13. *Polynucleotides. Part III.*[1] *Synthesis of Some Polyuridylic Acid Analogues and a Survey of Anhydride Reagents.*

By R. Letters and A. M. Michelson.

The synthesis of polyribonucleotides by the action of reagents such as diphenyl phosphorochloridate on the nucleoside-2′,3′ cyclic phosphate has been examined further for a variety of anhydrides and conditions in an attempt to determine the influences of these factors. 5-Chloro-, 5-bromo-, 5-iodo-, and N^1-methyl-uridine-2′(3′) phosphate have been prepared and converted into 2′,3′-cyclic phosphates by treatment with ethyl chloroformate, and have then been polymerised to oligonucleotides.

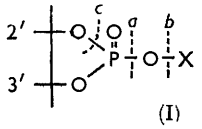
Halogenation of uridine-2′(3′) phosphate under appropriate conditions gives excellent yields of 5-halogenouridylic acids. Treatment of a solution of the nucleotide in acetic acid with chlorine rapidly gave 5-chlorouridylic acid at room temperature, without acetylation;[2] but when the reaction period was prolonged or an excess of chlorine used the ultraviolet absorption of the solution decreased, presumably owing to the formation of a 4,5-dihydro-derivative since the absorption characteristics were restored on heating the crude material in acid. Bromination of uridylic acid according to the procedure described by Levene and La Forge,[3] or with an excess of dioxan dibromide was not satisfactory, but treating the nucleotide in aqueous dioxan with bromine in the presence of a small amount of nitric acid gave 5-bromouridylic acid rapidly at room temperature. A similar procedure, with iodine [4] instead of bromine and at 100°, gave the iodo-derivative. Both the dioxan and the nitric acid were necessary in these reactions and formation of an intermediate halogen nitrate from dioxan dibromide (or di-iodide) and nitric acid is tentatively postulated. The halogenouridylic acids were then converted into the respective 2′,3′ cyclic phosphates and polymerised by methods previously described.[1] Small increases in the apparent pK's of the bases occurred on polymerisation but the apparent pK's (8·7—9·0 for the polymers) were still significantly lower than those of uridylic acid and polyuridylic acid (pK 9·6). The effect of oligo-halogenouridylic acids on the visible absorption of Acridine Orange was similar to that observed with polyuridylic acid.[5]

With an excess of diazomethane uridine-2′(3′) phosphate yielded the neutral dimethyl ester of N^1-methyluridylic acid. An aqueous ethanolic solution of this, when kept at 0° for some months, underwent spontaneous hydrolysis to N^1-methyluridine-2′(3′) phosphate in excellent yield. Polymerisation in the usual way gave the corresponding oligonucleotides. These possessed physical and biochemical properties identical with those recently described by Szer and Shugar [6] for similarly prepared material.

[1] Part II, Michelson, *J.*, 1959, 3655.
[2] Fukuhara and Visser, *J. Biol. Chem.*, 1951, **190**, 95.
[3] Levene and La Forge, *Ber.*, 1912, **45**, 615.
[4] Prusoff, *Biochim. Biophys. Acta*, 1959, **32**, 295.
[5] Michelson, *J.*, 1959, 1371.
[6] Szer and Shugar, *Acta Biochim. Polon.*, 1960, **7**, 491.

Polymerisation of nucleoside-2′,3′ cyclic phosphates by the action of diphenyl phosphorochloridate provides an efficient and widely applicable method for the synthesis of small polyribonucleotides containing 2′–5′ and 3′–5′ internucleotide linkages.[1,5] Although treatment of uridine-2′,3′ cyclic phosphate with diphenyl phosphorochloridate and an excess of benzyl alcohol gave a mixture containing 75% of uridine-3′ benzyl phosphate and 25% of the isomeric 2′-ester,[5] equal proportions of 2′–5′ and 3′–5′ linkages occur in the synthetic polynucleotides. Examination of a variety of solvent and pH conditions for cleavage of the intermediate polymeric triester showed that the ratio of 2′–5′ and 3′–5′ linkages was unaffected by such factors, but acidic hydrolysis of the triester gave polymers with longer average chain length than was obtained by hydrolysis in the presence of tributylamine.

