Nucleotide Exchange Reactions Catalysed by Ribonuclease and Spleen Phosphodiesterase

2. SYNTHESIS OF POLYNUCLEOTIDES

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In the preceding paper (Heppel & Whitfeld, 1955) evidence was presented that at least two different phosphodiesterases, namely ribonuclease and spleen phosphodiesterase, can catalyse synthetic and exchange reactions involving nucleotide residues. The simple alcohols, methanol and ethanol, were shown to be capable of acting as acceptors in these reactions in which nucleoside alkyl phosphates are formed from the corresponding benzyl phosphate and an alcohol, and also, in the case of ribonuclease only, from pyrimidine nucleoside-2′:3′ phosphates (pyrimidine cyclic nucleotides) and an alcohol.

The possibility of compounds other than simple alcohols acting as acceptors in this type of reaction has now been examined. It will be demonstrated that cyclic nucleotides and ribonucleosides can act as acceptors for donor nucleotide residues in the presence of ribonuclease to form compounds such as cyclic dinucleotides, cyclic trinucleotides, dinucleoside phosphates and trinucleoside diphosphates. The spleen enzyme catalyses analogous reactions with the exception of those involving nucleoside-2′:3′ cyclic phosphates, which do not participate in spleen phosphodiesterase-catalysed transfer reactions. Furthermore, in these reactions both enzymes exhibit the same substrate specificity as they do in their hydrolytic reactions.

A preliminary report of part of this work has already been published (Heppel, Whitfeld & Markham, 1954).

Nomenclature

For the sake of convenience, a shorthand notation will be used to denote small ribopolynucleotides and their derivatives. It is slightly different from that which has been used previously in this *Journal* (cf. Markham & Smith, 1952*b*), but offers certain advantages in the present work. The four ribonucleosides will be represented by A (adenosine), C (cytidine), G (guanosine) and U (uridine). A phosphate group will be denoted by p and when written to the right of the nucleoside is esterified to the hydroxyl group of either $C_{(2')}$ or $C_{(3')}$. (If the phosphate residue is involved in an internucleotide bond it is esterified to the $C_{(3')}$-hydroxyl group exclusively.) If the p is written to the left of the nucleoside it is esterified to the hydroxyl group of $C_{(5')}$. An exclamation mark after the terminal p denotes a 2′:3′ (cyclic) phosphate (cf. Markham & Smith, 1952*b*). Thus, Cp! is cytidine-2′:3′ phosphate or cyclic cytidylic acid. The symbol CpA represents a dinucleoside monophosphate in which the phosphate group is linked to $C_{(5')}$ of adenosine and to $C_{(3')}$ of cytidine. CpCp! represents a cyclic dicytidylic acid (Fig. 1).

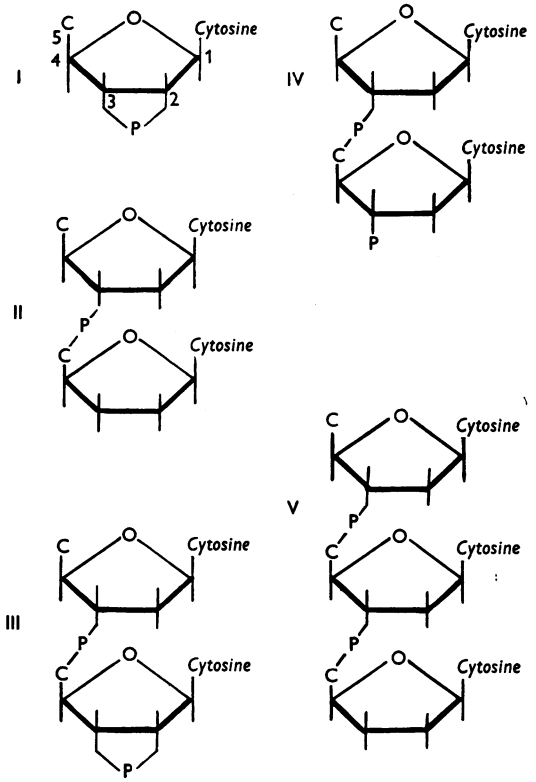

Fig. 1. (I) Cytidine-2′:3′ phosphate (cyclic cytidylic acid, Cp!), (II) Dicytidine phosphate (cytidylyl-cytidine, CpC), (III) Cyclic dicytidylic acid (CpCp!), (IV) Dicytidylic acid (CpCp), (V) Tricytidine diphosphate (CpCpC).

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MATERIALS AND METHODS

Materials and methods were as in the preceding paper with the following additions. Cytidine, uridine, adenosine, yeast cytidylic and uridylic acids were commercial preparations, none of which showed on paper chromatography any more than a trace of ultraviolet-absorbing impurity. Thymidine was a gift from Dr A. M. Michelson, and purine riboside was kindly supplied by Dr G. B. Brown. Various dinucleotides were isolated from ribonuclease digests of ribonucleic acid (RNA) by ion-exchange chromatography, according to the method of Volkin & Cohn (1953). Dinucleoside monophosphates were obtained by dephosphorylating these dinucleotides with phosphomonoesterase purified from human prostate glands (Markham & Smith, 1952*b*).

