Electrophoretic mobility referred to uridine 2′(3′)-phosphate; [b] E_3 (referred to uridine): *I* 1·65, *II* 1·50, *XII* 1·00, *XIII* 0·47, *XXII* 0·10.

is responsible for the interaction with the heterocyclic moiety. The overall effect will depend on many factors, particularly on the character of forces engaged in the interaction of the base with the active center of the enzyme and on concentration of the positive charge (strongly basic amino acids) at the active center and its very neighbourhood. The change of the substrate activity of uridine nucleotides due to substitution by an acidic function may vary in dependence on the sum of these factors. The considerable change in the susceptibility of nucleotide derivatives substituted by acidic groups to both the snake venom enzymes indicates the presence of a strongly basic medium of the enzyme active center. The explanation by means of a bond between the nucleotide and an arbitrary enzyme region with a high concentration of the positive charge through the mediation of an acid function of the substituent is hardly satisfactory since, *e.g.*, the nucleotides derived from 5-carboxyuridine (*II*) or 6-carboxyuridine (*I*) could not be dephosphorylated by nonspecific phosphomonoesterases where only the phosphate function of the monoester is bonded to the enzyme without any perceptible interaction of the enzyme with the heterocyclic base or the nucleoside residue. Another explanation based on the absence of the positive charge region is less probable.

Conclusively, the complex formation between the enzyme and the substrate in the case of enzymes requiring an interaction of the heterocyclic base with the active enzyme center may to a large extent depend – in addition to the steric influence of substitution, polarisibility increase of the heterocyclic base, and other effects accompanying the changes in the heterocyclic system – on introduction of a substituent carrying an electronegative charge.

EXPERIMENTAL

Melting points were taken on a heated microscope stage (Kofler block) and are uncorrected. Solutions were taken down on a rotatory evaporator at 35°C/15 Torr unless stated otherwise. Compounds were dried over phosphorus pentoxide at 0·1 Torr and room temperature.

Methods

Paper chromatography was performed by the descending technique on paper Whatman No 1 (preparative runs on paper Whatman No 3 MM) in the solvent systems S_1, 2-propanol–concentrated aqueous ammonia–water (7 : 1 : 2); S_2, ethanol–1M ammonium acetate (5 : 2); and S_3, 1-butanol–acetic acid–water (5 : 2 : 3).

Paper electrophoresis was performed by the technique according to Markham and Smith[27] on paper Whatman No 3 MM at 25 V/cm in the buffer solutions E_1, 0·1M triethylammonium hydrogen carbonate (pH 7·5); E_2, 0·05M sodium hydrogen citrate (pH 3·5); and E_3, 0·2M triethylammonium borate (pH 7·5). For R_F values and electrophoretic mobilities see Table VI.

Thin-layer chromatography on silica gel. Analytical determinations were performed on ready-for-use Silufol UV_{254} plates (Kavalier Glassworks, Votice, Czechoslovakia) in the solvent systems S_4, chloroform–ethanol (95 : 5); S_5 (90 : 5); and S_6 (90 : 10). Preparative runs were performed on loose layers (35 × 15 × 0·3 cm) of silica gel (30–60 mesh) with incorporated luminescent indicator (produced by Service Laboratories of our Institute).

Separations on DEAE-*cellulose* (Cellex D, standard capacity, Calbiochem, U.S.A.) in the HCO_3^- cycle. Column, 100 × 4 cm. The elution was performed with water (rate, 5 ml per min) to the drop of ultraviolet absorption of the neutral fraction and then (rate, 3 ml per min) with a linear gradient of triethylammonium hydrogen carbonate pH 7·5 (2 l of water in the mixing chamber and 2 l of the buffer solution of the final concentration in the reservoir). The course of evolution was checked continuously on a Uvicord apparatus (LKB, Uppsala, Sweden), the fractions being taken in 10 min intervals. The corresponding fractions were combined, evaporated, and the residue coevaporated with two 50 ml portions of methanol.

