

745. Polynucleotides. Part II.¹ Homopolymers of Cytidylic and Pseudouridylic Acid, Copolymers with Repeating Subunits, and the Stepwise Synthesis of Polyribonucleotides.

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Cytidylic and pseudouridylic acid have been polymerised by the action of diphenyl phosphorochloridate. The properties of the polymers are in accord with those of polyadenylic, polyguanylic, and polyuridylic acid previously reported. A definitive preparation of glycol cyclic phosphates using ethyl chloroformate in aqueous solution is described. Oligonucleotides with a defined order of bases have been prepared by polymerisation of three dinucleotides and a trinucleotide isolated as pure solids from a ribonuclease digest of yeast ribonucleic acid. A number of dinucleoside phosphates have been synthesised in good yield by treatment of a 2',3'-di-*O*-acetyl-nucleoside and a 5'-*O*-acetyl-nucleoside-2',3' cyclic phosphate with diphenyl phosphorochloridate and base. By further stepwise addition of nucleotide, five oligonucleotides containing three different bases and six containing four different bases have been prepared. The physical properties of these compounds confirm a restricted rotation about the internucleotide linkage even in small oligonucleotides.

IN Part I¹ was described the synthesis of a number of polynucleotides by the action of diphenyl phosphorochloridate on the nucleoside-2'(or 3') phosphate. Extension to the polymerisation of pseudouridylic acid² was straightforward, giving polypseudouridylic acids of different chain lengths. However, initial approaches to the polymerisation of cytidylic acid indicated that protection of the amino-group was necessary. While treatment of cytidine-2',3' cyclic phosphate with diphenyl phosphorochloridate in the usual way gave polymers, as shown by paper chromatography and dialysis of the products, these products were stable to treatment with mild acid or alkali and were unaffected by ribonuclease or the mixture of enzymes present in rattlesnake venom. Further, the ultraviolet absorption spectra in acid and alkali were virtually identical, whereas cytidylic acid shows a characteristic change due to ionisation of the amino-group. It is likely therefore that a large part of this material contained internucleotide linkages from the 2'- or 3'-phosphate to the 6-amino-group of the pyrimidine ring. Attention was therefore directed to selective acetylation of this amino-group. Cytidine-2',3' cyclic phosphate with 1 mol. of acetic anhydride gave a 95% yield of the required *N*⁶-acetylcytidine-2',3' cyclic phosphate with only traces of unchanged material and *N*⁶*O*⁵-diacetylcytidine-2',3' cyclic phosphate. The monoacetylated material was not a mixture of *N*⁶- and *O*-acetyl derivatives because the ultraviolet absorption spectra in acid and neutral solution were identical with those of *N*⁶*O*⁵-diacetylcytidine-2',3' cyclic phosphate (Fig. 1) and showed completely the changes previously³ noted as characteristic of acylation of the amino-group of cytosine nucleosides. Preliminary experiments indicated that the amino-acetyl group was readily removed by very mild treatment with alkali (pH 9.6) (this also partially hydrolysed the cyclic phosphate to 2'- and 3'-phosphates), while spectral studies of the compound in 0.04*N*-sodium hydroxide at room temperature showed 80% deacetylation in 10 min. and complete deacetylation in 30 min.

Treatment of *N*⁶-acetylcytidine-2',3' cyclic phosphate with diphenyl phosphorochloridate, followed by removal of the acetyl group, gave polycytidylic acid in good yield.

The properties of polypseudouridylic and polycytidylic acid were in general agreement with those of the homopolymers of adenylic, guanylic, and uridylic acid.¹ Alkali-treatment gave a mixture of the 2'- and the 3'-phosphates; crude rattlesnake venom degraded

¹ Part I, *J.*, 1959, 1371.

² Cohn, *Biochim. Biophys. Acta*, 1959, **32**, 569.

³ Michelson and Todd, *J.*, 1954, 34.

the polymers to nucleoside and nucleoside-2'(3'),5' diphosphate, and ribonuclease effected partial breakdown to the mononucleotide and a series of oligonucleotides containing 2'-5'-linkages exclusively. Although polypseudouridylic acid had very slight effect on the absorption spectra of Toluidine Blue and Acridine Orange, the action of polycytidylic acid was more pronounced. The influence of chain length on this interaction with dyes is

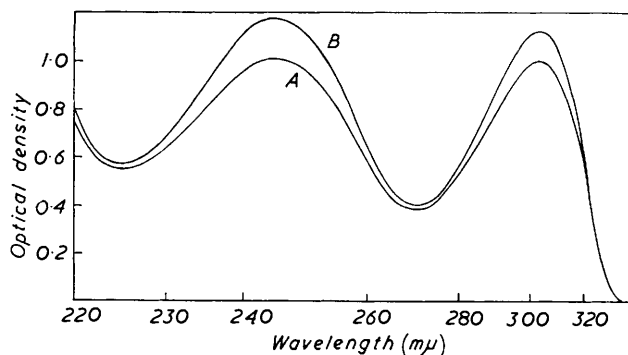
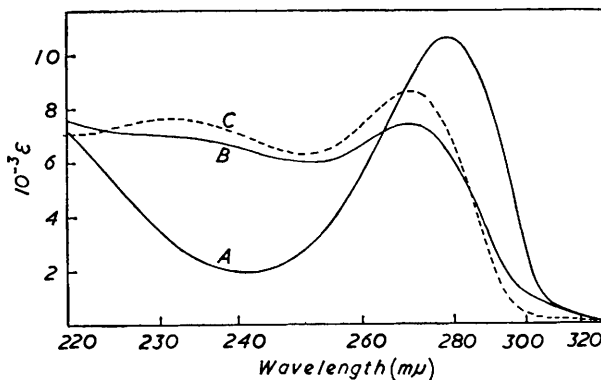


FIG. 1. N^6 -Acetylcytidine-2',3' cyclic phosphate (A) and N^6O^5' -diacetylcytidine-2',3' cyclic phosphate (B) in 0.01N-HCl.

indicated in Fig. 3; minimum chain lengths of 8—10 nucleotides would seem to be necessary for significant interaction.

Some optical properties of the polymers are given in Table 1. Comparison of the hyperchromic effects shown by the homopolymers and by various dinucleoside phosphates shows that the generalisation that "the diminution in ϵ_{\max} for the purine containing compounds is considerably higher than that for the compounds containing only pyrimidines" ⁴ is not valid. The small hypochromic values for apurinic acid are probably due, not

FIG. 2. Polycytidylic acid (average chain length ca. 10 units). A, in 0.01N-HCl; B, in 0.1N-NaOH; C, alkaline hydrolysis products in 0.1N-NaOH.



so much to removal of purines, as to dilution of the effect due to tracts of two, three, or more pyrimidines by single pyrimidines flanked by deoxyribose units (effectively mononucleotide material).

Like yeast ribonucleic acid, all the synthetic homopolymers (and copolymers) gave precipitates with protamine sulphate in neutral aqueous solution. These precipitates were partially soluble in 2M-sodium (or lithium) chloride, but were reprecipitated on dilution to an ionic strength of 0.2M or less.

Dr. K. S. Kirby, of the Chester Beatty Institute, has examined the countercurrent distribution of the homopolymers of adenylic, cytidylic, guanylic, and uridylic acid (all of average chain length ~ 6) and of an improved preparation of poly ACGU. The results

⁴ Gilham and Khorana, *J. Amer. Chem. Soc.*, 1958, **80**, 6212.

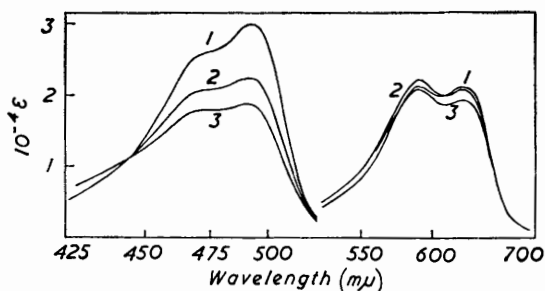
are shown in Fig. 4. An indication of partial interaction between polycytidylic acid and polyguanylic acid was observed. Paper electrophoresis also indicated partial interaction, but only a slight decrease in absorbance (3%) occurred when solutions of the two polymers were mixed. Since no interaction occurred between the synthetic polyadenylic and polyuridylic acid, it may be concluded that hydrogen bonds between the cytosine 6-amino-groups and guanine NH·CO groups are stronger than those between adenine and uracil.

