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Stepwise Enzymatic Synthesis of the Specific $C_{(3')} - C_{(5')}$ Internucleotide Bond

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The review deals with the methods of synthesis of low-molecular-weight oligoribonucleotides based on the use of various ribonucleases and polynucleotide phosphorylase, whereby oligonucleotides can be obtained rapidly and in fairly high yields by a gradual (stepwise) growth of the oligonucleotide chain. The possibilities of this comparatively new field in the synthesis of oligonucleotides and the prospects for its development are discussed.
The bibliography includes 72 references.

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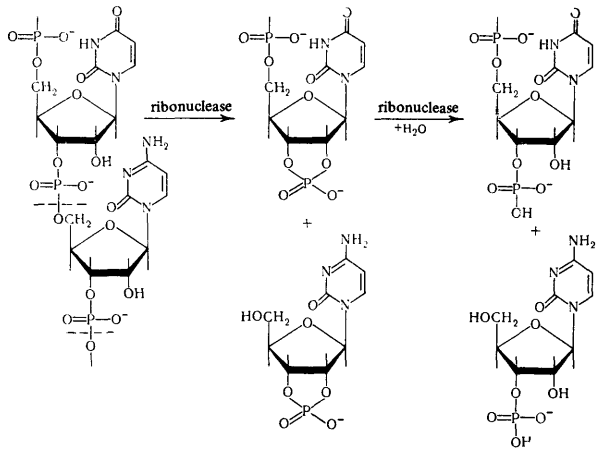
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I. INTRODUCTION

Since the publication of the review of methods for the stepwise synthesis of the specific $C_{(3')}-C_{(5')}$ internucleotide bond[1] several years have elapsed. In this period studies on further elaboration and improvement of these methods have developed widely and effectively (see, for example, Refs. 2–13) and very important results have been obtained. In particular 64 trinucleoside diphosphates containing the usual nucleotides in all possible combinations were synthesised[14], which permitted the final elucidation of the aminoacid code[2]. Studies on the synthesis of the gene coding for an alanine *t*RNA are also being successfully continued. However, it should be noted that the chemical methods for the synthesis of oligonucleotides consist of many stages and are fairly laborious. At the same time the need for oligonucleotides increases continuously in connection with the expansion of research in which they are employed: the study of the mechanism of protein synthesis, the study of substrate specificity and kinetics of enzymatic reactions catalysted by various nucleotidases, etc. Therfore the search for simpler and more effective methods of synthesis of oligonucleotides is continued and at present, together with chemical procedures, enzymatic methods are being increasingly employed. This review deals only with such enzymatic reactions, involving the *stepwise* synthesis of the $C_{(3')}-C_{(5')}$ internucleotide bond—(the synthesis of low-molecular oligonucleotides), while reactions leading to the synthesis of polynucleotides (synthesis with RNA and DNA polymerases[2], polymerisation using polynucleotide phosphorylase[15], etc.) are not considered.

II. SYNTHESIS OF OLIGORIBONUCLEOTIDES USING RIBONUCLEASES

It is well know that the cleavage of the phosphodiester bonds in ribonucleic acids or polynucleotides catalysed by ribonucleases takes place in two stages: (1) cleavage of the bond between the phosphorus atom and the 5′-O atom of ribose with subsequent transphosphorylation and the formation of 2′,3′-cyclic phosphates; (2) hydrolysis of 2′,3′-cyclic phosphates with formation of 3′-phosphates[16].



Usually ribonucleases catalyse the cleavage of the phosphodiester bond with a specific type of nucleoside whose 3′-oxygen atom is involved in this bond. Thus pyrimidylribonucleases cleave at the highest rate the bond in which a pyrimidinenucleoside donates the 3′-oxygen atom. For example, pancreatic ribonuclease is a pyrimidylnuclease. Similarly guanylribonucleases require that the 3′-oxygen atom be donated by guanosine. For example, the T1 ribonuclease from Takadiastase is of this type. However, non-specific ribonucleases also exist. As early as 1955 Heppel et al.[17] showed that the first stage of this reaction is reversible and under certain conditions pancreatic ribonuclease can catalyse the synthesis of compounds containing the phosphodiester bond. Somewhat later it was established that T1-ribonuclease also exhibits transesterifying activity and is capable of synthesising oligonucleotides[18]. Analogous properties were found also in guanylribonuclease isolated from *Actinomyces aureoverticilatus*[19].

At the present time studies on the application of pancreatic T1 ribonuclease in oligonucleotide synthesis are developing fairly rapidly[20-25]. However, it must be emphasised that the use of specific enzymes permits the synthesis of oligoribonucleotides in which the sequence of monomeric units is determined by the specificity of the enzyme. Recently it was shown that in the synthesis of oligonucleotides and in particular dinucleotides it is desirable to employ ribonucleases without definite specificity, since it is then possible to obtain dinucleotides with any sequence of bases[26,27].

In a discussion of methods for the synthesis of oligonucleotides using ribonucleases it is convenient to examine separately the synthesis of dinucleotides and trinucleotides, although both these reactions are of the same type and have much in common. Longer oligonucleotides have not so far been obtained by this method (see p. 700).

1. Synthesis of Dinucleotides (Dinucleoside Mono- and Di-phosphates)

Since ribonucleases of all the types known at present cleave the phosphodiester bond by the same mechanism, it follows that the synthesis of the phosphodiester bond using ribonucleases also involves substrates of the same type, namely nucleoside 2′,3′-cyclic phosphates. Nucleoside 2′,3′-cyclic phosphates are obtained as a rule from the corresponding nucleoside 2′,3′-phosphates with the aid of dicyclohexylcarbodi-imide or ethyl chloroformate[28]. In certain cases they are isolated from a ribonuclease hydrolysate[29].