Enzymatic degradations. A solution of the substrate (2 μmol) in 50 μl of 0·05M-TRIS-HCl (pH 8·0) was incubated at 37°C for 4 hours with *a*) 20 μg of pancreatic ribonuclease (Calbiochem, A grade), *b*) 10 μg ribonuclease T 2 (prepared and kindly provided by Professor Dr Dr H. Witzel, University Münster, Germany; in this case the buffer solution of pH 7 was used), *c*) 10 μg of purified snake (*Crotalus terr. terr.*) venom phosphodiesterase (Boehringer, Germany), *d*) 10 μg of intestinal alkaline phosphatase (Boehringer, Germany), *e*) 20 μg of snake (*Crotalus adamanteus*) venom 5′-nucleotidase (Worthington, U.S.A.), *f*) 10 μg of bacterial (*Escherichia coli*) alkaline phosphatase (Worthington, U.S.A.).

Spectroscopic measurements. Ultraviolet spectra were taken on a Beckman DU apparatus in 1 cm cells (water). In quantitative determinations, the molar extinction coefficients given in Table III were used; uridine, ε_{260}^{pH2} 10 000; guanosine, ε_{260}^{pH2} 11 800 (ref.[28]). The ultraviolet characteristics are listed in Table III. Infrared spectra were measured on a double-beam spectrophotometer UR-10. For characteristic frequencies see Table II. NMR spectra were measured on a Varian 100 apparatus in hexadeuteriodimethyl sulfoxide (hexamethyldisiloxane as internal standard). The exchange of labile hydrogen atoms was performed by the addition of deuteriated acetic acid. For chemical shifts and interaction constants see Table I. CD-Spectra were measured on a Jouan Model CD-185 dichrograph in water (concentration, 0·2 mg per ml). For the corresponding data see Table IV. The pK values were determined at 20°C in aqueous solutions on a Beckmann Model 1019 pH-meter.

Ethyl Malonyl Urethane (*V*)

A mixture of the chloride[8] *III* (200 g; 1·35 mol) and urethane *IV* (250 g; 2·8 mol) is stirred at 100°C for 10 hours, cooled down, poured into 3 liters of water, and extracted with three 500 ml portions of ether. The extracts are washed with three 200 ml portions of water, cold saturated aqueous sodium hydrogen carbonate (three 200 ml portions) and water (two 200 ml portions), dried over magnesium sulfate, filtered, and evaporated. The refluxing solution of the residue in ether (300 ml) is treated with light petroleum (50–60°C) until turbid and then is allowed to cool slowly. The precipitate is collected with suction, washed with light petroleum, and dried to afford 126 g (45%) of compound *V*, m.p. 57–58°C (1 : 1 ether–light petroleum). For $C_8H_{13}NO_5$ (203·2) calculated: 47·28% C, 6·44% H, 6·89% N; found: 47·95% C, 6·39% H, 6·74% N. Infrared spectrum (CCl_4): $\nu(C{=}O)_{asym}$ 1 760 cm^{-1}, $\nu(C{=}O)_{sym}$ 1 703 cm^{-1} (N-acylcarbamate), $\nu(C{=}O)_{free}$ 1 746 cm^{-1}, $\nu(C{=}O)_{bonded}$ 1 720 cm^{-1} (ester); $\nu(NH)_{free}$ 3 405 cm^{-1}, $\nu(NH)_{bonded}$ 3 280 cm^{-1} (sh), 3 225 cm^{-1}, 3 160 cm^{-1}.

Ethoxymethylene Derivative *VI* (*E*-Isomer)

A mixture of compound *V* (69 g; 0·34 mol), acetic anhydride (100 ml), and triethyl orthoformate (100 ml) was refluxed under exclusion of atmospheric moisture (calcium chloride guard tube) for one hour, treated with additional 50 ml of acetic anhydride and 50 ml of triethyl orthoformate, refluxed for one hour more, and allowed to stand overnight in a refrigerator to deposit the pro-