Pancreatic ribonuclease and spleen phosphodiesterase were the same as in the preceding paper. Purified snake venom phosphodiesterase, free of 5′-nucleotidase activity, was a gift from Dr L. Astrachan.

Chromatographic solvents. For convenience we list again the solvent systems discussed in the preceding paper. *Solvent* 1: *iso*propanol–water (70:30, v/v) with NH_3 in the vapour phase. *Solvent* 2: saturated $(NH_4)_2SO_4$–*iso*propanol–M sodium acetate (80:2:18, v/v/v). *Solvent* 3: *iso*propanol, 170 ml.; conc. HCl, 44 ml.; water to 250 ml.

Procedure for the identification of the products from enzymic reactions

After the reaction mixture had been incubated for the desired period it was chromatographed in solvent 1. By comparison of the experimental chromatogram with control chromatograms the formation of new substances in the reaction could be detected. The latter were eluted and then identified by applying several of the tests which are described below.

Analysis by chemical hydrolysis

Treatment (*a*). Ribopolynucleotides when hydrolysed with N-HCl at 100° for 1 hr. give free purines and pyrimidine nucleotides which can be separated by chromatography in solvent 3, identified and estimated quantitatively (Smith & Markham, 1950).

Treatment (*b*). The cyclic phosphate bonds of nucleoside-2′:3′ phosphates are broken by mild acid hydrolysis (0·1 N-HCl, 20–25°, 4 hr.) to yield the monoester phosphate (Brown, Magrath & Todd, 1952), a change which is reflected by a decrease in the R_F value in solvent 1. Under the same conditions phosphodiester internucleotidic links are quite stable.

Treatment (*c*). Phosphodiester internucleotidic links in ribopolynucleotides are broken proximal to $C_{(5')}$ by alkaline hydrolysis (N-NaOH, 20°, 18 hr.) (Markham & Smith, 1951). The products may be identified, after neutralization of excess alkali, by paper electrophoresis at pH 3·5 or by chromatography in solvents 1 and 3.

Treatment (*d*). Susceptibility of nucleoside-containing compounds to oxidation by neutral periodate (excess 0·1 M-$NaIO_4$, 20°, 30 min.) shows that the hydroxyl groups on $C_{(2')}$ and $C_{(3')}$ of the terminal nucleoside residue are unsubstituted (Lythgoe & Todd, 1944). The oxidation product may be isolated by chromatography in solvent 1 and upon subsequent treatment at pH 10 at 37° for 18 hr. the oxidized nucleoside residue is split off to leave a (poly)nucleotide containing one less nucleoside residue than the original compound (Whitfeld, 1954).

Analysis by enzymic hydrolysis

Polynucleotides synthesized in the various enzymic reactions to be described in the Results were in turn degraded by the same enzyme using it at a much higher concentration for the purpose of hydrolysis.

Treatment (*e*). The polynucleotide (0·1–0·5 μmole) was incubated at 38° for 4 hr. with 20 μg. of ribonuclease in 0·03 ml. of 0·05 M phosphate buffer, pH 7·4 (total vol., 0·08 ml.). The products were identified by chromatography in solvent 1 or solvent 2 and estimated quantitatively by elution in 0·1 N-HCl and measurement of the optical density of the eluate at 260 mμ.

Treatment (*f*). Dinucleoside monophosphates, synthesized by the action of spleen phosphodiesterase, were reincubated with the same enzyme, only in a much higher concentration (280 μg. of enzyme/μmole of substrate), in

Table 1. *Procedure for the identification of various cytidine-containing compounds by specific degradation methods*

The nomenclature is as given in the text. Subscripts are shown in certain cases to denote specific isomers of cytidylic acid when this information has significance. The substance at the column head is subjected to the treatment shown on the left and gives rise to the compound or compounds shown in the table. The products are isolated by chromatography in solvent 1. Further identification is then made by applying the next appropriate treatment or by subjecting the material to electrophoresis on paper. A similar scheme may be applied to other polynucleotides, with the exception that compounds containing purine nucleotide residues are unstable to treatment (*a*) and most of them will be unaffected by ribonuclease. Only results which have been obtained experimentally are recorded in this table. A — denotes that the treatment had no effect. A blank indicates that that particular reaction has not been carried out.