A small increase in the maximal chain length of the synthetic polynucleotides was achieved by continuous addition of concentrated solutions of the mononucleotide to diphenyl phosphorochloridate and it is likely that the intermediate monomer anhydride (P^1-nucleoside-2′,3′ cyclic P^2-diphenyl pyrophosphate) is more active than oligomer–diphenyl phosphate anhydride. The effect of different anhydrides on nucleoside-2′,3′ cyclic phosphates in dioxan in the presence of tributylamine (a hindered tertiary base) and in pyridine was then examined. In a broad survey of anhydride reagents, no increase in chain length of polymers beyond a maximum of 15—20 nucleotides was obtained, as shown by chromatography on ECTEOLA and DEAE papers or by column chromatography. In certain cases the same intermediate nucleotide anhydride resulted, under different conditions, either in polymerisation or in sulphonylation (or acylation) of the free 5′-hydroxyl. Indeed, all three possible reactions indicated by cleavage of bonds *a*, *b*, and *c* in (I) may occur, as previously postulated.[7]



(I)

X = $-\overset{\overset{O}{\|}}{P}\begin{matrix}OR\\OR\end{matrix}$; $-SO_2R$; $-CO\cdot R$

Nucleophilic attack at the nucleotide P atom and cleavage of bonds *a* or *c* yields polynucleotide material, while attack at the non-nucleotide moiety with cleavage of bond *b* results in non-polymeric esterification of the 5′-hydroxyl group.

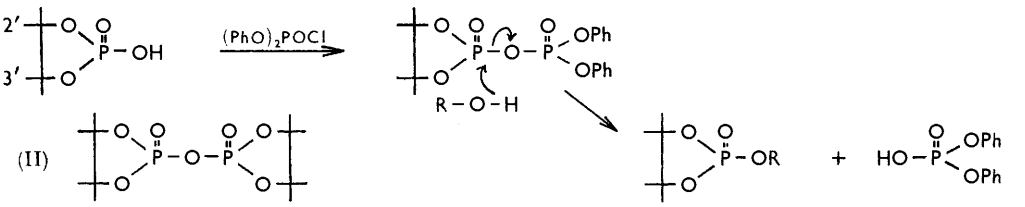
Uridine-2′,3′ phosphate, in dioxan or in pyridine, is polymerised quantitatively on treatment with an acid anhydride from a group which includes diphenyl phoshorochloridate, tetraphenyl pyrophosphate, tetra-*p*-bromophenyl pyrophosphate, phenyl phosphorodichloridate, and toluene-*p*-sulphonyl chloride. Presumably with all these reagents mechanism (*a*), that is, nucleophilic attack by the 5′-hydroxyl group of one monomer unit at the nucleotide phosphorus of an adjacent nucleotide anhydride with rear displacement of the more stable anion of the stronger acid of the intermediate anhydride,[8] is involved. It may be emphasised that in dioxan solution no significant phosphorylation of 5′-hydroxyl groups by diphenyl phosphorochloridate occurred under the conditions employed. Further, the products were soluble in the reaction medium, and insolubility of higher oligonucleotides cannot be advanced as a limiting factor for the synthesis of polynucleotides.

Tetra-*p*-nitrophenyl pyrophosphate was not an effective reagent for the polymerisation of uridine-2′,3′ cyclic phosphate. A possible explanation is that the initially formed P^1-nucleoside-2′,3′ P^2-di-*p*-nitrophenyl pyrophosphate is extremely unstable and is rapidly converted into a P^1,P^2-dinucleoside-2′,3′ pyrophosphate (II) that is, a relatively stable symmetrical tetra-alkyl pyrophosphate. Subsequent hydrolysis would then give nucleoside-2′,3′ cyclic phosphate. (This may also be the mechanism of chain termination when