TABLE I. *Ultraviolet spectra.*

	In 0.01N-HCl		In 0.1N-NaOH		Alkaline hyperchromicity (%)
	λ_{\max} (m μ)	λ_{\min} (m μ)	λ_{\max} (m μ)	λ_{\min} (m μ)	
Dicytidylic	278	240	270	250	8.7
Tricytidylic	278	241	270	251	13.9
Tetracytidylic	278	241	270	251	15.3
C2'P5'CP	278	241	270	251	11.0
C2'P5'C2'P5'CP	278	241	270	251	15.7
Polycytidylic (4.4)	278	241	270	251	13.9
Polycytidylic (9.8)	278	241	270	251	15.9
Polypseudouridylic (6.2)	262	231	285	246	10.5
"Stable" polycytidylic	273	244	273	246	0

Various workers⁵ have used cellulose anion-exchange materials for fractionation of polynucleotides. The use of "Ecteola" cellulose (Brown Co., Berlin, New Hampshire) for fractionation of nucleotides of low molecular weight is demonstrated by the ready separation of adenosine-2',3' cyclic phosphate, adenosine-2' phosphate, and adenosine-3' phosphate at neutral pH by gradient elution with aqueous lithium chloride (Fig. 5). Representative chromatographic analyses of synthetic polyuridylic and polyguanylic acids (before dialysis) are shown in Fig. 6. Similar elution diagrams were obtained for the other homopolymers.

FIG. 3. Action of polycytidylic acid [3×10^{-5} M (P)] on the absorption spectra of Acridine Orange (3×10^{-5} M) (left) and Toluidine Blue (3×10^{-5} M) (right) in 0.01M-ammonium acetate at pH 6.8. 1, Dye alone; 2, and 3, with added polycytidylic acid of average chain length ~ 6 and ~ 10 nucleotides respectively.



As part of a programme directed to the polymerisation of nucleotides in aqueous solution the use of ethyl chloroformate as a cyclising agent has been examined. In view of the general solvent difficulties in this field it is of particular interest that quantitative yields of all the nucleoside-2',3' cyclic phosphates (and of glycerol-1,2 cyclic phosphate and glycol-1,2 cyclic phosphate) were obtained by treating the appropriate phosphate with ethyl chloroformate and base in aqueous solution. The reagent was also effective in anhydrous media.

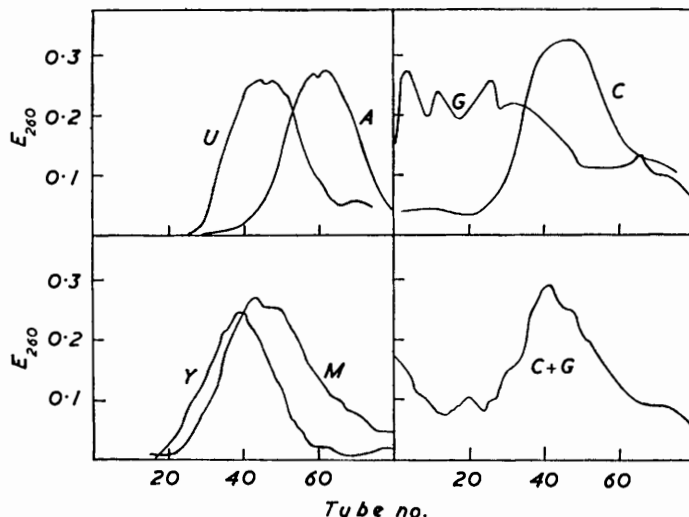
Enzymic degradation of ribonucleic acid to oligonucleotides ending with a pyrimidine nucleoside-3' phosphate, the remainder of the molecule consisting of purine nucleotides, provides a convenient preparation of small polynucleotides of defined structure. Volkin and Cohn described⁶ the fractionation of such mixtures using Dowex anion-exchange resin. By repeating this work on a large scale, 5'-(3'-adenylyl)cytidine-3' phosphate,

⁵ Bendich, Fresco, Rosenkranz, and Beiser, *J. Amer. Chem. Soc.*, 1955, **77**, 3671; Bendich, Pahl, Korngold, Rosenkranz, and Fresco, *J. Amer. Chem. Soc.*, 1958, **80**, 3949; Bradley and Rich, *ibid.*, 1956, **78**, 5898; Tener, Khorana, Markham, and Pol, *ibid.*, 1958, **80**, 6223.

⁶ Volkin and Cohn, *J. Biol. Chem.*, 1953, **205**, 767.

5'-(3'-guanylyl)cytidine-3' phosphate, 5'-(3'-adenylyl)uridine-3' phosphate, and 5'-[5'-(3'-adenylyl)-3'-adenylyl]cytidine-3' phosphate (*i.e.*, A3'P5'A3'P5'C3'P) were obtained as pure solids and characterised by the usual methods.⁶ While the dinucleotides were inert to the enzymes in rattlesnake (*Crotalus atrox*) venom, the trinucleotide was slowly hydrolysed

FIG. 4. Countercurrent distribution of polyadenylic (A), polycytidylic (C), polyguanylic (G), polyuridylic (U), poly ACGU (M), and yeast ribonucleic (Y) acids, and of a mixture of polycytidylic and polyguanylic acids (C + G).



to adenosine, cytidine-3',5' diphosphate, and a trace of dinucleotide [presumably 5'-(3'-adenylyl)cytidine-3' phosphate].

Treatment of these oligonucleotides under suitable conditions with ethyl chloroformate converted the terminal phosphate into a 2',3'-cyclic phosphate with only slight degradation to compounds of lower molecular weight. Reaction of 5'-[2'(or 3')-adenylyl]uridine-2',3'

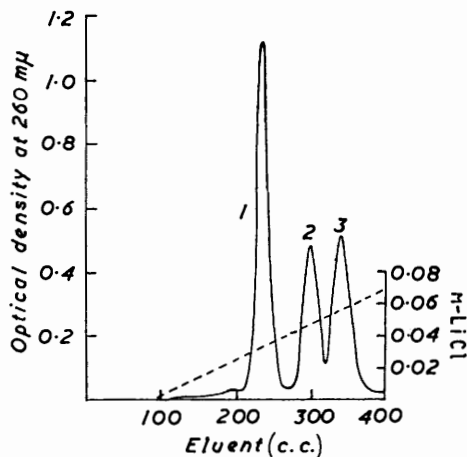


FIG. 5. Fractionation of adenosine-2',3' cyclic phosphate (1), adenosine-2' phosphate (2), and adenosine-3' phosphate (3) on an "Ecteola" cellulose column (10 × 1 cm.) with gradient elution with aqueous lithium chloride. Flow rate ~1 c.c./min.

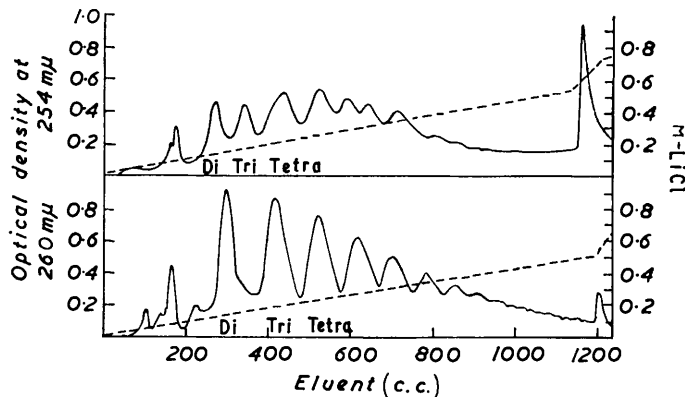
cyclic phosphate with diphenyl phosphorochloridate and tri-*n*-butylamine in anhydrous solution gave a series of polymers containing 2, 4, 6, etc., nucleotides, again with only slight apparent cleavage of the original internucleotide linkage. With cytosine-containing polynucleotides, the 6-amino-group was selectively acetylated after cyclisation of the terminal phosphate, and the protected compound was then polymerised, acetyl groups

being removed from the product by mild alkali-treatment. Optical properties of the oligonucleotides are given in Table 2.

A shift in the apparent pK_a 's (spectrophotometrically determined) of 5'-(3'-guanylyl)-cytidine-3' phosphate to 4.45 (280 $m\mu$) and 10.2 (265 $m\mu$) was observed on polymerisation of the dinucleotide. This type of shift has been noted previously⁷ for a number of deoxy-nucleic acids.

The polymers showed the expected behaviour towards alkali and rattlesnake venom.

FIG. 6. Chromatographic analysis of polyguanylic acid (upper curve) and polyuridylic acid (lower curve) (before dialysis) on an "Ecteola" cellulose column (12 x 1 cm.) with gradient elution with aqueous lithium chloride. Flow rate ~1 c.c./min. Approx. 10 mg. of polymer.



Pancreatic ribonuclease released no mononucleotide, but did not cause complete breakdown to the original oligonucleotide owing to the presence of 2'-5'-linkages. While no evidence is available at the moment, the original 3'-5' purine-pyrimidine linkages were presumably partially isomerised during polymerisation.

TABLE 2. Optical properties.