The synthesis of dinucleotides in the presence of ribonuclease can be represented by the following mechanism:

$$N{>}p + N' \xrightarrow{\text{ribonuclease}} NpN',$$

where N>p† is the substrate(nucleoside 2′,3′-cyclic phosphate) and N′ is an acceptor, which consists of a nucleoside derivative with a free 5′-hydroxy-group (nucleoside, nucleotide, nucleoside 2′,3′-cyclic phosphate). The reaction is usually carried out by incubating with the enzyme a mixture of the substrate and the acceptor dissolved in a buffer solution. At the same time nucleoside 2′,3′-cyclic phosphate is hydrolysed to nucleoside 3′-phosphate.

Substrates. The substrates employed in this reaction were the 2′,3′-cyclic phosphates of uridine[20-23,26,27], cytidine[17,20-23,26,27], adenosine[26,27], guanosine[18,19,24-27], inosine, and xanthosine[25] and also 5′-*O*-substituted nucleoside 2′,3′-cyclic phosphates: 5′-*O*-phosphoryl- and 5′-*O*-methylphosphoryl-uridine 2′,3′-cyclic phosphates[31], 5′-*O*-(α-n-butoxyethyl)-substituted 2′,3′-cyclic phosphates of uridine, cytidine, adenosine, and guanosine[32,33], 5′-*O*-acetyl-, 5′-*O*-methylphosphoryl-, 5′-*O*-sulphonyl-, and 5′-*O*-pyrophosphoryl-guanosine 2′,3′-cyclic phosphates[34], and the methyl ester of 2′,3′-cyclophosphoguanylyl-(5→N)-phenylalanine[35]. Pancreatic ribonuclease is known to be capable of cleaving phosphodiester bonds the 3′-oxygen atom of which belongs to minor (4-thiouridine[36]) or non-natural (6-azauridine, 6-azacytidine[37], 5-halogenouridine[38], etc.) nucleosides and also nucleosides containing an anomalous carbohydrate residue[39]. Therefore it may be supposed that the corresponding nucleoside 2′,3′-cyclic phosphates may function as substrates in the synthesis of oligonucleotides. Indeed it has recently been shown that 5-bromouridine 2′,3′-cyclic phosphate gives rise to 5-bromouridilyl-3′→5′)-uridine after incubation with uridine in the presence of pancreatic ribonuclease[40]. Evidently the number of substrates will increase as the study of the specificity of already known ribonucleases is extended and also as new ribonucleases, differing in the type of their activity from those already known, are discovered.

Acceptors. The second component of this reaction, the phosphate acceptor, may be a nucleoside containing a free 5′-hydroxy-group, a nucletide, and in the case of specific ribonucleases also a nucleoside 2′,3′-cyclic phosphate. Since the specificity of ribonucleases is determined by the donor of the 3′-hydroxy-group, the possibility of the reaction occurring should be independent of the type of heterocyclic base in the acceptor. Thus in syntheses with participation of pancreatic ribonuclease, one may use as phosphate acceptors, together with natural nucleosides, also the aza-analogues of uridine[40], guanosine[41], and 5-chlorouridine[40] and, in the case of T1 ribonuclease, 5-bromouridine, 6-azauridine, $N_{(3)}$-methyluridine, and 5-methyluridine[40]. Heppel et al.[17] noted that in the case of pancreatic ribonuclease nucleotides are poor acceptors and nucleosides and cyclic phosphates are most effective. On the basis of these data and later studies[19], it is asserted that pancreatic ribonuclease uses nucleotides as acceptors to only a slight degree. However, this is erroneous, since Tikhomirova-Sidorova and coworkers[22,23] synthesised under analogous conditions UpUp (10–30%), UpCp (40%), CpCp (9%), and CpUp (29%). Heppel's conclusion that thymidine cannot serve as an acceptor in this reaction also proved to be incorrect: uridilyl-(3′→5′)-thymidine was obtained in about 12% yield from uridine 2′,3′-cyclic phosphate and thymidine[40].

Substrate concentration, ratio of substrate and acceptor concentrations, and effect of solvent. The substrate concentration and also the ratio of the substrate and acceptor concentrations in the reaction mixture are very important for the synthesis of dinucleotides in this reaction. In the studies of various investigators the substrate concentration varied from 0.02–0.08 to 1 M. In a study of the effect of substrate concentration on the synthesis of dinucleoside monophosphates in the presence of pancreatic ribonuclease A, Bernfield[20] established that the yield is proportional to substrate concentration below 0.3 M and is a maximum at a concentration of 0.4 M. It is quite likely that in the presence of other enzymes a different substrate concentration will be optimum, but such data are not at present available.

† To abbreviate the naming of the nucleosides, nucleotides, and oligonucleotides, the rules developed by the IUPAC Commission on Biochemical Nomenclature[30] and also the notation proposed by Holý and Smrt[9] have been employed: A = adenosine, C = cytidine, G = guanosine, U = uridine, X = xanthosine, I = inosine, N = nucleoside, and aza N = aza-analogue of nucleoside; p = phosphoric acid residue; when p (or the symbol for any other substituent) is written to the right of the nucleoside symbol, the phosphate group is in the 3′-position or ribose and when it is written to the left of the nucleoside symbol the phosphate group is in the 5′-position; p interposed between two nucleoside symbols denotes a 3′→5′ internucleotide bond; the symbol >p denotes a 2′,3′-cyclic phosphate; HSO_3 = sulphonate group; 5ClU = 5-chlorouridine; 5BrU = 5-bromouridine; T = thymidine; Me = methyl; Be = α-n-butoxyethyl; MeOPhe = methyl ester of phenylalanine.