Treatment*	CpCpCp!	CpCpCp	CpCpC	CpCp!	CpCp	CpC	Cp!
(*a*) N-HCl, 100°, 1 hr.	Cp	Cp	2Cp + C	Cp	Cp	Cp + C	Cp
(*b*) 0·1 N-HCl, 20°, 4 hr.	CpCpCp	—	—	CpCp	—	—	Cp
(*c*) N-NaOH, 20°, 18 hr.	Cp	Cp	2Cp + C	Cp	Cp	Cp + C	Cp
(*d*) 0·1 M-$NaIO_4$, 20°, 30 min.	—	—	Oxidized	—	—	Oxidized	—
(*e*) Ribonuclease	Cp	Cp	2Cp + C	Cp	Cp	Cp + C	Cp
(*f*) Spleen phosphodiesterase	$2C_3p + C_2p$	Cp	2Cp + C	$C_3p + C_2p$	C_3p	$C_2p + C$	C_2p
(*g*) Whole snake venom			C	C + pCp		C	Cp
(*h*) Snake-venom phosphodiesterase			C + 2pC	C + pCp	—	C + pC	Cp
(*i*) Prostate phosphomonoesterase	—	CpCpC	—	—	CpC	—	—

* For experimental details of treatment see text.

0·1 M phosphate buffer, pH 7·4 (total vol., 0·08 ml.), for 6 hr. at 38°. Products were identified and estimated as in treatment (*e*).

Spleen phosphodiesterase was also used to detect the presence of nucleoside-2′:3′ cyclic phosphates. These compounds are hydrolysed by the enzyme to give nucleoside-2′ phosphates (Whitfeld, Heppel & Markham, 1955), whereas the internucleotidic links in ribopolynucleotides are broken by the same enzyme to give nucleoside-3′ phosphates (Heppel, Markham & Hilmoe, 1953). The 2′- and 3′- isomers of cytidylic, adenylic and guanylic acids were resolved by chromatography in solvent 2.

Treatment (*g*). Unfractionated snake (*Crotalus adamanteus*) venom, which contains a phosphodiesterase and a 5′-nucleotidase, was used to detect the presence of nucleoside-5′ phosphates and their esters. Incubation was for 6 hr. at 37° in 0·1 M glycine buffer, pH 8·6 (100 μg. of venom/μmole of nucleotide) and the products were separated by chromatography in solvent 1.

Treatment (*h*). Pure snake-venom phosphodiesterase (5 hr., 37°, pH 9·6, 200 μg. of enzyme/μmole of nucleotide) was used to detect $C_{(3')}$–$C_{(5')}$ internucleotidic links which it splits to produce nucleosides and nucleoside-5′ phosphates which can then be isolated by chromatography in solvent 1.

Treatment (*i*). Prostate phosphomonoesterase was used to determine phosphomonoester groups. The conditions of incubation have been given by Markham & Smith (1952*c*). The R_F value of the compound in solvent 1 is increased considerably by removal of the terminal phosphate group.

In the case of the di- and tri-nucleotides, identification often depended upon the successive application of two or three of the preceding reactions. The product of each reaction was isolated by chromatography in solvent 1 before the next reaction was carried out.

Table 1 shows the results of the application of these various treatments to all the cytidine-containing compounds encountered in this investigation.

Phosphorus analyses were made by the method of Allen (1940).

RESULTS

Synthesis of polynucleotides and related substances from pyrimidine cyclic mononucleotides by ribonuclease

When cytidine-2′:3′ phosphate in low concentrations (about 0·02 M) was incubated with ribonuclease it was hydrolysed to cytidine-3′ phosphate, and no other compounds were detected by paper chromatography during this hydrolysis. If, however, cytidine (0·1 M) was also present in the solution, another substance in addition to the mononucleotide was formed (Fig. 2). This new substance, which had an R_F value in solvent 1 similar to that of dinucleoside monophosphates containing adenine, cytosine or uracil (Table 2), was identified as cytidylyl-cytidine (CpC) (see below).

Further experiments were carried out in which the relative concentrations of both the cytidine-2′:3′ phosphate and the cytidine and also the time and temperature of the incubation were varied. With high concentrations of both substances (> 0·1 M) ribonuclease catalysed the formation of at least four other discrete compounds in addition to CpC and cytidylic acid (Fig. 3). These various products were separated by chromatography in solvent 1, and enough of each compound was isolated by combining several incubations to permit its identification by the series of tests indicated below. The substances which have been identified as

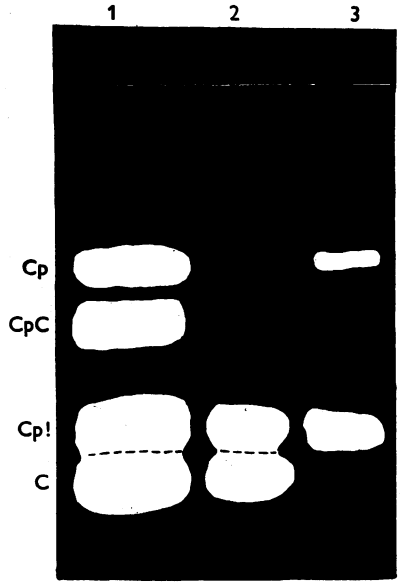


Fig. 2. Synthesis of dinucleoside monophosphate from cyclic cytidylic acid and cytidine by ribonuclease. Experimental conditions are as given in Table 3, Expt. 1. Descending chromatogram run in solvent 1. Col. 1, complete system; Col. 2, no enzyme; Col. 3, no cytidine.