Compound	In 0.01N-HCl			In 0.1N-NaOH			Alkaline hyperchromicity (%)
	λ_{max} (m μ)	λ_{min} (m μ)	280/260 m μ	λ_{max} (m μ)	λ_{min} (m μ)	280/260 m μ	
A3'P5'A3'P5'C3'P	259	232	0.52	259	229	0.36	22.5
A3'P5'A3'P5'C	259	232	0.53	259	230	0.39	23.3
A3'P5'A	257	230	0.22	258	229	0.19	10.7
A3'P5'C3'P	265	234	0.75	260	229	0.41	8.0
A3'P5'C	265	234	0.76	261	230	0.44	7.6
G3'P5'C3'P	276	233	1.10	268	231	0.70	3.8
G3'P5'C	276	233	1.25	268	232	0.76	3.0
A3'P5'U3'P	258	230	0.26	259	233	0.20	4.1
A3'P5'U	257	230	0.30	259	233	0.23	1.4
Poly (AAC) 2M-NaCl residue ...	260	233	0.54	259	229	0.43	28.8
Poly (AU) 2M-NaCl residue ...	260	235	0.26	259	237	0.24	17.2
GPCPGPCP	273	235	1.17	267	235	0.70	3.1

As shown by chromatography on "Ecteola" cellulose (Fig. 7) polymerisation was not so effective as with the mononucleotides. Indeed, with 5'-(3'-guanylyl) cytidine-3' phosphate the major product was the tetranucleotide, with unchanged material and small amounts of the hexanucleotide.

Three types of nucleotide co-polymer (random A and B, tracts of A and B, and alternating A and B) have been described. The next stage of the work concerned the stepwise synthesis of a number of oligonucleotides containing up to four different bases in a defined order. Although, partly for historical reasons, further addition has not been attempted,

⁷ Lee and Peacocke, *J.*, 1951, 3361.

extension to the stepwise synthesis of higher oligonucleotides may be expected to offer little difficulty.

Adenosine-2'(or 3') phosphate was converted into the cyclic phosphate by the action of ethyl chloroformate and base in aqueous solution. The anhydrous tri-*n*-butylammonium salt was then acetylated to give 5'-*O*-acetyladenosine-2',3' cyclic phosphate; under the conditions employed the *N*⁶-acetyl derivative was not obtained, as shown by ultraviolet absorption spectra of the product. Addition of diphenyl phosphorochloridate and base to a mixture of this protected nucleotide and 2',3'-di-*O*-acetyluridine in anhydrous solution gave a good yield of the protected dinucleoside phosphate from which acetyl groups were removed with mild alkali, to give a mixture of 5'-(2'- and 3'-adenylyl)uridine, separated by ion-exchange chromatography. A similar reaction of *N*⁶-*O*^{5'}-diacetylcytidine-2',3' cyclic phosphate with 2',3'-di-*O*-acetyluridine gave 5'-(2'- and 3'-cytidylyl)uridine, while treatment of 2',3'-di-*O*-acetyladenosine and diphenyl phosphorochloridate with 5'-*O*-acetyladenosine-2',3' cyclic phosphate, *N*⁶*O*^{5'}-diacetylcytidine-2',3' cyclic phosphate, 5'-*O*-acetylguanosine-2',3' cyclic phosphate and 5'-*O*-acetyluridine-2',3' cyclic phosphate

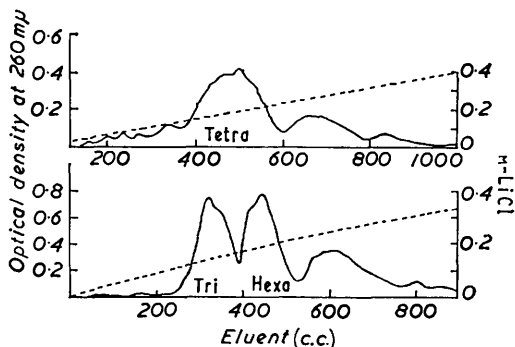


FIG. 7. Chromatographic analysis of poly (AU) (upper curve) and poly (AAC) (lower curve) (after dialysis against water) on an "Ecteola" cellulose column (12 × 1 cm.) with gradient elution with aqueous lithium chloride. Flow rate ~1 c.c./min. Approx. 7 mg. of polymer.

gave 5'-[2'(3')-adenylyl]-, 5'-[2'(3')-cytidylyl]-, 5'-[2'(3')-guanylyl]-, and 5'-[2'(3')-uridylyl]-adenosine respectively, in high yield and, in a number of cases, as crystalline compounds.

While separation of the 5'-2'- and 5'-3'-isomers of A2'(3')P5'A, G2'(3')P5'A, C2'(3')P5'A and A2'(3')P5'U on Dowex 1 anion-exchange resin was achieved, no useful fractionation of C2'(3')P5'U or of U2'(3')P5'A occurred. A direct estimate of the proportion of 5'-3'-linkage in these products, and in C2'(3')P5'A, was obtained, however, on samples removed from the crude reaction mixtures. After preliminary purification by paper chromatography the mixture of isomers was treated with ribonuclease, then separated into nucleoside, nucleotide, and dinucleoside phosphate by paper chromatography or paper electrophoresis. Appropriate strips were eluted with dilute alkali, and the solutions were incubated at 37° to obviate the hypochromic effect, and analysed spectrophotometrically. The original mixture in each case contained some 50% of 5'-3'-linked dinucleoside phosphate, the remainder being 5'-2', this result being analogous to those obtained by ion-exchange chromatography.

Differences in electrophoretic mobility between isomeric dinucleoside phosphates suggest that the 5'-2'-compounds are more compact or "streamlined" than the 5'-3'-isomers, *i.e.*, that stronger interaction occurs between the bases to restrict rotation about the internucleotide linkage. The alternative, that the 5'-2'-compounds are stronger acids, does not seem reasonable in view of the ion-exchange (Dowex 1) characteristics of the isomers examined. From the order of elution, in each case the 5'-3'-derivative behaved as a stronger acid. That the interplanar interaction is stronger in the 5'-2'-compounds is further indicated by a comparison of the hyperchromic effects. In general, the 5'-2'-isomer has a greater hyperchromicity than the 5'-3'-derivative (Table 3) as well as a greater electrophoretic mobility. It is perhaps significant also, that 5'-(2'-adenylyl)uridine showed a hyperchromicity twice that of the isomeric 5'-(2'-uridylyl)adenosine, *i.e.*, the increase in

ultraviolet absorption on degradation of polynucleotides depends on the order as well as the nature of the bases.

For the synthesis of trinucleoside diphosphates advantage was taken of the greatly disparate reactivities of the primary and secondary hydroxyl groups of ribonucleosides and their derivatives. Treatment of 5'-[2'(3')-uridylyl]adenosine with 5'-*O*-acetylguanosine-2',3' cyclic phosphate and diphenyl phosphorochloridate, followed by removal of the acetyl group, gave a good yield of G2'(3')P5'U2'(3')P5'A, purified by ion-exchange chromatography and isolated as the free acid. As expected, acylation of the free 2'- and 3'-hydroxyl group of adenosine did not occur, nor was there significant cleavage of the dinucleoside phosphate—in the conditions employed, 5'-[2'(3')-guanylyl]adenosine would have been formed had any such cleavage occurred. In a similar fashion, G2'(3')P5'A2(3')P5'U, U2'(3')P5'G2'(3')P5'A, C2'(3')P5'U2'(3')P5'A, and C2'(3')P5'A2(3')P5'U were prepared from the appropriate acetylated nucleoside-2',3' cyclic phosphate and dinucleoside phosphate.

Repetition of this process, using an excess of *N*⁶*O*^{5'}-diacetylcytidine-2',3' cyclic phosphate on G2'(3')P5'A2(3')P5'U gave C2'(3')P5'G2'(3')P5'A2(3')P5'U, isolated as the free acid. In like manner, C2'(3')P5'G2'(3')P5'U2'(3')P5'A and C2'(3')P5'U2'(3')P5'G2'(3')P5'A were synthesised in good yield from the respective trinucleoside diphosphates.