The dependence of the yield of dinucleoside mono- or di-phosphates on the ratio of the substrate and acceptor concentrations has been investigated somewhat more thoroughly. It has been shown that a ratio close to 1/4 is optimum[25,27,33], but in some cases further increase of the excess of the acceptor increases the yield of dinucleotide. As an example, one may quote the syntheses of ApC and GpC catalysted by the non-specific ribonuclease from *Aspergillus clavatus*[27]. In the synthesis of ApC a large excess of the acceptor (tenfold and more) retards both the cleavage of the substrate and the formation of dinucleoside monophosphate, while in the second case an increase in acceptor concentration leads only to an increase of the yield of dinucleoside monophosphate (27% for a threefold excess and 46% for a tenfold excess). On the other hand, if the reaction mixture contains an excess of the substrate, then monotonic oligonucleotides of various lengths are obtained together with the dinucleotide[20,21].

It is noteworthy that in the case of sparingly soluble nucleosides or nucleotides (adenosine, guanosine, etc.) it is virtually impossible to obtain the required concentrations of the substrate and acceptor, which leads to low yields of dinucleosides. The present author believes that, in order to increase the concentration, it is necessary to employ acceptors in the form of their more soluble derivatives. Such derivatives must meet the following requirements: (1) high solubility in the appropriate buffer; (2) the substituent used to enhance the solubility must be easily introduced into the nucleoside (nucleotide) and easily eliminated; (3) the substituent must not affect the reaction. Preliminary studies showed that one may employ as compounds of this kind the *N*-dimethylaminomethylene derivatives, which are obtained in quantitative yield when the nucleoside (nucleotide) is treated with the dimethyl acetal of dimethylformamide[43,44], are readily soluble in various solvents, and easily lose the dimethylaminomethylene group under mild conditions, for example during chromatography in solvent systems containing ammonia. UpG, [32] ApG, GpG, and GpA [27] have been synthesised using as acceptors the *N*-dimethylaminomethylene derivatives of guanosine and adenosine.

Another method for increasing the acceptor concentration (and if necessary also the substrate concentration) consists in the replacement of aqueous buffers by buffer solutions containing organic solvents. Usually oligonucleotides are synthesised in the presence of ribonucleases in an aqueous buffer at pH 7–8. Trishydroxymethylaminomethane (Tris) or phosphate salts are normally employed as buffers, the buffer concentration varying in the range 0.05–0.15 M (Tris) and 0.01–0.025 M (phosphate). The synthesis of UpazaG and CpazaG in the presence of pancreatic ribonuclease in 50% pyridine was described recently[41]. In the present author's laboratory the effect of various solvents (dimethylformamide, pyridine, acetonitrile) on the synthesis of CpC was investigated. The results presented in Table 1 show that ribonuclease catalyses the synthesis of dinucleoside monophosphate in all cases but in the presence of an organic solvent the reaction is slower and as a rule the yield is lower. The hydrolysis of the substrate is also retarded in the presence of dimethylformamide and acetonitrile, while pyridine accelerates it.

The use of buffer systems containing various organic solvents may prove to be very interesting and useful also in another connection. As already mentioned, when the reaction mixture is incubated with the enzyme, the synthesis of the dinucleotide is accompanied by the hydrolysis of the nucleoside 2,3′-cyclic phosphate to the 3′-phosphate, the latter reaction often predominating. Therefore it is of interest to find ways of inhibiting the hydrolysis while maintaining the synthesis. All such procedures involve the use of organic solvents. Pancreatic ribonuclease is known to retain its activity at high concentrations of alcohols, which, however, inhibit the enzymatic hydrolysis of cyclophosphate substrates[45]. Alcoholysis of the substrate catalysed by ribonuclease takes place only in the presence of the alcohols satisfying certain structural requirements for the formation of the ester. In particular, it is known that secondary alcohols do not participate in the alcoholysis[45,46]. Therefore a study has been made of the synthesis of oligoribonucleotides in the presence of ribonuclease A in isopropyl alcohol[20], i.e. under conditions where the hydrolysis of cytidine 2,3′-cyclophosphate is inhibited and alcoholysis does not occur. It was found that the yield of oligonucleotide increases with the concentration of the alcohol, but falls sharply at a concentration of 8.7 M. In 7.2 M isopropyl alochol the yield of oligonucleotide is 72% compared with the aqueous buffer and the hydrolysis takes place to the extent of only 42%. It is also extremely interesting that the effect of temperatures on the synthesis of oligonucleotides in isopropyl alcohol and water is not the same: in 7.2 M isopropyl alcohol at 10°C a greater amount of oligonucleotide is formed than at 0°C and more than at 0°C in water. At 24°C the yield of the synthesis in isopropyl alcohol is lower than at 0°C but higher than in water at the same temperature. In the presence of various amounts of methanol, glycerol, ethylene glycol, butane-2,3-diol and also mixtures of isopropyl alcohol with dioxane or formamide the yield in the synthesis did not exceed the level attained in water[20].

Table 1. Effect of organic solvent on the synthesis of CpC in the presence of pancreatic ribonuclease.

