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Enzymatic synthesis of oligoribonucleotides of defined base sequence

Mark J. Rowe
Brigham Young University - Provo

ENZYMATIC SYNTHESIS OF OLIGORIBONUCLEOTIDES OF DEFINED BASE SEQUENCE

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Mark J. Rowe

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GENERAL INTRODUCTION

Ribonucleic acid occupies the central position in what has been termed the central dogma of molecular biology. That is, genetic information stored in the unique base sequence of deoxyribonucleic acid (DNA) is transcribed to a complementary, and still unique, base sequence in ribonucleic acid (RNA). Subsequently, the RNA base sequence is translated with absolute fidelity into biologically active compounds, such as enzymes possessing unique catalytic specificity. During the past few years the mechanism of this flow from stored information into active compounds has begun to be understood. With this understanding has come a recognition of the importance of synthetic oligoribonucleotides of specified base sequence.

In recent years synthetic oligo- and poly-ribonucleotides have been useful in elucidating many facets of the structure and function of RNA, especially in relation to certain aspects of the genetic code. For example, synthetic oligonucleotides have been instrumental in (a) establishing the ratio of the three nucleotides which codes for each of the common amino acids (Jones and Nirenberg, 1962; Wahba et al., 1963), (b) the nucleotide sequences which code for each of the amino acids (Brimacombe et al., 1965; Nirenberg et al., 1965), (c) the nucleotide sequences which code for initiation and termination of polypeptide synthesis (for example, see Last, et al., 1967; Morgan, et al., 1966), (d) the direction of translation of the genetic message (Salas et al., 1965; Thach et al., 1965), and (e) the requirement for specific protein factors in the steps

involved in the translation of the genetic message, i.e., initiation, translocation and termination (for example, see Capecchi, 1967). In addition, oligonucleotides of defined sequence have been used in the study of the relationship of the primary structure of oligonucleotides to the secondary structure, a relationship important in such properties of nucleic acids as the folding of tRNA molecules (Martin et al., 1971; Uhlenbeck et al., 1971).

Despite all that has been learned, synthetic oligonucleotides of specified sequence continue to provide one of the best approaches to the study of certain problems which involve the structure and/or function of RNA. Many of the questions concerning specific mechanisms involved in converting information contained in the base sequence of RNA into biologically active sequences of amino acids can be answered by specific model oligonucleotides. Defined oligonucleotides will also be of interest in determining more about the role of specific sequences of nucleotides in the secondary structure of tRNA (and probably messenger and ribosomal RNA) in relation to specific associations with other macromolecules (such as enzymes or ribosomes). Specific sequences of oligonucleotides may also find use in medicine as inducers, virus mimickers, and agents for genetic therapy and transformation.

Synthesis of Defined Oligonucleotides

The necessity of fabricating more or less complex nucleotide sequences in oligonucleotides, tailored to answer precise questions involved in understanding some of the above mentioned processes, has resulted in the study of a variety of techniques. These may be classified in the following groups: (a) chemical synthesis involving stepwise condensation of appropriately protected nucleotides followed

by removal of the protecting groups (Lohrmann et al., 1966); digestion of RNA or random synthetic polynucleotides by specific nucleases, followed by separation of the desired sequences (Rushisky and Knight, 1960; Staehelin, 1964); (c) chemical synthesis of short DNA sequences which may be used as templates in the "rachet" mechanism of DNA polymerase, followed by transcription of the resulting high molecular weight DNA with RNA polymerase to give a defined repeating sequence (Khorana, 1968); (d) enzymatic sequential addition of a nucleotide to the 3'-end of a specific primer with polynucleotide phosphorylase (Leder et al., 1965); and (e) specific polymerization using the reversible transesterification step of certain nucleases (Heppel et al., 1955). These methods have been developed to varying degrees of sophistication. Their further development, individually or in combination with one or more other techniques will hopefully permit the synthesis of complex sequences of nucleotides.

Chemical synthesis of specific oligonucleotides

Synthetic oligonucleotides prepared by strictly chemical means have played an important role in the development of knowledge concerning the genetic code. This was made possible largely through work in Khorana's laboratory (Lohrmann et al., 1966), in which techniques were developed for the synthesis of all 64 possible trimers containing the four common RNA bases. A few tetranucleotides have also been synthesized (Lapidot and Khorana, 1963). Chemical synthesis of short-chain oligonucleotides has also been carried out by Smrt (1967), Chladek and Smrt (1964), Holy (1968), and Cramer (1966).

The chemical techniques involved are quite laborious and require

methods and materials not available to the average investigator. Complications stem, for the most part, from the presence of the 2'-hydroxyl on the ribose moiety of ribonucleotides. If the exclusive presence of 3'-5' phosphodiester linkages is not required in the synthetic oligonucleotide, synthesis is relatively simple. Unfortunately, oligonucleotides with unnatural 2'-5' phosphodiester linkages do not behave as natural messengers (Nishimura et al., 1969). Therefore, selective protection of the 2'-hydroxyl before condensation is essential. Since the 2'-hydroxyl is chemically very similar to the 3'-hydroxyl, selective protection is difficult, as is selective removal of the protecting group to facilitate a subsequent polymerization step. Conditions required for removal of the 2'-protecting group following completion of the oligonucleotide, in many cases render the phosphodiester linkage susceptible to isomerization or hydrolysis.

As a result of the complexity of the situation, only very short oligoribonucleotides can be synthesized before the problems of protecting certain groups while activating similar groups for condensation become unreasonable.

Digestion of natural or synthetic RNA

Although not strictly a synthetic method, digestion of natural RNA is a valid technique for the preparation of certain specific nucleotide sequences (Rushisky, 1960). A sophistication of this method involves enzymatic synthesis of high molecular weight RNA followed by digestion with a nuclease specific for depolymerization following a particular base. Yields of a desired sequence may be enhanced by varying the concentration of nucleotide substrate in the synthetic

reaction. Since incorporation is a random process, the ratio of bases incorporated is a function of their ratio in the synthetic reaction mixture.

Following digestion, the desired oligonucleotide may be separated from other digestion products by suitable combinations of paper chromatography and electrophoresis, and ion exchange chromatography (Staehelin, 1964).

Although very satisfactory for the preparation of short-chain oligonucleotides, this method suffers from the unfavorable probability of desirable complex sequences lending themselves to proper depolymerization and separation.

Transcription of defined sequences of DNA

Because deoxyribonucleotides do not possess a 2'-hydroxyl, short oligodeoxyribonucleotides can be chemically synthesized much more easily than oligoribonucleotides. Khorana's laboratory developed the technique for chemical synthesis of such oligodeoxyribonucleotides with defined sequences (Hayatsu and Khorana, 1967). They found that these oligonucleotides could be used as templates in the DNA polymerase reaction, and under appropriate conditions the enzyme acted as a "rachet," resulting in the formation of high molecular weight, double stranded DNA molecules with a repeating base sequence. The repeating units of these molecules were identical to the sequence of the template, and thus exactly defined (Bird et al., 1965) with the limitation that initiation was random, so that the 5'-end was not exactly defined. The polymerized DNA was found to be suitable as a template in the RNA polymerase reaction, permitting the synthesis of complementary RNA strands (Khorana

et al., 1967). The overall result of this process is an artificial, high molecular weight, totally defined (except at the 5'-end) messenger suitable for use in cell free protein synthesizing systems. Repeating di-, tri-, and tetra-oligoribonucleotides prepared in this fashion have been used extensively in various types of experiments dealing with the genetic code.

Sequential addition of nucleotides via polynucleotide phosphorylase

The enzyme most widely used for the preparation of synthetic oligonucleotides is polynucleotide phosphorylase. This enzyme catalyzes the sequential addition of ribonucleoside 5'-monophosphates to the 3'-end of an oligonucleotide primer possessing a free 3'-hydroxyl or de novo synthesis of polyribonucleotides, with liberation of inorganic phosphate. The four common ribonucleoside 5'-diphosphates, as well as some of the less common ones, act as substrates (Grunberg-Manago, 1959).

Literature concerning the preparation and properties of polynucleotide phosphorylase is extensive and not the purpose of this summary (Grunberg-Manago, 1963; Steiner and Beers, 1961; Abrams, 1961). Of importance is the fact that complex non-random base sequences in oligonucleotides may be synthesized only if addition to an oligonucleotide primer is limited to one nucleotide, i.e., in a stepwise manner. In this way suitable conditions can be arranged for addition of the desired nucleotide at each step.

Conditions have been determined which somewhat favor addition of only one nucleotide to a primer (Leder et al., 1965). An improved method which absolutely limits addition to one nucleotide involves use of a nuclease which specifically recognizes the nucleotide being added to

the primer and hydrolyzes the oligonucleotide product such that only one residue is added to the primer. In order for this method to be effective for the synthesis of complex sequences, it is necessary to protect any phosphodiester linkages in the primer which are susceptible to the specific nuclease being used. The protecting group must lend itself to removal under conditions which do not jeopardize the oligonucleotide. Some investigations have been made into such processes (Gilham, 1962; Ho et al., 1965).

Another approach to limiting the addition of nucleotides to a primer involves blocking the 3'-hydroxyl of the nucleoside diphosphate being used as the substrate. Since the initial addition to the primer results in an oligonucleotide which does not possess a free 3'-hydroxyl, the oligonucleotide product does not participate as a primer in further polymerization. A desirable blocking group must again lend itself to easy removal so that the oligonucleotide may be converted to an active primer for the next addition in stepwise synthesis. A successful example of such a protecting agent has been demonstrated by Mackey and Gilham (1971).

Reversible transesterification by nucleases

The initial step in the digestion of RNA by many nucleases is a transesterification involving transfer of the phosphate ester bond from the 5'-position of the adjacent nucleotide, resulting in a 2',3'-cyclic phosphodiester in place of the 3'-5'phosphodiester linkage. The second step is a hydrolysis of the cyclic phosphodiester with formation of a 3'-phosphomonoester on the terminal nucleotide. The latter step is, of course, virtually irreversible, but the initial transesterification

is reversible. Therefore, under conditions favoring the reverse reaction, formation of a 3'-5' phosphodiester linkage is possible by the condensation of a nucleotide or oligonucleotide possessing a 2',3'-cyclic phosphate with a nucleotide or oligonucleotide possessing a free 5'-hydroxyl (Heppel et al., 1955). Although the reaction is theoretically possible in the absence of a nuclease, the enzyme catalyzed reaction is much more favorable.

Reactions of this type provide a method for the synthesis of oligonucleotides which has the advantage of being very specific with regard to the nucleotide incorporated since many nucleases are specific only for phosphodiester linkages following a certain base. Bovine pancreatic ribonuclease and its derivatives, and ribonuclease T_1 have been most extensively used in such reactions, the former being specific for transesterification after uridine and cytidine, and the latter after guanosine nucleotides. Another advantage seems to be the absolute synthesis of the natural 3'-5' phosphodiester linkages under appropriate conditions.

There are two obvious disadvantages to this technique, both involving competing reactions. First, the transesterification equilibrium favors depolymerization. Second, the hydrolysis of the cyclic phosphate continually removes essential substrate from the reaction. Conditions must be employed which minimize the effect of these competing reactions.

Emphasis of Dissertation

During the period of time in which the work reported in this dissertation has been carried out, the research in Dr. Marvin A. Smith's laboratory has been concerned with the use of enzymes as instruments for

the synthesis of oligoribonucleotides of defined sequence. In particular, two of the methods of synthesis mentioned previously, polynucleotide phosphorylase reactions and the synthetic reactions of ribonuclease T_1, have been of interest. The research reported in this dissertation will describe studies aimed at the development of (a) two aspects of the use of polynucleotide phosphorylase, and (b) the general use of ribonuclease T_1 for the synthesis of defined oligoribonucleotides.

Initially, research involved an attempt to enzymatically synthesize a 3'-phosphorylated nucleoside 5'-diphosphate to be used as a blocked substrate for polymerization reactions of polynucleotide phosphorylase. If such a compound acted as a substrate for polynucleotide phosphorylase the 3'-phosphoryl group would limit addition onto a defined primer to one nucleotide, as mentioned previously. The reaction of adenylate kinase was studied with regard to its interaction with adenosine 2'(3'),5'-diphosphate in an attempt to synthesize a suitable substrate.

As research proceeded in the above area, the synthetic reaction of nucleases became more important as a result of reports of successful synthesis of several short oligonucleotide with pancreatic ribonuclease. Our interest in the synthetic reaction of ribonuclease T_1 was stimulated by these reports. It was hoped that suitable conditions for using ribonuclease T_1 in several aspects of synthesis could be obtained. During these investigations, we and several other laboratories published results of work with ribonuclease T_1.

At the conclusion of our study on ribonuclease T_1, interest was generated in a phosphorylation-dephosphorylation mechanism for the regulation of the primer dependence of polynucleotide phosphorylase. A

protein kinase and a protein phosphatase were isolated for the purpose of exploiting this mechanism as a simple method of preparing a completely primer dependent polynucleotide phosphorylase.

These areas of interest, i.e., preparation of a blocked substrate for polynucleotide phosphorylase, the synthetic activity of ribonuclease T_1, and preparation of a completely primer dependent polynucleotide phosphorylase form the basis for the investigations described in this dissertation. Sections reporting the results of these studies are preceded by an introductory chapter or subsection, including a review of literature pertinent to the section. Portions of this dissertation are presented as published or submitted for publication in scientific journals.

PART I

THE SYNTHETIC REACTION OF RIBONUCLEASE T_1

CHAPTER I

INTRODUCTION TO THE SYNTHETIC REACTION OF RIBONUCLEASE T_1

Reversible Nuclease Reactions

Early demonstrations of synthesis with nucleases

It is well known that many ribonucleases form 3'-monophosphorylated nucleosides or oligonucleotides during the degradation of RNA by a two step mechanism. During the first step the ribonuclease catalyses the depolymerization of RNA by a transesterification at a susceptible 3'-5'phosphodiester linkage. One ester bond of the phosphate is transferred from the 5'-position of the adjacent nucleoside to the 2'-hydroxyl resulting in the formation of a 2',3'-cyclic phosphodiester and a free 5'-hydroxyl. (Figure 1). The depolymerization step is followed by slow specific hydrolysis of the 2',3'-cyclic phosphodiester to a 3'-phosphomonoester, also catalyzed by the nuclease.

Before 1955, ribonucleases were not associated with any type of reaction other than the depolymerization by transesterification, and hydrolysis reactions mentioned above. During that year, Heppel and Whitfeld (1955) demonstrated that pancreatic ribonuclease also participates in synthetic and exchange reactions. They found that ribonuclease can catalyze the formation of certain alkylesters from nucleoside 2',3'-cyclic phosphates in the presence of primary alcohols, which act as phosphate acceptors. The products of the ribonuclease catalyzed

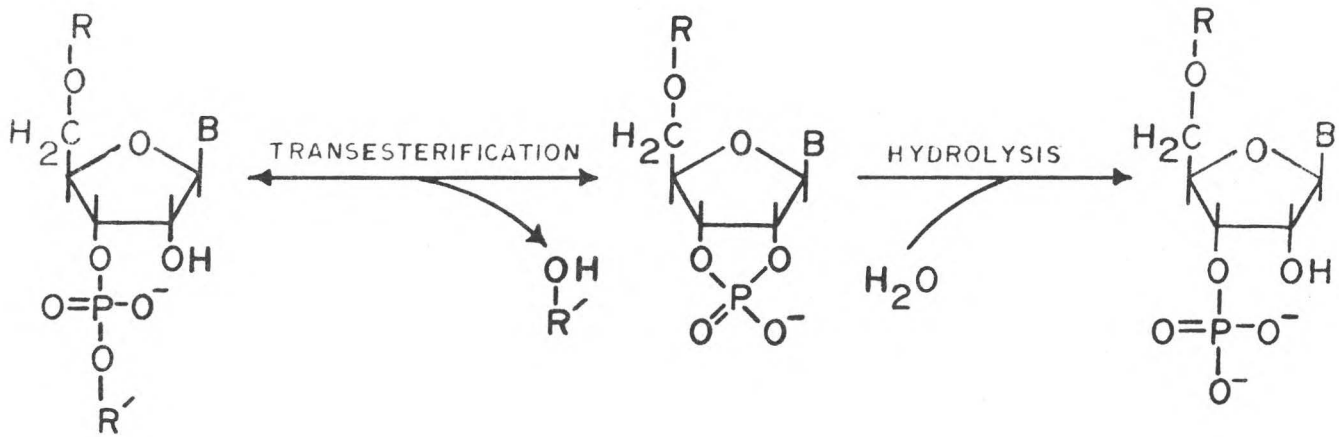

Figure 1.--Reactions catalyzed by the "cyclizing" nucleases.

reactions were nucleoside 3'-methylphosphates and nucleoside 3'-ethylphosphates, using methanol and ethanol respectively as acceptors. These reactions can be thought of either as the reverse of the transesterification reaction, or as a type of hydrolysis reaction (alcoholysis) using primary alcohols in place of water as the phosphate acceptor. Another reaction, which may be considered a transesterification, and also shown to be catalyzed by pancreatic ribonuclease, was the formation of nucleoside 3'-methylphosphates from nucleoside 3'-benzylphosphates and methanol. The nuclease was inactive toward the nucleoside 2'-benzylphosphates.

An extension of these results (Heppel et al., 1955) demonstrated that it was possible to use other primary alcohols in the reverse transesterification reaction of pancreatic ribonuclease. In this instance the primary alcohol used as an acceptor was the 5'-hydroxyl of nucleosides and nucleoside 2',3'-cyclic phosphates. Such condensation results in the synthesis of dinucleotides.

Specific Ribonucleases With Synthetic Properties

The work done by Heppel et al., (1955) was the first indication that under suitable conditions reverse transesterification catalyzed by a nuclease could be used for the synthesis of oligonucleotides. Since that time, conditions favoring the synthetic reactions of several nucleases have been developed to varying degrees of sophistication. Ribonuclease T_1 (Sato-Asano and Egami, 1958) ribonuclease U_2 (Kioke et al., 1971a), ribonuclease N_1 (Koike et al., 1971b), a ribonuclease from Actinomyces aureoverticillatus (Abrosimova-Amelyanchik et al., 1967) and a ribonuclease from Aspergillus clavatus (Zhenodarova and Habarova,

1968), in addition to pancreatic ribonuclease have been used in reactions for the synthesis of oligoribonucleotides.

Synthetic reactions of these nucleases have been exploited somewhat in proportion to the advantages and disadvantages they possess with regard to synthesis of specific oligonucleotides. The Aspergillus clavatus ribonuclease is nonspecific with regard to the base adjacent to the 3'-5' phosphodiester linkage which it attacks. It has therefore been used only for the synthesis of a variety of dinucleoside monophosphates such as A-C, G-C, C-C, and U-C from the condensation of cytidine and the appropriate nucleoside 2',3'-cyclic phosphate. Synthesis of larger oligonucleotides is prohibited since any substrate larger than a nucleoside or nucleotide will possess nuclease susceptible linkages.

The nuclease from Actinomyces aureoverticillatus, on the other hand, is specific for transesterification (and therefore reverse transesterification) at the phosphodiester linkage following a guanosine nucleoside. Such specificity is intrinsically desirable in most situations involved in the synthesis of defined sequences in oligonucleotides because only specific nucleotides can be added to a sequence by the enzyme. In addition, oligonucleotide substrates have less probability of containing susceptible linkages. This nuclease has been used for the synthesis of dinucleoside monophophates and diphosphates with a 5'-terminal guanylic acid.

Ribonucleases N_1 and U_2 are not only specific for transesterification at phosphodiester linkages following guanosine (or inosine) and adenosine respectively, but they have the added advantage that the hydrolytic activity catalyzed by the enzymes is much slower than the transesterification activity. Such a property is useful since any

hydrolytic activity serves to remove the substrate possessing the cyclic phosphate from the reaction mixture by converting it to the 3'-monophosphate. Ribonuclease N_1 has been successfully used for the synthesis of dinucleotides with terminal 5'-guanylic acids, oligoguanylic acids (Koike et al., 1969), the repeating dinucleotide oligo (AG), and specific di-, tri-, and tetra-nucleotides containing guanosine and inosine (Koike et al., 1971b). Ribonuclease U_2 has been applied to the synthesis of specific adenine containing dinucleotides and oligoadenylic acids (Koike et al., 1971a).

Since the original demonstration of the synthetic reaction of pancreatic ribonuclease, the synthetic properties of this enzyme have been studied and exploited only be Bernfield (1965 and 1966) and Bernfield and Rottman (1967). Ribonuclease A, being specific for the common pyrimidine ribonucleosides was shown to be useful in synthetic reactions involving these compounds. This nuclease became even more useful as a synthetic tool when it was shown by Bernfield (1965) that modified forms of the enzyme were less active than the native enzyme in depolymerization and hydrolysis reactions, while retaining comparable synthetic activity. The modified forms of ribonuclease A studied included (a) the ribonuclease S-protein which is a polypeptide residue of ribonuclease A derived from limited proteolytic digestion of the native enzyme (Richards, 1958); (b) the 1-carboxymethylhistidine-119-ribonuclease (Crestfield et al., 1963); and (c) the ϵ-dinitrophenylaminolysine-41-ribonuclease (Hirs, 1962).

Bernfield (1965 and 1966) and Bernfield and Rottman (1967) compared the synthetic properties of each of these forms of ribonuclease A with those of the native enzyme and found them different with respect

to optimal pH, temperature, and enzyme concentration.

The ribonuclease A derivatives were used for the synthesis of four dinucleoside monophosphates and twenty-five trinucleoside diphosphates (Bernfield, 1966). In addition, the kinetics of synthesis of the ϵ-dinitrophenylaminolysine-41-ribonuclease were compared with those of the native enzyme using 5'-blocked nucleoside cyclic phosphates as non-selfpolymerizing substrates (Bernfield and Rottman, 1967). Although optimum conditions for synthesis at fixed substrate concentrations were determined in these studies, important considerations concerning possible incorporation maxima at high concentrations of one substrate were not studied. The fixed concentrations used for synthesis (amounting to three and four fold excesses of the nucleoside cyclic phosphate) resulted in 15 to 27% incorporation of nucleoside acceptors, and 2 to 10% incorporation of dinucleoside acceptors.