TABLE 3. *Ultraviolet spectra.*

Compound *	In 0.01N-HCl			In 0.01N-NaOH			Alkaline hyperchromicity (%)
	λ_{\max} (m μ)	λ_{\min} (m μ)	280/260 m μ	λ_{\max} (m μ)	λ_{\min} (m μ)	280/260 m μ	
U2'(3')P5'A	258	230	0.25	259	233	0.20	4.2
U2'P5'A	258	230	0.24	259	231	0.19	5.2
U3'P5'A	—	—	—	—	—	—	3.2
A2'P5'U	258	230	0.31	260	234	0.27	11.1
A3'P5'U	259	230	0.28	260	231	0.22	4.1
A5'P5'U	258	230	0.30	260	234	0.24	—
C2'(3')P5'U	269	236	1.0	265	246	0.65	8.3
C2'P5'U	270	236	1.0	265	246	0.64	7.9
C3'P5'U	—	—	—	—	—	—	8.7
C2'P5'C	278	241	1.85	270	251	0.90	10.8
C3'P5'C	—	—	—	—	—	—	7.4
G2'P5'A	257	229	0.42	259	232	0.37	5.0
G3'P5'A	257	229	0.40	259	230	0.37	3.3
A2'P5'A	257	231	0.23	258	229	0.22	18.4
A3'P5'A	257	230	0.25	258	229	0.20	13.3
C2'P5'A	264	234	0.72	261	228	0.40	9.7
C3'P5'A	264	234	0.77	261	229	0.44	10.2
A3'P5'C (natural)	265	234	0.76	261	230	0.44	7.6
UpGpA	257	230	0.39	259	232	0.36	6.8
GpApU	257	229	0.40	259	232	0.37	11.3
GpUpA	257	229	0.39	259	231	0.34	5.3
CpApU	263	233	0.64	261	232	0.42	12.5
CpUpA	263	233	0.62	261	229	0.38	7.5
CpUpGpA	261	232	0.63	260	232	0.45	7.7
CpGpApU	261	232	0.63	260	231	0.45	12.9
CpGpUpA	261	232	0.62	260	233	0.45	6.2
GpCpApU	260	231	0.63	261	232	0.47	12.3
GpCpUpA	260	231	0.63	260	231	0.45	6.6
ApUpGpC	260	232	0.63	261	232	0.47	8.6
Poly ACGU	262	232	0.68	262	228	0.54	12.1

An alternative approach to tetranucleoside triphosphates with a defined order of bases, using acylation of a dinucleoside phosphate with a dinucleotide, was also successful. Small-scale preliminary experiments on the acylation of 2',3'-di-*O*-acetyluridine and 2',3'-di-*O*-acetyladenosine with 5'-(3'-adenylyl)cytidine-3' phosphate, 5'-(3'-guanylyl)cytidine-3' phosphate, and 5'-(3'-adenylyl)uridine-3' phosphate (isolated from an enzymic hydrolysate of yeast ribonucleic acid) demonstrated the feasibility of the method for the partial synthesis of six trinucleoside diphosphates, ApCpA, ApCpU, GpCpA, GpCpU, ApUpA, and ApUpU.* Although this work was not further pursued, extension to

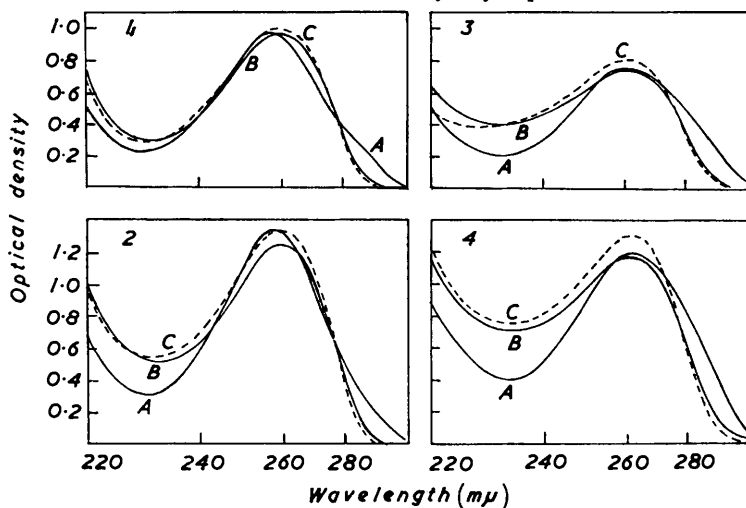
* p denotes the 2'P5' or 3'P5' linkage.

tetranucleoside triphosphates was equally successful. 5'-(3'-Guanylyl)cytidine-3' phosphate was treated with ethyl chloroformate, to give a dinucleotide with a terminal 2',3'-cyclic phosphate. This was then fully acetylated and the product treated with diphenyl phosphorochloridate and 5'-[2'(3')-uridylyl]adenosine. Acetyl groups were removed with mild alkali, and the crude tetranucleoside triphosphate purified by ion-exchange chromatography to give a good yield of G2'(3')P5'C2'(3')P5'U2'(3')P5'A, isolated as the free acid. Similar reactions of GpCp with ApU and of ApUp with GpC (acetylated at the cytosine N⁶-position) yielded GpCpApU and ApUpGpC respectively.

Finally, copolymerisation of adenylic, cytidylic, guanylic, uridylic, and pseudouridylic acid with 5'-[2'(3')-cytidylyl]adenosine yielded co-polymer ending with C2'(3')P5'A, that is, a model of the "soluble RNA" concerned in protein biosynthesis.⁸

Degradation of the oligonucleotides was as expected. Treatment with alkali gave the respective mononucleotides and end nucleoside, while rattlesnake venom caused rapid breakdown to nucleosides, since a terminal 2'(or 3')-phosphate was absent. The action of

FIG. 8. Ultraviolet absorption spectra of 1, G3'P5'A; 2, UpGpA; 3, CpUpGpA; 4, poly ACGU; A, in 0.01N-HCl; B, in 0.1N-NaOH; C, alkaline hydrolysis products in 0.1N-NaOH.



ribonuclease provided a final check on the structures of the synthetic compounds, the nature of the products being in accord with the specificity of this enzyme.⁹ Thus, of the dinucleoside phosphates, only the pyrimidine nucleoside-3'P5'-purine(or -pyrimidine) nucleoside derivatives were degraded to 3'-phosphate and nucleoside, the others being unchanged; of the trinucleoside phosphates GpApU was undegraded, the others giving a range of products including unchanged material (2'P5' linkage), dinucleotide, dinucleoside phosphate, nucleotide, and nucleoside, depending on the nature of the linkages and order of the bases. The tetranucleoside triphosphates also gave different products depending on the substrate; *e.g.*, CpGpApU gave C2'P5'GpApU, GpApU, and C3'P, while six products including adenosine and cytidine-3' phosphate were obtained from CpGpUpA.

Ultraviolet absorption spectra illustrating the build up of a typical polynucleotide are shown in Fig. 8; Table 3 lists some optical properties of the synthetic compounds. A comparison of the hyperchromicities and other properties of the compounds described here and elsewhere^{1,10} confirms the view that the anomalous ultraviolet absorption of a polynucleotide is a function, not only of nucleotide composition and chain length, but also of the order of bases, *i.e.*, it is a property of the entire molecule; that specious generalisations

⁸ Hoagland, Stephenson, Scott, Hecht, and Zamecnik, *J. Biol. Chem.*, 1958, **231**, 241.

⁹ Brown and Todd, in "The Nucleic Acids," ed. Chargaff and Davidson, Academic Press, New York, 1955, Vol. I, p. 409.

¹⁰ Michelson, *Acta Biochem. Polon.*, 1959, **6**, 335.

concerning the effect of purines or pyrimidines are incorrect; that the effect is probably due to interaction between the purine and/or pyrimidine bases; and that because of this interaction there is restricted rotation about the internucleotide linkage even at the dinucleotide level. Any process which increases this interaction (*e.g.*, contraction of the macromolecule, metal chelation between rings) will increase the hypochromic effect, while a further diminution in absorption is caused by hydrogen-bonding between purine and pyrimidine rings of different chains or separate parts of the same chain.

Because of this restricted rotation,* oligonucleotides could act as templates (*cf.* the interaction of polyguanylic and polycytidylic acids of low molecular weight), a factor of possible significance for the chemical origin of life. Since these small polynucleotides give precipitates with protamine and bind planar basic dyes, it is possible that protection by small peptides would occur at relatively short chain lengths, giving rise to a process of natural selection at the molecular level and evolution from primitive oligonucleotide-peptides, to the present-day nucleoproteins. Some biological properties of proteins and nucleic acids are not entirely dependent on extremely high molecular weight.

EXPERIMENTAL

Polypseudouridylic Acid.—Mono(tri-*n*-octylammonium) pseudouridine-2'(3') phosphate (from 50 mg. of the calcium salt) in dioxan (0.5 c.c.) was treated with diphenyl phosphorochloridate (0.08 c.c.) and tri-*n*-butylamine (0.17 c.c.), and the clear solution kept at room temperature for 3 hr. under anhydrous conditions. The polypseudouridylic acid was isolated as the *calcium salt* (30 mg.) in the usual manner (Found, in material dried at 110°/10⁻³ mm.: N, 8.4; P, 9.6. [C₉H₁₀O₈N₂PCa₄]_x requires N, 8.6; P, 9.5%).