Buffer solution	Content of CpC and C>p in reaction mixture, %							
	0.5 h		1 h		2 h		4 h	
	CpC	C>p	CpC	C>p	CpC	C>p	CpC	C>p
0.05 M tris-HCl in water (pH 7.6)	19.5	65.4	17.2	60.6	17.0	57.0	12.4	45.8
0.05 M tris-HCl in 50% dimethylformamide (pH 7.5)	9.6	82.0	11.9	71.4	11.6	66.4	10.2	54.1
0.05 M tris-HCl in 50% acetonitrile (pH 7.6)	15.6	72.6	18.0	61.0	14.9	64.9	18.7	53.2
10% pyridine	14.3	67.0	15.1	59.0	15.2	50.9	10.5	38.3
30% pyridine	12.2	66.7	11.9	60.5	12.0	52.3	10.1	40.0

In the discussion of problems associated with the solubility of the reaction components we must finally draw attention to the fact that the substrate may be used in the form of various salts: ammonium[20,22,25,27], sodium[26,27], potassium[19], dicyclohexylguanidinium, pyridinium, tributylammonium[27], and triethylammonium[27,41]. However, preliminary data obtained in our laboratory showed that the nature of the cation in the substrate influences not only its solubility in the buffer but also the course of the synthesis. In particular, the pyridinium salt of adenosine 2′,3′-cyclic phosphate is cleaved more rapidly under the conditions of synthesis of ApC.

Temperature. The synthesis of oligonucleotides with participation of ribonucleases takes place usually at temperatures close to 0°C. In Heppel's studies[17] it was

already noted that the reaction takes place better at a low temperature, while at a higher temperature (37°C) the substrate is rapidly cleaved. These data were confirmed later in other investigations[20,22] for pancreatic ribonuclease and for the non-specific ribonuclease from *Aspergillus clavatus*[27].

Concentration of ribonuclease. The concentration of ribonuclease used in the synthesis is as a rule low and varies in the range from 10 to 40 μg ml^{-1}. In the presence of pancreatic ribonuclease the synthesis varies linearly with enzyme concentration in the range not exceeding 40 μg ml^{-1} [20] and falls on further increase of concentration. An increase in the concentration of guanylribonuclease from *actinomycetes* accelerates the synthesis and increases the yield of oligonucleotides[19]. In the case of 5-*O*-substituted substrates the synthesis of the oligonucleotide in the presence of pancreatic ribonuclease reaches a plateau at an enzyme concentration of 40 μg ml^{-1}.[31]

Duration of reaction. The dependence of the yield of oligonucleotides on the duration of incubation of the reaction mixture has been investigated. Heppel noted an increase of the yield of dinucleotide with incubation time[17]. Later studies of reactions with participation of pancreatic ribonuclease showed that the synthesis of dinucleotides largely terminates after 3–4 h when the substrate is cytidine 2′,3′-cyclic phosphate[20,23] and after 5–6 h when the substrate is uridine 2′,3′-cyclic phosphate[22]. However, the hydrolysis of the substrates continues when the acceptor is a nucleoside. When the acceptor is a nucleotide, the synthesis and the hydrolysis terminate simultaneously. Therefore, it has been suggested that the cytidylic or uridylic acid formed in the reaction inhibits the synthetic function of ribonuclease in the first place[22]. It must be emphasised that these results are valid only for this particular enzyme. The synthesis of dinucleotide monophosphate catalysed by the non-specific ribonuclease from *Aspergillus clavatus* is slower and reaches a maximum after 24–48 h, depending on the nature of the substrate[27]. Moreover it has been found that the curves relating the yield of dinucleoside monophosphate to time are influenced by the ratio of the concentrations of the substrate and acceptor in the reaction mixture[27].

Effect of the pH. According to Heppel's data[17], the synthesis in the presence of pancreatic ribonuclease is independent of the acitidity of the medium in the pH range between 5 and 9. In the cases of guanylribonuclease from *Actinomyces*, at pH 5.5 the synthesis takes place at a lower rate than at pH 7.2.[19] In a study of the non-specific ribonuclease from *Aspergillus clavatus* in the synthesis of GpC it was shown that the optimum pH for the synthesis and hydrolysis is the same[27].

Thus it follows from the data examined that it is now possible to synthesise dinucleotides of any composition and with any sequence of nucleic acid bases using a set of ribonucleases or a single non-specific ribonuclease. The synthesis is achieved in one stage and may be carried out on a preparative scale. Table 2 lists all the dinucleoside mono- and di-phosphates synthesised in this way.

The compounds synthesised by this procedure were isolated by preparative paper chromatography in standard solvent systems: 2-propanol–conc. ammonia–water (7 : 1 : 2),[33] 1-propanol–conc. ammonia–water (55 : 10 : 35),[20–23] 1-butanol–acetic acid–water (5 : 2 : 3),[25] and isobutyric acid–0.5 *N* ammonia (10 : 6),[19] with a subsequent check of purity by paper electrophoresis. The converse procedure has also been used: the reaction mixture was separated by paper electrophoresis in 0.05 M triethylammonium bicarbonate and then additionally purified by chromatography in a particular standard system[23,26,32]. The separation of the reaction mixture by chromatography on ion-exchange resins or molecular sieves is promising for syntheses on a preparative scale.

Table 2. Dinucleoside mono- and di-phosphates synthesised in the presence of ribonucleases*.