Table 2. *The chromatographic and electrophoretic properties of mono-, di- and tri-nucleotides containing cytidine*

R_C is the ratio of the distance moved by the substance in solvent 1 to the distance moved by cytidine-3′ phosphate. Abbreviations are as given in the text.

Compound	R_C	Electrophoretic mobility at pH 3·5 and 20v/cm. (cm./2 hr.)
C	2·7	−6·0
Cp	1·0	6·5
Cp!	2·6	7·0
CpCp	0·55	8·5
CpCp!	1·2	9·1
CpCpCp	0·15	9·7
CpCpCp!	0·55	10·2
CpC	1·7	1·0
CpCpC	0·8	4·2
ApC	1·7	2·2
GpC	1·0	4·3

products of the reaction are CpC, CpCp!, CpCpC, CpCpCp! and CpCp.

Of these five compounds, the formation of CpCp!, CpCpCp! and CpCp was subsequently shown to occur in the absence of cytidine when high concentrations of cytidine-2′:3′ phosphate (>0·1 M) were incubated with a dilute solution of ribonuclease at 2°. The production of all these cytidine-containing compounds may therefore be represented by the following two relationships:

$$\text{Cp!} \xrightarrow{\text{ribonuclease}} \text{Cp} + \text{CpCp!} + \text{CpCpCp!} + \ldots + (\text{CpCp}), \quad (1)$$

$$\text{Cp!} + \text{C} \xrightarrow{\text{ribonuclease}} \text{Cp} + \text{CpC} + \text{CpCpC} + \ldots + (\text{CpCp}). \quad (2)$$

Dicytidylic acid (CpCp) has been written in brackets because it cannot have been formed directly from cytidine-2′:3′ phosphate itself but, in all probability, resulted from the condensation of the latter with cytidylic acid which appeared in the incubation mixture as a product of the hydrolytic reaction.

There is a certain amount of evidence that synthesis of the polynucleotides from cyclic mononucleotides by ribonuclease does not stop at the cyclic trinucleotide stage. Chromatograms of incubations which contained ribonuclease and high concentrations of cyclic cytidylic acid and cytidine (about 0·5 M) showed the formation of compounds which, although not present in sufficient quantities to permit their characterization, by virtue of their R_F values in solvent 1, could be tentatively regarded as polynucleotides containing more than three cytidylic acid residues (bands *A* and *B*, Fig. 3).

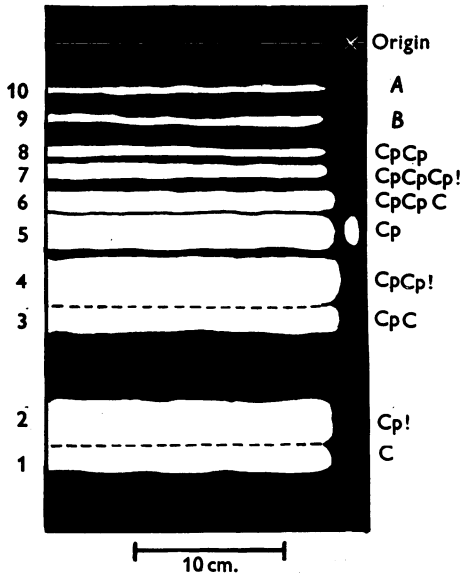


Fig. 3. Synthesis of polynucleotides from cyclic cytidylic acid and cytidine by ribonuclease. Experimental conditions are as given in Table 3, Expt. 5. Descending chromatogram run in solvent 1.

Table 3. *Polynucleotide synthesis by ribonuclease*

Incubations were carried out in 0·015 M phosphate buffer, pH 7·4, and the concentration of crystalline ribonuclease was 14 μg./ml. At the indicated time intervals a sample was removed, placed on Whatman no. 3 MM paper and chromatographed in solvent 1. Quantitative assays were done as described in the text. All concentrations are expressed as μmoles/ml. Abbreviations are as given in the text.

Expt. no.	Temp. (°)	Time (hr.)	Initial Cp!	Initial C	Reaction products: Cp	CpC	CpCp!	CpCpC	CpCpCp!* CpCp*
1	2	7	24	103	4	4	Trace	0	0
2	2	5·5	45	45	6·5	3·5	Trace	0	0
3	2	4	100	130	4·6	7·9	2·9	1·1	Trace
4	2	8	450	150	30	6·8	41	2·7	12·5
5	2	8	450	50	17·5	23	38	5	5·5
6	37	1·5	100	130	7·1	5·1	Trace	0	0
	37	5	100	130	14·2	5·3	Trace	0	0
7	2	0†	135	310	3·6	1·5	0·5	0	0
	2	3·2	135	310	4·2	8·2	2·5	Trace	0
	2	6·7	135	310	5·2	13·4	3·5	0·5	0
8	2	2	102	0	6·6	0	3·6	0	0
		4	102	0	10·3	0	3·6	0	0
		8	102	0	14·6	0	3·6	Trace	Trace
9	2	2	102	130	4·8	9·0	3·3	0·9	Trace
		4	102	130	7·7	10·9	3·2	0·9	Trace
		8	102	130	12·7	14·8	3·2	1·4	Trace

* Expressed as cytidylic acid in the form of these two compounds (they appeared in approximately equal concentrations).