Ribonuclease T_1 is the only other nuclease which has been used for synthetic purposes. This enzyme will be discussed in detail in the following section.

Synthetic Reaction of Ribonuclease T_1

Aspergillus oryzae was shown to be an exceptionally abundant source of ribonucleases by Kuninaka (1955) and Saruna (1957). These authors also described the general characteristics of the enzyme mixture. A commercial enzyme product of Aspergillus oryzae called Taka-Diastase was shown to contain, among other nucleases, two which by far accounted for the largest fraction of the nuclease activity of the preparation. These were designated ribonuclease T_1 and T_2, the former being the most abundant by a ratio of ten to one (Rushisky and Sober, 1963). Ribonuclease T_1 has been given the systematic name Ribonucleate Guanine-nucleo-

tide-2'-transferase (cyclizing) and was originally classified EC 3.1.4.8. but is now classified EC 2.7.7.26.

Ribonuclease T_1 has been well characterized, owing mainly to its abundance and stability. The first indication that a synthetic reaction was possible with ribonuclease T_1 was reported by Sato-Asano and Egami (1958) when they were able to show that under appropriate conditions the enzyme catalyzed the synthesis of G-G, G-A, G-U, and G-C.

Later, Sato-Asano (1960) was able to demonstrate synthesis of a variety of dinucleotides using nucleosides, nucleoside cyclic phosphates, and nucleoside (2'),3'-monophosphates as acceptors. In addition, oligoguanylic acids varying in length from two to twelve residues were synthesized by polymerization of guanosine 2',3'-cyclic phosphate in the presence of ribonuclease T_1 (Sato-Asano and Egami, 1960; Hiroshi and Egami, 1963).

Since the demonstration that the reverse transesterification reaction of ribonuclease T_1 was useful for synthesis of specific dinucleotides, this reaction has been used extensively but not studied in detail. Examples of its use in situations where the authors did not elect to study the reaction in depth are those in which a particular short oligonucleotide was desired for other investigations. These included: synthesis of poly-8-bromoguanosine to study the properties of that polymer (Yuki and Yoshida, 1971); the synthesis of 5'-substituted trinucleotides to study their effectiveness as templates for binding tRNA to ribosomes (Holy et al., 1970; Holy, 1969; Scheit and Cramer, 1964); synthesis of trinucleotide analogues for similar purposes (Lisy et al., 1968); synthesis of oligonucleotides for the study of phosphodiester linkage specificity of the synthetic reaction (Podder and Tinoco,

1969; Podder, 1970); and synthesis of oligoguanylic acids for the study of aggregations of these compounds and the effect of mercuric salts on such aggregations (Lipsett, 1964).

In addition to the studies mentioned above in which the synthetic reaction of ribonuclease T_1 was used mainly as a synthetic tool, there have been several studies attempting to define the optimum conditions for maximum synthesis. Grunberger et al., (1968) described conditions for the synthesis of 46 trinucleotides containing guanosine, inosine, 8-azaguanosine and xanthosine in predetermined positions. A brief study was made to determine conditions for maximum incorporation of the donor (substrate containing the cyclic phosphate). Incorporation of the donor reached 27% with a molar ratio of acceptor to donor of 5 to 1. These authors did not attempt a detailed study of conditions for maximum incorporation of acceptor.

Sekiya et al., (1968) also attempted to determine optimum conditions for the synthesis of di- and tri-ribonucleosides and synthesized twelve trinucleotides possessing a guanylyl residue at the 5'-end. In these reactions yields up to 20% were obtained using equimolar ratios of the donor and acceptor substrates. Again, a detailed study of maximum incorporation of either substrate as a function of substrate concentration was not attempted. In addition, it was noted that under conditions of equimolar substrate concentrations, polymerization of guanosine 2',3'-cyclic phosphate occurred, lowering yields of the desired product.

Although these studies were able to demonstrate that various oligonucleotides of defined sequence could be synthesized with ribonuclease T_1, and that certain physical conditions (e.g., pH and temperature) favor the synthetic reaction, they do not include studies concerned with

obtaining good yields of the oligonucleotides. Mohr and Thach (1969) were the first to show that 66% incorporation of the guanosine cyclic phosphate could be obtained in the presence of large excesses of the acceptor (600 fold). In addition to this significant yield, Mohr and Thach (1969) also showed that oligonucleotides could be used as donors as well as acceptors in the synthetic reaction. They were able to synthesize thirty-two different oligonucleotides ranging in chain length from two to eight nucleotide residues.

Before the work of Mohr and Thach appeared, we had also been able to demonstrate good incorporation of guanosine 2',3'-cyclic phosphate (58%) in the presence of a large excess of the acceptor (Rowe and Smith, 1970, see Chapter II). In addition, our paper reported the demonstration that under these conditions, natural 3'-5' phosphodiester linkages were the exclusive product, in contrast to synthesis under the conditions of Podder and Tinoco (1969) and Podder (1970).

It became obvious from our results and those of Mohr and Thach that good incorporation of the desired substrate could be obtained by driving the synthetic reaction with large excesses of the other substrate. With regard to driving the synthetic reaction in this way, it is useful to note that there are three general types of synthesis in which ribonuclease T_1 may be useful as a tool for synthesis of oligonucleotides possessing guanosine in a predetermined position:

I. Addition of a particular nucleoside to the 3'-end of an oligonucleotide,

$$(N-)_nG>p + X \xleftarrow{\text{ribonuclease T1}} (N-)_nG-X$$

II. Addition of a guanosine nucleotide to the 5'-hydroxyl of an oligonucleotide,

$$G>p + (N-)_n \xleftrightarrow{\text{ribonuclease T1}} G-(N-)_n$$

III. Condensation of two oligonucleotides,

$$(N-)_n\ G>P + (N-)_m \xleftrightarrow{\text{ribonuclease T1}} (N-)_nG-(N-)_m$$

Reactions of type I above may be driven with an excess of the acceptor, X, since nucleosides are relatively inexpensive and available in large amounts. As mentioned, this was shown to be the case (Mohr and Thach, 1969; Rowe and Smith, 1970, see chapter II). However, since oligonucleotides of defined sequence are very expensive, high yields are not economically obtained using large excesses of these substrates in reactions of type II. Indeed, reaction conditions should favor maximum yield calculated on the basis of incorporation of such acceptors since they are expensive or difficult to prepare. With the aim of obtaining maximum incorporation of acceptor the donor concentration was increased to drive the reaction. Chapters III and IV describe the problems associated with such reaction conditions, and the results of synthesis using methods which overcome these problems. Chapters II, III, and IV are presented as they have been published or submitted for publication (Rowe and Smith 1970; Rowe and Smith 1971; and Rowe and Smith, 1972).

Reactions of type III, in which both substrates are oligonucleotides, are considerably more complex. Clearly, using high concentrations of either substrate is not a satisfactory approach to increasing the synthetic yield. Methods of overcoming the problems involved in reactions of this type are not included in the studies reported in this dissertation, but are important since one of the real values of the synthetic reaction of ribonuclease T_1 may be the condensation of blocks of oligonucleotides with a known base sequence.

CHAPTER II

SYNTHESIS OF 3'-5' DINUCLEOTIDES WITH RIBONUCLEASE T_1

(As published by M.J. Rowe and M.A. Smith, 1970)

Introduction

Studies on the mechanism of protein synthesis have demonstrated the need for oligonucleotides of predetermined sequence. The synthetic reactions of several nucleases have been used in the synthesis of such oligonucleotides (Bernfield, 1965 and 1966; Bernfield and Rottman, 1967; Abrosimova et al., 1967). Ribonuclease T_1 (EC 2.7.7.26) has been used to form guanosine containing oligonucleotides (Sato-Asano, 1960; Sato-Asano and Egami, 1960; Hiroshi and Egami, 1963; Sekiya et al., 1968; Grunberger et al., 1968; Scheit and Cramer, 1964). However, Podder and Tinoco (1969) and Podder (1970) demonstrated that the reverse reaction of ribonuclease T_1 also catalyzes the formation of unnatural 2'-5' phosphodiester bonds. The usefulness of ribonuclease T_1 in the synthesis of oligonucleotides of predetermined base sequence therefore becomes questionable, especially if they are to be used as model compounds in studying the mechanism of protein synthesis, since oligoribonucleotides containing 2'-5' bonds fail to stimulate the binding of tRNA to ribosomes (Nishimura et al., 1969). It would therefore be beneficial to outline conditions in which exclusively 3'-5' phosphodiester linkages are formed. Data in this communication indicate that G-C containing only 3'-5' phos-

phodiester bonds can be synthesized in the ribonuclease T_1 catalyzed reaction using G>p and cytidine as substrates.

Materials and Methods

Guanosine 2',3' cyclic phosphate (G>p) and all nucleosides were obtained from Schwarz Bioresearch. DEAE-cellulose (DE 23, 1.0 meq/g) was purchased from Whatman.

The solvent system used for descending paper chromatography was isopropanol/1% $(NH_4)_2SO_4$ in a ratio of 2/1 (v/v).

Column chromatography was carried out on DEAE-cellulose columns (1.5 mm X 25 cm) using an exponential gradient of NH_4HCO_3 (0-0.15 M, pH 8.6) and a 125 ml mixer. Four columns were eluted simultaneously using a single gradient producing device. The flow rate was 460 ml/ cm^2/hr. Column effluents were monitored continously at 260 nm with a Gilford multiple sample recording spectrophotometer.

The synthetic activity of ribonuclease T_1 was assayed by the following method. Aliquots of reaction mixtures were spotted on 1 in. X 7 in. strips of Whatman #1 paper. These were subsequently dipped in a 0.05 M ammonium bicarbonate buffer (pH 8.6), excess moisture removed by blotting, and placed in a Gelman electrophoresis chamber containing the same buffer. A potential of 20 V/cm was applied. Ultraviolet absorbing regions sugsequently obtained were cut out and eluted overnight in 1.2 ml of 0.01 M NH_4OH. The absorbance of each sample was measured at 260 nm. The molar extinction coefficient for G-C was assumed to be identical to that of G-Cp (Stanley and Bock, 1966).

Results and Discussion

Conditions for optimum synthesis with ribonuclease-T_1

The nature of the phosphodiester linkage formed under conditions of maximum dinucleotide synthesis offers a starting point from which conditions favoring 3'-5' diester linkages may be found. Maximum yields are important since substrates are expensive and time consuming to prepare. Since the kinetics of the reaction are complex, it was necessary to control time, temperature, and enzyme concentration carefully. The influence of each of these factors was determined by varying each separately.

Ribonuclease T_1 concentration.--As shown in Figure 2, the amount of G-C formed in two hours goes through a maximum at ribonuclease T_1 concentration of 0.35 µg/ml. At lower concentrations a typical "enzyme versus time" relationship is obtained. At higher enzyme concentrations the depolymerization reaction dominates the addition reaction. In concentrations greater than about 59 µg/ml no G-C is synthesized, and the formation of 3'-GMP (G>p $\longrightarrow$ 3'-GMP) is increased. GMP seen at low ribonuclease T_1 concentrations is a contaminant in the G>p (about 10%). On the basis of G>p incorporation into dinucleotide, the yield of G-C (Figure 2) is about 55%.

Influence of temperature.--Figure 3 shows the effect of temperature on the synthesis of G-C. The yield decreased almost linearly with increasing temperature, from 0 to 37° C. At the higher temperatures depolymerization is favored, resulting in the breakdown of G-C. At 37° C about a 20% incorporation of G>p into G-C was obtained. The yield of G-C at 37° C goes through a maximum at 30 minutes but never reaches a value comparable to that obtained at lower temperatures.

Reaction kinetics.--Figure 4 shows the distribution of substrates

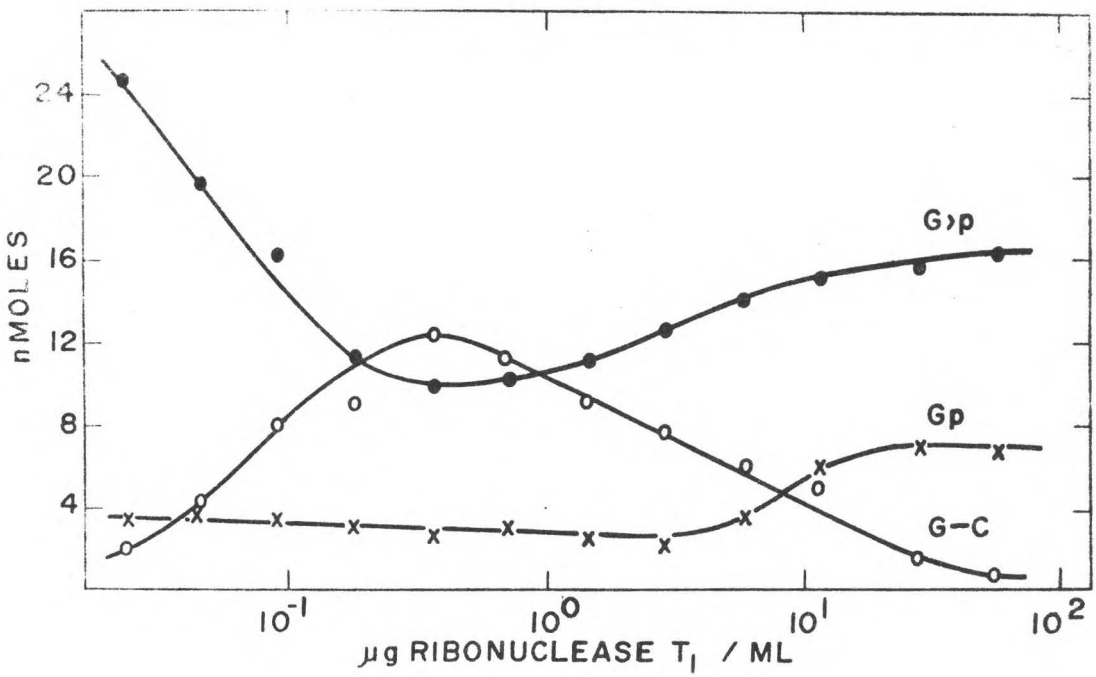

Figure 2.--Influence of ribonuclease T_1 concentration on the synthesis of G-C. Reaction mixtures (17 μl) contained 59 mM Tris-HCl buffer (pH 7.3), 0.35 M cytidine, 2.75 mM G>p. Ribonuclease T_1 was varied from less than 0.1 to 100 μg / ml. Reactions were carried out at 0° C for two hours.

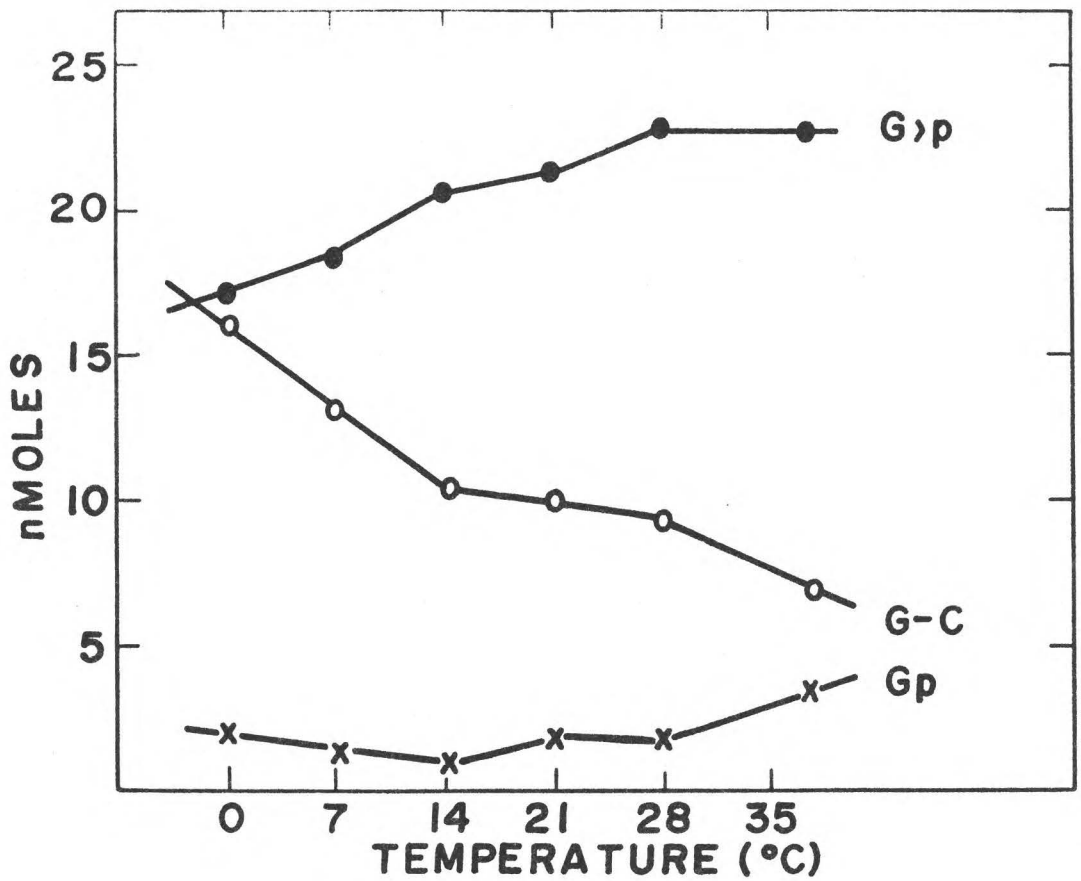

Figure 3.--The effect of temperature on the synthesis of G-C from G>p and cytidine. The reaction mixtures (17 µl) contained 59 mM Tris-HCl buffer (pH 7.3), 0.35 M cytidine, 2.75 mM G>p and 0.30 µg/ml of ribonuclease T_1. Reactions were carried out at temperatures ranging from 0 to 37° C for six hours.

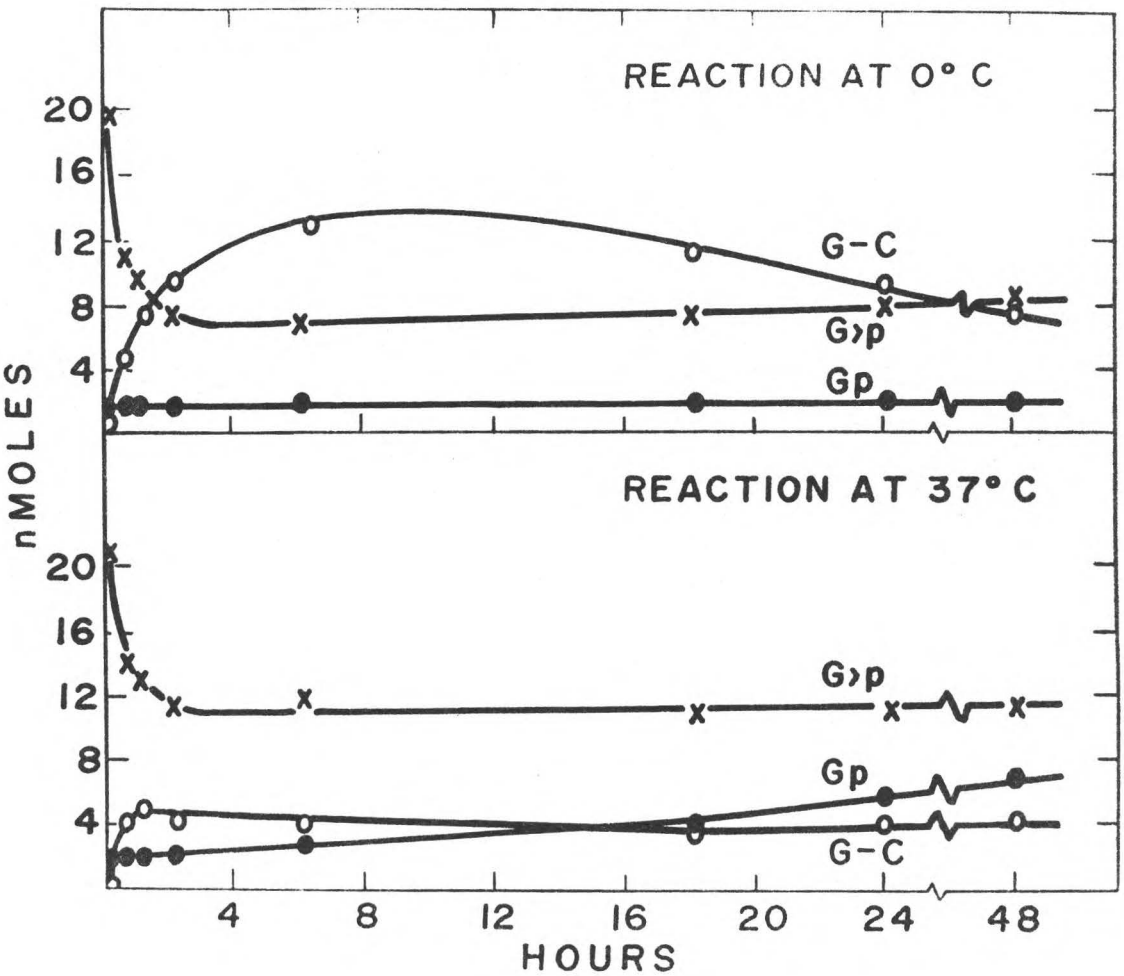

Figure 4.--Synthesis of G-C over a 48 hour period at 0 and 37° C. The reaction mixtures (102 µl) contained 59 mM Tris-HCl buffer (pH 7.3), 0.35 M cytidine, 2.75 mM G>p and 0.30 µg ml of ribonuclease T_1. Ten µl aliquots were removed at the times indicated.

and products at intervals over a 48 hour period, at both 0 and 37° C. At 0° the yield of G-C was maximal at six hours, and 58% of the cyclic phosphate was incorporated into dinucleoside monophosphate. At 37° C a maximum yield of G-C occurred in 30 minutes and was three fold less than at 0° C. Although considerably less hydrolysis occurred at 0°, 3'-GMP was produced at a constant rate at both temperatures.