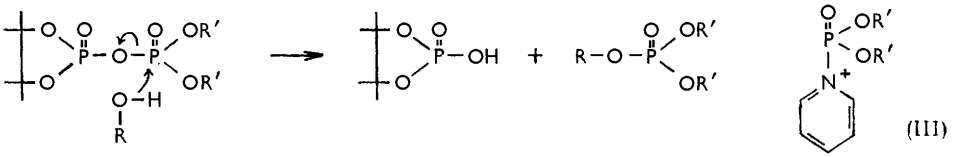
[7] Michelson, *Nature*, 1958, **181**, 375.
[8] Todd, *Proc. Nat. Acad. Sci. U.S.A.*, 1959, **45**, 1389.

diphenyl phosphorochloridate is employed.) Thus nucleoside-2′,3′ cyclic phosphates are polymerised by the action of anhydrides of acids which are stronger, but not too much stronger, than the cyclic phosphates.

When the anhydride reagent contains an acid which is not significantly stronger than the cyclic phosphate as in the case of tetrabenzyl pyrophosphate, tetra-allyl pyrophosphate, and P^1,P^2-diphenyl P^1P^2-dibenzyl pyrophosphate, then non-polymeric phosphorylation of

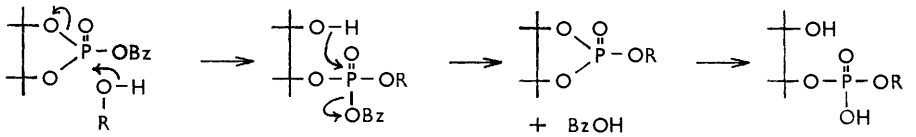


the 5′-hydroxyl group occurs by nucleophilic attack at the non-nucleotide phosphorus and cleavage of bond *b* of the intermediate anhydride. This reaction occurs to a significant extent in pyridine only and is markedly slower than the polymeric phosphorylation of the 5′-hydroxyl group that was observed with the stronger anhydrides. Since little, if any, reaction occurs in dioxan (in the presence of tributylamine) the pyridine-catalysed reaction may involve the formation of a pyridinium phosphate complex such as (III). Likewise, treatment of uridine-2′,3′ cyclic phosphate



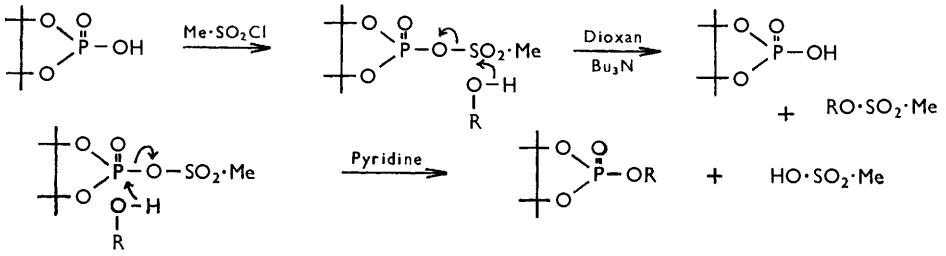
with 3,5-dinitrobenzoyl chloride gave 5′-*O*-(3,5-dinitrobenzoyl)uridine-2′,3′ cyclic phosphate quantitatively, acylation being assisted by the additive properties of the carbonyl group so that the reaction occurred readily in dioxan in the absence of base catalysis, as well as in pyridine. In the same way acetic anhydride gave the 5′-*O*-acetyl derivative.[1]

However, with benzoyl chloride, the third mechanism (cleavage at bond *c*) may be involved since, despite the additive properties of the carbonyl group, considerable polymerisation occurred in dioxan solution. Direct nucleophilic attack by the 5′-hydroxyl group at the phosphorus atom would possibly give an intermediate oligo(nucleotide–benzoyl anhydride) that is subsequently hydrolysed to oligonucleotide. Such a mechanism would avoid direct displacement of the less stable anion.



The same reagent in pyridine effects benzoylation of uridine-2′,3′ cyclic phosphate together with some polymerisation, that is, mechanism *b* is also operative, as would be expected. However, treatment of adenosine-2′,3′ cyclic phosphate with benzoyl chloride gave mainly oligonucleotide material in both solvents. A more powerful directive effect was observed with methanesulphonyl and ethanesulphonyl chloride as the anhydride reagents. In dioxan in the presence of tributylamine only the 5′-*O*-sulphonyluridine-2′,3′ cyclic phosphate was obtained (mechanism *b*), while in pyridine both reagents caused extensive polymerisation comparable with that obtained by the action of diphenyl phosphorochloridate.