"*Stable*" *Polycytidylic Acid.*—Diphenyl phosphorochloridate (4.6 c.c.) and tri-*n*-butylamine (8.0 c.c.) were added to a solution of tri-*n*-butylammonium cytidine-2',3' cyclic phosphate (from 2.75 g. of cytidylic acid) in dimethylformamide (10 c.c.) and dioxan (15 c.c.), and the mixture was kept at room temperature for 24 hr. Solvent was removed and the polymer precipitated by ether (200 c.c.). The crude material was dissolved in aqueous ethanol, the pH adjusted to 3.0 by 2*N*-hydrochloric acid, and the solution kept at room temperature for 36 hr. Excess of ethanol and hydrochloric acid were then added and the precipitated polymer was centrifuged off, washed with ethanol, then ether, and dried (1.35 g.) (Found, in material dried at 110°/10⁻³ mm.: P, 10.5%).

Polycytidylic Acid.—Ethyl chloroformate (1.0 c.c.) was added to cytidine-2'(3') phosphate (1.615 g.) and tri-*n*-butylamine (3.6 c.c.) in water (15 c.c.). The mixture was shaken vigorously for 5 min., then solvent was removed under reduced pressure and the residue dried by repeated evaporation with dioxan. The tri-*n*-butylammonium cytidine-2',3' cyclic phosphate was dissolved in dimethylformamide (15 c.c.) and dioxan (15 c.c.), redistilled acetic anhydride (0.5 c.c., 1.1 mol.) was added, followed by tri-*n*-butylamine (2.25 c.c.), and the solution kept at room temperature for 24 hr. Solvent was removed under reduced pressure, ether (75 c.c.) added to the residue with shaking, and the precipitated tri-*n*-butylammonium *N*⁶-acetylcytidine-2',3' cyclic phosphate dried and dissolved in dimethylformamide (1.5 c.c.) and dioxan (10 c.c.). To this solution were added diphenyl phosphorochloridate (1.5 c.c.) and tri-*n*-butylamine (3.0 c.c.), and the mixture was kept at room temperature for 6 hr., then at 0° overnight. Solvent was removed under reduced pressure, ether (75 c.c.) added to the residue, and the precipitated polymer dissolved in water (45 c.c.). Ammonia was added to pH 9.6 and the solution kept at 37° for 18 hr. The solution was then concentrated to small volume, and ethanol and hydrochloric acid were added to precipitate the *polycytidylic acid* (1.46 g.) (Found, in material dried at 110°/10⁻³ mm.: N, 13.4; P, 9.9. [C₉H₁₂O₇N₃P]_x requires N, 13.8; P, 10.2%).

No significant change in the spectrophotometrically determined (at 290 mμ), apparent p*K*_α (4.3) was observed on polymerisation of cytidylic acid.

Polycytidylic Acids of Low Molecular Weight.—A solution of tri-*n*-butylammonium *N*⁶-acetylcytidine-2',3' cyclic phosphate [from 320 mg. of cytidine-2'(3') phosphate] in dimethylformamide (1 c.c.) and dioxan (8 c.c.) was treated with diphenyl phosphorochloridate (0.3 c.c.) and tri-*n*-butylamine (0.6 c.c.) at room temperature for 3 hr., and the product isolated as previously described¹ (220 mg.).

* Confirmatory evidence is provided by the photochemical behaviour of the synthetic polyuridylic acids (D. Shugar, personal communication) and by the ultraviolet absorption changes induced in the synthetic polyadenylic acids by interaction with mercuric chloride (C. A. Dekker, personal communication).

Poly ACGU.—Equimolar quantities of the tri-*n*-butylammonium salts of adenosine-2',3' cyclic phosphate, guanosine-2',3' cyclic phosphate, uridine-2',3' cyclic phosphate, and *N*⁶-acetylcytidine-2',3' cyclic phosphate [from 323 mg. of cytidine-2'(3') phosphate] were dissolved in dimethylformamide (2 c.c.) and dioxan (10 c.c.) and treated with diphenyl phosphorochloridate (1.2 c.c.) and tri-*n*-butylamine (2.4 c.c.) in the usual way. The final product was dissolved in water, ammonia was added to pH 9.6, and the solution was kept at 37° for 18 hr. to remove acetyl groups. The copolymer was isolated in the normal manner, as the free acid (1.17 g.).

Countercurrent Distribution (By Dr. K. S. KIRBY).—A two-phase system of Cellosolve-2-methoxyethanol-2-ethoxyethanol (2 : 8 : 3) (16 parts by volume), water (25 parts) and phosphate buffer (25 parts) was used with 96 transfers in each case. The phosphate buffer used was aqueous dipotassium hydrogen phosphate (*d* 1.32)–33% (v/v) phosphoric acid (50 : 3). With this system, countercurrent distribution of adenosine-3' phosphate, cytidine-3' phosphate, guanosine-3' phosphate, uridine-3' phosphate, and 5-(3'-adenylyl)cytidine-3' phosphate (A3'P5'C3'P) gave curves in good agreement with those determined theoretically, with peaks at tube numbers 51, 39, 42, 42, and 52 respectively.

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

	<i>R_F</i>			<i>R_F</i>	
	<i>A</i>	<i>B</i>		<i>A</i>	<i>B</i>
Cytidine-2'(3') phosphate	0.16	0.41	CpCpC	0.12	0.13
CpCp	0.04	0.23	CpCpCpC	0.06	0.06
CpCpCp	0.01	0.12	Polycytidylic acid	0.0	0.0–0.1
CpCpCpCp	0.0	0.03	Pseudouridine-2'(3') phosphate	0.13	0.32
CpC	0.32	0.29	Polypseudouridylic acid	0.0	0.0

Paper Electrophoresis.—Movements towards the anode on Whatman No. 1 paper at 10 v/cm. for 2 hr. in (I) *m*/50-*Na*₂HPO₄, and (II) *m*/50-KH₂PO₄, respectively were: pseudouridylic acid 10.3, 7.9; polypseudouridylic acid 11.4, 10.1; cytidylic acid 10.5, 8.3; polycytidylic acid 11.3, 9.3; "stable" polycytidylic acid 7.7–10.5, 3.3–7.6; polyguanylic acid 9.0; mixture of polyguanylic and polycytidylic acid 10.5 cm.

General Method for the Preparation of Nucleoside-2',3' Cyclic Phosphates.—To a solution of the nucleoside-2'(3') phosphate (1 mmole) in water (3 c.c.) was added tri-*n*-butylamine (0.72 c.c., 5 mmoles), followed by ethyl chloroformate (0.19 c.c., 2 mmoles), and the mixture was shaken vigorously for 5–10 min. Solvent was then removed under reduced pressure and the residue dried by evaporation with ethanol. The nucleotide was isolated by precipitation with ether or as the barium salt on addition of a methanolic solution of barium bromide. Identity with authentic specimens was established by paper chromatography, paper electrophoresis, and behaviour towards acid, alkali, and ribonuclease. For biochemical purposes the tri-*n*-butylamine in the above preparation may be replaced by triethylamine or sodium hydrogen carbonate (Dr. W. F. Hemmens). Replacement of the nucleotide by β-glycerophosphate or 2-hydroxyethyl phosphate gave quantitative yields of the corresponding cyclic phosphates.

Isolation of Natural Oligonucleotides from Yeast Ribonucleic Acid.—Yeast ribonucleic acid (50 g.) was exhaustively treated with ribonuclease and the mixture then dialysed against water. The dialysate, containing largely mononucleotides, dinucleotides, and trinucleotides, was fractionated on Dowex 1 × 2 (chloride form) as described by Volkin and Cohn.⁶ Appropriate fractions were combined and refractionated on smaller columns with the eluting agents as shown:

A3'P5'A3'P5'C3'P	0.01 <i>N</i> -hydrochloric acid, 0.0125 <i>M</i> -lithium chloride
G3'P5'C3'P	0.01 <i>N</i> -hydrochloric acid, 0.0125 <i>M</i> -lithium chloride
A3'P5'U3'P	0.01 <i>N</i> -hydrochloric acid, 0.025 <i>M</i> -lithium chloride

Each compound was isolated as the free acid by precipitation with ethanol and hydrochloric acid, identified by the usual methods, and further characterised by treatment with prostatic monoesterase to remove the terminal phosphate. Purified monoesterase (4 mg.) was added to a solution of the substrate (400 mg.) in citrate buffer (40 c.c.) at pH 5.3 and the mixture incubated at 37° for 9 hr. The solution was then diluted to 100 c.c., adjusted to pH 9, and run on to a column of Dowex 1 × 2 (formate form). Elution with dilute formic acid followed by evaporation of the appropriate fractions to dryness under reduced pressure gave the free acids, which were precipitated from concentrated aqueous solution by the addition of ethanol. The normality of formic acid used for removal of the various compounds from the Dowex 1 × 2 resin was:

A3'P5'C (0.015); G3'P5'C (0.04); A3'P5'U (0.05); A3'P5'A3'P5'C (0.075). The G3'P5'C crystallised from water as clusters of long thin needles.

Analyses are tabulated.