Characterization of G-C

Dinucleoside monophosphate (G-C) synthesized at 0° and 37°, was characterized with regard to the nucleosides present, their sequence and the nature of the phosphodiester linkage, i.e., 3'-5' or 2'-5'. The 37° C product was characterized because formation of 2'-5' diester linkages was shown to occur at temperatures greater than 0° C. (Podder and Tinoco, 1969).

Alkaline hydrolysis.--Alkaline hydrolysis was used to identify the component nucleosides or nucleotides and their sequence. This was carried out in 0.5 M KOH for 25 minutes at 100° C. G-C synthesized at both 0 and 37° and was converted to equal amounts of cytidine and 2', (3')-GMP as shown in Table 1. The products of the hydrolysis were separated by column and paper chomatography and identified using authentic markers.

Sensitivity of G-C to ribonuclease T_1.--The 2'-5' phosphodiester bond in oligonucleotides is known to be resistant to ribonuclease T_1 (Lipsett, 1964). This provides a tool for characterization of the diester linkage in G-C. Aliquots of G-C formed at both 0 and 37° were incubated for 16 hours at 37° with 32 units of ribonuclease T_1 in 1 mM

TABLE 1

CHARACTERIZATION OF RIBONUCLEASE T_1 SYNTHESIZED G-C

Treatment	Initial nmoles G-C	Products (nmoles)[a]			C/G	Recovery[b]
		2'-GMP	3'-GMP	C		
0.5 M KOH	(0°) 154	75	75	158	1.05	97%
	(37°)158	76	76	164	1.08	96%
RNase T_1	(0°) 155	0	154	155	1.01	99%
	(37°) 71	0	63	71	1.12	94%
IO_4, Lysine	(0°) 170	0	166	...	....	97%
	(37°)168	0	165	...	....	98%

[a]Analysis of the products was done by column chromatography as described in materials and methods.

[b]The recovery is based on the nmoles of GMP recovered from the initial G-C.

Tris-HCl (pH 7.3). Products were separated by column chromatography. As shown in Table 1, G-C was quantitatively hydrolyzed to 3'-GMP and C. This gave convincing evidence that 3'-5' G-C was the exclusive product, at 0 and 37° C.

Periodate oxidation of G-C.--Additional evidence of the nature of the phosphodiester linkage was obtained by oxidation of the 3'-cytidine portion of G-C followed by amine catalyzed β-elimination of the 5'-linked nucleoside (Neu and Heppel, 1964).

This was accomplished with periodate followed by cleavage with lysine. Products from such reactions were analyzed by column chromatography as described above. 3'-GMP was quantitatively recovered from G-C synthesized at both 0° and 37° (Table 1), and was shown to cochromatograph with authentic 3'-GMP. No 2'-GMP was detected.

These results are extremely important since they demonstrate that under conditions of maximum polymerization ribonuclease T_1 can be catalytically used in the synthesis of oligonucleotides containing entirely 3'-5' phosphodiester linkages. Although Podder and Tinoco (1969) have been successful in demonstrating the formation of 2'-5' oligoguanylic acid using high concentrations of ribonuclease T_1, the synthesis of G-C can be carried out under conditions such that entirely 3'-5' linkages are obtained. Preliminary experiments carried out in this laboratory under our conditions indicate that pG>p and cytidine are also converted to 3'-5' pG-C in high yields. The apparent discrepancy might be a consequence of peculiarities observed in the absence of added nucleosides, or the combination of higher ribonuclease T_1 concentrations and temperatures, used in the formation of oligoguanylic acid. Since we have not extended our studies beyond those conditions

supporting maximal conversion of G>p into dinucleotide we are unable to clarify the question further.

CHAPTER III

SYNTHESIS OF 3'-5' DINUCLEOSIDE DIPHOSPHATES WITH RIBONUCLEASE T_1

(As published by M.J. Rowe and M.A. Smith, 1971)

Introduction

Numerous enzymatic methods have been used for the synthesis of oligoribonucleotides, including several nucleases (Leder et al., 1965; Bernfield, 1965 and 1966; Bernfield and Rottman, 1967; Koike et al., 1969; Abrosimova-Amelyanchik et al., 1967; Grunberger et al., 1968; Sekiya et al., 1968; Mohr and Thach, 1969; Rowe and Smith, 1970). Synthetic reactions of nucleases provide a method for the synthesis of oligoribonucleotides which has the advantage of being very specific with regard to the nucleotides incorporated, and the internucleotide linkage formed (i.e., 3'-5') (Rowe and Smith, 1970). Ribonuclease T_1 (EC 2.7.7.26) under appropriate conditions catalyzes the condensation of guanosine 2',3'-cyclic phosphate (G>p) with the 5'-hydroxyl group of various nucleosides or oligonucleotides (Sata-Asano, 1960; Sato-Asano and Egami, 1960; Sato-Asano and Egami, 1958; Hiroshi and Egami, 1963; Scheit and Cramer, 1964). This reaction provides a tool for the synthesis of oligoribonucleotides possessing a guanosine nucleotide in a predetermined position.

The synthetic reaction is, however, complicated by the competing hydrolysis of guanosine 2',3'-cyclic phosphate. The formation of

oligoguanylic acids can also complicate the reaction (Sekiya et al., 1968; Sato-Asano 1960).

$$G>p \xleftarrow{\text{ribonuclease } T_1} (G-)_nG>p$$

In condensations of G>p with dinucleoside monophosphates, G-Gp was obtained as a major product (Sekiya et al., 1968). These complications may be avoided using a large excess of the phosphate accepting substrate which contains a free 5'-hydroxyl group (acceptor) (Mohr and Thach, 1969; Rowe and Smith, 1970), or by blocking the 5'-hydroxyl of the phosphate donor substrate which contains a 2',3'-cyclic phosphate (donor). The latter is the preferred approach when the phosphate acceptor is an oligonucleotide which is difficult to prepare. This communication describes data obtained from the reaction of cytidine with pG>p. The results indicate that increasing concentrations of the donor may be used to force the reaction in the direction of synthesis, without the complication of oligoguanylic acid formation. Conditions which favor synthesis of pG-C have been determined.

Materials and Methods

Nucleosides were obtained from Schwarz Bioresearch. Ribonuclease T_1 and E. coli alkaline phosphatase were obtained from Sigma and Worthington Biochemicals respectively, and polyphosphoric acid from Matheson, Coleman, and Bell. Carbobenzoxychloride was purchased from Aldrich Chemical Company. DEAE-cellulose (0.66 meq/gm) was purchased from BioRad.

Synthesis of pG>p was accomplished by cyclization of pGp with benzylchloroformate in a procedure similar to the ethlychloroformate method described by Michelson (1966). Others have prepared this com-

pound by phosphorylation of 5'-GMP (Holy and Pischel, 1967). pGp used in the above synthesis was prepared by the phosphorylation of guanosine with polyphosphoric acid (Bernfield, 1966; Hall and Khorana, 1955; Waehneldt and Fox, 1967; Michelson, 1958), and by the phosphorus trichloride method of Yoskikawa et al., (1970). It was purified by chromatography on a DEAE-cellulose column (1.5 X 50 cm) in the HCO_3^- form. The column was eluted with an exponential gradient (0--0.15 M), of NH_4HCO_3 (pH 8.6) using a one liter mixer. The product of the cyclization reaction was separated from unreacted pGp by chromatography on Whatman 3MM paper using solvent A. The pG>p was further purified by high voltage paper electrophoresis (120 V/cm, 0.05 M NH_4HCO_3, pH 8.6).

The identity of the above compounds was established by analysis of products obtained after treatment with bacterial alkaline phosphatase (BAP) and/or ribonuclease T_1. Results are shown in Table 2. Products in Table 2 were identified by comparative chromatography with authentic compounds using one or more of the following solvent systems: solvent A, isopropanol/1.5% ammonium bicarbonate, 2/1; solvent B, isopropanol/water, 7/3; solvent C, isobutric acid/ NH_4OH / water, 57/4/39; solvent D, 95% ethanol/ 1 M ammonium acetate, 5/2; solvent E, isopropanol/ 1% ammonium sulfate, 2/1. Inorganic phosphate was determined by the method of Fiske and Subbarow (1925). Ultraviolet absorption data are summarized in Table 3.

Column chromatography used in analytical characterization experiments involving pGp, pG>p and pG-C was carried out on DEAE-cellulose columns (15 X 25 cm) using an exponential gradient of NH_4HCO_3 (0-0.15 M, pH 8.6) and a 125 ml mixer. Four columns were eluted simultaneously using a single gradient producing device. The flow rate

TABLE 2

ENZYMATIC CHARACTERIZATION OF pGp AND pG>p[a]

Compounds		Treatment	Product		
Abrev.	nmoles		Abrev.	nmoles	P/G[b]
pGp	268	None	pGp	268	1.97
pGp	268	BAP (exhaustive)	G	265	....
pGp	1160	BAP (limited)	pGp,pG,Gp,G	1160[c]	....
pGp	268	RNase T1	pGp	268	....
pGp	340	5'-Nucleotidase	pGp	340[d]	....
pG>p	36.0	None	pG>p	36.0	1.05
pG>p	36.0	BAP	G>p	35.0	....
pG>p	36.0	RNase T1	pGp	36.8	2.0
pG>p	36.0	RNase T1 & BAP	G	33.5	....

[a]The Compounds shown above were characterized by enzymatic hydrolysis with several different enzymes, followed by chromatographic comparison of Products with authentic markers.

[b]P/G is the ratio of inorganic phosphate to guanosine (or guanosine 2',3'-cyclic phosphate) after treatment of the product with bacterial alkaline phosphatase.

[c]Total nmoles of the products and reactant present in the reaction mixture remained constant during the course of the hydrolysis reaction (see Figure 5).

[d]2' or 3' substituted nucleotides are known to be resistant to hydrolysis by 5'-Nucleotidase (Heppel, 1961).

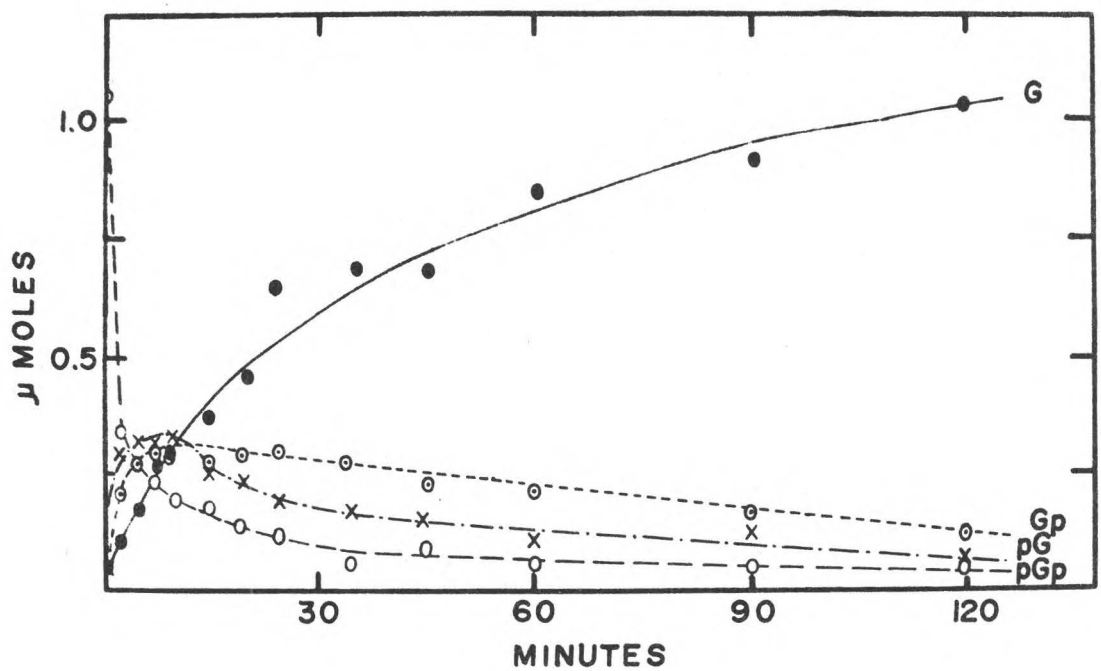

Figure 5.--Characterization of pGp by hydrolysis with BAP. A reaction mixture (52 μl) containing 22.3 mM pGp, 916 mM Tris-HCl buffer (pH 7.8), and 0.64 units of bacterial alkaline phosphatase, was incubated at 37° C. Two μl aliquots were withdrawn at intervals increasing from 2.5 minutes to 30 minutes for a period of 120 minutes, spotted on Whatman #1 paper, and developed in Solvent E with appropriate markers. UV absorbing spots were cut out and extracted overnight in two ml of 0.01 M NH_4OH. The absorbance of each was determined at 260 nm. The total absorbance of each aliquot remained constant. (This figure was not included in this paper as published, Rowe and Smith, 1971).

TABLE 3

SPECTRAL CHARACTERISTICS OF PHOSPHORYLATED GUANOSINE COMPOUNDS

Compound	λ Max.	λ Min.	280/260	250/260
5'-GMP[a]	256 nm	228 nm	0.70	0.99
pGp[b]	256 nm	227 nm	0.68	0.99
pG>p[b]	256 nm	229 nm	0.70	0.97

[a]Published spectral characteristics for 5'-GMP are shown (Beaven et al., 1955).

[b]All values reported were measured at pH 2.

was 460 ml/cm^2/hr. Column effluents were monitored continously at 260 nm with a Gilford Model 2000 spectrophotometer.

The synthetic activity of ribonuclease T_1 was assayed as follows. Substrates and enzyme were reacted in Tris-HCl Buffer (pH 7.3) under various conditions as described in the legends to the figures. Aliquots were removed and diluted with equal volumes of 0.05 M dithiothreitol in 7.5 M ammonium hydroxide and heated to 37°C for 15 minutes to terminate the reaction (Mohr and Thach, 1969). Major components of the reaction mixture were separated by electrophoresis (40 V/cm) on Whatman #1 paper in 0.05 M ammonium bicarbonate buffer (pH 8.6), in a Gelman electrophoresis apparatus. Separation was complete in about 30 minutes. Ultraviolet absorbing regions were cut out and eluted overnight in 1.2 ml of 0.01 M NH_4OH. The absorbance of each sample was measured at 260 nm. Blanks were obtained by eluting identical sections of paper strips to which no sample had been added.

Results

That pG>p is a substrate for the synthetic reaction of ribonuclease T_1 has been demonstrated by Holy et al., (1970). However, conditions which permit optimum yields have not been reported. Because synthesis is complicated by ribonuclease T_1 catalysed hydrolysis of the cyclic phosphate, the effect of varying the pH, temperature, time, and enzyme and substrate concentrations has been studied in detail. Our data demonstrate that the non-self-polymerizing substrate pG>p is as effective in the synthesis of guanylyl cytidine as G>p, and that improved incorporation of the cytidine into pG-C can be obtained in the presence of increased concentrations of pG>p.

Dependence of synthetic activity on pH

Synthetic activity of ribonuclease T_1 was studied at pH values ranging from 5.7 to 9.0. Phosphate and Tris-HCl buffers were used below and above pH 7.0 respectively. The pH profile of the synthetic activity was very similar to that obtained for the depolymerization (Rushisky and Sober, 1962) and hydrolysis activities (Irie, 1967) with a maximum near pH 7.5 (See Figure 6).

Dependence of synthetic activity on temperature

Earlier work with ribonuclease T_1 has shown the enzyme to exhibit a greater net synthetic activity at low temperatures (Rowe and Smith, 1970). The data presented in Figure 7 confirms this observation for the substrate pG>p. The synthetic activity of ribonuclease T_1 was found to be a linear function of temperature between 0 and 37° C. Low yields obtained at higher temperatures probably result from competition by hydrolytic and depolymerization reactions. These results are similar to those observed for the synthetic activity of Ribonuclease A (Bernfield, 1965).

Relative rates of hydrolysis and synthesis

The rate of hydrolysis of the cyclic phosphate is an important consideration for obtaining maximum incorporation because of its effect on the synthetic equilibrium. As shown in Figure 8, pG>p is hydrolyzed relatively slowly during the reaction. Under the conditions used the rate of the synthetic reaction is an order of magnitude greater than the rate of hydrolysis. Early disappearance of pG>p can be attributed almost entirely to synthesis of pG-C. Although considerable hydrolysis

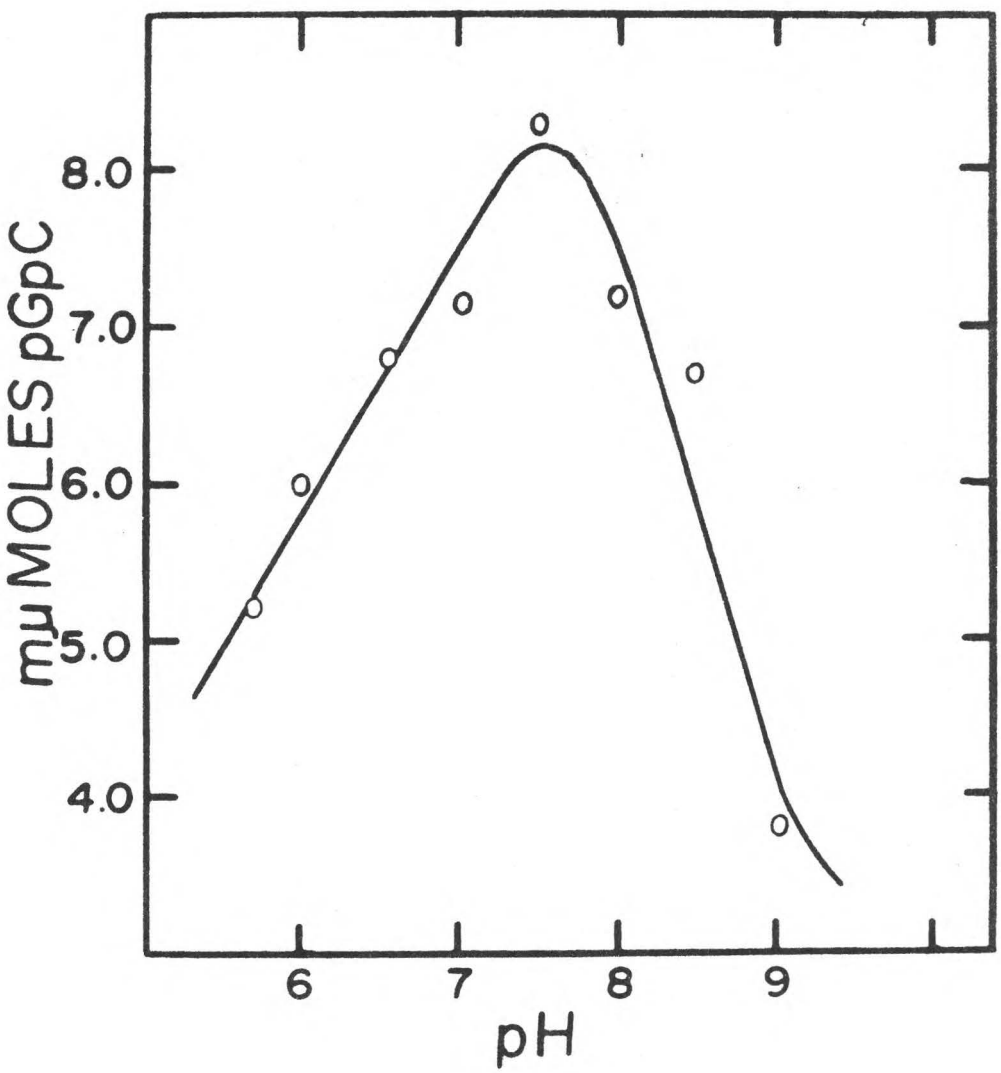

Figure 6.--The effect of pH on the ribonuclease T_1 catalyzed synthesis of pG-C from pG>p and cytidine. Reaction mixtures (17 μl) contained 2.65 mM pG>p, 97 mM cytidine, 59 mM buffer, and 0.3 μg of ribonuclease T_1 per ml. Reactions were carried out at 0° C for 75 minutes, at which time ten μl aliquots were removed and analyzed. Buffers for pH 7.0 and below were phosphate. Tris-HCl was used above 7.0. (This figure was not included in this paper as published, Rowe and Smith, 1971).