The nature of the products from fully esterified nucleotide anhydrides of the type described is thus a function of the components of the anhydride (and in particular of the non-nucleotide portion), of the nature of the solvent (presumably the dielectric properties



of the environment affect the electronic distribution in the anhydride), and of the presence or absence of base catalysis.

Experimental

Factors Controlling the Polymerisation of Nucleoside-2′,3′ Cyclic Phosphates.—The polymerisation procedure was as follows: Uridine-2′,3′ cyclic phosphate (0·17 mmole) was prepared by the ethyl chloroformate method and dried overnight. The solvent (1·5 c.c.), tributylamine (0·5 mmole), and the anhydride (0·25 mmole) were added, and the mixture was left at room temperature for 3 hr. and then overnight at 0°. Solvent was removed and the polymer was precipitated with 1 : 1 ether–cyclohexane (3 c.c.), then dissolved in water and neutralised with tributylamine. Addition of ethanolic calcium chloride and an excess of ethanol precipitated the calcium salt of the polymer which was washed once with ethanol and then twice with ether.

(a) *Influence of the anhydride and solvent.* Polymerisation occurred with the following anhydride–solvent combinations in order of preference: tetra-*p*-bromophenyl pyrophosphate and dioxan; diphenyl phosphorochloridate and dioxan; toluene-*p*-sulphonyl chloride and pyridine; methanesulphonyl chloride and pyridine; benzoyl chloride and dioxan; phenyl phosphorodichloridate and dioxan. In pyridine solution the 5′-hydroxyl group was phosphorylated by P^1P^2-dibenzyl P^1P^2-diphenyl pyrophosphate, tetra-allyl pyrophosphate, and tetrabenzyl pyrophosphate in that order of reactivity. Quantitative acylation of the 5′-hydroxyl group was obtained with 3,5-dinitrobenzoyl chloride in pyridine, whereas benzoyl chloride in the same solvent resulted in acylation (50—70%) and polymerisation. The 5′-*O*-methane- and 5′-*O*-ethane-sulphonylnucleoside-2′,3′ cyclic phosphates were obtained quantitatively when the nucleoside-2′,3′ cyclic phosphates, in dioxan, were treated with methane- or ethane-sulphonyl chloride and tributylamine.

(b) *Order and rate of addition of reactants.* Uridine-2′,3′ cyclic phosphate (0·5 mmole of the tributylammonium salt) in dioxan (1·5 c.c.) was polymerised in the usual way with diphenyl phosphorochloridate (0·75 mmole). After being kept at room temperature for 15 min. the mixture was cooled in ice and further quantities of diphenyl phosphorochloridate (0·75 mmole) and tributylamine (1·5 mmoles) were added, followed by the dropwise addition (in 15 min.) of tributylammonium uridine-2′,3′ cyclic phosphate (0·5 mmole) in dioxan (1·5 c.c.). The mixture was then allowed to warm to room temperature and kept for 3 hr. Polyuridylic acid was isolated as a calcium salt. Ion-exchange analysis of this salt showed that chain lengths greater than 10 nucleotides were obtained by this method but still in comparatively low yield.

(c) *Cleavage of the intermediate polymeric triester.* Uridylic acid (1 mmole) was polymerised with diphenyl phosphorochloridate in dioxan in the usual way, and the mixture kept at room temperature for 3 hr., then divided into four equal portions. Solvent was removed in each case and the polymer precipitated by the addition of ether–cyclohexane. The following treatments were then used: (i) Water (1 c.c.) was added and the acidic solution left for 30 min. before being neutralised. (ii) Water (1 c.c.) and tributylamine (0·24 c.c.) were added. (iii) Dioxan (2 c.c.), water (0·045 c.c.), and tributylamine (0·24 c.c.) were added with shaking, and the solution was kept at room temperature for 1 hr. and then adjusted to 15 c.c. with 95% ethanol. (iv) t-Butyl alcohol (2 c.c.), water (0·045 c.c.), and tributylamine (0·24 c.c.) were added, then the procedure in (iii) was followed. The products were isolated as calcium salts in