Substance	Yield (in relation to substrate), %	References	Substance	Yield (in relation to substrate), %	References
UpU	25; 13	21; 22	UpT	12.0	40
	14.0	40	5BrUpU	14.5	40
UpC	23.0	22	UpUp	10—30	22
CpC	25.0	23	UpCp	40.0	22
	27.0	21	CpCp	9.0	23
CpU	2.3	27	CpUp	29.0	23
ApC	24.0	27	GpUp	40.0	19
ApU	4.5	27	GpCp	50.0	19
ApG	13.8	27	GpAp	25.0	19
ApA	6.1	27	pUpU	7.0	31
GpC	56.0	27	MepUpU	8.0	31
	50.0	19	BeUpU	13.0	33
GpU	50.0	19	BeUpC	15.0	33
	13.0	27	BeApC	13.0	33
	24.5	34	BeCpC	20.0	33
GpG	4.3	27	BeGpC	19.0	33
GpA	13.4	27	MepGpU	24.0	34
IpU	25.0	61	PGpU	60.0	34
XpU	10.0	61	MepGpU	25.0	34
Up8azaG	13.5	41	ppGpU	1.5	34
Cp8azaG	13.5	41	HSO_3GpU	1.5	34
Up 6aza U	11.3	40	MeOPhepGpU	10.0	35
Up 5ClU	15.6	40			

*The Table does not list the dinucleotides for which the yield is not quoted in the original paper.

2. Synthesis of Trinucleotides (Trinucleoside Di- and Tri-phosphates)

The stepwise synthesis of trinucleotides may be achieved in two ways:

$$N > p + N'pN'' \rightarrow NpN'pN'', \quad (I)$$

$$NpN' > p + N'' \rightarrow NpN'pN''. \quad (II)$$

One of the principal characteristics of this synthesis is the presence of the phosphodiester bond in the acceptor [reaction (I)] or in the substrate [reaction (II)], which makes it necessary in such cases to choose the enzyme catalysing the synthesis, or other reaction conditions, in such a way that the cleavage of the bond is reduced to a minimum.

The mechanisms quoted show that the substrates should be 2′,3′-cyclic phosphates of nucleosides or of dinucleoside monophosphates. Compounds of the first type are readily available[28,29], but the introduction of the 2′,3′-cyclic phosphate group into nucleotides is more difficult. Three methods have now been proposed for the synthesis of compounds of the type NpN′>p: (1) selected phosphorylation of dinucleoside monophosphates with triethyl phosphite and subsequent oxidation with hexachloroacetone[47]; (2) cyclisation of the terminal 3′-phosphate in dinucleotides with the aid of cyclohexyl-β-(*N*-methylmorpholino)ethylcarbodi-imide *p*-toluenesulphonate[48]; (3) cyclisation of the terminal 3′-phosphate with ethyl chloroformate[69].

There exists also a third method for synthesising trinucleotides: incubation of an excess of a nucleoside 2′,3′-cyclic phosphate (N>p) with a nucleoside, which leads to the formation of trinucleoside diphosphates of the type NpNpN′ [reaction (III)]. The first trinucleotides were in fact obtained by this procedure in enzymatic reactions with participation of ribonucleases:

$$2N>p + N' \rightarrow NpNpN'. \qquad \text{(III)}$$

Heppel et al.[17] already noted that, at high concentrations of cytidine 2′,3′-cyclic phosphate (> 0.1 M) and cytidine, their incubation with ribonuclease at 2°C leads to the formation (together with CpC, CpCp, CpC>p) of CpCpC and CpCpC>p. When reaction mixtures containing about 0.5 M substrate and acceptor are incubated, oligonucleotides with longer chains are formed. Bernfield and Nirenberg[49] reported the synthesis of UpUpU (12%), UpUpC (8%), and CpCpC (10%), and later[21] of CpCpU (8%) and CpCpA (2%). Each synthesis involved the reaction of two molecules of pyrimidine 2′,3′-cyclic phosphate with a nucleoside.

In 1964 the first communication[24] on the synthesis of the trinucleoside diphosphate GpUpC from a nucleoside 2′,3′-cyclic phosphate (G>p) and a dinucleoside monophosphate (UpC [see reaction (I)] was published. The synthesis was achieved by thermostatting 6.5 micromoles of G>p and 20 micromoles of UpC in 0.1 ml of 0.05 M tris buffer (pH 7) with 1 μg of T1 ribonuclease at 2°C and 24 h.

A series of papers by Grünberger, Holý, and Šorm[25,41,50-54] described the synthesis of 66 different trinucleoside diphosphates with participation of T1 ribonuclease. The synthesis was achieved by reactions (I) and (II) and the substrates were the 2′,3′-cyclic phosphates of guanosine, inosine, 8-azaguanosine, and xanthosine; the reactions of dinucleoside monophosphates with guanosine, inosine, and 8-azaguanosine at the 3′-end were also employed. It was found that in reaction (I) G>p and I>p were more effective substrates than 8-azaG>p or X>p. The acceptors in these reactions were dinucleoside monophosphates without guanosine, inosine, 8-azaguanosine, or xanthosine at the 5′-end and consequently incapable of being cleaved by T1 ribonuclease.

The synthetic activity of T1 ribonuclease depends on the nature of both nucleosides in the dinucleoside monophosphate acceptor, but the effect of the nucleoside at the 5′-end is more pronounced. Other conditions being equal, the yield of the oligonucleotide diminishes in the series C > U > A when the 5′-terminal nucleoside is varied. These results differ from the known sequence of the rates of cleavage of the corresponding internucleotide bonds[55]. GpGpU could not be obtained from G>p and GpU, since in the course of incubation GpU is cleaved more rapidly than the trinucleoside diphosphate synthesised. When the 3′-terminal nucleoside is altered, the yield varies in the series C > U > A > I > G.

Certain other types of oligonucleotides containing anomalous bases (5-methyluracil, $N_{(3)}$-methyluracil, $N_{(1)}$-methylinosine, 5-bromouracil, and 5-iodouracil)[54] or analogues of phosphodiester bonds have been obtained by the same procedure[56].