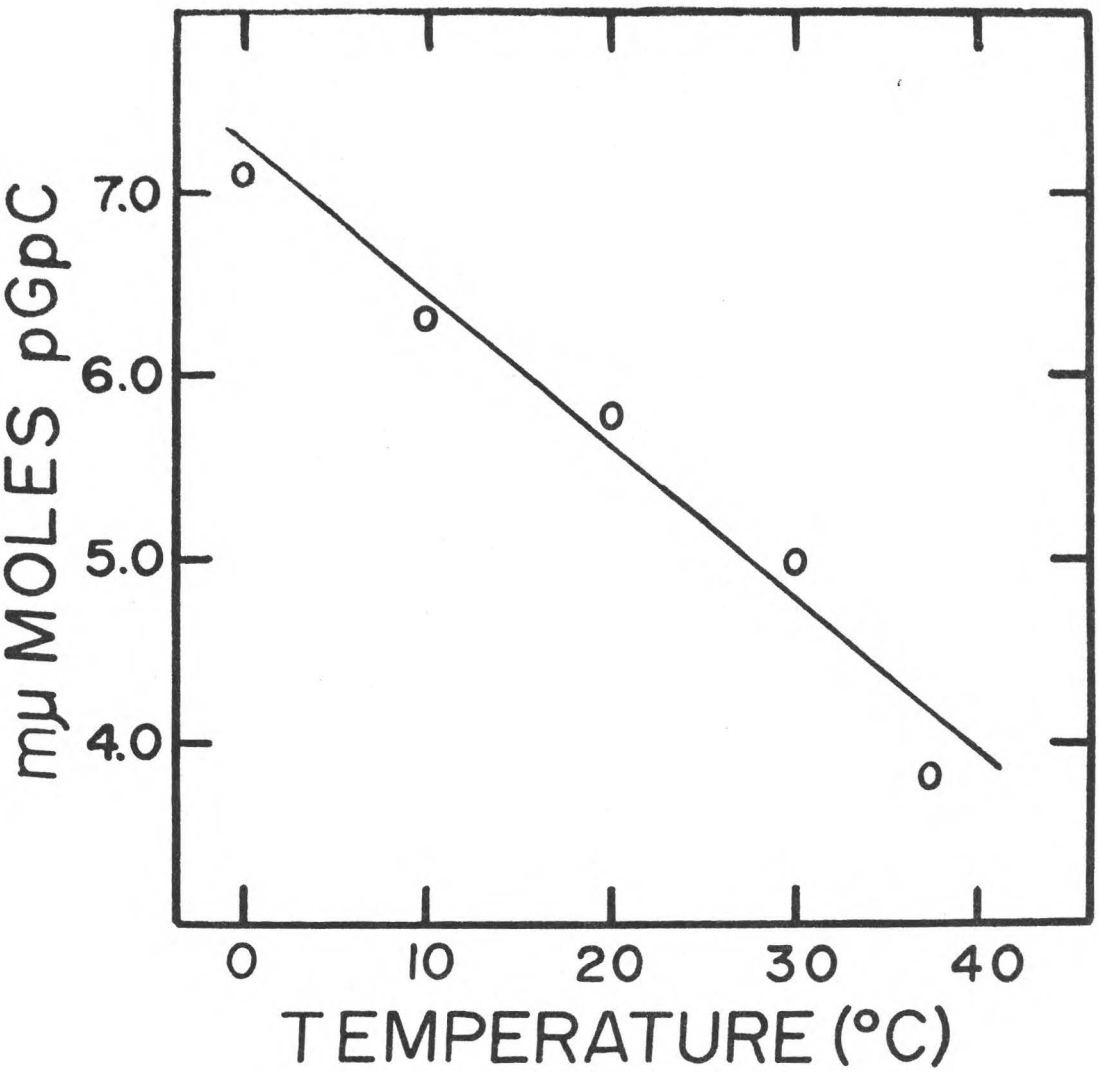

Figure 7.--The effect of temperature on the synthesis of pG-C from pG>p and cytidine. Reaction mixtures (17 μl) contained 59 mM Tris-HCl buffer (pH 7.3), 0.35 M cytidine, 2.4 mM pG>p, and 0.15 μg ribonuclease T_1 per ml. Reactions were carried out at temperatures ranging from 0 to 37° C for two hours. Ten μl aliquots were removed from each and analyzed as in methods.

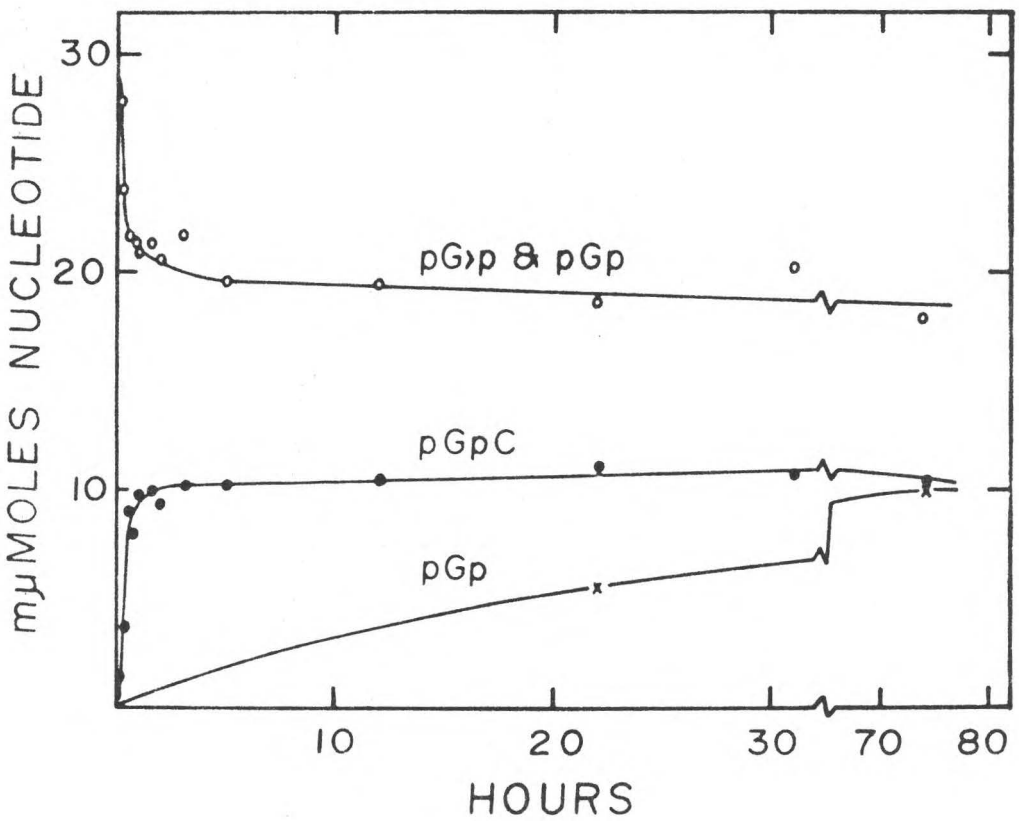

Figure 8.--Comparative rates of synthesis and hydrolysis. The reaction mixture (170 μl) contained 59 mM Tris-HCl buffer (pH 7.3), 0.35 M cytidine, 3.68 mM pG›p and 0.30 μg ribonuclease T_1 per ml. The reaction was carried out at 0° C. Ten μl aliquots were removed at the times indicated and analyzed as described in methods. The upper curve represents pG›p plus pGp, its hydrolysis product. The amount of pGp present was determined by chromatography on DEAE-cellulose columns as described in methods.

of pG>p occurs during prolonged reaction periods, the pseudo equilibrium concentration of pG-C reached during the early part of the reaction does not decrease appreciably even after 74 hours. This plateau region however, does not represent attainment of a true equilibrium, since the hydrolysis of pG>p will eventually shift the reaction toward depolymerization. Similar results were observed for the synthesis of dinucleotide monophosphate from G>p and cytidine (Rowe and Smith, 1970).

Influence of enzyme concentration

The synthesis of dinucleotide was followed as a function of ribonuclease T_1 concentration at several fixed concentrations of pG>p and a constant amount of cytidine (Figure 9). A typical family of hyperbolic curves was obtained. One would expect the amount of pG-C to increase to a maximum and subsequently decrease because of the competing hydrolysis reaction. This does not occur under our conditions even when the ribonuclease T_1 concentration is increased more than 3 fold above that shown in Figure 9. The decrease in synthesis at high enzyme concentrations reported previously (Rowe and Smith, 1970; see chapter II) was a result of inefficient enzyme quenching. The incorporation of pG>p was about 50% at higher cytidine concentrations. This compares favorably with yields obtained using G>p as donor (Rowe and Smith, 1970).

Effect of substrate concentration

The influence of the concentrations of the 5'-phosphorylated guanosine cyclic phosphate donor, and the nucleoside acceptor on the yield of the synthetic reaction was determined by varying each independently (Figure 10 and Figure 11). The cytidine to pG>p ratio (C/pG>p)

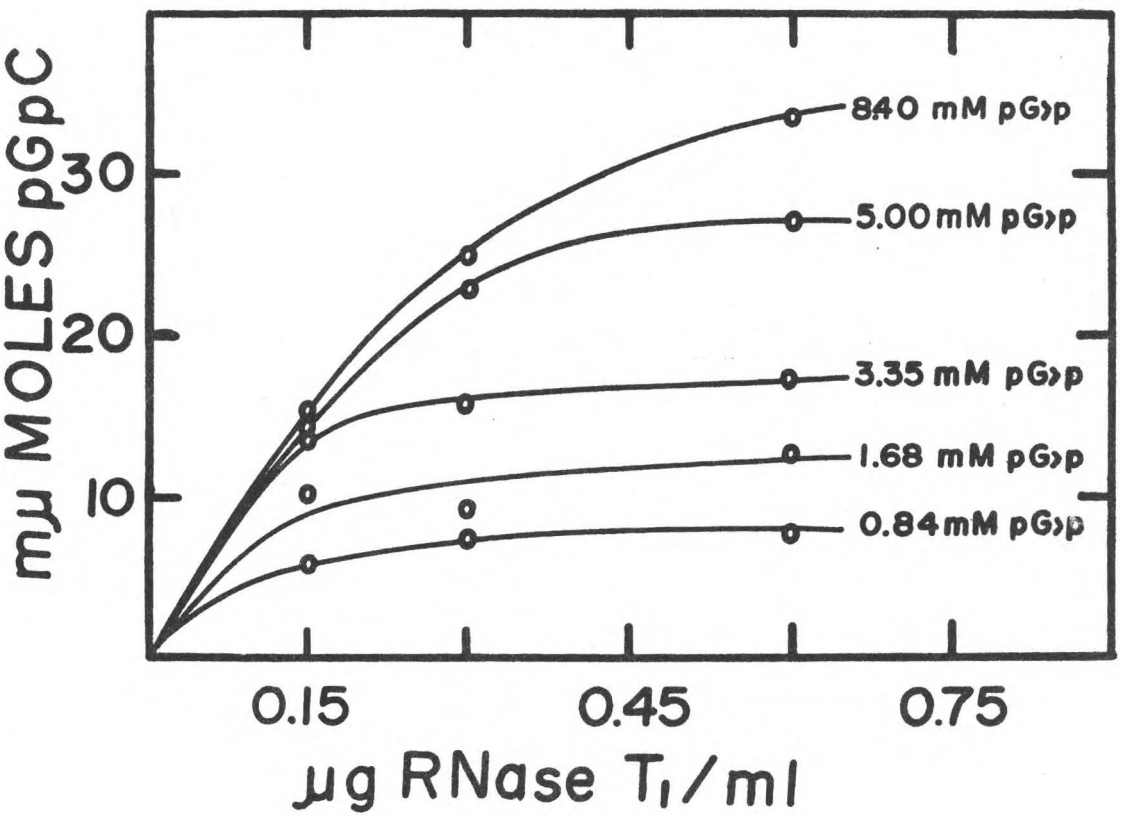

Figure 9.--Influence of ribonuclease T_1 concentration on the synthesis of pG-C. Reaction mixtures (17 μl) contained 59 mM Tris-HCl buffer (pH 7.3), 97 mM cytidine, and pG>p concentrations as shown. Reactions were carried out at 0° C for two hours.

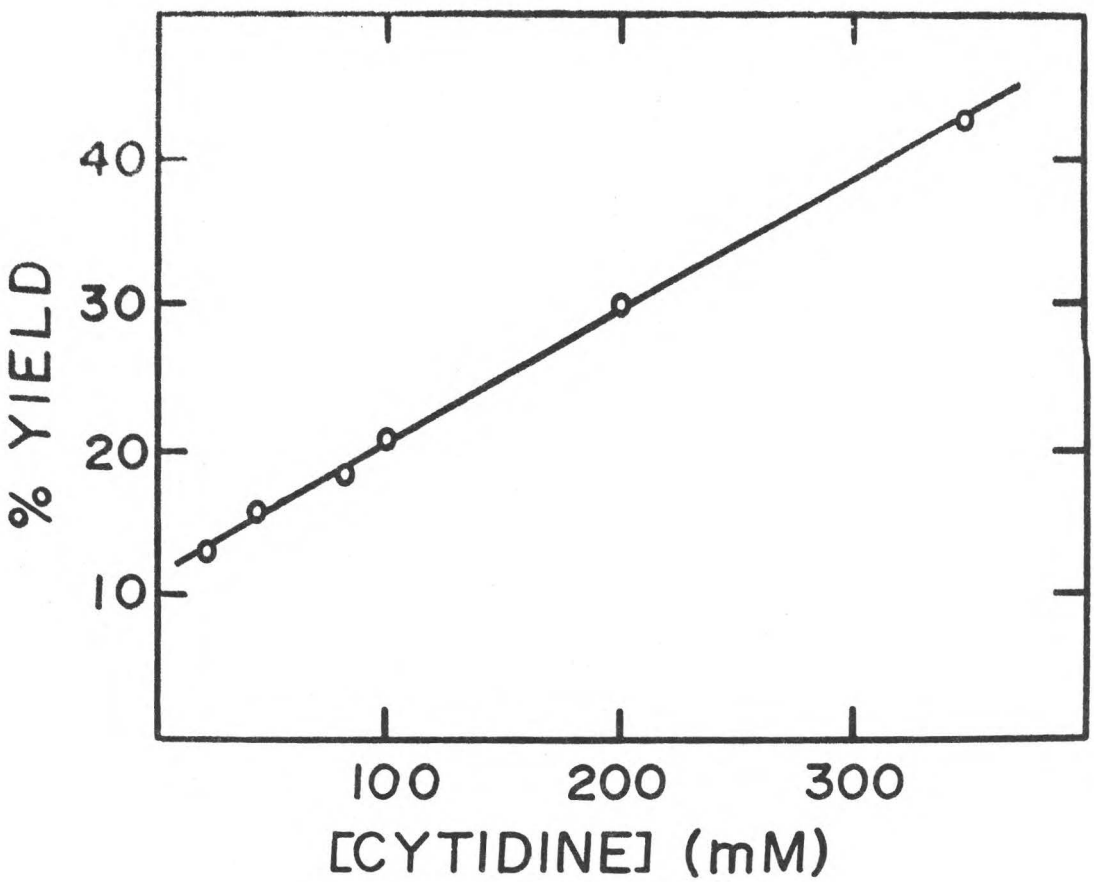

Figure 10.--The effect of cytidine concentration on the percentage incorporation of pG>p into dinucleotide. Reaction mixtures (17 µl) contained 59 mM Tris-HCl buffer (pH 7.3), 2.6 mM pG>p, and 0.3 µg of ribonuclease T_1 per ml. Reactions were carried out at 0° C for two hours.

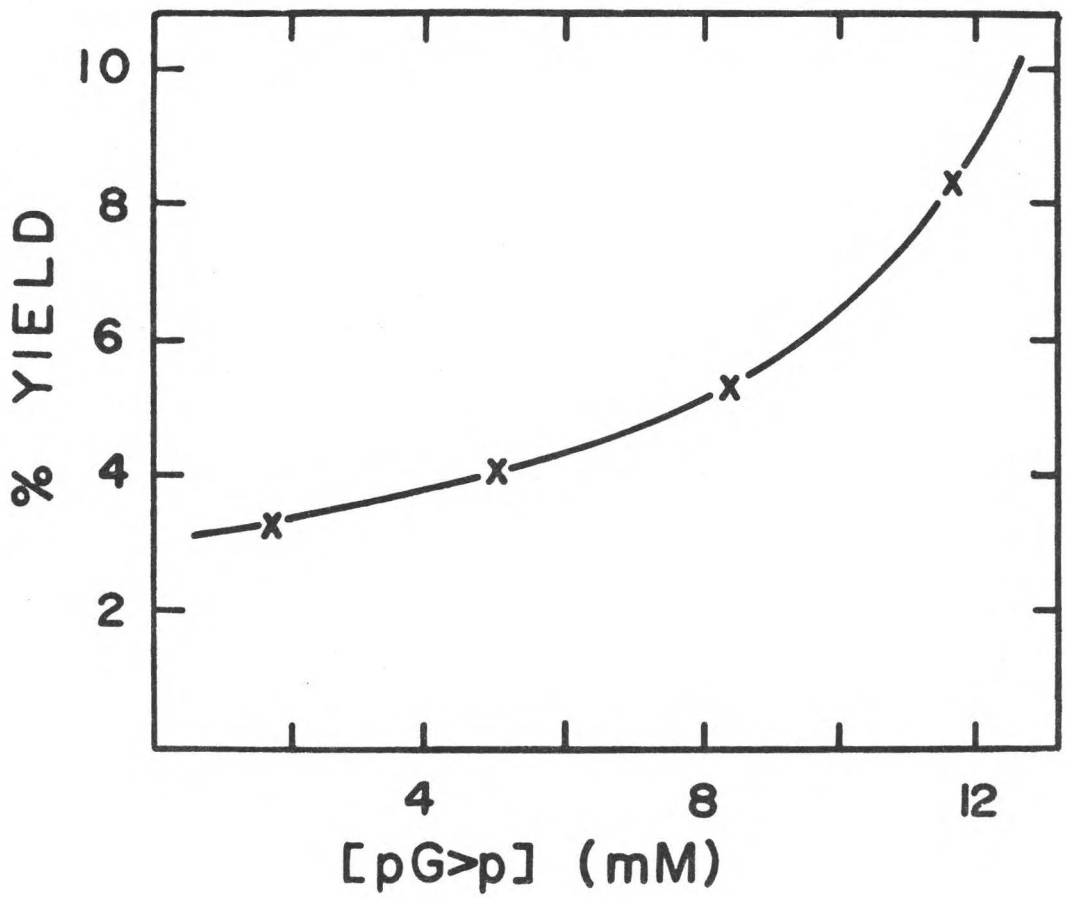

Figure 11.--Effect of pG>p concentration on the percentage incorporation of cytidine into dinucleotide. Reaction mixtures (17 µl) contained 59 mM Tris-HCl buffer (pH 7.3), 11.7 mM cytidine, 6 µg of ribonuclease T_1 per ml. Reactions were carried out at 0° C for two hours.

was varied from 1 to 135. The results obtained are consistent with those predicted by LeChatlier's principle. The percentage incorporation of pG>p into dinucleotide (yield) increased proportionally with increasing concentrations of cytidine (Figure 10). Although the present availabilty of pG>p precluded its use in large excesses, the percentage incorporation of cytidine into dinucleotide (yield) was shown to increase with increasing concentrations of the cyclic phosphate (Figure 11). Since the incorporation of cytidine increases rapidly at higher concentrations of pG>p, yields comparable to those obtained using large excesses of cytidine are expected when the reaction is driven with a 5--10 fold excess of the cyclic phosphate donor. In these reactions, pG-C was the only synthetic product. There was no evidence for the presence of unwanted synthetic products similar to those found when G>p was used as donor in low concentrations of acceptor (Sekiya et al., 1968).

Nature of phosphate acceptor

Adenosine, guanosine, inosine, and uridine were investigated to determine the effect of the base on the synthetic reaction. Uridine proved to be as efficient as cytidine in dinucleotide formation. Purine nucleosides, however, were less efficient. Adenosine, guanosine, and inosine were incorporated into dinucleotide in yields of about 10%, due in part to solubility limitations and possibly to inhibition by these purine nucleosides (Irie, 1964). These results are consistent with those observed for G>p.

Discussion

Ribonuclease T_1 may be used in three general synthetic reactions:

I. Addition of a particular nucleoside to the 3'-end of an oligonucleotide,

II. Addition of a guanosine nucleotide to the 5'-hydroxyl of an oligonucleotide,

III. Condensation of two oligonucleotides.

The apparent equilibrium constant (K_{app}) for the simplest synthetic reaction (calculated from data in Figure 10) is about 5.1 M^{-1}. This low value points to the necessity of using large excesses of one of the substrates for good synthetic yields. Reactions of type I above may be driven with an excess of the nucleoside acceptor, since these acceptors are relatively inexpensive and available in large amounts. Indeed, previous investigations have also shown that an incorporation of the cyclic phosphate near 60% is obtained using large excesses of the nucleoside acceptor (Mohr and Thach, 1969; Rowe and Smith, 1970; Holy, 1969).

In reactions of types II and III however, high oligonucleotide acceptor concentrations cannot readily be obtained. In II the concentration of the donor may be increased to obtain better incorporation of the acceptor, providing the 5'-hydroxyl is blocked, avoiding selfpolymerization. The data presented in this paper show that the substrate, pG>p is useful for this purpose. Since formation of oligoguanylic acids is entirely prevented the synthetic potential of the ribonuclease T_1 reaction is used only in the formation of the desired product. Reaction type III, in which both substrates are oligonucleotides, is considerably more complex. Clearly, using high concentrations of either substrate is not a practical approach to forcing the synthetic reaction.

The effect of large excesses of pG>p on the incorporation of oligonucleotide acceptors is currently under investigation. Preliminary

data show that high yields can be obtained, but that the reaction is considerably more complex and requires different reaction conditions. This is due in part to the low affinity of the acceptor for the enzyme relative to pG>p, and perhaps to inhibition of the synthetic reaction by high concentrations of the substrate pG>p, or its hydrolysis product, pGp. Similar effects are also seen with pUp and pCp using ribonuclease A.

CHAPTER IV

SYNTHESIS OF OLIGORIBONUCLEOTIDES WITH RIBONUCLEASE T_1

(As submitted for publication by M.J. Rowe, and M.A. Smith, 1972)

Introduction

Synthetic reactions of nucleases provide a method for the synthesis of oligoribonucleotides which has the advantage of being very specific with regard to the nucleotide incorporated (Bernfield and Rottman, 1967; Koike et al., 1969; Koike et al., 1971; Mohr and Thach, 1969) and the internucleotide linkage formed (i.e., 3'-5') (Rowe and Smith, 1970). Ribonuclease T_1 (EC 2.7.7.26) under appropriate conditions catalyzes the condensation of guanosine 2',3'-cyclic phosphate (G>p, phosphate donor) with the 5'-hydroxyl group of various nucleosides or oligonucleotides (phosphate acceptors) (Grunberger et al., 1968; Sekiya et al., 1969; Mohr and Thach, 1969). This reaction provides a tool for the synthesis of oligonucleotides possessing a guanosine nucleotide in a predetermined position.