Compound	Found		Formula	Required	
	N (%)	P (%)		N (%)	P (%)
A3'P5'A3'P5'C3'P	18.8	9.4	C ₂₈ H ₃₈ O ₂₀ N ₁₃ P ₃	18.6	9.5
A3'P5'A3'P5'C	20.0	7.0	C ₂₉ H ₃₇ O ₁₇ N ₁₃ P ₂	20.2	6.9
G3'P5'C3'P	16.4	9.3	C ₁₉ H ₂₆ O ₁₅ N ₈ P ₂	16.8	9.3
G3'P5'C	18.9	5.3	C ₁₉ H ₂₅ O ₁₂ N ₈ P	19.0	5.3
A3'P5'U3'P	15.2	9.4	C ₁₉ H ₂₅ O ₁₅ N ₇ P ₂	15.0	9.5
A3'P5'U	16.9	5.3	C ₁₉ H ₂₄ O ₁₂ N ₇ P	17.1	5.4
A3'P5'C	19.6	5.4	C ₁₉ H ₂₅ O ₁₁ N ₈ P	19.6	5.4

Apparent p*K*_a's were spectrophotometrically determined at the wavelengths indicated: G3'P5'C3'P, 4.4 (280 mμ), 9.65 (265 mμ); G3'P5'C, 4.15 (280 mμ), 9.6 (265 mμ); A3'P5'C3'P, 4.25 (290 mμ); A3'P5'C, 4.15 (290 mμ).

5'-(3'-Adenylyl)adenosine (*Natural*).—Stepwise degradation of A3'P5'A3'P5'C3'P by prostatic monoesterase and periodate oxidation¹¹ gave diadenylic acid. The terminal phosphate was removed and the product purified by ion-exchange chromatography on Dowex 1 (formate form) with 0.025*N*-formic acid as eluting agent. 5'-(3'-Adenylyl)adenosine crystallised from water as rosettes of needles, softening at 184—186°, decomp. 204° (Found, in material dried at 100°/1 mm. for 24 hr.: N, 23.1; P, 5.2. C₂₀H₂₅O₁₀N₁₀P requires N, 23.5; P, 5.2%).

Poly (AU).—Ethyl chloroformate (0.08 c.c.) was shaken with tri-*n*-butylamine (0.30 c.c.) and 5'-(3'-adenylyl)uridine-3' phosphate (135 mg., 0.2 mmole) in water (1.5 c.c.), for 10 min. Solvent was then removed under reduced pressure, and the residue dried by evaporation with dimethylformamide and kept overnight at room temperature at 10⁻² mm. over phosphoric oxide. A solution of this tri-*n*-butylammonium adenylyluridine cyclic phosphate in dimethylformamide (1 c.c.) and dioxan (0.5 c.c.) was treated with tri-*n*-butylamine (0.2 c.c.) and diphenyl phosphorochloridate (0.1 c.c.) at room temperature for 6 hr. Solvent was removed under reduced pressure, and the polymer precipitated with ether and worked up as a calcium salt in the usual way (130 mg.). Paper chromatography showed unchanged dimer, tetramer, and higher oligonucleotides and traces of monomer and trimer.

Poly (AC) and Poly (GC).—The dinucleotide (0.2 mmole) was first treated with ethyl chloroformate as described above. To a solution of the anhydrous product in dimethylformamide (3 c.c.) and dioxan (1 c.c.) were then added tri-*n*-butylamine (0.1 c.c.) and acetic anhydride (0.022 c.c., 0.22 mmole), and the solution was kept at room temperature for 36 hr. Solvent was removed under reduced pressure and the residue shaken with cyclohexane (5 c.c.), then with ether (5 c.c.). The precipitated material was dried, then dissolved in dimethylformamide (1 c.c.) and dioxan (0.5 c.c.) and treated with diphenyl phosphorochloridate (0.1 c.c.) and tri-*n*-butylamine (0.2 c.c.) at room temperature for 6 hr. Ether (10 c.c.) was added, and an aqueous solution of the precipitated polymer was kept at pH 9.8 at 37° for 12 hr. Precipitation with ethanol and hydrochloric acid gave the crude polymer mixture (100 mg.).

Poly (AAC).—The phosphate A3'P5'A3'P5'C3'P (110 mg., 0.1 mmole) was treated successively with ethyl chloroformate (0.05 c.c.), acetic anhydride (0.015 c.c.), and diphenyl phosphorochloridate (0.085 c.c.) as described for the polymerisation of 5'-(3'-adenylyl)cytidine-3' phosphate. The *product* was isolated in the usual way (99 mg.) (Found, in material dried at 100°/1 mm. for 24 hr.: N, 18.5; P, 9.3. [C₂₉H₃₆O₁₉N₁₃P₃]₂ requires N, 18.9; P, 9.7%).

Paper Chromatography.—Ascending chromatograms on Whatman No. 1 paper were used with solvent systems: *A*, ethanol-*m*-ammonium acetate (5 : 2); *B*, *t*-pentyl alcohol-formic acid-water (3 : 2 : 1). Results are tabulated as R_F's.

	<i>A</i>	<i>B</i>		<i>A</i>	<i>B</i>
A3'P5'A3'P5'C3'P	0.02	0.11	A3'P5'C3'P	0.06	0.22
A3'P5'A3'P5'C	0.08	0.14	A3'P5'C	0.25	0.27
A3'P5'A3'P	0.04	0.22	G3'P5'C3'P	0.04	0.16
A3'P5'A	0.23	0.30	G3'P5'C	0.20	0.22
A3'P	0.14	0.44	A3'P5'U3'P	0.06	0.24
Adenosine	0.60	0.64	A3'P5'U	0.31	0.29
N ⁶ -Acetyl (ApApC2',3'p) ...	—	0.19	N ⁶ -Acetyl (GpC2',3'P)	—	0.26
N ⁶ -Acetyl (ApC2',3'p)	—	0.32			

¹¹ Whitfield and Markham, *Nature*, 1953, **171**, 1151; Brown, Fried, and Todd, *J.*, 1955, 2206.

Paper Electrophoresis.—Movement (cm.) towards the anode is tabulated, for Whatman No. 1 paper, with (I) $m/50\text{-Na}_2\text{HPO}_4$ and (II) $m/50\text{-KH}_2\text{PO}_4$, both at 10 v/cm. for 2 hr.

	I	II		I	II
A3'P5'A3'P5'C3'P	10.1	8.3	A3'P5'C3'P	9.8	8.1
A3'P5'A3'P5'C	6.4	6.2	A3'P5'C	4.8	4.7
A3'P5'A3'P	9.9	8.1	G3'P5'C3'P	10.1	8.1
A3'P5'A	4.3	3.7	G3'P5'C	4.8	4.0
A3'P	9.3	6.9	A3'P5'U3'P	10.0	8.1
Poly (AAC)	11.2	9.2	A3'P5'U	5.2	4.9
Poly (AC)	10.5	8.9	Poly (AU)	10.8	9.3
Poly (GC)	10.6	8.5			

Acetylated Nucleoside-2',3' Cyclic Phosphates.—Ethyl chloroformate (0.2 c.c.) was shaken with a solution of the nucleotide (1 mmole) and tri-*n*-butylamine (0.75 c.c.) in water (3 c.c.) for 10 min., to prepare the 2',3'-cyclic phosphate. Solvent was removed under reduced pressure, and the anhydrous residue dissolved in dimethylformamide (3 c.c.) and dioxan (3 c.c.). Acetic anhydride (0.5 c.c.) and tri-*n*-butylamine (1.45 c.c.) were added and the solution was kept at room temperature for 48 hr.; paper chromatography then showed that acetylation was complete. Solvent was removed and anhydrous ether (50 c.c.) added to the residue. The precipitated tri-*n*-butylammonium 5'-*O*-acetylnucleoside-2',3' cyclic phosphate (N^6O^5' -diacetyl in the case of cytidylic acid) was washed with ether by decantation and dried. Paper chromatography showed that the product was homogeneous with respect to ultraviolet-absorbing material. Yields were quantitative. Absorption maxima were as tabulated.