duct *VI* which was collected with suction, washed with ether (100 ml), light petroleum (200 ml), and dried. The filtrate was diluted with 5 volumes of light petroleum (50—60°C) and kept at —10°C to deposit the second crop which was worked up similarly. The crops were combined, dissolved in ether (200 ml), the solution treated with ether until turbid, seeded, and allowed to crystallise at —10°C. The crystals were collected with suction, washed with a mixture (50 ml) of ether and light petroleum (1 : 1), and dried to afford 42 g (0·16 mol; 47%) of compound *VI*, m.p. 98—99°C (1 : 1 benzene–light petroleum). For $C_{11}H_{17}NO_6$ (259·3) calculated: 50·95% C, 6·61% H, 5·40% N 52·13% ethoxyl; found: 51·05% C, 6·70% H, 5·21% N, 49·2% ethoxyl. Ultraviolet spectrum (methanol): λ_{max} 252 nm (ε_{max} 11 700), λ_{min} 230 nm (ε_{min} 6300), ε_{260} 10 900, $A_{250/260}$ 1·04, $A_{280/260}$ 0·30, $A_{290/260}$ 0·10. Infrared spectrum (CCl_4): $\nu(C{=}O)_{asym}$ 1781 cm^{-1}, $\nu(C{=}O)_{sym}$ 1666 cm^{-1} (N-acylcarbamate), $\nu(C{=}O)_{bonded}$ 1706 cm^{-1}, $\nu(C{=}C)$ 1594 cm^{-1}, $\nu(NH)_{bonded}$ 3260 cm^{-1}, 3190 cm^{-1} (sh). NMR spectrum (hexadeuteriodimethyl sulfoxide): 10·86 p.p.m. (bs, NH), 8·23 p.p.m. (s, CH=), 4·35 p.p.m., 4·28 p.p.m., 4·21 p.p.m. (q, 3 CH_2, ethyl), 1·41 p.p.m., 1·32 p.p.m., 1·29 p.p.m. (t, 3 CH_3, ethyl).

2,3-O-Isopropylidene-β-D-ribofuranosylamine *p*-Toluenesulfonate (*VIII*)

A mixture of the ribopyranosylamine[7] *VII* (54 g; 0·36 mol), triethyl orthoformate (270 ml), acetone (135 ml), and *p*-toluenesulfonic acid monohydrate (86 g; 0·45 mol) was stirred at room temperature overnight. The solution was then treated under stirring with ether (2·5 l) until turbid and allowed to crystallise under stirring. The solid was suspended in acetone (300 ml) and the suspension was treated with ether (1 000 ml) under stirring. The precipitate was collected with suction, washed with ether, and dried to afford 52 g (40%) of the salt *VIII*, m.p. 138—139°C (reported[7], m.p. 138—139°C). When condensed with compound *VI*, the present salt affords the same reaction product as the authentic salt obtained by the reported procedure[7].

Compound *XIV*

A suspension containing compound *VI* (1·05 g; 4 mmol), ammonium chloride (250 mg; 4·7 mmol), and methanol (7 ml) was treated under stirring with triethylamine (0·5 g *i.e.* 0·7 ml; 5 mmol). The mixture was stirred at room temperature overnight, evaporated, the residue dissolved in hot ethanol (50 ml), the solution filtered, the filtrate evaporated, and the residue crystallised twice from 25% aqueous ethanol (20 ml each). The solid was collected with suction, washed with water, and dried to afford 700 mg (76%) of compound *XIV*, m.p. 159—160°C. For $C_9H_{14}N_2O_5$ (230·2) calculated: 46·95% C, 6·13% H, 12·17% N; found: 47·13% C, 5·86% H, 12·16% N. Ultraviolet spectrum (methanol): λ_{max} 232 nm (ε_{232} 18 000), 276 nm (ε_{276} 19 800), ε_{260} 10 500, λ_{min} 248 nm (ε_{248} 5000), $A_{250/260}$ 0·48, $A_{280/260}$ 1·87, $A_{290/260}$ 0·90. Compound *XIV* is homogeneous in the solvent system S_5 and does not undergo any change when refluxed in 20% ethanolic triethylamine for 20 hours.

Isopropylidene Derivative *IX*

A solution of compound *VI* (7·7 g; 30 mmol) and compound *VIII* (11·0 g; 30·5 mmol) in methanol (60 ml) was treated with methanolic 1M sodium methoxide (35 ml). As shown by chromatography in the solvent system S_4, the reaction mixture is after 3 min free of any compound *VI* (R_F value 0·85). The mixture was kept at room temperature for 15 min, evaporated, the residue shaken with chloroform (100 ml), the insoluble sodium *p*-toluenesulfonate filtered off, and washed with chloroform (100 ml). The filtrate and washing were combined, evaporated, the residue dissolved in chloroform (50 ml), and the solution applied to a column of 400 g of silica gel (40—60 mesh), packed in chloroform. The column was eluted with chloroform, 500 ml fractions being taken and

the residues checked by chromatography in the solvent system S_4. Compound *IX* is present in fractions 10—22. Yield, 7·73 g (19·2 mmol; 80%) after drying over phosphorus pentoxide at 0·1 Torr, a light yellow nonhygroscopic foam, homogeneous on chromatography in the solvent system S_4 (R_F value 0·45) and S_5 (R_F 0·62). For $C_{17}H_{26}N_2O_9$ (402·4) calculated: 50·73% C, 6·51% H, 6·96% N; found: 50·97% C, 6·32% H, 7·12% N.