Grunberger et al. (1968) and Holy et al. (1970) obtained trinucleotides for tRNA-ribosome binding studies using the synthetic reaction of ribonuclease T_1, and Sekiya et al. (1968) described reaction conditions which lead to synthesis of trinucleotides. However, in both of these instances incorporation of phosphate donor was seldom more than

20%, and the incorporation of acceptor was usually much less, about 2%. More recent reports have shown that the equilibrium[1] concentration of the desired product can be greatly increased by driving the reaction with high concentrations of phosphate acceptor (Mohr and Thach, 1969; Rowe and Smith, 1970). This results in a donor incorporation maximum of about 60%. Mohr and Thach (1969) have also shown that oligonucleotides may be used either as the donors or acceptors in ribonuclease T_1 catalyzed synthetic reactions. However, when adding guanosine 3'-monophosphate to the 5'-hydroxyl of a dinucleotide via this reaction, incorporation of dinucleotide acceptor was less than 1%, except in one instance when a 12 fold excess of donor resulted in 6.8% acceptor incorporation. In a similar reaction catalyzed by ribonuclease N_1, only a 2% incorporation of acceptor was obtained (Koike et al., 1971b).

Results similar to those obtained when driving the reaction with a large excess of acceptor could be expected when the reaction is driven with an excess of donor, thus permitting comparable incorporation of acceptor into oligonucleotide. However, with donor to acceptor ratios greater than one, the reaction is complicated by the simultaneous formation of unwanted oligoguanylic acids, formed by enzymatic condensation of G>p (Sekiya et al., 1968). Such oligoguanylic acid formation may be blocked using 5'-substituted donors. Several such compounds have been shown to act as donors in the synthetic reaction of ribonuclease T_1 (Holy, 1969; Rowe and Smith, 1971). Preliminary experiments suggested

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[1]The term "equilibrium" used throughout this paper refers to reversible reactions of the type pG>p + N $\leftrightarrow$ pG-N. The synthetic plateau or maximum referred to is only temporary and does not represent a true equilibrium because of the irreversible hydrolysis of the cyclic phosphate by ribonuclease T_1.

that the 5'-phosphorylated donor pG>p might well be used in large amounts to obtain favorable equilibrium concentrations of the desired product (Rowe and Smith, 1971). However, reaction conditions which gave a maximum incorporation of donor in the presence of a large excess of acceptor (Rowe and Smith, 1970) failed to give comparable incorporation of acceptor in the presence of excess donor. Therefore, relative effects of substrate concentrations on the rate of the synthetic reaction have been studied. On the basis of results obtained from these studies, conditions necessary for obtaining maximum incorporation of phosphate acceptor into dinucleotide by driving the reaction with an excess of donor were determined. These conditions were applied to reactions involving individual members of a family of oligocytidylic acid acceptors. The effects of acceptor chain length and the competing hydrolytic reaction on the yield of desired product were determined.

Materials and Methods

Nucleosides were obtained from Schwarz Bioresearch. Ribonuclease T_1 and bacterial alkaline phosphatase (*E. coli*) were obtained from Sigma and Worthington Biochemicals, respectively. G-C was purchased from Sigma and polycytidylic acid from Miles Chemical Co. DEAE-cellulose (DE 23) (1.0 meq/g) was purchased from Whatman.

The compounds pGp and pG>p were synthesized as described previously (Rowe and Smith, 1971). Chicken pancreatic nuclease was prepared through the DEAE-sephadex step according to the method of Eley and Roth (1966), and assayed as described previously (Rowe and Smith, 1968).

Cytidylic acid oligomers were prepared by enzymatic hydrolysis of polycytidylic acid with chicken pancreatic nuclease in the presence

of bacterial alkaline phosphatase. Reaction mixtures (5.2 ml) contained 50 mg of poly-C, 50 mM glycine-NaOH buffer (pH 10.0), 4.0 mM $MgCl_2$, 56 units of the nuclease and 1.5 units of bacterial alkaline phosphatase. Hydrolysis products obtained from 48 hr, 37° C reactions were added to a DEAE-cellulose column (2 cm X 50 cm). The column was eluted with an exponential gradient of NH_4HCO_3 (pH 8.6, 0--0.4 M) using a one liter mixer. The flow rate was 38 ml/cm^2/hr. The absorbance of the effluent was monitored at 290 nm (because of the large amount of absorbing material present) with a Gilford model 2000 recording spectrophotometer. Approximately 75% of the poly-C was recovered in a family of peaks subsequently shown to be di- through deca-cytidylic acid. Only oligomers containing no phosphatase-susceptible phosphate were obtained, and no cytidine or CMP was detected. Oligonucleotides were recovered from NH_4HCO_3 column effluents by lyophilization, and brought to the desired concentration by the addition of water. The chain lengths of several adjacent peaks were established by experimental determination of the ratio of CMP to cytidine in alkaline hydrolysates.

The synthetic activity of ribonuclease T_1 was assayed as follows: Substrates were allowed to react with enzyme in Tris-HCl buffer (pH 7.3) at 0° C for various times as indicated in the legends to the figures. Reactions were terminated by the addition of one volume of 0.05 M dithiothreitol in 7.5 M ammonium hydroxide, and heated to 37° C for 15 minutes (Mohr and Thach, 1969) Reaction products were separated by high voltage electrophoresis (300V/cm) on Whatman #3MM paper strips (1 in X 15 in) in 0.05 M ammonium formate buffer (pH 3.5), in a Camag HVE apparatus. Separation was complete in about 45 minutes. Ultraviolet absorbing regions were cut into small squares and eluted overnight in

2.5 ml of 0.01 M NH_4OH. The absorbance of each sample was measured at 260 nm. Blanks were obtained by eluting similar regions from paper electrophoresis strips to which no sample had been added.

Rate experiments were carried out as indicated above, stopping reactions after 30 minutes. Substrate concentrations used were within the limits which allowed synthesis to proceed linearly during this time interval.

Ion exchange chromatography used in chain length determination of oligocytidylic acids and the separation of guanosine nucleotides was carried out on DEAE-cellulose columns (0.15 X 25 cm) using an exponential gradient of NH_4HCO_3 (0--0.15 M, pH 8.6) and a 125 ml mixer. Four columns were eluted simultaneously using a single gradient producing device. The flow rate was 460 $ml/cm^2/hr$. Column effluents were monitored continously at 260 nm with the Gilford recording spectrophotometer.

Results

Rate of synthesis

In preliminary experiments involving ribonuclease T_1 catalyzed synthesis of pG-C it became obvious that conditions which gave maximum incorporation of donor in the presence of a large excess of acceptor (Rowe and Smith, 1970) failed to give comparable incorporation of acceptor in the presence of excess donor. These results could be explained if reaction rate were greatly affected by changes in substrate concentrations. Of particular concern in this regard was the effect of high pG>p and low cytidine concentrations on the rate of the reaction, since under these conditions maximum incorporation of acceptor is expected.

The effect of pG>p (phosphate donor) and cytidine (phosphate acceptor) concentrations on the initial rate of synthetic reactions was determined by varying the concentration of one substrate at a fixed concentration of the other. As opposed to conditions required for classical kinetic studies, we were interested in rate changes with non-saturating concentrations of the fixed substrate, since these conditions are required for optimum incorporation of acceptor. Figure 12 shows the influence of increasing cytidine concentrations on the synthetic rate at 6.62 mM pG>p. These data reveal the expected hyperbolic relationship between reaction rate and cytidine concentration.

Cytidine concentrations ranging from 6 to 23 mM were used in subsequent experiments to obtain conditions favoring maximum cytidine incorporation. From Figure 12 it can be seen that at these low cytidine concentrations the rate of the synthetic reaction is only 3--10% that obtained using acceptor concentrations which favor maximum donor incorporation (300 mM).

Figure 13 shows the rate of the synthetic reaction at several pG>p concentrations. In reactions containing 165 mM cytidine the rate of pG-C synthesis plateaus near 6 mM pG>p. From an extrapolation of the plateau region of this hyperbolic curve it is expected that higher concentrations of pG>p used to increase incorporation of acceptor will not overcome the reduced reaction rate. The difference in the concentration effects of pG>p and cytidine on the reaction rate reflect a high affinity of the enzyme for the guanosine containing donor, as compared to acceptor.

Synthetic yield with increasing donor

Since the rate of synthesis is retarded at low cytidine levels,

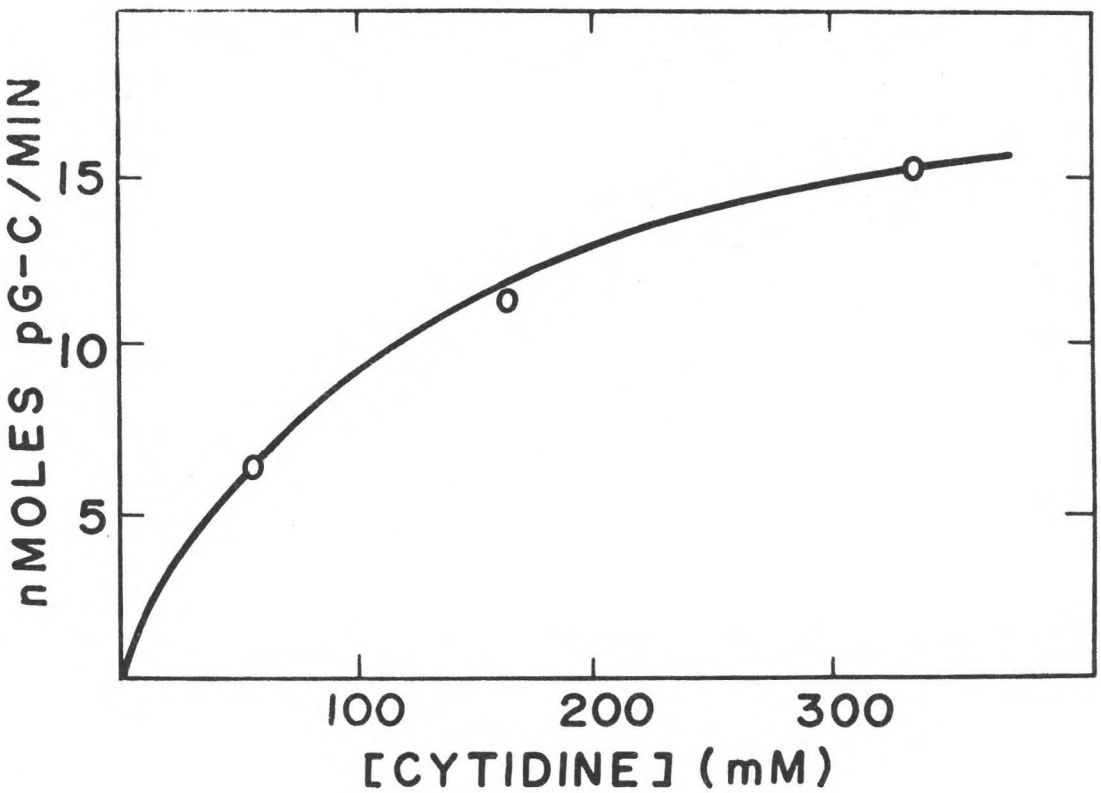


Figure 12.--Influence of increasing cytidine concentration on the rate of pG-C synthesis. Reaction mixtures (17 µl) contained 59 mM Tris-HCl buffer (pH 7.3), 0.15 µg ribonuclease T_1 per ml, 6.62 mM pC>p, and cytidine concentrations as indicated. Reactions were carried out at 0° C. With the relatively small amounts of enzyme used, the amount of product obtained at all concentrations of cytidine was a linear function of time for at least 90 minutes. Rates were calculated from data obtained well within this linear region using 30 minute reactions.

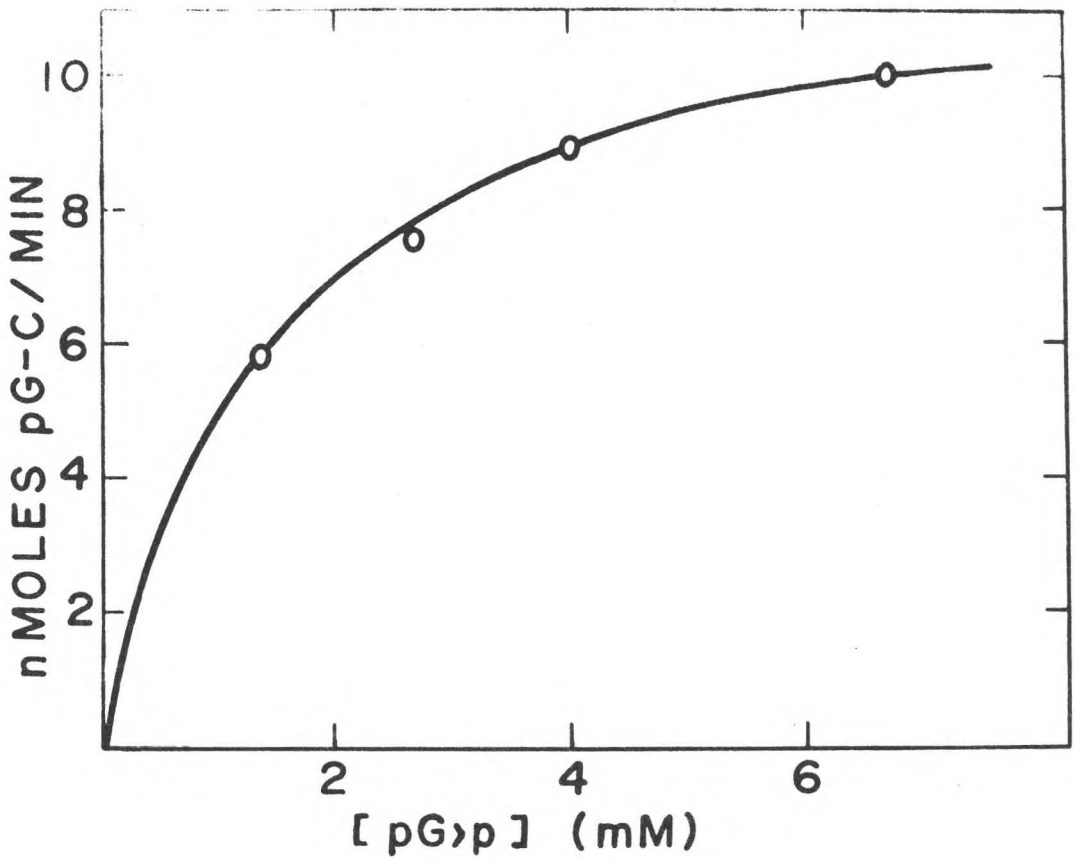


Figure 13.--Influence of increasing pG›p concentration on the rate of pG-C synthesis. Reaction mixtures (17 µl) contained 59 mM Tris-HCl buffer (pH 7.3) 0.15 mg ribonuclease T_1 per ml, 165 mM cytidine, and pG›p concentrations as indicated.

longer reaction times or larger amounts of enzyme are required to reach equilibrium than at higher cytidine levels. In order to be confident of the attainment of equilibrium at various fixed levels of pG>p, it was necessary to follow the course of the synthetic reaction over prolonged periods of time. Figure 14 shows the synthesis of pG-C as a function of time using concentrations of pG>p increasing from 13.2 to 93 mM, at constant cytidine concentration (23.5 mM). As expected, the extent of cytidine incorporation and the time required for the attainment of equilibrium, depends on the amount of pG>p present.

As shown in the inset to Figure 14, the incorporation of acceptor at 48 hours is a linear function of concentration between 13.2 and 93 mM pG>p. Whereas cytidine incorporation approaches 65% with a 4-fold excess of donor, a 170-fold excess of cytidine was required to attain 50% incorporation of pG>p (Rowe and Smith, 1971). This comparison between donor and acceptor in driving the synthetic reaction (with regard to percentage incorporation of the other substrate) is shown in Figure 15. The donor pG>p is thus about 40 fold more effective in driving the synthetic reaction than is the acceptor, cytidine.

Other nucleosides as acceptors

Uridine, guanosine, and adenosine were compared with cytidine with regard to accepting ability in the presence of excess donor. Concentrations were adjusted to provide a three-fold excess of donor in each reaction mixture. The percentage of acceptor incorporated was as follows: C, 35%; U, 20%; A, 12.5%; and G, 10%. The order of accepting ability is identical to that found in reactions driven with high concentrations of each acceptor at low donor levels (Rowe and Smith, 1971),

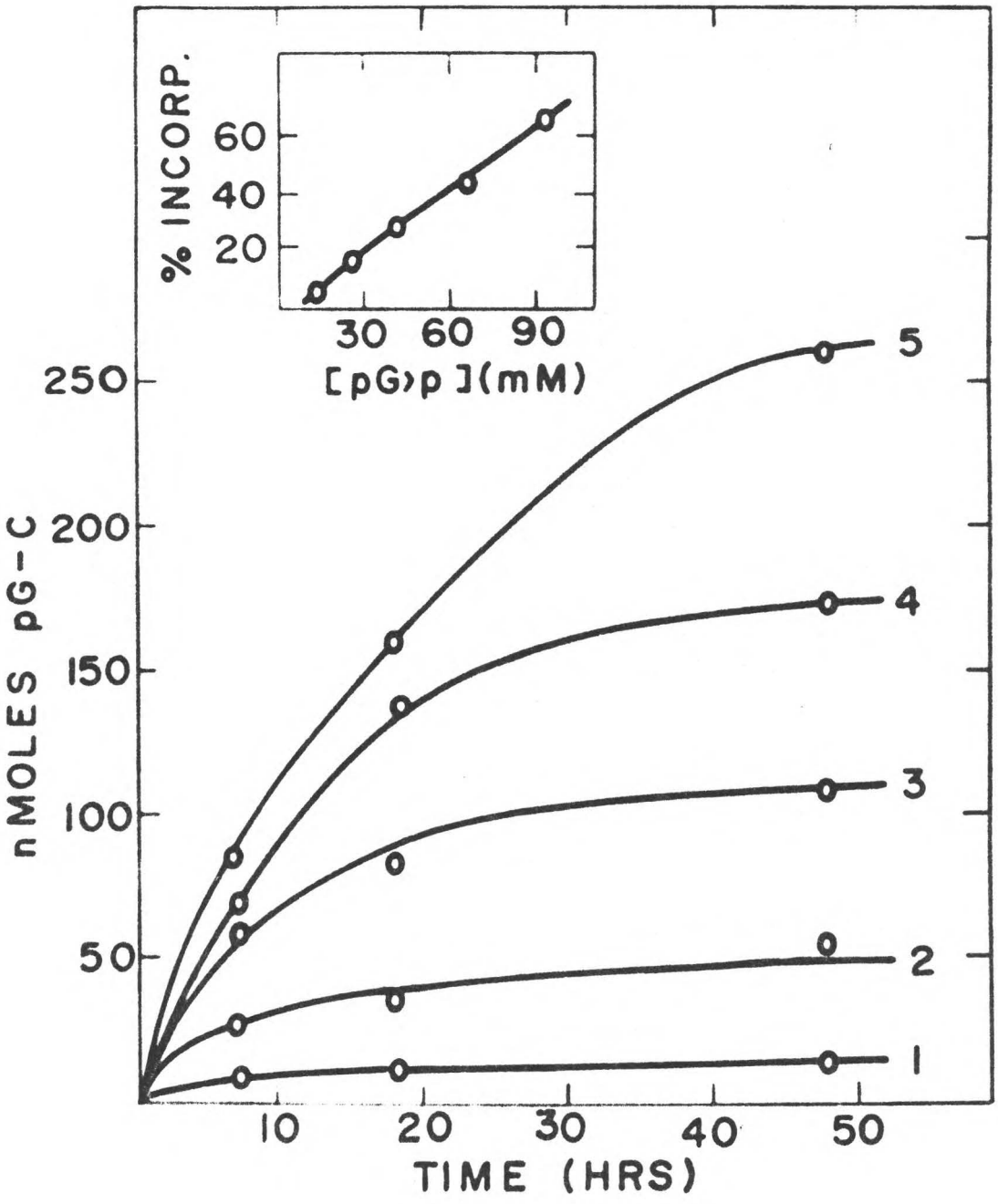


Figure 14.--Synthesis of pG-C at a fixed concentration of cytidine and increasing concentration of pG>p. Reaction mixtures (17 µl) contained 59 mM Tris-HCl buffer (pH 7.3), 0.60 µg ribonuclease T_1 per ml, 23.5 mM cytidine, and pG>p concentrations of 13.2, 26.5, 40.0, 66.0 and 93 mM (pG>p to C ratios increasing from 0.56 to 4.0). Reaction mixtures were carried out at 0° C. Five µl aliquots were removed at the times indicated and analyzed as described in methods.

Inset: Percent incorporation of cytidine as a function of pG>p concentration at 48 hours.

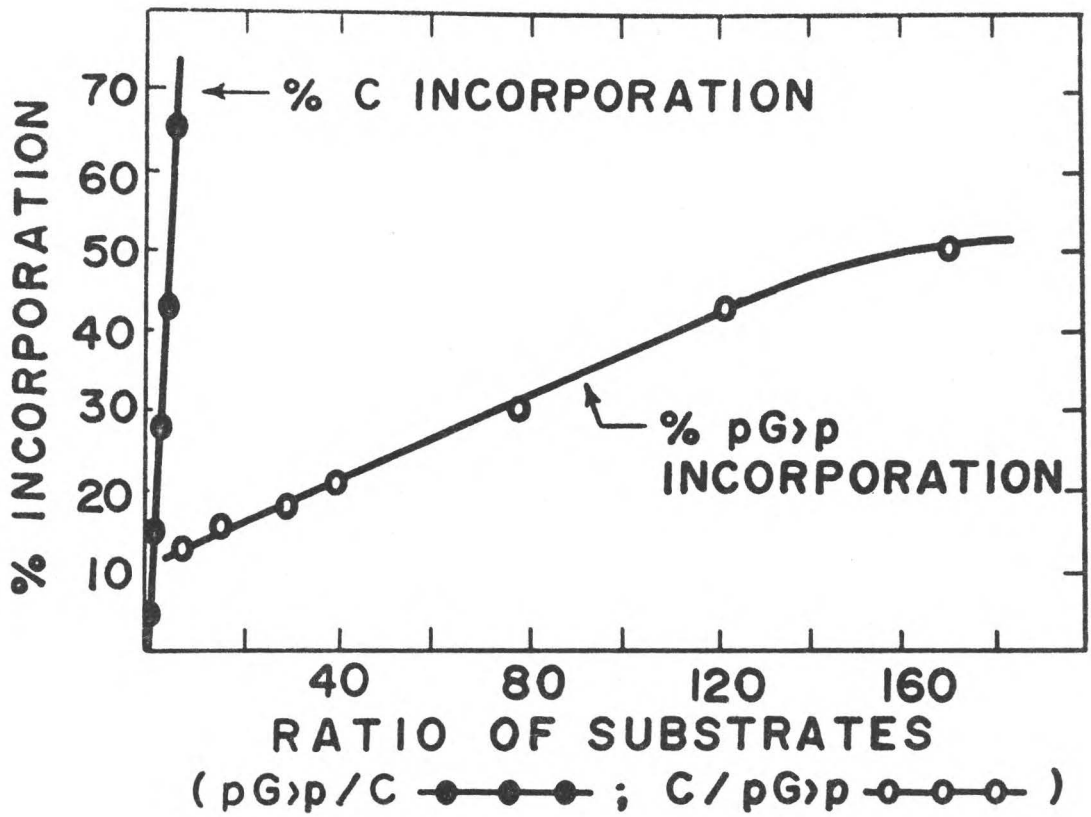


Figure 15.--Comparative ability of pG›p (donor) and cytidine (acceptor) to drive the synthetic reaction. Results involving C/pG ratios are those of Rowe and Smith (1971). Data involving pG›p/C ratios are described in Figure 14.

and is consistent with several other properties of these nucleosides and their interactions with ribonuclease T_1, i.e., binding strengths (Takahashi, 1970) and inhibitory properties (Irie, 1964).