† The mixture was prepared in a tube kept in ice. After mixing, a sample (0·02 ml.) was placed on paper and rimmed with acetone to inhibit enzyme action during drying. Cytidine-2′:3′ phosphate and cytidine were pure except for traces of cytidylic acid.

Certain quantitative aspects of reactions (1) and (2) are presented in Table 3. It can be seen that, of the synthetic products, CpC and CpCp! are formed in the greatest amounts. No exact kinetic studies were undertaken to determine the conditions most favourable for synthesis but it was found that there was no pronounced effect of pH change over the range of pH 5–9 on the relative extent of synthesis and hydrolysis. Lower temperatures appeared to favour synthesis (compare Expts. 3 and 6, Table 3). The synthetic reactions become progressively slower with time, but the reason for this was not investigated.

Identification of the products formed in reactions (1) *and* (2)

Each compound is related to its position in a chromatogram which has been run in solvent 1 (see Fig. 3). Wherever quantitative data are included in the identification procedure they are expressed in the terms of molar ratios.

Dicytidine monophosphate (CpC, band 3, Fig. 3) was identified in the following way: (1) The molar ratio of cytosine to phosphorus was 2·1:1. (2) Treatment (*e*) gave cytidine (1·0) and cytidylic acid (1·05). (3) Treatment (*f*) gave cytidine (1·05) and cytidylic acid (1·0). (4) It was oxidized by $NaIO_4$ (treatment *d*) showing the presence of vicinal hydroxyl groups. (5) Treatment (*h*) converted it into cytidine-5′ phosphate (1·0) and cytidine (1·0). (6) Neither treatment (*b*) nor treatment (*i*) had any effect. (7) The electrophoretic mobility at pH 3·5 was small (1·0 cm./2 hr./20 v/cm.) as would be expected for a substance of this composition (the amino groups are about half dissociated at this pH and thus cancel the effect of the fully dissociated primary phosphate group).

Cyclic dicytidylic acid (CpCp!, band 4, Fig. 3): (1) The R_F values in solvent 1 corresponded to those of the pyrimidine cyclic dinucleotides (Markham & Smith, 1952*b*). (2) Treatment (*a*) gave cytidylic acid only. (3) Treatment (*g*) gave cytidine-3′:5′ diphosphate (1·0) and cytidine (1·25) which were separated by electrophoresis at pH 3·5. The deviation in the value for the molar ratio from the theoretical figure of 1:1 is probably due to a high blank due to the venom. (4) Treatment (*i*) had no effect, but when steps (*b*), (*i*) and (*e*) were carried out in succession, each intermediate product being isolated by chromatography in solvent 1, cytidine and cytidylic acid were formed in the molar ratio 1·1:1. This result may be interpreted by the following reaction sequence:

$$\text{CpCp!} \xrightarrow{\text{step } (b)} \text{CpCp} \xrightarrow{\text{step } (i)} \text{CpC} \xrightarrow{\text{step } (e)} \text{Cp} + \text{C}.$$

(5) Treatment (*f*) yielded cytidine-2′ phosphate (1·1) and cytidine-3′ phosphate (1·0). (6) The electrophoretic mobilities were measured at pH 3·5 and pH 7·0 before and after treatment (*b*); these showed that a weak acidic grouping was liberated by this treatment.

Tricytidine diphosphate (CpCpC, band 6, Fig. 3): (1) The electrophoretic mobility at pH 3·5 and 20 v/cm. was 4·2 cm./2 hr., a value which agrees closely with that expected for CpCpC on theoretical grounds. (2) Treatment (*e*) gave cytidine (1·0) and cytidylic acid (1·9). (3) Treatment (*h*) gave cytidine-5′ phosphate (2·0) and cytidine (1·0).

Cyclic tricytidylic acid (CpCpCp!, band 7, Fig. 3): (1) The electrophoretic mobility was 10·2 cm./2 hr./20 v/cm. compared with a value of 9·1 cm. for CpCp!. (2) Treatment (*a*) gave cytidylic acid only. (3) Treatment (*b*) gave a substance which had an R_F value of 0·05 in solvent 1. This substance when subjected to steps (*i*) and (*e*) in succession produced cytidylic acid (2·0) and cytidine (1·0). This may be represented by the following:

$$\text{CpCpCp!} \xrightarrow{\text{step } (b)} \text{CpCpCp} \xrightarrow{\text{step } (i)} \text{CpCpC} \xrightarrow{\text{step } (e)} 2\text{Cp} + \text{C}.$$

Dicytidylic acid (CpCp, band 8, Fig. 3). The R_F value of this compound in solvent 1 frequently corresponded with that of CpCpCp! (band 7), but the two substances could be separated by electrophoresis at pH 3·5 at 20 v/cm., under which conditions the CpCp moved 8·5 cm./2 hr., whereas CpCpCp! moved 10·2 cm./2 hr. (1) Treatment (*b*) had no effect on its R_F value in solvent 1 or on its electrophoretic mobility at pH 7. (2) Treatment (*i*) gave a substance whose R_F value in solvent 1 was the same as that of CpC and which was then converted into cytidylic acid (1·0) and cytidine (1·0) by treatment (*e*).