A reaction of type (II) has been carried out with the substrates CpG>p, UpG>p, CpI>p, and UpI>p in the presence of a tenfold excess of a nucleoside acceptor (C, U, or A‡). Higher yields were obtained in those cases where the 3′-terminal nucleoside was inosine. In a discussion of the synthesis of oligonucleotides with participation of T1 ribonuclease, mention should be made of the report of Podder and Tinoco[57], who observed that under certain conditions (incubation of G>p with T1 ribonuclease at room temperature) T1 ribonuclease catalyses the formation of 2′–5′ phosphodiester bonds. If this result is not an artifact, it is necessary to ensure a particularly rigorous control of the conditions of synthesis and to analyse carefully the oligonucleotides obtained with participation of ribonucleases§.

Comparison of all three versions of the synthesis of trinucleotides shows that pathway (III) is simplest but is also the most restricted, since it yields only trinucleotides containing two identical nucleosides at the 3′-end of the molecule. Moreover, the reaction mixture contains in this case a considerable amount of monotonic oligonucleotides, which makes their separation difficult. Pathways (I) and (II) both require preformed dinucleotides and in the second case there is an additional requirement of the introduction of a 2′,3′-cyclic phosphate group into the dinucleotide. Therefore we believe that pathway (I) is to be preferred, and, for a more complete utilisation of the acceptor dinucleotide, it is desirable to introduce an excess of the substrate into the reaction mixture. In order to avoid the formation in a side process of a series of monotonic oligonucleotides, it is suggested that substrates with a blocked 5′-OH-group be used[31-33]. The synthesis of oligonucleotides in the presence of ribonucleases using substrates of this type has so far been comparatively thoroughly investigated only at the level of dinucleoside monophosphates[31-35]. In these investigations it has been shown convincingly that 5′-*O*-substituted nucleosides 2′,3′-cyclic phosphates may serve as substrates for pancreatic[31-33], guanyl-[34,35] and non-specific ribonuclease from *Aspergillus clavatus*[33] and that the use of an excess of a 5′-*O*-substituted substrate really does not lead to the formation of a mixture of oligomers[34]. The synthesis of pGpApU and pGpUpU from pG>p and the corresponding acceptor in the presence of T1 ribonuclease has also been briefly reported[42].

As already stated, in the synthesis of trinucleoside diphosphates via pathways (I) and (II), under certain conditions the formation of the oligonucleotide and the cleavage of the 2′,3′-cyclic phosphate group in the substrate may be accompanied by the cleavage of the phosphodiester bond in the acceptor [reaction (I)] or the substrate [reaction (II)], which complicates the composition of the reaction mixture and hinders the preparation of the trinucleotide. To avoid this, Bernfield[20] suggested the use of derivatives of pancreatic ribonuclease with synthetic activity but hydrolytically inactive. Thus, it has been established that S-protein[58], 1-carboxymethylhistidine-119-ribonuclease[59], and ε-dinitrophenyl-lysine-41-ribonuclease[60] exhibit synthetic activity (Table 3) but virtually do not hydrolyse C>p and RNA with a high molecular weight.

In the study of the synthesising activity of these derivatives it was established that the substrates for S-protein may be cytidine and uridine 2′,3′-cyclic phosphates. The optimum pH in the syntheses with participation of S-protein and 1-carboxymethylhistidine-119-ribonuclease proved to be 8.0 and the optimum reaction temperature is 23°C.[20] The synthesising activity of the derivatives is

‡ In the case of adenosine, a tenfold excess could not be achieved because of the low solubility of adenosine in tris buffer.

§ Later, in a special study Rowe and Smith[71] showed that, under conditions optimum for synthesis, T1 ribonuclease gives rise to the formation of 3′–5′ internucleotide bonds only.

inhibited by lower concentrations of nucleoside 3′-phosphate than in the case of ribonuclease A. The relative initial rates of synthesis for ribonuclease A, S-protein, and 1-carboxymethylhistidine-119-ribonuclease are 100, 21, and 7 respectively. In all cases the dependence of the synthesis on enzyme concentration is linear in the range up to 60 $\mu g\ ml^{-1}$.

Table 3. Depolymerase and synthetic activities of derivatives of ribonuclease A.[20]

Enzyme or derivative	Depolarisation, %	Synthesis, %	Ratio of synthesis to depolymerisation
Ribonuclease A	100	100	1.0
Ribonuclease S	108	103	0.96
S-Protein	5.2	17	3.3
S-Peptide	0	0	0
Carboxymethylhistidine-119-ribonuclease	0.98	5.5	5.6
Ribonuclease inactivated by pepsin	0	0	0
Poly-DL-alanylribonuclease prepared in bicarbonate buffer	10—21	9—22	1
Poly-DL-alanylribonuclease prepared in phosphate buffer	12	14	1.2
ε-dinitrophenyl-lysine-41-ribonuclease	4.4	38	8.6

Comparison of the hydrolytic activities of ribonuclease A and S-protein in the reaction mixtures U>p−UpC and C>p−UpC showed that S-protein cleaves UpC 4−5 times more slowly than ribonuclease A and may be used in the synthesis of trinucleotides via pathway (I) using as an acceptor a dinucleotide with a pyrimidine nucleoside at the 5′-end of the molecule[21].

The activities of ribonuclease A and S-protein have also been compared in the reaction with U>p + ApA and C>p + ApA. It has been established that S-protein catalyses the synthesis of UpApA about three times more effectively than ribonuclease A—the reaction is rapid and begins to slow down after 4 h. The yield of UpApA reaches 10% after 48 h.