Elution with chloroform–methanol (95 : 5) afforded 530 mg (1·5 mmol; 5%) of compound *XI* (R_F in S_4, 0·20) (identical with a specimen obtained by an independent procedure) and 800 mg of a non-identified compound, m.p. 118—120°C (ethanol–cyclohexane), R_F in S_4, 0·30, analyzing 46·35% C, 6·27% H, 7·56% N. Ultraviolet spectrum (methanol): λ_{max} 230 nm, 280 nm; λ_{min} 247 nm, $A_{250/260}$ 0·58, $A_{280/260}$ 1·87. Infrared spectrum (chloroform): ν(C=C) 1 602 cm^{-1}, 1 620 cm^{-1} (sh), $\nu(NH)_{free}$ 34 800 cm^{-1}, 3 370 cm^{-1} (amine), $\nu(NH)_{bonded}$ 3 240 cm^{-1}, 3 155 cm^{-1} (amine), ν(C=O) 1 761 cm^{-1}, 1 677 cm^{-1} (sh), $\nu(OH)_{free}$ 3 620 cm^{-1} (sugar). The NMR spectrum of the non-identified compound shows in contrast to that of compound *XI* one ethyl group at 4·13—4·18 p.p.m. (3 CH_2, ethyl, q) and 1·25—1·32 p.p.m. (t, 3 CH_3, ethyl).

Acidic hydrolysis of compound IX. A solution of compound *IX* (2·7 g; 6·7 mmol) in 80% aqueous acetic acid was refluxed for 30 min, evaporated, and the residue coevaporated with three 20 ml portions of ethanol. As shown by chromatography in the solvent system S_5, the residue was contaminated with a small amount of compound *IX*. The preparative chromatography was performed on two plates of loose silica gel in the same solvent system. Band of product *X* (R_F value 0·48) was eluted with methanol (200 ml), the eluate evaporated, and the residue crystallised from a mixture of ethanol and cyclohexane (1 : 1) to afford 1·25 g (51·5%) of compound *X*, m.p. 156—157°C. For physicochemical data see the Tables. For $C_{14}H_{22}N_2O_9$ (362·3) calculated: 46·41% C, 6·12% H, 7·73% N, 24·9% ethoxyl; found: 46·31% C, 6·12% H, 7·73% N, 24·8% ethoxyl.

2′,3′-O-Isopropylidene-5-ethoxycarbonyluridine (*XI*)

A. A mixture of compound *IX* (1·0 g; 2·5 mmol), methanol (20 ml), and triethylamine (5 ml) was refluxed for 4 hours (the reaction was quantitative after this period of time, as shown by chromatography in the solvent system S_5), evaporated, the residue coevaporated with ethanol (20 ml) and recrystallised from ethanol to afford 0·80 g (90%) of the chromatographically pure derivative *XI*, m.p. 191—192°C. For $C_{15}H_{20}N_2O_8$ (356·2) calculated: 50·56% C, 5·65% H, 7·86% N, 12·6% ethoxyl; found: 50·26% C, 5·85% H, 8·06% N, 13·2% ethoxyl.

B. A mixture of compound *VI* (13 g; 50 mmol), compound *VIII* (18 g; 50 mmol), and methanol (60 ml) was treated with 60 ml of methanolic 1M sodium methoxide, kept at room temperature for 30 min and processed analogously to compound *IX* (*vide supra*). The residue was refluxed with a mixture of 99% ethanol (200 ml) and triethylamine (50 ml) for one hour (the conversion of compound *IX* was quantitative, as shown by chromatography in the solvent system S_5). The mixture was evaporated, the residue coevaporated with two 50 ml portions of ethanol, the final residue dissolved in chloroform (50 ml), and the solution applied to a column of 100 g of silica gel (30—60 mesh). The column was eluted with 500 ml of chloroform and 500 ml of a mixture chloroform–ethanol (9 : 1). The corresponding eluates were combined, evaporated, the residue dissolved in boiling ethanol (50 ml), treated with cyclohexane (100 ml), and the mixture kept in a refrigerator overnight to deposit a solid which was collected with suction, washed with ether, and recrystallised from a mixture of ethanol and cyclohexane. Yield, 7·0 g (39·5%) of the isopropylidene derivative *XI*, identical with the specimen obtained by the procedure *A* on chromatography (solvent system S_5) as well as melting point and mixed melting point determination. Work-up of the mother liquor on a column of silica gel (500 g) in chloroform–ethanol (95 : 5) afforded additional 3·2 g of compound *XI*. Overall yield, 10·2 g (57%).