Other compounds as acceptors

The small quantities of CpCp which appeared in reactions (1) and (2) could not have been formed directly from the initial reactants. One of the several possible mechanisms for its formation would be a condensation of cyclic cytidylic acid and cytidine-3′ phosphate. However, as the yield of CpCp was not increased by adding large amounts of cytidylic acid to an incubation mixture of cytidine-2′:3′ phosphate and ribonuclease, it was concluded that nucleoside-3′ phosphates with a free monoester phosphate group function as poor acceptors at best. Nucleosides, in which this group is removed, and cyclic nucleotides, in which the phosphate group is diesterified, appear to be much more effective acceptor molecules.

Adenosine and adenosine-2′:3′ phosphate also reacted with cytidine-2′:3′ phosphate in the presence

of ribonuclease although, in neither case, was the yield of ester as favourable as that from cytidine-2′:3′ phosphate and cytidine (Table 4):

$$\text{Cp!} + \text{A} \xrightarrow{\text{ribonuclease}} \text{CpA}, \quad (3)$$

$$\text{Cp!} + \text{Ap!} \xrightarrow{\text{ribonuclease}} \text{CpAp!}. \quad (4)$$

The procedure for identification of CpA and CpAp! followed that used for the identification of CpC and CpCp!

electrophoretic mobility at pH 3·5 was the same as that of ApC. (3) Treatment (*f*) gave adenylic acid (1·0) and cytidine (1·02).

Spleen diesterase acting on RNA 'core' formed guanosine-3′ phosphate and adenosine-3′phosphate. If, however, the reaction was carried out in the presence of cytidine, dinucleoside monophosphates were produced as well as the mononucleotides. The incubation mixture (2 ml.) contained 49 mg. of 'core', 0·5 mg. of spleen enzyme, 0·6 ml. of 0·2 M phosphate buffer, pH 7·4, and 52 mg. of cytidine (0·1 M final concentration). After 6 hr. at 38° the mixture was chromatographed in solvent 1. The ultraviolet-absorbing material which moved in the same position as dinucleoside phosphates not containing guanosine (1·7 times as fast as cytidylic acid) was eluted. When subjected to treatment (*f*) it yielded adenylic acid (1·0) and cytidine (0·92), and was therefore assumed to be ApC. The material which had an R_F value the same as that of cytidylic acid was also eluted and subjected to electrophoresis at pH 3·5. It showed the presence of a component which had a mobility of approximately 4 cm./2 hr./20 v/cm. and which could be recrystallized from hot water as fine needles. A sample was digested with spleen phosphodiesterase (treatment *f*) and gave guanylic acid (1·0) and cytidine (0·93). On this evidence it was concluded that guanylyl-cytidine, GpC, was also formed by the action of spleen enzyme on 'core' in the presence of cytidine.

Table 4. *Enzymic formation of CpA and CpAp!*

The incubation mixture contained 0·015 M phosphate buffer, pH 7·4, and 14 μg. of ribonuclease/ml. Incubation was for 8 hr., after which the mixture was chromatographed in solvent 1. All concentrations are expressed as μmoles/ml.

Temp. (°)	Initial Cp!	Initial Ap!	Initial adenosine	Residual Cp!	Reaction products			
					Cp	CpCp!	CpAp!	CpA*
0	101	—	—	87	5·1	4·2	—	—
0	102	109	—	67	32	—	2·9	—
37	102	107	—	49	51	—	2·1	—
37	45	—	100	(Not measured)	(Not measured)	Trace	—	1·5

* This compound was eluted, concentrated and reincubated with 100 μg. of ribonuclease/ml. of mixture. The products were analysed by chromatography in solvent 1 and there was found 0·1 μmole of cytidylic acid and 0·1 μmole of adenosine.

Similar experiments with adenosine-3′ phosphate, purine nucleoside (purine 9-β-D-ribofuranoside) and the deoxyribonucleoside, thymidine, as possible acceptors were all negative.

The other pyrimidine cyclic nucleotide, uridine-2′:3′ phosphate, was found to be capable of acting as a donor molecule in an analogous series of reactions. For instance, in the presence of cytidine and cyclic uridylic acid ribonuclease catalysed the formation of uridylyl-cytidine (UpC). In general, however, the various uridine-containing products are harder to characterize and consequently the major part of the investigation was confined to an examination of the cytidine derivatives.