Oligonucleotides as acceptors

Conditions giving maximum incorporation of cytidine were applied to the synthesis of oligonucleotides using oligocytidylic acid acceptors. These oligocytidylic acids included a continuous series from monomer to hexamer. Experiments were carried out in the presence of a 9.5 fold excess of pG>p, that is, more than twice the excess required to give 65% incorporation of cytidine. Reactions were followed as a function of time to determine the point of maximum synthetic product, as shown in Figure 16. Two very striking characteristics of these results, as compared with those obtained using a large excess of cytidine (Rowe and Smith, 1971), are: First, as expected from the rate studies, much longer times are required to reach equilibrium, using comparable amounts of enzyme. Secondly, each curve exhibits a maximum, and is bell-shaped rather than hyperbolic. Bell-shaped curves were not observed in Figure 14 because higher acceptor concentrations and shorter times were used.

It can also be seen from Figure 16 that the maximum amount of oligonucleotide, pG-(C)n, synthesized is greatly influenced by the chain length of the cytidylic acid oligonucleotide acceptors. Figure 17 shows that maximum incorporation of acceptor, under these conditions, decreases from 67% to 18% as the acceptor chain length increases from the monomer to the hexamer.

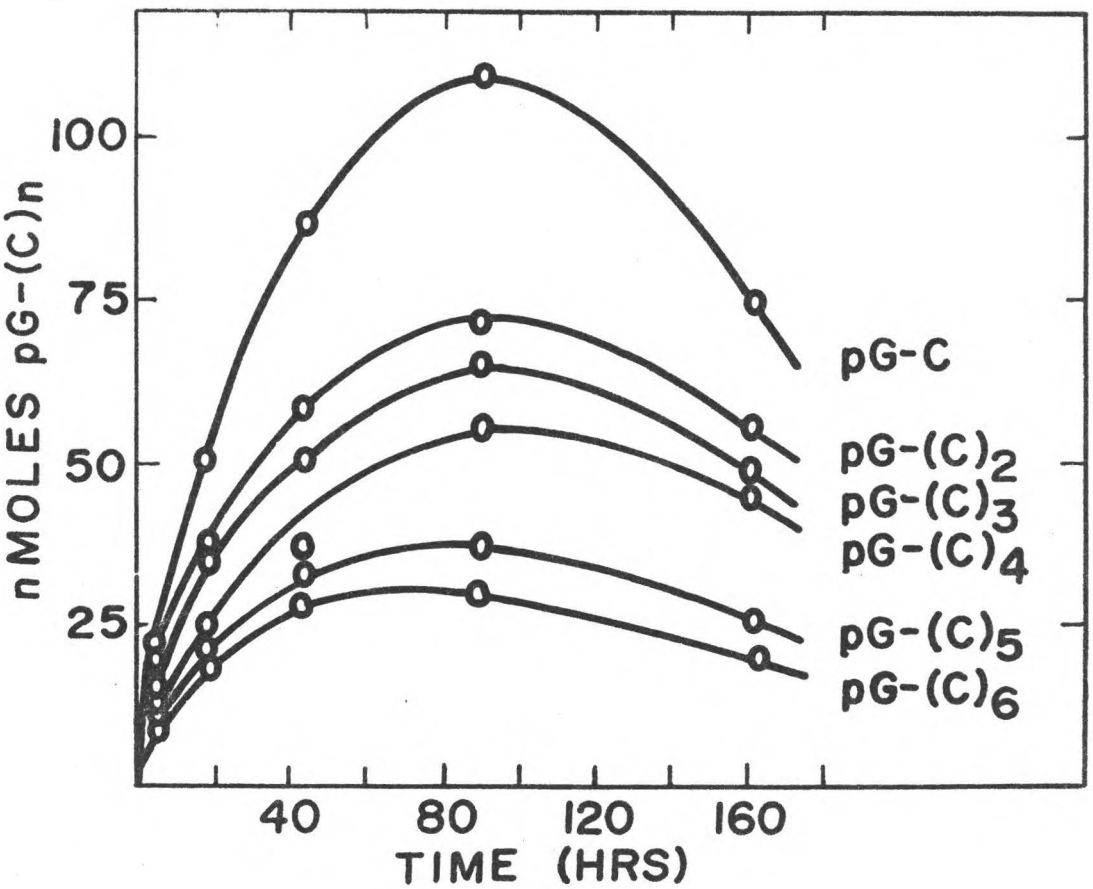


Figure 16.--Addition of pG>p to the 5'-end of oligocytidylic acid acceptors, one to six nucleotides in length. Reactions (25 µl) contained 80 mM Tris-HCl buffer (pH 7.3), 4 µg ribonuclease T_1 per ml, 63 mM pG>p, and 6.6 mM oligocytidylic acid acceptor. Reactions were carried out at 0° C. Five µl aliquots were withdrawn at the times indicated and analyzed as described in methods.

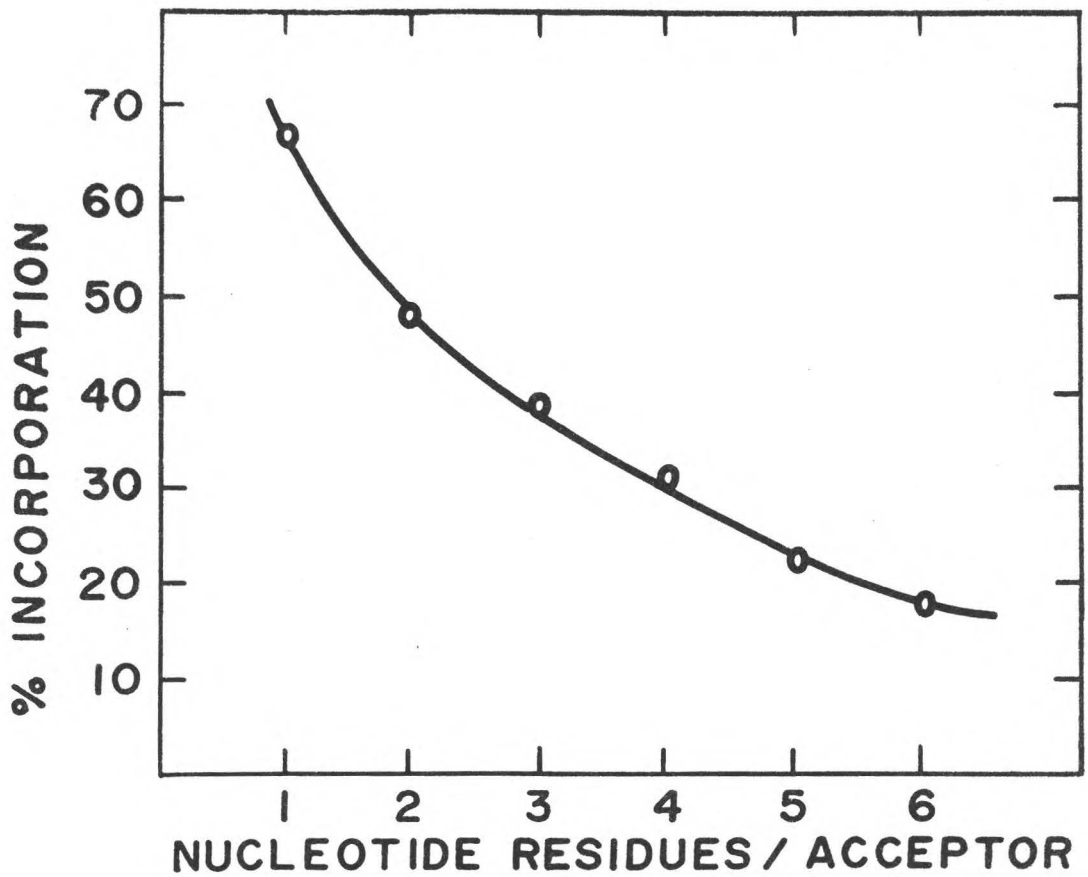


Figure 17.--Effect of chain-length on oligocytidylic acid acceptor incorporation into guanosine containing oligonucleotides. Points represent percent incorporation of the oligonucleotide at the maxima shown in Figure 5.

Effect of hydrolysis on synthetic yield

Although for convenience we have defined equilibrium only in terms of the synthetic reaction, a side reaction involving the hydrolysis of donor (pG>p ⟷ pGp) could decrease the concentration of pG>p to the point where it is no longer present in sufficient amounts to allow maximum incorporation of acceptor. This is particularly true when oligonucleotide acceptors are used because under conditions of maximum incorporation, reaction rates are significantly retarded. Indeed, bell-shaped curves observed in Figure 16 suggest that pG>p does become limiting before equilibrium is attained.

To test this possibility, synthesis of pG-C-C-C was followed using a 7.4 and 22.2 fold donor excess (Figure 18). The results substantiated the hypothesis that pG>p hydrolysis leads to a shift in equilibrium toward depolymerization, and consequently bell-shaped curves.

Ribonuclease T_1 susceptible acceptors

The susceptibility of guanosine containing donors or acceptors to depolymerization presents a limitation in the use of ribonuclease T_1 for the synthesis of oligonucleotides. Large excesses of pG>p could however have a protective effect on such susceptible oligonucleotides by saturating enzyme depolymerization sites with pG>p. The extent of protection was determined by following the degradation of G-C during the synthesis of pG-C from the condensation of pG>p and cytidine (Figure 19). The ratios of pG>p/C and pG>p/G-C were 4.5 and 13.6 respectively.

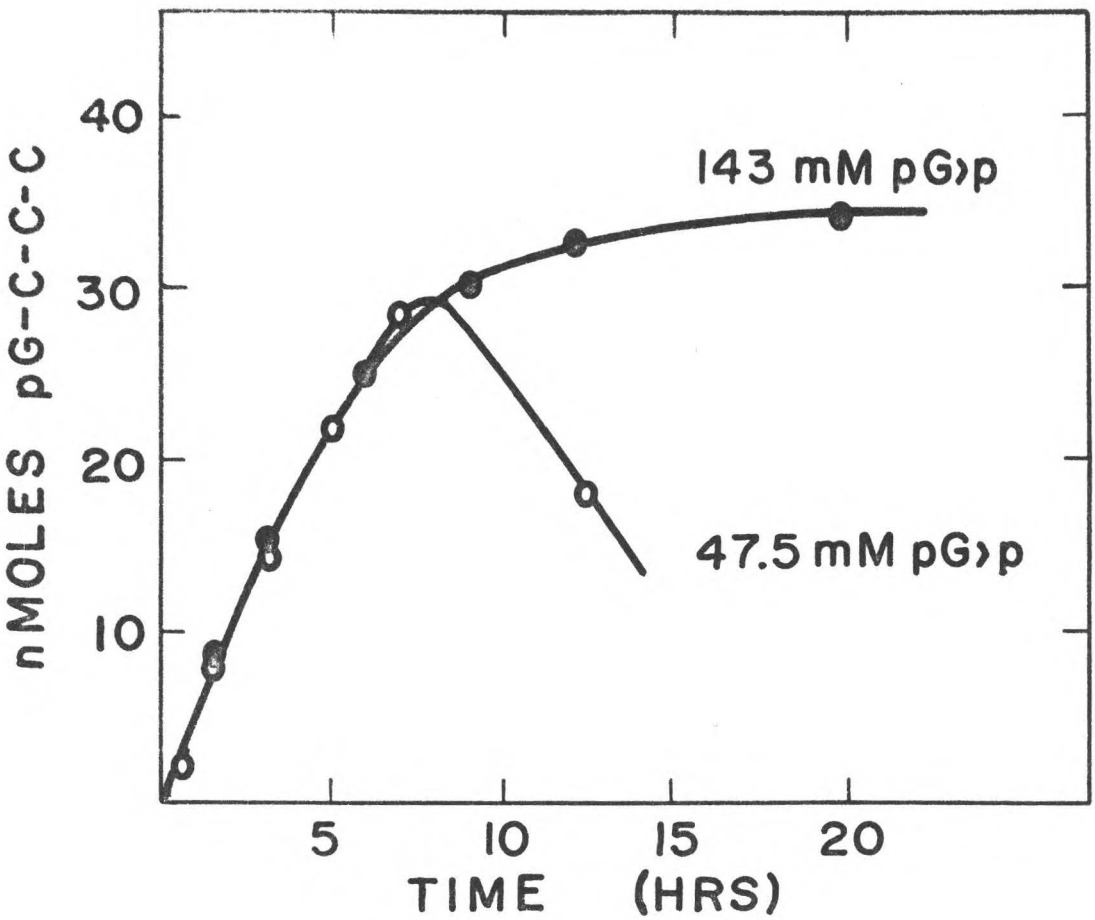


Figure 18.--Synthesis of pG-C-C-C with 7.4 and 22.2 fold excesses of pG>p. Reaction mixtures (12 µl) contained 0.25 M Tris-HCl buffer (pH 7.3), 6.4 mM C-C-C, 167 µg ribonuclease T_1 per ml, and 47.5 or 143 mM pG>p. Two ul aliquots were removed at the times indicated and analyzed as in methods.

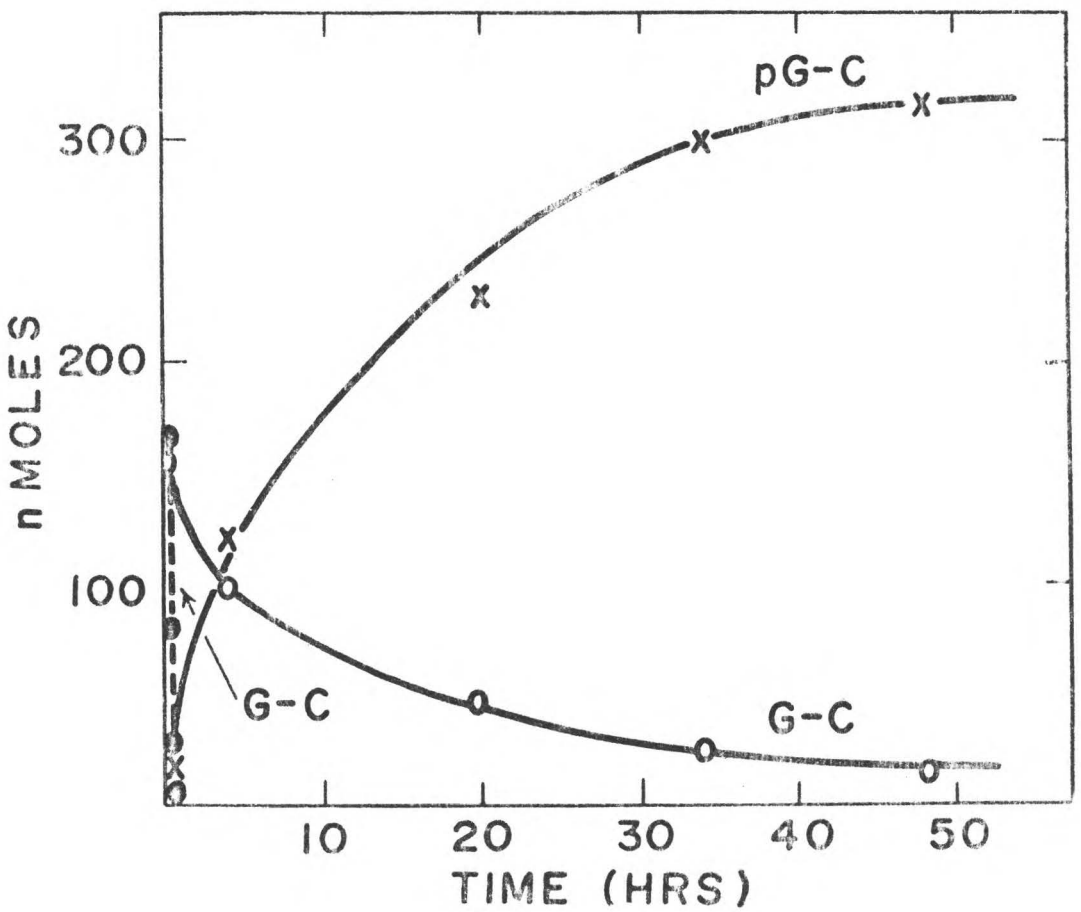


Figure 19.--Time course for the hydrolysis of G-C in the presence of excess donor. The reaction mixtures (35 µl) contained 0.14 M Tris-HCl buffer (pH 7.3), 4.5 ug ribonuclease T_1 per ml, and 4.7 mM G-C. Closed circles (dotted line) represent depolymerization of G-C in the absence of pG>p and cytidine. Open circles represent depolymerization of G-C in the presence of 64 mM pG>p and 14.3 mM cytidine. Also shown is the synthesis of pG-C in the latter reaction mixture. Reactions were carried out at 0° C. Five µl aliquots were removed at the times indicated and analyzed as described in methods.

Whereas the rate of pG-C synthesis was only negligibly affected by G-C depolymerization, the initial rate of G-C depolymerization rate decreased from 240 nmoles/hr in the absence of pG>p, to 20 nmoles/hr, in the presence of 13.6 fold molar excess of pG>p. Therefore, it appears possible to arrange synthetic reaction conditions such that equilibrium can be attained before significant depolymerization occurs.

Discussion

Synthetic oligonucleotides having specific base sequences have been used extensively to elucidate details of the genetic code and protein synthesis, and have potential use as interferon inducers, as agents for genetic therapy, and in studies involving the secondary structure of RNA. Ribonuclease T_1 is potentially useful for the synthesis of guanosine containing oligonucleotides, however the unfavorable synthetic equilibrium ($K \approx 5\ M^{-1}$) requires elevation of the concentration of one of the reactants to affect maximum incorporation of the other. Incorporation of G>p near 60% has been achieved while driving the reaction with high concentrations of several acceptors (Mohr and Thach, 1969; Rowe and Smith, 1970). However, it has not been previously demonstrated that it is possible to achieve a similar level of acceptor incorporation in the presence of excessive amounts of donor. Attempts to carry out the synthetic reaction with even equimolar amounts of guanosine cyclic phosphate donor and various nucleoside and oligonucleotide acceptors, have been hampered by polymerization of G>p, resulting in the formation of oligoguanylic acids (Sekiya et al., 1968). Recent data (Rowe and Smith, 1971) indicate that the 5'-phosphorylated donor, pG>p, eliminates oligoguanylic acid formation and therefore might be an effective substrate for attain-

ing high incorporation of acceptor. The results reported in this manuscript demonstrate that 65% incorporation of cytidine can be achieved in the synthesis of pG-C with only a four fold excess of pG>p, and that reaction conditions necessary for accomplishing this differ from those giving maximum incorporation of the donor at high acceptor levels.

These differences in reaction condition are a consequence of a 90--97% decrease in reaction rate because low acceptor concentrations are needed to obtain high pG>p/C ratios, which facilitate good incorporation of cytidine. Higher acceptor concentrations may or may not be useful in obtaining more favorable reaction rates, depending not only on the availability of such acceptors but also on the ratio of donor to acceptor required to give maximum incorporation of the derived substrate. Such ratios vary as a consequence of decreasing reaction rates with increasing oligonucleotide acceptor chain lengths. As these reaction rates decrease the enzymatic hydrolysis of donor (pG>p) becomes increasingly more important in the overall reaction. Decreasing the ratio of pG>p to acceptor beyond certain limits results in a shift in the equilibrium toward depolymerization, and lower yields. Such shifts are characterized by bell-shaped rather than hyperbolic product versus time curves. Similar results were observed by Mohr and Thach (1969) when oligonucleotides terminating in G>p were used as donors. These donors are hydrolyzed more rapidly than G>p resulting in a lower synthesis to hydrolysis ratio. Therefore, donor must be used in sufficient excess to insure maximum incorporation of acceptor.

The extent of synthesis also appears to decrease with increasing oligonucleotide acceptor chain length. This could well reflect a decrease in the synthetic equilibrium constant for larger substrates.

In reactions which contained a 70 fold excess of acceptor, Mohr and Thach (1969) also observed a decrease in the extent of synthesis with increasing chain length.

As the concentration of donor increases from 13.2 to 93 mM the equilibrium constant increases from about 5 M^{-1} (near that reported for high acceptor concentrations) to 25 M^{-1}. This increase is reflected in the ability of pG>p to drive synthetic reactions while present in much smaller excesses than those required when driving the reaction with acceptor. Since guanosine containing compounds are known to form complexes in solution (Lipsett, 1964; Gelbert, et al., 1962) aggregation between pG>p and pG-C could account for the observed results, especially at the temperatures (0°) at which our reactions were carried out. Furthermore, aggregation would tend to increase with increasing concentrations of pG>p, and render this substrate much more effective in driving the reaction toward maximum incorporation of cytidine, as observed. Mohr and Thach (1969) also observed concentration dependant variations in the equilibrium constant for ribonuclease T_1 synthetic reactions.