5-Ethoxycarbonyluridine (*XII*)

A solution of the isopropylidene derivative *XI* (7·1 g; 20 mmol) in 80% aqueous acetic acid was refluxed for 40 min, evaporated, the residue coevaporated with three 50 ml portions of ethanol, and the final residue recrystallised from ethanol (200 ml). The solid was collected with suction, washed with ethanol and ether, and dried to afford 5·4 g (85%) of compound *XII*, m.p. 199—201°C. For $C_{12}H_{16}N_2O_8$ (316·2) calculated: 45·56% C, 5·09% H, 8·85% N; found: 45·26% C, 4·96% H, 8·71% N. For the physicochemical data see the corresponding Tables.

2′,3′-O-Isopropylidene-5-carboxyuridine Ammonium Salt (*XIII*)

A. A solution of compound *IX* (0·50 g; 1·24 mmol) in methanol (10 ml) was treated with 10% aqueous lithium hydroxide (5 ml) and the whole heated at 50°C for one hour (after this period of time, the reaction is quantitative, as shown by chromatography in the solvent system S_5). The mixture was diluted with methanol (50 ml), neutralised with Dowex 50 (H^+) ion exchange resin, filtered, the resin washed with 50 ml of methanol, the filtrate and washings evaporated, and the residue dissolved in water (10 ml). The aqueous solution was applied to a column (25 ml) of Dowex 50 (H^+) ion exchange resin and the column eluted with water to the drop of ultraviolet absorption. The eluate was adjusted with concentrated aqueous ammonia to pH 8·0—8·5, evaporated, and the residue coevaporated with ethanol. The final residue was briefly refluxed with ethanol (10 ml), the solution cooled down, the solid collected with suction, washed with ethanol and ether, and dried to afford 320 mg (75%) of the salt *XIII*, homogeneous on chromatography (solvent system S_1) and electrophoresis. For $C_{13}H_{19}N_3O_8$ (345·3) calculated: 45·21% C, 5·54% H, 12·17% N; found: 45·25% C, 5·35% H, 12·43% N.

B. The isopropylidene derivative *XI* (0·50 g; 1·4 mmol) was processed analogously to paragraph *A*. Yield, 442 mg (92·5%) of the salt *XIII*, identical with the specimen obtained by procedure *A* on chromatography (S_1) and electrophoresis (E_1). For the physicochemical data see the corresponding Tables.

5-Carboxyuridine (*II*)

A. *From 5-ethoxycarbonyluridine* (XII). A solution of the nucleoside *XII* (3·2 g; 10 mmol) in 3% aqueous lithium hydroxide (75 ml) was kept at room temperature overnight and then applied to a column of Dowex 50 (H^+) ion exchange resin (200 ml). The column was eluted with water to the drop of ultraviolet absorption, the eluate adjusted with cyclohexylamine to the value of pH 8·5, evaporated, and the residue coevaporated with three 50 ml portions of ethanol. The final residue was recrystallised from a mixture of ethanol and acetonitrile (4 : 1), the solid collected with suction, washed with ether, and dried to afford 3·50 g (90%) of 5-carboxyuridine (*II*) cyclohexylammonium salt, m.p. 195—196°C. For $C_{16}H_{25}N_3O_8$ (387·4) calculated: 49·60% C, 6·50% H, 10·85% N; found: 50·01% C, 6·84% H, 10·87% N. The salt is homogeneous on chromatography (solvent systems S_{1-3}) and electrophoresis (buffer solutions E_1 and E_2). For physicochemical data see the corresponding Tables.