Thus in the synthesis of trinucleotides via reaction (I) S-protein has certain advantages over ribonuclease A. Bernfield noted[21] that, for a given substrate, the yield of the trinucleotide depends on the nature of the nucleosides at the 3′-end of the acceptor molecule and is low when the cyclic nucleotide and the nucleoside at the 3′-end of the acceptor molecule are complementary (for example, U>p + CpA). The low yields in those cases where the pyrimidine nucleoside is at the 5′-end of the molecule are probably due to the admixtures of ribonuclease in the S-protein.

Evidently the cleavage of the dinucleotide introduced into the reaction may be avoided also by other methods, for example by introducing a blocking group in the 2′-position of the 5′-terminal nucleotide, since it is known that in this case ribonuclease does not cleave the phosphodiester bond. As usual, in the blocking of a particular functional group it is necessary that the blocking group should be readily introduced into the oligonucleotide, should be stable under the conditions of enzymatic synthesis, and should be readily eliminated. However, no studies on these lines have so far been published.

The trinucleotides synthesised with participation of ribonucleases (Table 4) are isolated and identified in the same way as the dinucleotides (see p. 698).

Summarising the discussion of the methods of synthesis of trinucleotides in the presence of ribonucleases, it should be said that in order to obtain trinucleotides of any composition and any sequence of nucleic acid bases, it is necessary to have at one's disposal a set of highly specific ribonucleases (such as guanylribonucleases) or to find conditions under which the preferential synthesising activity of the enzyme will be shown and its hydrolytic activity will be suppressed. Already, using only the known and more or less available ribonucleases or their derivatives, it is possible to synthesise a large number of various trinucleoside diphosphates via pathways (I), (II), or (III), depending on which sequence of nucleic bases it is to be obtained.

Table 4. Trinucleotides synthesised in the presence of ribonucleases.

Substance	Yield, % (reaction)	References	Substance	Yield, % (reaction)	References
CpCpC	10.0 (III)	21	GpCpU	23.0 (I)	25
UpUpU	12.0 (III)	21	GpCpA	14.0 (I)	25
CpCpU	8.0 (III)	21	GpCpI	11.2 (I)	25
CpCpA	2.0 (III)	21	GpCpG	8.3 (I)	25
UpUpC	8.0 (III)	21	IpCpC	20.0 (I)	25
GpUpC	6.5 (I)	24	IpCpU	19.2 (I)	25
	19.2 (I)	22	IpCpA	15.0 (I)	25
GpCpC	27.0 (I)	25	azaGpUpG	2.4 (I)	25
IpCpI	7.1 (I)	25	azaGpApC	6.5 (I)	25
IpCpG	6.8 (I)	25	azaGpApU	2.5 (I)	25
GpUpU	18.0 (I)	25	XpUpU	7.0 (I)	25
GpUpA	8.4 (I)	25	XpApC	2.2 (I)	25
GpUpI	5.2 (I)	25	GpCp8azaG	3.7 (I)	41
GpUpG	4.1 (I)	25	GpUp8azaG	1.3 (I)	41
GpApC	8.2 (I)	25	aza GpUp aza G	1.0 (I)	41
GpApU	6.0 (I)	25	CpGpC	8.0 (II)	25
IpUpC	16.9 (I)	25	CpGpU	7.8 (II)	25
IpUpU	17.0 (I)	25	CpGpA	2.9 (II)	25
IpUpA	7.5 (I)	25	CpIpC	23.0 (II)	25
IpUpI	4.1 (I)	25	CpIpU	12.0 (II)	25
IpUpG	3.2 (I)	25	UpGpC	6.0 (II)	25
IpApC	7.0 (I)	25	UpGpU	3.1 (II)	25
IpApU	5.0 (I)	25	UpIpC	17.0 (II)	25
azaGpCpC	8.2 (I)	25	UpIpU	14.5 (II)	25
azaGpCpU	7.0 (I)	25	UpIpA	2.3 (II)	25
azaGpCpA	5.0 (I)	25	CpazaGpU	2.2 (II)	41
azaGpCpG	3.0 (I)	25			
azaGpUpC	8.5 (I)	25			
azaGpUpU	8.0 (I)	25			
azaGpUpA	5.0 (I)	25			

Table 5. Trinucleoside diphosphates synthesised in the presence of S-protein[21].

Substance	Yield, %	Substance	Yield, %
CpApA	8.0	UpCpU	5.0
UpApA	5.0	CpGpA	8.0
CpApC	10.0	UpGpA	6.0
UpApC	7.0	CpUpC	2.0
CpApG	4.0	CpUpG	2.0
UpApG	6.0	CpUpU	5.0
UpCpC	4.0		

Evidently, by combining any of the methods and approaches under discussion, it is possible to synthesise in the presence of ribonucleases also longer oligonucleotides, although the experimental data concerning such syntheses are still unavailable, apart from the reports of the formation of tetranucleotides and longer oligonucleotides by incubating an excess of the substrate with pancreatic ribonuclease[17,20].

After the present review had been sent to the editors, a paper was published by Mohr and Tach[69] in which they report the synthesis of 8 tetranucleotides (ApUpGpU, ApUpGpC, ApUpGpA, ApApGpU, ApUpGpUp, ApUpGpCp, and ApUpGpAp) in yields of 5–37%, 5 pentanucleotides (ApUpGpCpC, ApUpGpUpC, ApUpGpApA, ApUpGpApC, and ApUpGpApU) in yields of 11–25%, 3 hexanucleotides (ApUpGpApApA, ApUpGpApUpG, and ApUpGpUpUpUp) in yields of 8–15%, 2 heptanucleotides (ApUpGpApApApApA and ApApApGpApApA) in 5% yields, and 1 octanucleotide ApUpGpApApApApApA in 3% yield from oligonucleotide donors and acceptors in the presence of guanyl-specific T1 ribonuclease.