Our results indicate that the substrate pG>p is suitable for the synthesis of oligonucleotides containing guanosine at the 5'-terminal position. The usefulness of this substrate in specific oligonucleotide synthesis is limited by a decrease in incorporation of acceptor with increasing chain length and by the presence of a ribonuclease T_1-susceptible guanosine linkage in the desired acceptor. The first of these may or may not be serious depending on the availability of acceptor. Fortunately, unreacted acceptor can be recovered intact. Susceptible guanosine linkages are partially protected by large excesses

of pG>p. However, a better approach might involve protection with specific blocking groups, e.g., a carbodimide (Ho and Gilham, 1967).

PART II

SEQUENTIAL ADDITION OF NUCLEOTIDES TO PREFORMED OLIGONUCLEOTIDE PRIMERS WITH POLYNUCLEOTIDE PHOSPHORYLASE

CHAPTER V

3'-BLOCKED SUBSTRATES IN THE POLYNUCLEOTIDE PHOSPHORYLASE REACTION

Introduction

Stepwise synthesis with polynucleotide phosphorylase

Polynucleotide phosphorylase catalyzes the synthesis of polyribonucleotides from nucleoside 5'-diphosphates, with the elimination of inorganic phosphate (Grunberg-Manago and Ochoa, 1955; Grunberg-Manago et al., 1956). This is accomplished either de novo, or by initiation of synthesis at the 3'-end of an oligonucleotide primer. In addition, the enzyme catalyses a reaction which is the reverse of synthesis (phosphorolysis), and a reaction related to both synthesis and phosphorolysis (transnucleotidation).

The enzyme has been isolated from many bacterial sources (Grunberg-Manago, 1963) but the enzymes most commonly used for the synthesis of specific polyribonucleotides are those from Azotobacter vinelandii (Grunberg-Manago and Ochoa, 1955), E. coli (Littauer and Kornberg, 1957), and Micrococcus luteus (Beers, 1956).

The use of polynucleotide phosphorylase for the synthesis of oligonucleotide and polynucleotides is not new. Its properties relative to the synthesis of homopolymers and copolymers are not suitable for many studies which require defined base sequences, since the defined

oligonucleotides need to be of a complex nature. Polymers with complex nucleotide sequences may be synthesized using polynucleotide phosphorylase if addition at the 3'-end of a defined primer is strictly limited to one nucleotide. This addition may then be followed by isolation of the product, and its use as a primer in the polynucleotide phosphorlase reaction under reaction conditions which again limit addition onto the primer to one desired nucleotide. Repetition of this process results in the stepwise synthesis of a defined oligonucleotide.

Several possible approaches exist for limiting nucleotide addition to primers in the polynucleotide phosphorylase reactions. One approach utilizes the observation that the chain length of the polymeric product obtained from the polynucleotide phosphorylase reaction may be controlled by the inclusion of appropriate concentrations of sodium chloride, magnesium, or urea (Thach and Doty, 1965 and 1965b). This method of limiting polymer growth suffers from two main disadvantages. It is not absolutely specific for the addition of a single nucleotide, and the effects of sodium chloride, magnesium, and urea vary with the nucleoside diphosphate being polymerized so that a given set of conditions is optimal only in a given polymerization (Thach and Doty, 1965b). Both of these disadvantages decrease the yield of desired oligonucleotide with each successive nucleotide addition step.

Another approach to limiting addition of nucleotides to a defined primer in the polynucleotide phosphorylase reaction is discussed by Thach (1966). In this method the chain length is determined by the ratio of the nucleoside diphosphate to primer in the reaction mixture, if the reaction is allowed to reach thermodynamic equilibrium. However, if the primer is larger than a dinucleotide, phosphoryolysis and transnucleoti-

dation occur, resulting in a mixture of products. This method provides between 20 and 35% yield for the synthesis of a defined trinucleotide, and between 10 and 20% for synthesis of tetra- and penta-nucleotides.

A technique which specifically limits addition of nucleotides onto an oligonucleotide primer to a single nucleotide involves the use of a nucleoside diphosphate substrate esterified at the 3'-hydroxyl. The advantage of this method is that oligonucleotides formed by condensation of a primer and a 3'-blocked nucleoside diphosphate have a blocked 3'-hydroxyl, making subsequent polymerization impossible. The method has other advantages. First, oligonucleotides containing a 3'-phosphate are resistant to phosphorolysis catalyzed by polynucleotide phosphorylase (Heppel et al., 1965). Secondly, since no polymerization is possible at the 3'-position of the nucleoside diphosphate, de novo synthesis of polymers is not possible. Therefore, tedious preparation of a primer dependent enzyme is not required (see chapter VI).

An excellent and successful example employing the principles of this approach has recently been described by Mackey and Gilham (1971). Nucleoside 5'-diphosphates were blocked at the 2'- or 3'-position by direct acid catalyzed reactions with methyl vinyl ether. The products, 2'(3')-O-(α-methoxethyl) nucleoside 5'-diphosphates, were substrates for the polynucleotide phosphorylase reaction, and upon addition to a primer, terminated polymerization. The blocking group satisfies necessary conditions of being stable under the conditions of the enzyme reaction, and removed easily under conditions which do not jeopardize the structure of the polymer.

Since a 3'-phosphoryl group on an oligonucleotide is stable, but can also be easily removed via bacterial alkaline phosphatase,

3'-phosphorylated nucleoside 5'-diphosphates (ppNp) were also good candidates for use in single addition reactions as discussed above. Such compounds have been synthesized by Michelson (1958), studied as inhibitors of the polynucleotide phosphorylase reaction (Michelson et al., 1962), and shown not to be polymerized into acid insoluble polynucleotides (Grunberg-Manago, 1961). These studies indicated that ppUp and ppAp are not substrates, at least for incorporation into acid insoluble polymers, but interact with the enzyme since they inhibit polynucleotide phosphorylase. However, their use as potential chain-terminating substrates in polymerization reactions has not been studied in detail.

With a study of this nature in mind we undertook the enzymatic synthesis of ppAp. This was attempted by enzymatic hydrolysis of the homopolynucleotide, polyadenylic acid, to mono-, di-, and tri-nucleotides each terminating with a 5'-phosphate. These were hydrolyzed in dilute alkaline solution, and adenosine 2'(3'),5'-diphosphate (pAp) separated from the products.

The phosphorylation of pAp was then attempted by substitution of pAp for AMP in a transphosphorylation reaction catalyzed by adenylate kinase:

$$\text{ATP} + \text{pAp} \xleftrightarrow{\text{adenylate kinase}} \text{ppAp} + \text{ADP}$$

Since no phosphorylation of pAp occurred, the interaction of pAp with adenylate kinase was studied in detail as described later in the chapter, to determine possible reasons for the failure of the reaction to occur, and to study possible methods of forcing transphosphorylation in the presence of pAp.

Adenylate kinase

History and occurance.--Adenylate kinase is one of many enzymes which transfers a phosphate group from a nucleoside triphosphate to a nucleoside monophosphate. The earliest work on this type of enzyme was done by Colowick and Kalchar (1943) and Kalchar (1943), who described the enzyme from rabbit skeletal muscle extracts. Because the enzyme was first found in muscle, it was termed myokinase. Later, as more enzymes of its type were demonstrated, with varying specificities, it became known as adenylate kinase (adenosine triphosphate-adenosine monophosphate phosphotransferase).

In addition to skeletal muscle, adenylate kinase has been found in bovine liver mitochondria by Markland and Wadkins (1966), swine liver by Chiga and Plant (1960), red bone marrow by Kenlow and Lichter (1957), erythrocytes (Cerletti and Bucci, 1960), kidney (Kalchar and Kenlow, 1954), yeast (Libermann et al., 1955), pigeon breasts (Bowen and Kerwin, 1956), rat diaphragm and liver (Alertson, 1958), rat heart (Lenti and Brillo, 1956), spleen (Ashwell and Hickman, 1953), human placenta (Cerletti and Zichella, 1960), and several plant species (Mazelus and Stumpf, 1955). In addition, adenylate kinase isozymes were demonstrated in the several different subcellular compartments of several rat tissues (Criss, 1971; Keilley and Keilley, 1951).

Co-factor concentration.--Most workers agree on the optimum reaction condition for the enzyme especially with regard to the magnesium co-factor concentration. Noda (1958) found that maximum activity for the reaction is obtained when the ratio of magnesium to ADP is 0.5. In the opposite direction the maximum is obtained when the concentrations of magnesium and ATP are equal. This was interpreted as indicating

that the active substrates are MgADP and ADP in one direction, and MgATP and AMP in the other. Markland and Wadkins (1966) supported this observation for the bovine liver mitochondria enzyme, finding that the ATP concentration governs the optimum magnesium concentration. Lindberg (1967) found that the enzyme was inhibited if the molar ratio of magnesium to ATP was not close to one.

These results were confirmed in proton relaxation rate studies by O'Sullivan and Noda (1968) which indicated that there was little interaction between manganous ion (which can substitute for magnesium) and rabbit muscle adenylate kinase. The studies further indicated that the enzyme combines with a manganous-ATP complex rather than directly with manganous ion. It was assumed that in the other direction the reaction requires a combination with a metal ion-ADP complex for the substrate binding to the ATP site.

The latter assumption was shown to be the case by Khoo and Russell (1970) for the yeast enzyme, when they were able to obtain excellent initial velocity patterns considering MgADP and ADP as the substrates. Concentrations of MgADP and ADP were calculated based on the stability constant of the MgADP complex. In addition to these results, Blair (1970) demonstrated that magnesium ion is a significant feedback signal in the adenine nucleotide pool because of its effect on the adenylate kinase equilibrium.

These results are interesting in view of our results, mentioned later, which indicated that pAp binds at the MgATP site, and varying the magnesium concentration does not alter the enzymes exclusion of pAp as a substrate.

Substrate specificity.--Synthesis of ppAp with adenylate kinase requires the use of a substrate analogue(pAp) in the reaction. For this reason it is of interest to note other substrates and substrate analogues which have been used with the enzyme. Most of the adenylate kinase enzymes have been studied with regard to their base specificity and varying degrees of latitude have been observed (Libermann et al., 1955; Strominger et al., 1959). However, such latitude is not important in this instance, since the desired substrate contains an adenine base. More important is the degree of substitution allowed on the ribose moiety of a substrate.

Markland and Wadkins (1966) found that rabbit skeletal muscle adenylate kinase, as well as the bovine and swine enzymes, lack discrimination between AMP and 2'-deoxy AMP. This same specificity was observed by Su and Russell (1968) for baker's yeast adenylate kinase. In addition, 2'-deoxy ATP can serve as a substrate in the adenylate kinase reaction. This was the extent of studies on changes in the ribose portion of adenylate kinase substrates.

Since the enzyme does not require a hydroxyl on the 2'-position, we were encouraged that it may also allow a phosphate substitution at that position, or at the 3'-position. Also encouraging was the report of Dounce and Kay (1953) that a 5'-terminally phosphorylated RNA molecule could accept phosphate from ATP via the adenylate kinase reaction. Such a phosphorylation would have to take place on a nucleotide substituted at the 3'-hydroxyl with a large RNA molecule. However, later work by Stahl and Ebel (1964) indicated that phosphorylation could not occur at the 5'-end of RNA.

Our results, indicating that pAp does not act as a substrate for

the adenylate kinase reaction, and reporting the results of inhibition studies which indicate the reason it does not act as a substrate, follow.

Inhibition of Adenylate Kinase by 3',5'-Nucleoside Diphosphates

(As published by M.J. Rowe and M.A. Smith, 1968)

Introduction

The chemical synthesis of nucleoside 3'-phosphate, 5'-pyrophosphate has recently been reported using diphenyl- and dibenzyl-phosphochloridate as phosphorylating agents (Michelson, 1964 and 1966). In biological systems phosphorylation by ATP is common and generally catalyzed by a large variety of kinases which exhibit a wide range of specificities (Nordlie and Lardy, 1962). ATP-AMP phosphotransferase (myokinase or adenylate kinase) catalyzes the phosphorylation of AMP (pA) by ATP (pppA) producing ADP (ppA) (Colowick and Kalchar, 1943). This manuscript presents the results of experiments aimed at determining whether nucleoside 3'(2'), phosphate, 5'-pyrophosphate (ppAp) can be enzymatically synthesized in the presence of myokinase by the direct phosphorylation of 3'(2'),5'-nucleoside diphosphate (pAp) with ATP. Uridine- and adenosine-3'(2),5'-diphosphates (pUp and pAp respectively) have been chemically prepared (Michelson, 1966; Hall and Khorana, 1955), however in this study pAp was obtained by enzymatic, followed by alkaline hydrolysis of poly A. Nucleoside 5'-pyrophosphates esterified at the 3'-hydroxyl position with phosphate are of considerable interest because of their similarity to pyrophosphates of biological importance. It has been suggested that they may be useful as possible chain-terminating nucleotides (Michelson, 1958) in the enzymatic synthesis of polynucleotides from pyrophosphates (Grunberg-Manago and Ochoa, 1955).

However, they have been found to inhibit the polynucleotide phosphorylase-catalyzed exchange reaction between nucleoside diphosphates and inorganic phosphate (Grunberg-Manago, 1961). A readily available source of such compounds would permit a more detailed investigation of ways in which modified nucleoside pyrophosphates might be useful in the enzymatic synthesis of oligoribonucleotides of predetermined base sequence (Stanley et al., 1966; Thach et al., 1966).

Experimental procedure

Materials.--DEAE-cellulose was obtained from Bio-Rad, (0.66 meq./g.). All nucleotides were obtained from Schwarz Bioresearch. Bacterial alkaline phosphatase (E. coli) was obtained from Worthington, and rabbit muscle adenylate kinase from General Biochemicals. Yeast RNA was purchased from Matheson, Coleman and Bell. Polyadenylic acid was the product of Miles Chemical Company.

Chromatography.--Ascending paper chromatography was performed on Whatman No. 1 paper, using isobutyric acid, ammonium hydroxide and water (66:1:33). Column chromatography was carried out on DEAE-cellulose using exponential gradients of NaCl in Tris buffer (0.01 M, pH 7.8). Ammonium bicarbonate gradients were used when recovery of various fractions was necessary. Such fractions were recovered by repeated flash evaporation. Columns were repacked after each use.

Chicken pancreatic ribonuclease assay.--The method of assaying the ribonuclease activity was an adaption of the method of Eley and Roth (1966). The reaction mixture contained, in a total of 0.2 ml, 10 μmoles of glycine-NaOH buffer (pH 9.5), 1 μmole of $MgCl_2$, and enzyme. The assay

was initiated with 0.1 ml of 1% RNA. The reaction was carried out at 37° for 30 minutes, and it was terminated by the addition of 0.3 ml of a 1 N HCl solution in 76% ethanol. After centrifugation, the supernatant fraction was diluted 50-fold. The extent of reaction was determined by following an increase in the ethanol-HCl soluble absorption at 260 nm.

Adenylate kinase assay.--The adenylate kinase assay contained in the order of mixing the following components (in μmoles/ml unless otherwise indicated) in a total volume of 0.2 ml: $MgCl_2$, 5; Tris-HCl (pH 7.8), 50; ATP, 0.81; BSA, 1.5 mg/ml; AMP from 0.28 to 1.38; and pAp from 0 to 1.68. The reaction was initiated with 0.02 units of adenylate kinase. The reaction was terminated by the addition of 0.1 volume of 30% trichloroacetic acid and subsequently centrifuged at 5,400 x g for 10 minutes. A 50-fold dilution of each supernatant fraction was added to a DEAE-cellulose column (0.5cm x 20 cm). The products of the reaction were identified by their position of elution relative to those of authentic AMP, ADP,and ATP, eluted under identical conditions. Quantitative data was obtained by collecting 10 minute fractions and determining their absorption at 260 nm. In the concentration range of AMP mentioned, the production of ADP was found to be linear for at least four minutes. The protein and inorganic phosphate content were determined by the methods of Folin and Ciocalteau (1927) and Fiske and Subbarow (1925) respectively.

Partial purification of chicken pancreatic nuclease.--Chicken pancreas nuclease was isolated by an adaption of the method of Eley and Roth (1966). Pancreases were immersed in ice water and, after chilling,

extraneous tissue was removed and the pancreases stored at -22°. Fatty tissue was cut from 100 g of thawed pancreases, and the remaining tissue immersed in ice cold 0.05 M glycine-NaOH buffer (pH 9.5). The total volume was 100 ml. All steps in the purification were carried out at 4°. In this state the tissue was homogenized in a Potter-Elvejhem homogenizer. The homogenate was centrifuged at 27,000 x g for 1 hour and the precipitate and lipid layer discarded. The remaining turbid supernatant fraction was brought to 50% saturation by the addition of 29.1 g of ammonium sulfate, stirred for 30 minutes and subsequently centrifuged at 34,000 x g for 30 minutes.

To 75 ml of this supernatant fraction were added 20.1 g of ammonium sulfate, bringing it to 90% saturation. After stirring for 30 minutes, the 90% precipitate was obtained by centrifugation at 34,800 x g for 30 minutes. This precipitate was suspended in a minimum volume of the buffer and dialyzed against 0.05 M glycine buffer (pH 9.5).

The 50-90% ammonium sulfate fraction was further purified on a DEAE-cellulose column (1.8 cm x 55 cm) with an exponential gradient (0.04-0.4 M NaCl) in 0.01 M Tris-HCl (pH 7.8). A 1 liter mixer was used, and the flow rate was 1 ml/min. Ribonuclease activity was found in fractions (10 ml) 20 through 44 (Figure 20). The enzyme was concentrated more than 70-fold by adding it to a second DEAE-cellulose column (0.8 cm x 20 cm), and subsequently eluting it with 2.0 M NaCl in 0.01 M Tris (pH 7.8). A summary of the purification is given in Table 4.

Preparation of pAp.--DEAE-cellulose purified chicken pancreas nuclease (9.5 units) was used to hydrolyze 0.1 mg of polyadenylic acid. The reaction mixture (0.3 ml) contained the following components

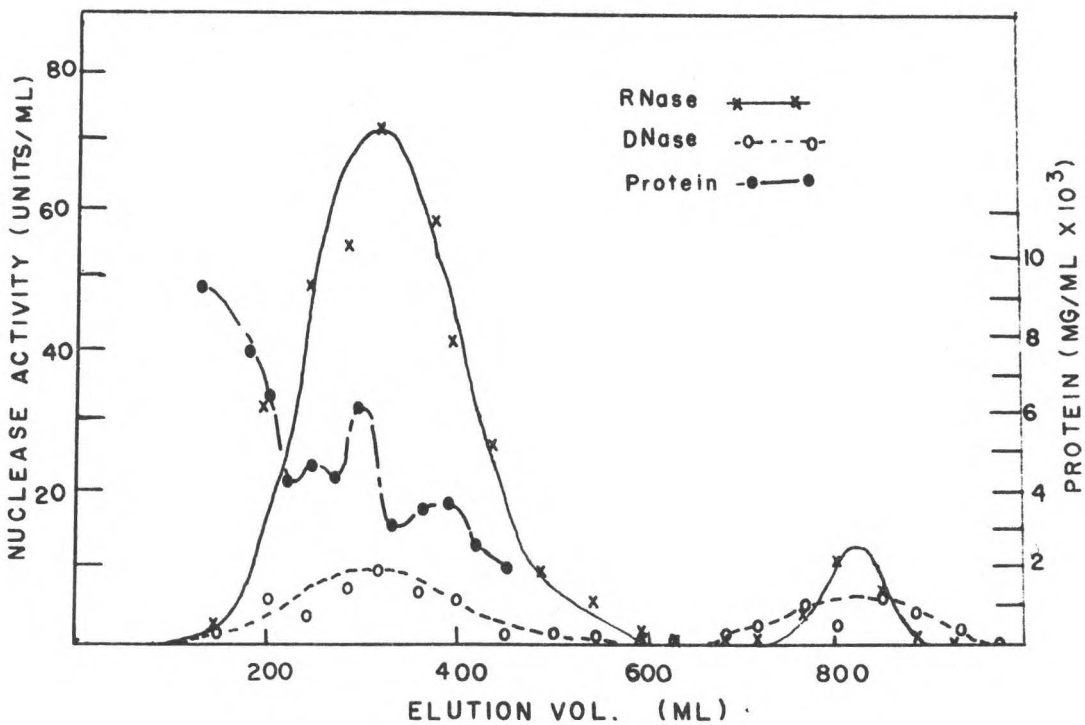


Figure 20.--Partial purification of chicken pancreas nuclease on DEAE-cellulose. Sample: 160 mg, 50-90% ammonium sulfate fraction. Column: 1.8 x 55 cm. Gradient: exponential, 0.04-0.4 M NaCl in 0.01 M Tris, 1 liter mixer. Flow rate: 1 ml/min. Fractions: 10 minutes. (See text for details). (This figure not included in this paper as published, Rowe and Smith, 1968).