Transfer reactions catalysed by spleen phosphodiesterase.

The spleen phosphodiesterase preparation failed to catalyse exchange reactions involving cyclic nucleotides, but it was active in promoting other types of transfer reactions. Thus, in the presence of cytidine as an acceptor, adenylyl-uridylic acid (ApUp) reacted to form the dinucleoside monophosphate, ApC, and uridylic acid. The conditions and results of two experiments are given in Table 5.

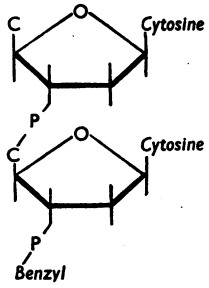
A sample of the dinucleoside phosphate was isolated by chromatography in solvent 1 and identified as ApC in the following way: (1) Its R_F value in solvents 1 and 2 was the same as that of ApC derived from the dinucleotide, ApCp. (2) Its

Table 5. *Formation of adenylyl-cytidine (ApC) from ApUp and cytidine by spleen phosphodiesterase*

Incubation was at 38° for 90 min. and the reaction mixture (0·08 ml.) contained 16 μmoles of cytidine, 0·8 μmole of phosphate buffer, pH 7·4, and 30 μg. of enzyme. Results are expressed as μmoles/ml.

ApUp		ApUp hydrolysed	ApC formed
Initial	Final		
3·6	2·7	0·45	0·45
8·5	6·6	1·1	0·8

Spleen phosphodiesterase also catalysed a transfer reaction in which a nucleotide benzyl ester acted both as donor and acceptor molecule. When a high concentration of cytidine-3′ benzyl phosphate (0·5 M) was incubated with spleen enzyme (30 μg.) in phosphate buffer (pH 7·4, 0·1 M) for 24 hr. at 37° and the mixture chromatographed in solvent 1, in addition to the hydrolysis product, cytidylic acid, the presence of a new compound with an R_F value 2·6 times that of cytidylic acid was detected. This material was eluted and digested with dilute ribonuclease (2 μg.) for a short time (1 hr., 37°). The products were identified as cyclic cytidylic acid and cytidine-3′ benzyl phosphate. It would therefore appear that the new compound was the benzyl ester of dicytidylic acid and would be represented by the following structure (VI).



VI

A similar compound was formed when adenosine-3′ benzyl phosphate was incubated with spleen phosphodiesterase.

DISCUSSION

The experiments described in this paper show that two enzymes, which have hitherto been regarded as having purely degradative properties, possess considerable synthetic abilities. As can be seen from Table 3, polynucleotide synthesis occurs to a marked extent with substrate concentrations which are as low as 0·1 M, or even less. Thus, in Expt. 2, in which the concentration of both cyclic cytidylic acid and cytidine was only 0·045 M, the amount of the former compound which was converted into CpC was equal to half the amount which was hydrolysed to cytidylic acid. In Expt. 5, of the initial 0·45 M cyclic cytidylic acid, 30 % was used, of which 87 % took part in the synthetic reaction and only 13 % was hydrolysed, in spite of the large excess of water (nearly 55 M).

The existence of these enzymes in cells may well be for the purpose of carrying out synthetic reactions rather than for effecting hydrolyses. The reason why such striking properties have been overlooked previously is that their substrates were not recognized.

Neither ribonuclease nor spleen phosphodiesterase appears to be able to synthesize phosphodiester bonds *de novo*. Ribonuclease, in fact, would appear to possess a unique mechanism for the preservation of such structures by means of the conversion of the normal type of internucleotidic bond into the analogous intranucleotidic cyclic 2′:3′ phosphate diester bond, which the enzyme is then able to reconvert into a diester bond of the more usual type. A mechanism of this kind is clearly able, in theory at least, to take part in reactions involving rearrangements in RNA's, and while we have only been able to show that such reactions take place among relatively small polynucleotide chains, there would appear to be no *a priori* reason why it should be restricted to such simple compounds. The only real limitation which would appear to be imposed in these reactions, is that any polynucleotide involved would have to be terminated by a pyrimidine nucleotide residue esterified at $C_{(3')}$, and it can only be joined at the other end to a chain ending in a similar residue. Breakage of the ester links would then give rise to chains having a primary alcoholic —OH group at one end and a pyrimidine nucleoside-2′:3′ phosphate residue at the other, structures which would appear to be the only essential reactants in the transfer reaction. The nature and number of the intervening nucleotide residues would not appear to impose a restriction.

This type of mechanism should also be capable of building up polynucleotide chains of a very restricted type if provided with the correct substrates, but it should probably be mentioned at this point that free cyclic nucleotides have not yet been found to occur naturally.

It is tempting to speculate whether a 'purine ribonuclease' exists which would provide a similar way for adding purine nucleotide residues to a polynucleotide chain, but so far no such enzyme has been found. The spleen phosphodiesterase could, however, provide a means for effecting this type of transfer. Neither this enzyme nor ribonuclease would appear to be able to synthesize a polynucleotide chain from simple mononucleotides, and it seems likely that any biological function of a synthetic type which these enzymes may have is restricted to the interconversion of existing polynucleotide chains.