B. *From the isopropylidene derivative* XIII. A solution of the salt *XIII* (345 mg; 1 mmol) in 80% aqueous acetic acid (10 ml) was refluxed for 40 min, evaporated, and the residue coevaporated first with three 20 ml portions of water and then three 20 ml portions of ethanol. The final residue was dissolved in water (5 ml), the solution deionised on a column (10 ml) of Dowex 50 (H^+) ion exchange resin to the drop of ultraviolet absorption (elution with water), and the eluate evaporated. The residue was coevaporated with two 20 ml portions of water, dissolved in water, the solution adjusted with concentrated aqueous ammonia to pH 9, and freeze-dried.

Yield, 250 mg (82%) of the ammonium salt *II*, homogeneous on chromatography (solvent systems S_{1-3}) and electrophoresis (buffer solutions E_1 and E_2) and identical with a specimen obtained according to the procedure *A*. Content > 95%, as determined spectrophotometrically.

5-Ethoxycarbonyluridine 2',3'-Cyclic Phosphate (*XVIa*)

A mixture of compound *XII* (0·94 g; 3 mmol), dimethylformamide (25 ml), triethyl phosphite (10 ml), and 6M-HCl in dimethylformamide (1·5 ml) was kept at room temperature overnight, treated with 0·4M triethylammonium hydrogen carbonate (50 ml) and water (50 ml), and evaporated. The residue was applied to a column of DEAE-cellulose and eluted (0·2M buffer solution in the reservoir). The fractions of the product (0·08—0·12M buffer solution) were evaporated, the residue dried by coevaporation with ethanol, and then stored over phosphorus pentoxide at 0·1 Torr overnight. The dry residue was shaken with a mixture of dimethylformamide (10 ml) and hexachloroacetone (5 ml), the whole kept at room temperature overnight, and treated with 0·4M triethylammonium hydrogen carbonate (50 ml). After one hour at room temperature, the mixture was diluted with water (50 ml) and washed with two 25 ml portions of ether. The aqueous phase was concentrated and applied to a column of DEAE-cellulose (elution as above). The fractions of compound *XVIa* (0·16—0·19M buffer solution) were evaporated and the residue rechromatographed on 6 sheets of paper Whatman No 3 MM in the solvent system S_1. Bands of compound *XVIa* were eluted with dilute aqueous ammonia (pH 8·5—9·0; total 100 ml), the eluates evaporated, and the residue freeze-dried. Yield, 650 mg (54%) of the ammonium salt *XVIa*, homogeneous on chromatography (solvent systems S_1 and S_2) and electrophoresis (buffer solutions E_1 and E_2). Content as determined spectrophotometrically, > 95%. For $C_{12}H_{18}N_3O_{10}P$ (395·3) calculated: 10·63% N, 7·85% P; found: 10·95% N, 7·56% P.

5-Carboxyuridine 2',3'-Cyclic Phosphate (*XVIb*)

The title compound was prepared from 5-carboxyuridine (*II*; 1 mmol) analogously to compound *XVIa*. After paper chromatography in the solvent system S_1, elution with dilute aqueous ammonia (pH 9), and freeze-drying, the yield of the diammonium salt *XVIb* was 174 mg (45%). The salt was homogeneous in the solvent systems S_1 and S_2 as well as in the buffer solutions E_1 and E_2. For $C_{10}H_{17}N_4O_{10}P$ (384·3) calculated: 14·58% N, 8·07% P; found: 15·20% N, 7·72% P. Pancreatic ribonuclease degradation, 60%; ribonuclease T 2 degradation, 35%. The degradation product is the 3'-isomer of compound *XVIIb*, identical (S_1, S_2, and E_1) with an authentic specimen; its alkaline phosphatase *E. coli* degradation affords the nucleoside *II* as the sole product.

5-Ethoxycarbonyluridine 2'(3')-Phosphate and 5-Carboxyuridine 2'(3')-Phosphate (*XVIIa,b*)

The corresponding ammonium salt *XVI* (50 mg) in 50% aqueous acetic acid (1 ml) was heated at 50°C for 3 hours and chromatographed on one sheet of paper Whatman No 3 MM in the solvent system S_1 for 2 days. Bands of the product were eluted with dilute aqueous ammonia (pH 9; 50 ml) and the eluates freeze-dried. Yield, 90% of the corresponding ammonium salt *XVII*, homogeneous in the solvent system S_1 and S_2 as well as in the buffer solutions E_1 and E_2.