2',3'-Cyclic phosphates	In 0.04N-HCl			In water (pH 7)			In 0.04N-NaOH		
	$\lambda_{\text{max.}}$ ($m\mu$)	$\lambda_{\text{min.}}$ ($m\mu$)	280/ 260 $m\mu$	$\lambda_{\text{max.}}$ ($m\mu$)	$\lambda_{\text{min.}}$ ($m\mu$)	280/ 260 $m\mu$	$\lambda_{\text{max.}}$ ($m\mu$)	$\lambda_{\text{min.}}$ ($m\mu$)	280/ 260 $m\mu$
N^6 -Acetylcytidine	244, 302	225, 270	0.92	246, 295	225, 271	0.60	298	237	1.51
N^6O^5' -Diacetylcytidine	244, 302	225, 270	0.90	246, 295	225, 271	0.60	298	237	1.45
5'- <i>O</i> -Acetyladenosine...	256	230	0.31	258	228	0.16	258	230	0.22
5'- <i>O</i> -Acetylguanosine...	255	227	0.71	252	222	0.71	261	231	0.63
5'- <i>O</i> -Acetylruridine ...	258	228	0.22	257	228	0.21	259	242	0.30

Dinucleoside(-3', -5') Phosphates.—A solution of tri-*n*-butylammonium 5'-*O*-acetyl-nucleoside-2',3' cyclic phosphate [from 1 mmole of the 2'(or 3') phosphate] in dioxan (4 c.c.) [in the case of guanylic acid a mixture of dimethylformamide (2 c.c.) and dioxan (2 c.c.) was used] was added to the anhydrous 2',3'-di-*O*-acetyl-nucleoside (1 mmole). Diphenyl phosphorochloridate (0.3 c.c.) was then added, followed by tri-*n*-butylamine (0.6 c.c.), and the clear solution was kept at room temperature under anhydrous conditions for 18 hr. Solvent was removed under reduced pressure and ether (50 c.c.) added to the residue with shaking. The precipitated material was dried, then dissolved in water (10 c.c.), ammonia being added to pH 9.8, and the solution was extracted twice with ether, the extracts being discarded. The aqueous solution was kept at pH 9.8 at 37° for 48 hr., then diluted and run on a column (12 × 1.2 cm.) of Dowex 1 (formate) resin which was then washed with water; the dinucleoside phosphate was eluted with dilute formic acid. Appropriate fractions were combined and evaporated to dryness under reduced pressure. The residue was dissolved in water, then ethanol or acetone was added to give the dinucleoside phosphate as the free acid, which in a number of cases readily crystallised from water or aqueous ethanol.

Yields of dinucleoside phosphate isolated in pure solid form were: ApA, 53%; CpA, 60%; GpA, 40%; UpA, 58%; ApU, 60%; CpU, 65%; from equimolar proportions of phosphate and diacetyl-nucleoside. These yields could be improved relative to the nucleoside by using an excess of the acetylated nucleoside cyclic phosphate.

The apparent pK_a of 5-(2'-cytidilyl)adenosine was 4.35, and of 5'-(3'-cytidilyl)adenosine 4.25, both determined spectrophotometrically at 290 $m\mu$.

Trinucleoside Diphosphates.—A solution of the tri-*n*-butylammonium dinucleoside phosphate (0.5 mmole) and tri-*n*-butylammonium 5'-*O*-acetyl-nucleoside-2',3' cyclic phosphate [or in the case of cytidylic acid the N^6O^5' -diacetyl derivative] (1 mmole) in dimethylformamide (2 c.c.) and dioxan (1.0 c.c.) was treated with diphenyl phosphorochloridate (0.45 c.c.) and tri-*n*-butylamine (0.90 c.c.) at room temperature for 12 hr. Solvent was removed under reduced pressure, ether (75 c.c.) added to the residue, and the precipitated material dissolved in water (10 c.c.). Ammonia was added to pH 9.8, amines were removed by extraction twice with ether,

and the aqueous solution was kept at pH 9.8 at 37° for 47 hr. The diluted solution, shown by paper chromatography to contain nucleotide, dinucleoside phosphate, and trinucleoside diphosphate, was run on a column (12 × 1.2 cm.) of Dowex 1 × 2 resin (chloride form), and the column was eluted with 0.01N-hydrochloric acid, then with lithium chloride in 0.01N-hydrochloric acid. Appropriate fractions were combined, neutralised with triethylamine, and concentrated to small volume under reduced pressure. Ethanol and dilute hydrochloric acid were then added, to precipitate the trinucleoside diphosphate as the free acid. The material was collected by centrifugation, washed with ethanol, then ether, and dried. Yields ranged from 20 to 35% or, after allowance for recovered material, 30–60% based on the dinucleoside phosphate used.

Tetranucleoside Triphosphates from Trinucleoside Diphosphates.—A solution of tri-n-butylammonium trinucleoside diphosphate (0.03 mmole) and tri-n-butylammonium N⁶O⁶-diacetyl-nucleoside-2',3' cyclic phosphate (0.12 mmole) in dimethylformamide (0.2 c.c.) and dioxan (0.4 c.c.) was treated with diphenyl phosphorochloridate (0.06 c.c.) and tri-n-butylamine (0.12 c.c.) at room temperature for 4 hr., then worked up in the usual way. Acetyl groups were removed at pH 9.8 and the crude products purified by ion-exchange chromatography on Dowex 1 resin (chloride form). Appropriate fractions were combined, neutralised with triethylamine, and evaporated to small volume under reduced pressure. Addition of ethanol and dilute hydrochloric acid precipitated the tetranucleoside triphosphates as fine white powders which were collected by centrifugation, washed with ethanol, then ether, and dried. The yields of isolated material were ~40%. As unchanged material was recovered, the yield based on the trinucleoside diphosphate used was 75–80%.

Tetranucleoside Triphosphates from Dinucleoside Phosphates.—Ethyl chloroformate (0.08 c.c.) was shaken with the dinucleotide (0.2 mmole) and tri-n-butylamine (0.3 c.c.) in water (1.5 c.c.) for 10 min. Solvent was removed and the anhydrous residue dissolved in dimethylformamide (3 c.c.) and dioxan (1 c.c.). Acetic anhydride (0.15 c.c.) and tri-n-butylamine (0.45 c.c.) were added and the solution was kept at room temperature under anhydrous conditions for 48 hr. Solvent was removed under reduced pressure and ether (10 c.c.) added to precipitate the acetylated dinucleotide containing a terminal cyclic phosphate. This product was dried, dissolved in dimethylformamide (1 c.c.) and dioxan (0.5 c.c.), and added to the tri-n-butylammonium dinucleoside phosphate (0.1 mmole). To the clear solution were added diphenyl phosphorochloridate (0.15 c.c.) and tri-n-butylamine (0.3 c.c.), and the mixture was kept at room temperature for 6 hr., then worked up as previously described. Acetyl groups were removed at pH 9.8 at 37° (48 hr.) and the crude tetranucleoside triphosphate purified by ion-exchange chromatography.

In the case of dinucleoside phosphates containing cytosine it was necessary to protect the 6-amino-group by partial acetylation in aqueous dimethylformamide, before reaction of the dinucleotide and diphenyl phosphorochloridate.

Yields of isolated material ranged from 25 to 50%; allowance for unchanged materials recovered from the ion-exchange fractionation increased the yields to 50–75% based on dinucleoside phosphate used.

Ion-exchange Chromatography.—For purification and separation of the various nucleotide derivatives ion-exchange chromatography on columns of Dowex 1 × 2 resin, both formate and chloride form, was used, with the eluting agents tabulated for the respective compounds.

	Normality of H-CO ₂ H used to elute compound from resin (formate form)	Molarity of LiCl in 0.01N-HCl used to elute compound after washing with 0.01N-HCl
ApA	0.025	UpGpA
CpA	0.01	GpUpA
GpA	0.09	GpApU
UpA	0.05	CpUpGpA
ApU	0.05	CpGpUpA
CpU	0.025	CpGpApU
CpApU	0.15	ApUpGpC
CpUpA	0.15	GpCpApU
		GpCpUpA

Separation of U2'(3')P5'A from A3'P5'U was obtained on Dowex 1 resin (formate form), the peaks being at fraction numbers 44 and 60 respectively.

Where necessary the oligonucleotide was further purified by paper chromatography before spectroscopic examination; some degradation appeared to occur during the working up of

the higher polymers, possibly because of the high salt concentration on evaporation to small volume and the acidic conditions used for precipitation.

Co-polymer Terminating with 5'-[2'(3')-Cytidylyl]adenosine.—Adenosine-2'(3') phosphate (116 mg.), guanosine-2'(3') phosphate (121 mg.), uridine-2'(3') phosphate (88 mg.), and pseudo-uridine-2'(3') phosphate (20 mg.) were added to water (3 c.c.) and tri-n-butylamine (0.75 c.c.) and treated with ethyl chloroformate (0.2 c.c.) at room temperature for 10 min. Solvent was removed, the anhydrous residue dissolved in dimethylformamide (1.5 c.c.) and dioxan (2 c.c.) and added to *N*⁶-acetylcytidine-2',3' cyclic phosphate (from 108 mg. of cytidylic acid) and 5'-[*N*⁶-acetyl-2'(3')-cytidylyl]adenosine (from monoacetylation of 200 mg. of the dinucleoside phosphate with 0.04 c.c. of acetic anhydride). The mixture was treated with diphenyl phosphorochloridate (0.5 c.c.) and tri-n-butylamine (1 c.c.) at room temperature for 4 hr. and worked up in the normal way, acetyl groups being removed at pH 9.8. The crude polymer mixture was dialysed against 2*M*-sodium chloride and isolated as the free acid (120 mg.). Light absorption: in 0.01*N*-HCl, λ_{\max} 264, $m\mu$, λ_{\min} 233 $m\mu$, density ratio 280/260 $m\mu$ 0.76; in 0.1*N*-NaOH, λ_{\max} 262 $m\mu$, λ_{\min} 233 $m\mu$, density ratio 280/260 $m\mu$ 0.52. Alkaline hyperchromicity, 13.3%.