each case and the distribution of chain lengths examined by chromatographic methods. Total yields were virtually identical but more material of higher molecular weight was obtained by procedure (i). Enzymic digestion with pancreatic ribonuclease followed by separation of the products showed no marked variation in the ratio (unity) of 2′–5′ and 3′–5′ internucleotide linkages. The same treatments applied to polyguanylic acid gave similar results, the product being isolated as the free acid.

Polymerisation of Nucleoside-2′,3′ Cyclic Phosphates with Benzoyl Chloride.—Benzoyl chloride (0·14 c.c.) and tributylamine were added to a solution of the tributylammonium nucleoside (adenosine, cytidine, guanosine, or uridine)-2′,3′ cyclic phosphate in dimethylformamide (0·25 c.c.) and dioxan (0·75 c.c.). After 30 min. at room temperature no significant polymerisation had occurred (as indicated by paper chromatography) but after 24 hr. the mixtures in each case contained mainly oligonucleotide material that was isolated by the standard procedures.

N^1-*Methyluridine-2′(3′) Phosphate.*—Uridine-2′(3′) phosphate (0·5 g.) was dissolved in 95% methanol (20 c.c.). An excess of diazomethane in ether was added (permanent yellow colour), then solvent was removed, the residue dissolved in 50% aqueous ethanol, and the solution kept for several months at 0° until paper chromatography showed complete hydrolysis of methyl ester groups. The solution was neutralised with tributylamine, ethanolic calcium chloride and an excess of ethanol were added, and the precipitated *calcium salt* of N^1-methyluridine-2′(3′) phosphate was collected (0·49 g.) (Found, in material dried at 120°/10^{-3} mm. for 24 hr.: N, 7·7; P, 8·0. $C_{10}H_{13}CaN_2O_9P$ requires N, 7·4; P, 8·2%).

5-*Chlorouridine-2′(3′) Phosphate.*—Chlorine (1·5 mmoles) in carbon tetrachloride (2·5 c.c.) was added to a solution of uridine-2′(3′) phosphate (1 mmole) in acetic acid (8 c.c.), and the mixture kept at room temperature for 5—10 min., then evaporated to dryness. The residue was dissolved in methanol, again taken to dryness, and finally dissolved in methanol (10 c.c.), and the solution was refluxed for 10 min. Ethanolic calcium chloride and pyridine were added and the precipitated *calcium salt* of 5-chlorouridine-2′(3′) phosphate was collected, washed with ethanol, then twice with ether, and dried (0·275 g., 69%). For analysis the salt was reprecipitated from aqueous solution by the addition of two volumes of ethanol (Found, in material dried at 125°/10^{-3} mm. for 24 hr.: N, 6·9; P, 7·9. $C_9H_{10}CaClN_2O_9P$ requires N, 7·1; P, 7·8%).

5-*Bromouridine-2′(3′) Phosphate.*—Bromine (1·5 mmoles) in carbon tetrachloride (2 c.c.) was added to a solution of uridine-2′(3′) phosphate (1 mmole) in 0·5N-nitric acid (2 c.c.) and dioxan (8 c.c.), and the mixture kept at room temperature for 1 hr. Solvent was removed under reduced pressure and the residue washed with dry ether. The *calcium salt* of 5-bromouridine-2′(3′) phosphate was prepared in the usual way (0·300 g., 68%) (Found, in material dried at 120°/10^{-3} mm. for 24 hr.: N, 6·4; P, 6·8. $C_9H_{10}BrCaN_2O_9P$ requires N, 6·3; P, 7·0%).