It should be mentioned, too, that it is by no means certain that either of these enzymes occurs in tissues other than those which have been used as their source. It is true that 'ribonucleases' have been reported as occurring in a variety of tissues, and, in fact, the spleen phosphodiesterase is one of these enzymes. So far, however, no other enzyme having the characteristic properties of pancreatic ribonuclease, namely the specificity for pyrimidine nucleotide esters and the ability to form cyclic phosphates from such diesters, has been identified.

The experiments which have demonstrated the formation of polynucleotides from cyclic mononucleotides have some bearing on the present views of RNA structure. The hydrolytic conditions used by Markham & Smith (1952*a*–*c*) were quite favourable for synthetic reactions to manifest themselves, and it is probable that part, at least, of the cyclic dinucleotides reported by these workers as occurring in partial ribonuclease digests of RNA's was synthetic in origin. It is thus impossible to conclude that any particular pair of pyrimidine nucleotide residues are adjacent in the RNA molecules, although from a statistical point of view it is certain that regions must occur where several pyrimidine nucleotide residues are adjacent. As far as the other polynucleotides which are liberated from RNA by ribonuclease are concerned, it should be noted that this enzyme appears to be unable to synthesize compounds which it cannot degrade, and so there is no reason to suspect that substances isolated from exhaustively digested RNA are artifacts.

SUMMARY

1. Ribonuclease was found to catalyse the synthesis of polynucleotides from cyclic cytidylic acid (cytidine-2′:3′ phosphate). Evidence for synthesis of the dinucleotide, cyclic dinucleotide and cyclic trinucleotide has been presented. With cytidine also present as an acceptor molecule, two other reaction products were isolated. They were the dinucleoside phosphate and the trinucleoside diphosphate. Under certain conditions the amount of synthesis exceeded by far the extent of hydrolysis of the cyclic mononucleotides. Uridine derivatives were also active in these reactions.

2. In the transfer reactions catalysed by ribonuclease, adenosine and adenosine-2′:3′ phosphate could also be used as acceptor molecules. In general, the nucleoside-3′ phosphates were very poor as acceptors.

3. A phosphodiesterase preparation purified from spleen was observed to catalyse exchange reactions involving ribopolynucleotides. This enzyme preparation, however, was inactive with cyclic nucleotides.

4. The possible significance of these reactions in ribonucleic acid interconversions has been discussed.

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REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Brown, D. M., Magrath, D. I. & Todd, A. R. (1952). *J. chem. Soc.* p. 2708.
Heppel, L. A., Markham, R. & Hilmoe, R. J. (1953). *Nature, Lond.*, **171**, 1151.
Heppel, L. A. & Whitfeld, P. R. (1955). *Biochem. J.* **60**, 1.
Heppel, L. A., Whitfeld, P. R. & Markham, R. (1954). *Biochem. J.* **56**, iii.
Lythgoe, B. & Todd, A. R. (1944). *J. chem. Soc.* p. 592.
Markham, R. & Smith, J. D. (1951). *Biochem. J.* **49**, 401.
Markham, R. & Smith, J. D. (1952*a*). *Biochem. J.* **52**, 552.
Markham, R. & Smith, J. D. (1952*b*). *Biochem. J.* **52**, 558.
Markham, R. & Smith, J. D. (1952*c*). *Biochem. J.* **52**, 565.
Smith, J. D. & Markham, R. (1950). *Biochem. J.* **46**, 509.
Volkin, E. & Cohn, W. E. (1953). *J. biol. Chem.* **205**, 767.
Whitfeld, P. R. (1954). *Biochem. J.* **58**, 390.
Whitfeld, P. R., Heppel, L. A. & Markham, R. (1955). *Biochem. J.* **60**, 15.

The Enzymic Hydrolysis of Ribonucleoside-2′:3′ Phosphates

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The 2′:3′ phosphate esters of ribonucleosides (referred to as cyclic nucleotides in this paper) arise as intermediate products in the course of hydrolysis of ribonucleic acids (RNA) by pancreatic ribonuclease and by dilute alkali. The mechanism of action of ribonuclease has been discussed recently by several authors (Markham & Smith, 1952*b*, *c*; Merrifield & Woolley, 1952; Brown & Todd, 1953; Volkin & Cohn, 1953). The enzyme hydrolyses pyrimidine cyclic nucleotides at a slow rate (Markham & Smith, 1952*b*) to produce exclusively pyrimidine nucleoside-3′ phosphates; thus, cytidine-2′:3′ phosphate is split to yield only cytidine-3′ phosphate (Brown, Dekker & Todd, 1952). In contrast, alkali degrades the same cyclic nucleotide to give a mixture of cytidine-2′ phosphate and cytidine-3′ phosphate. A mechanism for the alkaline hydrolysis of RNA which involves the

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