Degradation of Nucleotide Derivatives.—(a) *With ribonuclease.* Ribonuclease (0.05 c.c. of a solution containing 2 mg. of 5 times recrystallised ribonuclease per c.c.) was added to the substrate (1—1.5 mg.) in water (0.15 c.c.) and glycine buffer (0.05 c.c.; pH 7), and the mixture was incubated at 37° for 48 hr.

(b) *With rattlesnake (Crotalus atrox) venom.* Venom (0.05 c.c. of a solution containing 10 mg./c.c.) was added to the substrate (1—1.5 mg.) in water (0.15 c.c.) and glycine buffer (0.05 c.c.; pH 9), and the mixture was incubated at 37° for 48 hr.

(c) *With alkali.* The material (~1 mg.) was treated with 0.1*N*-sodium hydroxide at 37° for 48 hr.

Products were identified by paper chromatography in solvents A and B and in the propan-2-ol-hydrochloric acid solvent described by Wyatt.¹²

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

	<i>A</i>	<i>B</i>		<i>A</i>	<i>B</i>
A2'(3')P	0.14	0.44	A2'P5'A	0.23	0.30
C2'(3')P	0.16	0.41	A3'P5'A	0.24	0.30
G2'(3')P	0.10	0.33	A3'P5'A (natural)	0.24	0.30
U2'(3')P	0.21	0.43	C2'P5'A	0.27	0.29
ψ U2'(3')P	0.13	0.32	C3'P5'A	0.28	0.30
			A3'P5'C (natural)	0.25	0.27
U2'(3')P5'A	0.33	0.30	A2'P5'U	0.30	0.29
C2'(3')P5'U	0.33	0.28	A3'P5'U	0.31	0.29
G2'P5'A	0.20	0.26	A3'P5'U (natural)	0.31	0.29
G3'P5'A	0.20	0.24	A5'P5'U	0.26	0.24

In the following compounds *p* signifies a phosphate linkage from the 2'(or 3')-hydroxyl of the nucleoside on the left to a 5'-hydroxyl of the nucleoside on the right.

UpGpA	0.10	0.12	CpUpGpA	0.03	0.06
GpApU	0.09	0.12	CpGpApU	0.03	0.07
GpUpA	0.08	0.12	CpGpUpA	0.03	0.06
CpApU	0.12	0.14	GpCpApU	0.03	0.07
CpUpA	0.13	0.14	GpCpUpA	0.03	0.06
			ApUpGpC	0.02	0.05

2',3'-Cyclic phosphates

Adenosine	0.41	0.44
5'- <i>O</i> -Acetyladenosine	0.60	0.53
Cytidine	0.43	0.41
<i>N</i> ⁶ -Acetylcytidine	0.54	0.50
<i>N</i> ⁶ <i>O</i> ^{6'} -Diacylcytidine	0.68	0.58
Guanosine	0.37	0.33
5'- <i>O</i> -Acetylguanosine	0.53	0.46
Uridine	0.47	0.43
5'- <i>O</i> -Acetyluridine	0.62	0.56
5'-[<i>N</i> ⁶ -Acetyl-2'(3')-cytidylyl]adenosine	—	0.36

¹² Wyatt, *Biochem. J.*, 1951, **48**, 584.

Paper Electrophoresis.—Movement (cm.) towards the anode is tabulated, for Whatman No. 1 paper, with (I) M/50-Na₂HPO₄ (pH 8.9) and (II) M/50-KH₂PO₄ (pH 4.5), both at 10 v/cm. for 2 hr.

	I	II		I	II
A2'(3')P	9.3	6.9	A2'P5'A	5.0	4.5
C2'(3')P	10.5	8.2	A3'P5'A	4.3	3.7
G2'(3')P	9.6	7.4	A3'P5'A (natural)	4.3	3.7
U2'(3')P	11.1	8.8	C2'P5'A	5.4	4.7
ψU2'(3')P	10.8	8.0	C3'P5'A	4.8	4.6
			A3'P5'C (natural)	4.8	4.7
U2'(3')P5'A	5.7	5.0	A2'P5'U	5.3	6.0
C2'(3')P5'U	6.5	5.9	A3'P5'U	5.2	4.9
G2'P5'A	5.5	5.0	A3'P5'U (natural)	5.2	4.9
G3'P5'A	5.0	4.3	A5'P5'U	5.6	5.7
UpGpA	6.9	7.6	CpUpGpA	8.7	7.9
GpApU	6.9	7.3	CpGpApU	8.9	8.5
GpUpA	6.5	6.8	CpGpUpA	8.5	6.9
CpApU	7.7	7.6	GpCpApU	8.7	8.0
CpUpA	7.2	7.3	GpCpUpA	8.3	7.6
			ApUpGpC	8.3	7.6
2',3'-Cyclic phosphates			I	II	
Adenosine			5.3	6.9	
5'-O-Acetyladenosine			5.1	6.8	
Cytidine			6.3	8.2	
N ⁶ -Acetylcytidine			6.1	8.1	
N ⁶ O ^{5'} -Diacetylcytidine			5.6	7.3	
Guanosine			5.7	7.4	
5'-O-Acetylguanosine			5.3	7.4	
Uridine			6.8	8.8	
5'-O-Acetyluridine			6.5	8.0	

Analyses.—The free acids were dried at 110°/10⁻³ mm. for 24 hr. (see Table).

Compound	Found			Required	
	N (%)	P (%)		N (%)	P (%)
U2'(3')P5'A	16.9	5.3	C ₁₉ H ₂₄ O ₁₂ N ₇ P	17.1	5.4
A2'P5'U	17.2	5.4	C ₁₉ H ₂₄ O ₁₂ N ₇ P	17.1	5.4
A3'P5'U	16.8	5.6	C ₁₉ H ₂₄ O ₁₂ N ₇ P	17.1	5.4
C2'(3')P5'U	12.5	5.7	C ₁₈ H ₂₄ O ₁₁ N ₆ P	12.7	5.6
G2'P5'A	23.0	4.9	C ₂₀ H ₂₅ O ₁₁ N ₁₀ P	22.9	5.1
G3'P5'A	22.7	5.0	C ₂₀ H ₂₅ O ₁₁ N ₁₀ P	22.9	5.1
A2'P5'A	23.2	5.2	C ₂₀ H ₂₅ O ₁₀ N ₁₀ P	23.5	5.2
A3'P5'A	23.3	5.2	C ₂₀ H ₂₅ O ₁₀ N ₁₀ P	23.5	5.2
C2'P5'A	19.7	5.4	C ₁₉ H ₂₅ O ₁₁ N ₈ P	19.6	5.4
C3'P5'A	19.4	5.6	C ₁₉ H ₂₅ O ₁₁ N ₈ P	19.6	5.4
UpGpA	18.0	6.8	C ₂₉ H ₃₆ O ₁₉ N ₁₂ P ₂	18.3	6.8
GpApU	—	7.0	C ₂₉ H ₃₆ O ₁₉ N ₁₂ P ₂	18.3	6.8
GpUpA	—	7.1	C ₂₉ H ₃₆ O ₁₉ N ₁₂ P ₂	18.3	6.8
CpApU	—	7.3	C ₂₈ H ₃₆ O ₁₉ N ₁₀ P ₂	15.9	7.1
CpUpA	—	7.1	C ₂₈ H ₃₆ O ₁₉ N ₁₀ P ₂	15.9	7.1
CpUpGpA	—	7.7	C ₃₈ H ₄₈ O ₂₆ N ₁₆ P ₃	17.2	7.6
CpGpApU	—	7.4	C ₃₈ H ₄₈ O ₂₆ N ₁₆ P ₃	17.2	7.6
CpGpUpA	—	7.8	C ₃₈ H ₄₈ O ₂₆ N ₁₆ P ₃	17.2	7.6
GpCpApU	—	7.5	C ₃₈ H ₄₈ O ₂₆ N ₁₆ P ₃	17.2	7.6
GpCpUpA	—	7.6	C ₃₈ H ₄₈ O ₂₆ N ₁₆ P ₃	17.2	7.6
ApUpGpC	—	7.7	C ₃₈ H ₄₈ O ₂₆ N ₁₆ P ₃	17.2	7.6

When recrystallised from water, C2'P5'A and G2'P5'A formed long thin needles; A2'P5'A and A2'P5'U formed monoclinic prisms; and A3'P5'A formed rosettes of small needles. As means of characterisation m. p. were unsatisfactory, all of the compounds softening and darkening at 190—200° and decomposing at 200—220°; nor were mixed m. p.s valid for identification.

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