III. SYNTHESIS OF OLIGORIBONUCLEOTIDES USING POLYNUCLEOTIDE PHOSPHORYLASE

The possibility of adding one or more nucleotide residues from a nucleoside 5′-diphosphate to a preformed oligonucleotide in the presence of polynucleotide phosphorylase from *Micrococcus lysodeicticus* was first demonstrated by Singer et al.[62], who obtained and characterised nucleotides such as pApApApU and pApApApGpG.

Table 6. Trinucleoside diphosphates synthesised in the presence of polynucleotide phosphorylase from *Micrococcus lysodeicticus*[63].

Substrates for synthesis			
Dinucleoside monophosphate	Nucleoside diphosphate	Trinucleoside diphosphate	Yield, %
ApA	ppC	ApApC	6.0
ApA	ppG	ApApG	12.0
ApA	ppU	ApApU	32.0
ApU	ppC	ApUpC	6.0
ApU	ppG	ApUpG	4.0
ApU	ppU	ApUpU	15.0
ApC	ppG	ApCpG	3.0
ApG	ppC	ApGpC	21.0
ApC	ppU	ApCpU	12.0
ApG	ppU	ApGpU	27.0
ApG	ppA	ApGpA	13.0
GpA	ppU	GpApU	14.0
UpA	ppU	UpApU	5.0
GpC	ppU	GpCpU	6.0

Leder et al.[63] suggested that trinucleoside diphosphates be synthesised by the addition of a nucleotide residue from a nucleoside 5′-diphosphate to a dinucleoside monophosphate according to the following mechanism

$$N'pN'' + ppN \xrightarrow{\text{polynucleotide phosphorylase}} N'pN''pN + P_i ,$$

where ppN is a nucleoside 5′-diphosphate, N′pN″ a dinucleoside monophosphate, and P_i inorganic phosphate. This method may be used for the synthesis of oligonucleotides with any sequence of monomeric units. The reaction was carried out by incubating the substrates with an enzyme at 37°C for 2 h. The trinucleoside diphosphates were isolated from the reaction mixture by paper chromatography and paper electrophoresis. The trinucleoside diphosphates synthesised in this way are listed in Table 6.

The data in Table 6 show that the yields of trinucleoside diphosphates vary from 3 to 30%, depending, according to the authors, on several factors. Thus, at a high concentration of nucleoside 5′-diphosphate, polynucleotides with a long chain (10 or more units) accumulate and shorter oligonucleotides are not formed. Such tendency towards the formation of polynucleotides with a large number of units is particularly typical for adenosine 5′-diphosphate and less so for cytidine 5′-diphosphate. However, even under the most favourable conditions, tetra-, penta-, hexa-, and hepta-nucleotides are formed in addition to the trinucleoside diphosphate. The yield of trinucleotide depends also on the nature of the nucleoside 5′-diphosphate: uridine 5′-diphosphate gives the highest yield. In those cases where the substrates used in the synthesis contain complementary bases, the yield of trinucleotide may be improved by heating the reaction mixture to 70°C before adding the enzyme.

This method was subsequently used for preparative purposes[64]. After incubating for 12 h, the reaction is stopped by adding alkaline phosphatase and the reaction mixture is separated on a column with DEAE cellulose with subsequent purification of the oligonucleotide fractions by paper chromatography. The yields of trinucleoside diphosphates were 5–40% in relation to the initial dinucleoside monophosphate.

An advantage of the method consists in the fact that polynucleotide phosphorylase does not cleave dinucleoside monophosphates[66] and therefore, on the one hand, there can be no rearrangement of the nucleotide sequence in the dinucleoside monophosphate as a result of cleavage and resynthesis and, on the other hand, dinucleoside monophosphates may be regenerated. As already mentioned, a disadvantage of the method is the fact that the reaction mixture contains, together with the trinucleoside diphosphate, also longer oligonucleotides, which reduces the yield of the trinucleoside diphosphate and hinders its isolation.

Comparison of the method of synthesis of oligonucleotides in the presence of polynucleotide phosphorylase with methods based on the application of various ribonucleases shows that both types of method have their advantages and disadvantages; bearing these in mind, the procedures must be regarded as fully of equal value in the stage involving the preparation of oligonucleotides longer than dinucleotides and may be used depending on which substrates and which enzyme are more available in each particular situation. In the synthesis of dinucleotides only the "ribonuclease method" may be employed.

The data discussed above suggest that the methods of synthesis of oligonucleotides based on the use of enzymes, particularly ribonucleases, are more advantageous and therefore more promising than chemical methods. The principal advantages of enzymatic methods are as follows: the relative availability of the initial components (substrates and acceptors), the elimination of the need to block reversibly the "superfluous" functional groups of these components and hence a significant shortening of the time required for the synthesis, the simplicity of separation of the reaction mixture, and in the majority of cases a higher yield of the oligonucleotide.

Further development and propagation of these methods should be accompanied by an increase in the number of enzymes with suitable catalytic properties and also by the development of methods necessary for the rapid and simple separation of large amounts of reaction mixtures. Special mention should be made of the necessity to seek analogous approaches to the synthesis of oligodeoxyribonucleotides, which will significantly extend the scope of enzymatic methods for the stepwise synthesis of the $C_{(3')}-C_{(5')}$ bond between specific nucleotides.

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