5-Ethoxycarbonyluridine 5'-Phosphate (*XIX*)

A. *Phosphorylation of the nucleoside* XII. A suspension of compound *XII* (1·9 g; 6 mmol) in triethyl phosphate (15 ml) was treated at 0°C with phosphorus oxychloride (1·4 ml; 15 mmol) and the whole stirred at 0°C for 3 hours. Triethylammonium hydrogen carbonate (0·2M; pH 7·5; 150 ml) was then added, the mixture adjusted to pH 7·0 with triethylamine, and stirred at room

temperature for one hour the pH value being held in the range of 6—7 by additions of triethylamine (no change after 30 min). The pH was then brought to the value of 7·5—8·0, the mixture evaporated, and the residue applied to a column of DEAE-cellulose. Elution with water afforded the recovered nucleoside *XII* (2 mmol; 33%). Elution with a gradient of triethylammonium hydrogen carbonate (0·4M buffer solution in the reservoir) gave a fraction of product *XIX*. The corresponding residue was rechromatographed on 6 sheets of paper Whatman No 3 MM in the solvent system S_1. Bands were eluted with dilute aqueous ammonia (pH 9), the eluates concentrated, and the residue freeze-dried to afford 1·25 g (3 mmol, 50%) of the ammonium salt *XIX* (content, > 90%), homogeneous on chromatography (solvent systems S_1 and S_2) and electrophoresis (buffer solutions E_1 and E_2). For $C_{12}H_{20}N_3O_{11}P$ (413·4) calculated: 10·16% N, 7·51% P; found: 10·42% N; 7·20% P.

B. *Phosphorylation of the isopropylidene derivative* XI. A mixture of compound *XI*, triethyl phosphate (3·5 ml), and phosphorus oxychloride (0·3 ml) was stirred at room temperature overnight (as shown by chromatography in the solvent system S_1, the reaction is practically quantitative within 6 hours). Triethylammonium hydrogen carbonate (0·4M; 100 ml) was then added, the mixture kept at room temperature for 2 hours, a small amount of the insoluble *XI* filtered off, the filtrate evaporated, and the residue chromatographed on 6 sheets of paper Whatman No 3 MM in the solvent system S_1. Bands of compound *XVIII* were eluted with dilute aqueous ammonia (pH 9) and the eluate was evaporated. Intestinal alkaline phosphatase degradation of a sample of the residue afforded compound *XI* as the sole product. The residual compound *XVIII* was refluxed in 80% aqueous acetic acid (20 ml) for 30 min, the solution evaporated, and the residue coevaporated with three 20 ml portions of water. The final residue was chromatographed on 4 sheets of paper Whatman No 3 MM in the solvent system S_1 and bands of the product *XIX* were eluted with dilute aqueous ammonia (pH 9). The eluate (50 ml) was concentrated and the residue freeze-dried to afford 200 mg (48·5%) of the ammonium salt *XIX*, identical with the specimen obtained by procedure *A*.

5-Carboxyuridine 5′-Phosphate (*XX*)

A solution of the ammonium salt of compound *XIX* (105 mg; 0·25 mmol) in 10% aqueous lithium hydroxide (2 ml) was heated at 50°C for 2 hours and chromatographed on two sheets of paper Whatman No 3 MM in the solvent system S_1 for two days. Bands of the product *XX* were eluted with dilute (1 : 50) aqueous ammonia (50 ml) and the eluate was evaporated to afford, after freeze-drying, 66 mg (72%) of the diammonium salt *XX*, homogeneous on chromatography (solvent systems S_1 and S_2) and electrophoresis (buffer solutions E_1 and E_2).

Uridylyl-(3′→5′)-5-ethoxycarbonyluridine (*XXIV*) and Guanylyl-(3′→5′)-5-ethoxycarbonyluridine (*XXIX*)

A mixture of 5-ethoxycarbonyluridine (*XII*; 1·6 g; 5 mmol), dimethylformamide (10 ml), triethyl orthoformate (5 ml), and 6M-hydrogen chloride in dimethylformamide (0·5 ml) was kept at room temperature overnight (as shown by chromatography in the solvent system S_4, the conversion to compound *XXII* was quantitative). Triethylamine (1 ml) was then added, the mixture evaporated to dryness at 40°C/0·1 Torr, the residue dissolved in 10 ml of 50% aqueous pyridine and divided into two equal parts.