5-*Iodouridine-2′(3′) Phosphate.*—Iodine (0·51 g., 2 mmoles) was added to a solution of uridine-2′(3′) phosphate (1 mmole) in 0·5N-nitric acid (2 c.c.) and dioxan (8 c.c.), and the mixture kept at 100° for 1 hr. Solvent was removed and the residue dried by repeated dissolution in ethanol, followed by evaporation. Ether (15 c.c.) was added to the final residue and the precipitated nucleotide converted in the usual way into the *calcium salt* of 5-iodouridine-2′(3′) phosphate (0·356 g., 73%) (Found, in material dried at 120°/10^{-3} mm.: N, 6·0; P, 6·3. $C_9H_{10}CaIN_2O_9P$ requires N, 5·7; P, 6·4%).

Ultraviolet Absorption Properties of the 5-Halogenouridine-2′(3′) Phosphates.—Ultraviolet absorption characteristics are tabulated. Apparent pK values were determined spectroscopically at the wavelength indicated (all wavelengths in mμ).

Nucleoside-2′(3′) phosphate	In 0·01N-HCl			In 0·01N-NaOH			Apparent pK	
	$\lambda_{max.}$	$10^{-3}\varepsilon$	$\lambda_{min.}$	$\lambda_{max.}$	$10^{-3}\varepsilon$	$\lambda_{min.}$		
Uridine	262	—	230	261	—	242	9·6	(260 mμ)
5-Chlorouridine ...	274	9·2	237	274	6·6	250	8·5	(270 mμ)
5-Bromouridine ...	277	9·4	242	275	6·8	250	8·5	(280 mμ)
5-Iodouridine	286	8·7	248	278	6·1	253	8·8	(290 mμ)
N^1-Methyluridine	258	—	232	260	—	234	—	—
Polynucleotides								
5-Chlorouridylic ...	274	—	242	274	—	250	8·7	(270 mμ)
5-Bromouridylic...	275	—	243	275	—	250	8·8	(280 mμ)
5-Iodouridylic.....	286	—	249	278	—	254	9·0	(290 mμ)
N^1-Methyluridylic	259	—	233	259	—	233	—	—

Polymerisation of Uridylic Acid Analogues.—The nucleotide was converted into the 2′,3′-cyclic phosphate and then polymerised in dioxan by the action of diphenyl phosphorochloridate at room temperature for 3 hr., as previously described. In each case the product (oligonucleotides of different chain lengths) was isolated as the calcium salt. Conditions for degradation with alkali, pancreatic ribonuclease, and rattlesnake (*Crotalus atrox*) venom were also as described earlier. The poly-5-halogenouridylic acids were hydrolysed by ribonuclease; poly-N^1-methyluridylic acid was not. Alkaline hydrolysis of the polymers gave only the nucleoside-2′(and 3′) phosphates, as shown by paper chromatography and paper electrophoresis in several solvents.

R_F's in solvents *A* and *B*.

Nucleoside-2′,3′ cyclic phosphates			*Nucleoside-2′(3′) phosphates*		
	A	*B*		*A*	*B*
Uridine	0·44	0·48	Uridine	0·23	0·45
N^1-Methyluridine	0·63	0·62	N^1-Methyluridine	0·39	0·57
5-Chlorouridine	0·48	0·54	5-Chlorouridine	0·26	0·47
5-Bromouridine	0·48	0·50	5-Bromouridine	0·24	0·47
5-Iodouridine	0·49	0·51	5-Iodouridine	0·23	0·47

Paper Chromatography.—Ascending chromatograms on Whatman No. 1 paper were used with solvent systems: *A*, ethanol–1M-ammonium acetate (5 : 2); *B*, t-pentyl alcohol–formic acid–water (3 : 2 : 1). Results are tabulated.

Polynucleotide mixtures were examined by descending chromatography on ECTEOLA cellulose paper, with 10% or 20% saturated aqueous ammonium hydrogen carbonate as the developing solvent. The relative amounts of slower- and faster-moving material were estimated visually with ultraviolet illumination.

Ion-exchange Analysis of Polymers.—The polymers (10 mg. of calcium salt) in water (10 c.c.) were fractionated on a cellulose anion-exchange (ECTEOLA) column (14 × 1·3 cm.) with a linear concentration gradient of aqueous lithium chloride for elution at a flow rate of ~1 c.c./min.

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