A solution of the calcium salt (1·5 mmol) of compound *XXI* or *XXVII* in 30% aqueous pyridine (10 ml) was applied to a column (20 ml) of pyridinium Dowex 50 ion exchange resin, the column eluted with 30% aqueous pyridine (100 ml), the eluate concentrated at 30°C/15 Torr to the volume of about 20 ml, and the concentrate treated with the above stock solution of compound *XXII*

(2·5 mmol). The whole mixture was evaporated at 30°C/0·1 Torr and the residue dried by repeated coevaporations with pyridine (six 25 ml portions) under the same conditions. The final residue was dissolved in pyridine (10 ml) and the solution kept at room temperature with 2 g of N,N'-dicyclohexylcarbodiimide for 6 days under exclusion of atmospheric moisture. Water (50 ml) was then added and the mixture adjusted to pH 9 by the addition of dilute (1 : 1) aqueous ammonia. After one hour at 50°C, the mixture was diluted with water (50 ml), washed with two 25 ml portions of ether, and the aqueous phase concentrated to the volume of about 50 ml. The concentrate was processed on DEAE-cellulose, the column being eluted as above (0·2M buffer solution in the reservoir). The fractions (0·08—0·12M buffer) of the product were evaporated and the residue was chromatographed on six sheets of paper Whatman No 3 MM in the solvent system S_1. Bands of products *XXIII* and *XXVIII*, respectively, were eluted with dilute (1 : 100) aqueous ammonia (50 ml), the eluates evaporated, and the residue coevaporated with two 25 ml portions of ethanol. The final residue was dissolved in methanol (5 ml), the solution added dropwise under stirring into 100 ml of ether, the precipitate collected by centrifugation, washed with ether, and dried to afford the corresponding ammonium salts, homogeneous on chromatography in the solvent system S_1 and electrophoresis in the buffer solution E_1. *XXIII* (as ammonium salt), yield 36%; ultraviolet spectrum (pH 2): λ_{max} 268 nm, $A_{250/260}$ 0·62, $A_{280/260}$ 0·72, $A_{290/260}$ 0·25. *XXVIII* (as ammonium salt), yield 34%; ultraviolet spectrum (pH 2): λ_{max} 268 nm, $A_{250/260}$ 0·87, $A_{280/260}$ 0·80, $A_{290/260}$ 0·44.

A solution of compound *XXIII* or *XXVIII* (0·25 mmol) in 50% aqueous acetic acid (2 ml) was heated at 50°C for 30 min and chromatographed on two sheets of paper Whatman No 3 MM for two days in the solvent system S_1. Bands of products were eluted with dilute (1 : 100) aqueous ammonia (50 ml), the eluates concentrated, and freeze-dried to afford 76% of *XXIV* (ammonium salt) or 72% of *XXIX* (ammonium salt), both homogeneous on chromatography (solvent systems S_1 and S_2) as well as electrophoresis (buffer solution E_1). Their spectral data (pH 2) were identical with those of compounds *XXIII* and *XXVIII*, resp.

Uridylyl-(3'→5')-5-carboxyuridine (*XXVI*) and Guanylyl-(3'→5')-5-carboxyuridine (*XXXI*)

A solution of the ammonium salt (0·25 mmol) of the derivative *XXIII* or *XXVIII* in 10% aqueous lithium hydroxide (2 ml) was heated at 50°C for 30 min and applied to two sheets of paper Whatman No 3 MM. After chromatography in the solvent system S_1, the bands of products *XXV* or *XXX* were eluted with dilute (1 : 10) aqueous ammonia, the eluates evaporated, and the residues heated in 50% aqueous acetic acid (3 ml) at 50°C for 30 min. The solutions were then chromatographed on two sheets of paper Whatman No 3 MM in the solvent system S_1 for 3 days. Bands of compounds *XXVI* or *XXXI* were eluted with dilute (1 : 10) aqueous ammonia (50 ml), the eluates concentrated and freeze-dried. Yield (determined spectrophotometrically), 47% of the ammonium salt *XXVI* or 42% of the ammonium salt *XXXI*. Both compounds were homogeneous on chromatography (solvent systems S_1 and S_2) and electrophoresis (buffer solution E_1). Ultraviolet spectrum (pH 2), *XXVI*: λ_{max} 268 nm, $A_{250/260}$ 0·68, $A_{280/260}$ 0·79, $A_{290/260}$ 0·40; *XXXI*: λ_{max} 268 nm, $A_{250/260}$ 0·83, $A_{280/260}$ 0·80, $A_{290/260}$ 0·45.

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