TABLE 4

PARTIAL PURIFICATION OF PANCREATIC NUCLEASE

Fraction	Total Protein (mg)	Total Units[a]	Specific Activity (units/mg)
Crude supernatant	5,500	23,000	4.2
50% Precipitate	1,300	9,400	7.2
50% Supernatant	975	18,750	19.2
90% Precipitate	161	16,500	105
90% Supernatant	473	1,950	4.1
DEAE-cellulose Concentrate (3.5 ml)	114	16,800	147

[a]A unit of nuclease activity is defined as the amount of enzyme that gives an absorption increase of 1.0 (O.D.) in the ethanol-HCl soluble fraction at 260 nm in 30 minutes.

expressed in μmoles per ml. Glycine-NaOH buffer (pH 9.5), 33; $MgCl_2$, 3.3; BSA, 1.8 mg/ml, and a small drop of toluene. Hydrolysates of polyadenylic acid were added to small DEAE-cellulose columns (0.8 cm x 24 cm). A family of four peaks was obtained, the relative proportion of each being determined by the hydrolysis time (Figure 21). These results are similar to those obtained by other workers with a similar enzyme preparation, and each peak represents a short chain 5'-phosphorylated oligoadenylic acid (Eley and Roth, 1966). These are eluted in order of increasing chain length (Smith, 1964; Smith et al., 1966). In a preparative experiment 50 mg of poly A was subjected to a 42-hour enzymatic hydrolysis followed by alkaline hydrolysis in 0.25 M KOH at 100° for 1 hour. The resulting products were also separated on DEAE-cellulose columns as described above. Three major peaks were obtained. The first two peaks were eluted in the same position as authentic adenosine, and AMP respectively. The third peak was shown to be pAp, and as expected its elution position was not changed upon further treatment with 0.25 M KOH at 37° for 47 hours. The conditions of chromatography were chosen such that authentic nucleoside mono-, di-, and tri-phosphates of interest were well separated. Their order of elution is A, AMP, ADP, and pAp.

Results and discussion

The hydrolysis of poly A or poly U with chicken pancreas nuclease produces a family of short chain oligonucleotides with free 3'-hydroxyls and a phosphate monoesterified to the 5'-position (Eley and Roth, 1966). Similar products can also be obtained with other nucleases such as those isolated from guinea pig liver nuclei (Heppel et al., 1956), bacteria

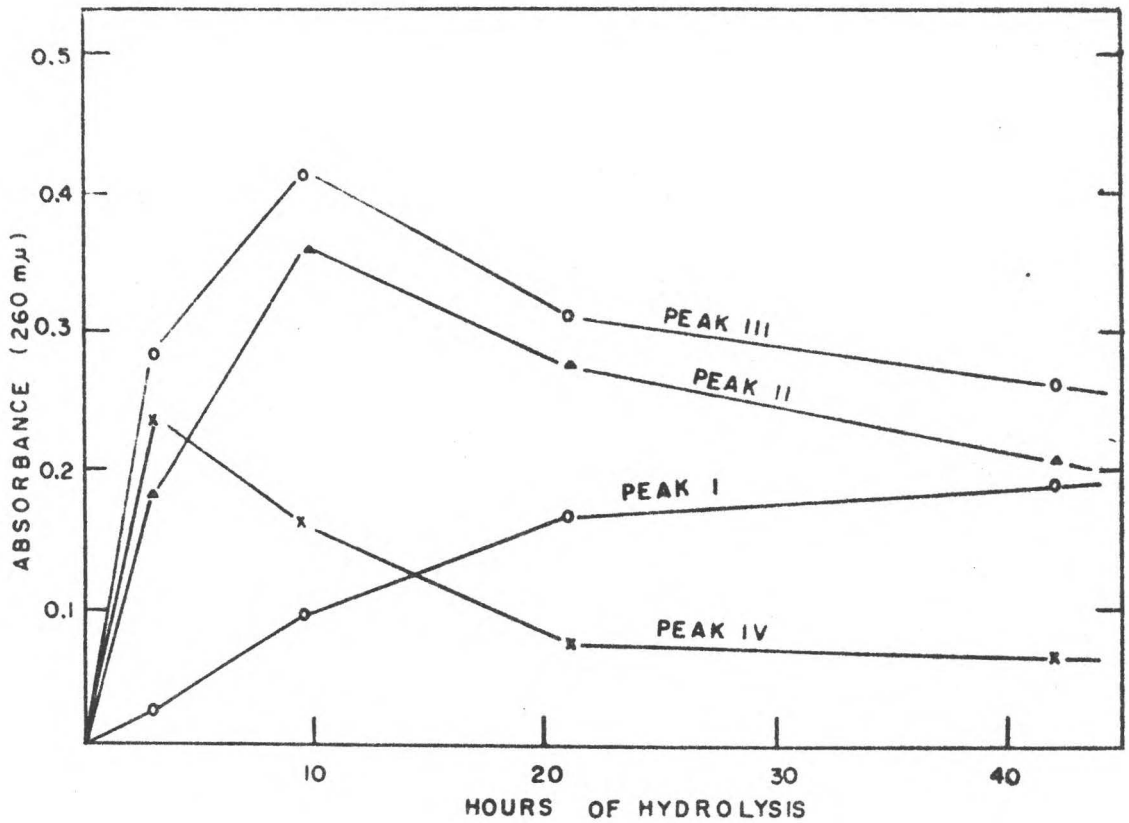


Figure 21.--Hydrolysis of poly A with chicken pancreas nuclease. 0.075 ml aliquots were taken from the reaction mixture at the times indicated and the reaction stopped by heating. Samples were diluted and added to DEAE-cellulose columns (0.8 cm x 24 cm). Elution was carried out by an exponential gradient, 0--0.4 M NaCl in 0.01 M Tris, using a 500 ml mixer. Peaks are numbered in order of elution and represent short oligoadenylic acids of increasing chain length. The total absorption (260 nm) of each of the peaks was determined as a function of time.

Note: Only negligible hydrolysis of pentaadenylic acid occurs when treated under conditions similar to those used for the above heat inactivation.

(Stevens and Hilmoe, 1960), and sheep kidney (Kasai and Grunberg-Manago, 1967). Since it is well known that mild alkaline hydrolysis of ribonucleic acids produces 2'- or 3'-phosphorylated nucleosides (Carter and Cohn, 1949), treatment of 5'-phosphorylated oligoadenylic acids should yield adenosine, 3'(2')-AMP, and 3'(2'),5'-adenosine diphosphate as follows:

$$\text{Poly A} \xrightarrow[\text{nuclease}]{\text{chicken}} \begin{cases} \text{pA} \xrightarrow{\text{KOH}} \text{pA} \\ \text{pA-A} \xrightarrow{\text{KOH}} \text{pAp + A} \\ \text{pA-A-A} \xrightarrow{\text{KOH}} \text{pAp + Ap + A} \end{cases}$$

Adenosine 3'(2'),5'-diphosphate (pAp) can easily be separated from adenosine (A) and AMP (pA or Ap) by ion exchange chromatography. The identity of pAp was confirmed by the following observations: (a) the elution position of pAp on DEAE-cellulose was unchanged upon prolonged treatment with alkali (must therefore be free of short chain oligoadenylic acids which are completely hydrolyzed under these conditions.) The position of elution was reproducible upon rechromatography, and clearly distinguishable from ADP. This observation is of importance since, (b) the Pi/Adenosine ratio of samples treated with alkaline phosphatase was 2 (Table 5). (c) Upon treatment with limited amounts of alkaline phosphatase the following products were obtained: adenosine, 5'-AMP, 3'-AMP, 2'-AMP, and unhydrolyzed pAp. Further evidence that 5'-AMP was obtained under these conditions was demonstrated by observing that treatment of pAp with limited amounts of alkaline phosphatase, in the presence of ATP and adenylate kinase resulted in the appearance of ADP, even when 5'-AMP was omitted from the initial reaction mixture, as will be described later. Note: pAp has also been obtained by other workers using this procedure (Kasai and Grunberg-Manago, 1967).

TABLE 5

TREATMENT OF pAp WITH E. COLI ALKALINE PHOSPHATASE[a]

Exp.	μmoles A	μmoles Pi	Pi/A
Exp. I	0.185	0.363	1.97
Exp. II	0.185	0.362	1.96

[a]The phosphatase reaction (0.6 ml) contained 1.85 mM pAp, 50 mM Tris-HCl (pH 7.8), and 6.4 units of alkaline phosphatase and was carried out at room temperature for two hours. The pAp concentration was determined by the absorption at 260 nm. Inorganic phosphate concentration was determined by the method of Fiske and Subbarow (1925).

Substitution of pAp for pA (5'-AMP) in the adenylate kinase reaction.--In an attempt to synthesize ppAp, pAp was substituted for 5'-AMP in the transphosphorylation reaction with ATP catalyzed by adenylate kinase.

$$ATP + AMP \leftrightarrow 2ADP$$

It was envisioned that the substitution of pAp for pA (5'-AMP) in the above reaction might result in the phosphorylation of the 5'-phosphate of pAp to yield ppAp, adenosine 3'(2')-phosphate, 5'-pyrophosphate.

$$pppA + pAp \leftrightarrow ppAp + ppA$$

This reaction was followed by chromatography, on paper and on DEAE-cellulose columns, using variable concentrations of Mg^{++} in the incubation mixture. No detectable phosphorylation of pAp occurred, as evidenced by the quantitative recovery of pAp at the conclusion of the reaction. Phosphorylation of the 5'-phosphate would have resulted in the elution of a peak at a higher salt concentration than pAp (Smith et al., 1966). Clearly such a peak would be distinguishable from pAp under our elution conditions because they are adequate not only for the separation of short chain oligonucleotides (Smith et al., 1966) but also AMP, ADP, ATP, and pAp, which differ in some cases by less than a single charge.

Inhibition of adenylate kinase by 3'-phosphorylated adenylic acid (pAp).--In order to further characterize the effect of pAp on adenylate kinase, kinetic studies were carried out to determine if it inhibited the phosphorylation of 5'-AMP. The concentrations of the adenylate kinase, co-factors, and substrates were adjusted to optimum condition (Callaghan and Weber, 1959), and the reaction terminated during the linear region of the transphosphorylation.

A Lineweaver-Burk plot of the data is shown in Figure 22. The Michaelis constant of the uninhibited reaction was found to be 0.3 x 10^{-3} M, in agreement with other reported values (Callaghan and Weber, 1959). These data show that pAp is a non-competitive or mixed inhibitor of the enzyme. It is of interest that the Mg^{++} optimum for this reaction is the same in the presence or absence of pAp, however, at higher concentrations of Mg^{++} pAp is a more effective inhibitor.

Enzymatic conversion of pAp to 5'-AMP.--It will be observed that pAp can be converted to 5'-AMP, a substrate of adenylate kinase, by treatment with limited amounts of bacterial alkaline phosphatase (BAP):

$$\text{pAp} \xrightarrow{\text{BAP}} \text{pA (5'-AMP)} + \text{Ap (3'-AMP)} \xrightarrow{\text{BAP}} \text{A}$$

This suggests that in the presence of alkaline phosphatase, adenylate kinase, pAp, and ATP, ADP should be produced (even in the absence of added authentic 5'-AMP). Bacterial alkaline phosphatase, in quantities ranging from 0 to 6.4 units, was added to the adenylate kinase reaction mixture in the absence of authentic 5'-AMP (Figure 23). The reaction was carried out at 30° for 30 minutes. Because of the inhibitory action of pAp, ten times the normal amount of adenylate kinase was used. In the presence of a large excess of alkaline phosphatase, pAp was quantitatively converted to a substrate for adenylate kinase (presumably 5'-AMP) by alkaline phosphatase, and subsequently to ADP by adenylate kinase. Inasmuch as the adenylate kinase reaction is reversible and the monoesterase reaction essentially irreversible, one would expect, in the presence of large amounts of alkaline phosphatase, that all of the pAp would be converted to adenosine.

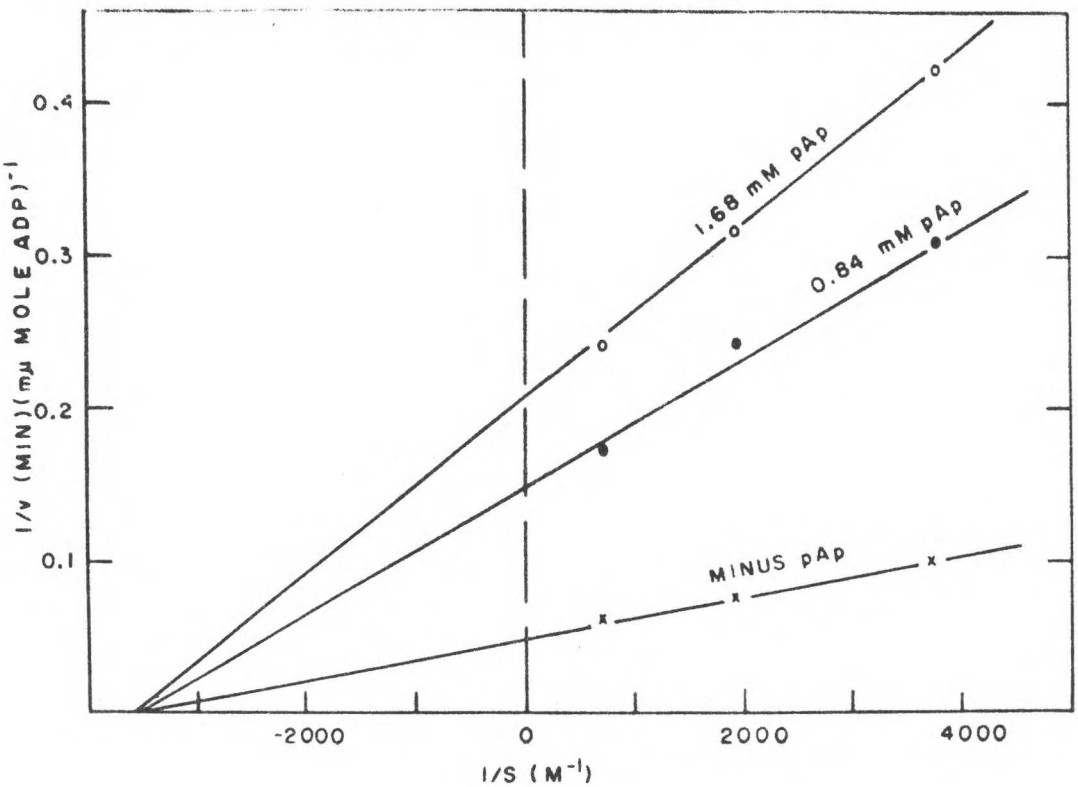


Figure 22.--Lineweaver-Burk plot of adenylate kinase with variable AMP. The concentration of ATP was held constant and AMP was varied. Increasing concentrations of pAp were used; 0, 0.84, and 1.68 mM. Ordinate shows the reciprocal of velocity (1/V) in nmoles of ADP produced per minute and the abscissa shows the reciprocal molar AMP concentration (1/S).

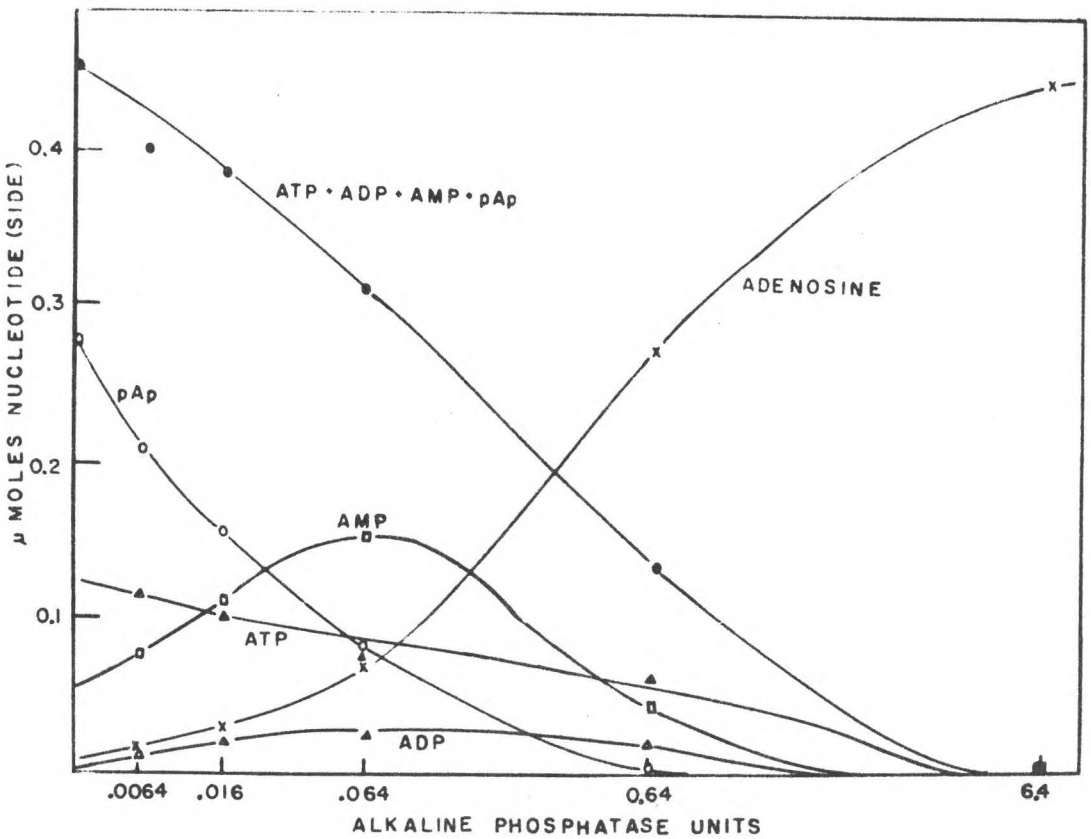
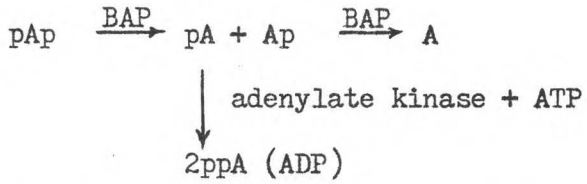


Figure 23.--Conversion of pAp to AMP in the presence of adenylate kinase. The ordinate shows the change in μmoles of nucleotide (or nucleoside) as determined by the absorption at 260 nm. A common molar extinction coefficient of 15.4×10^3 was assumed for the calculations. (Increasing concentrations of alkaline phosphatase are represented logrithmically on the abscissa.) Reaction conditions were identical to those in Figure 22 with the exception of a 10-fold increase in adenylate kinase and the exclusion of a 5'-AMP from the initial reaction mixture.



The simultaneous hydrolysis of ATP by alkaline phosphatase also favors the formation of adenosine. The instability of ATP to alkaline phosphatase raises the question concerning the interpretation of the Pi/Adenosine ratio of 2 referred to earlier, when pAp was treated with alkaline phosphatase. The same ratio could be obtained from ADP. However, our preparation was initially purified on columns of DEAE-cellulose as mentioned, and under the conditions used it was readily separable from authentic ADP. Pentaadenylic acid was found to be completely stable to alkaline phosphatase under these conditions.

These experiments give conclusive evidence that 3',5'-nucleoside diphosphates can readily be prepared using chicken pancrease nuclease, and alkaline hydrolysis, but that such diphosphates cannot be further phosphorylated using commercially available rabbit muscle adenylate kinase. Whether the inhibition of this enzyme by 3'(2'),5'-adenosine diphosphate is a consequence of the 2' or 3' isomer or both was not determined. However, all available evidence indicates that neither of the two isomers is a substrate for adenylate kinase.

(End of published article)

Inhibition of Adenylate Kinase With Respect to ATP

Introduction

The foregoing work relating to adenylate kinase contains a description of experiments designed to determine if adenosine 2'(3'),5'-diphosphate (pAp) was not phosphorylated as a result of inhibition of the

of the enzyme by pAp. As demonstrated, pAp is a non-competitive (or possibly mixed) inhibitor of the enzyme with respect to the substrate AMP. That is, pAp combines both with the same form of enzyme responsible for combination with AMP, and with a different form of the enzyme than that responsible for combination with AMP (Cleland, 1963). The result of such inhibitor-enzyme combinations with AMP as the variable substrate is an effect on both the slope and intercept of the reciprocal plot, as seen in the foregoing section.

The particular pattern of pAp inhibition with respect to AMP leads one to speculate that pAp may bind to the ATP site on the enzyme surface, since its inhibition is noncompetitive with AMP. Assuming the enzyme mechanism were random bi bi, such binding could explain the inhibition pattern since it could be both with the same form and also with a different form of the enzyme than that responsible for binding AMP (see discussion).

Further evidence for this mechanism of inhibition, and therefore the postulated mechanism of enzyme action, might be obtained by observing the inhibition patterns of pAp with respect to ATP as the variable substrate.

Materials and Methods

DEAE-cellulose was obtained from Whatman (DE 23) (1.0 meq/g). Nucleotides were obtained from Swartz Bioresearch with the exception of pAp, which was prepared as described previously (Rowe and Smith, 1968). Rabbit muscle adenylate kinase was purchased from General Biochemicals.

The adenylate kinase assay contained the following components (in μmoles/ml unless otherwise indicated) in a total volume of 0.2 ml:

$MgCl_2$, 5; Tris-HCl (pH 7.8), 50; AMP 1.3; BSA, 1.5 mg/ml; ATP from 0.175 to 1.75; and pAp concentrations of 0, 0.24, and 0.73 mM. The reaction was initiated with 0.02 units of adenylate kinase. Under these conditions the formation of ADP was linear for at least five minutes. The reaction was terminated by the addition of 0.1 volume of 30% trichloroacetic acid and subsequently centrifuged at 5,400 x g for 10 minutes. A 50 fold dilution of each supernatant fraction was added to a DEAE-cellulose column (0.15 cm x 25 cm). Elution was accomplished using an exponential gradient of NaCl (0--0.2 M in 0.01 M Tris-HCl, pH 7.8) and a 125 ml mixer. Four columns were eluted simultaneously using a single gradient producing device. The flow rate was 460 ml/cm^2/hr. Column effluents were monitored continously at 260 nm with a Gilford multiple sample recording spectrophotometer.

The products of the reaction were identified by their position of elution relative to those of authentic AMP, ADP and ATP, eluted under identical conditions. Quantitative data was obtained by calculating optical density under each peak and converting the data to nmoles of product.

Results and Discussion

pAp inhibition with respect to ATP.--The results of varying the ATP concentration at several fixed concentrations of pAp and a fixed concentration of AMP are shown in Figure 24 as a reciprocal plot. The data indicate that with respect to ATP, pAp is a competitive inhibitor. According to Cleland (1963), this means that pAp combines only with the same form of adenylate kinase as does ATP.

Reaction mechanism.--There are several possibilities for the

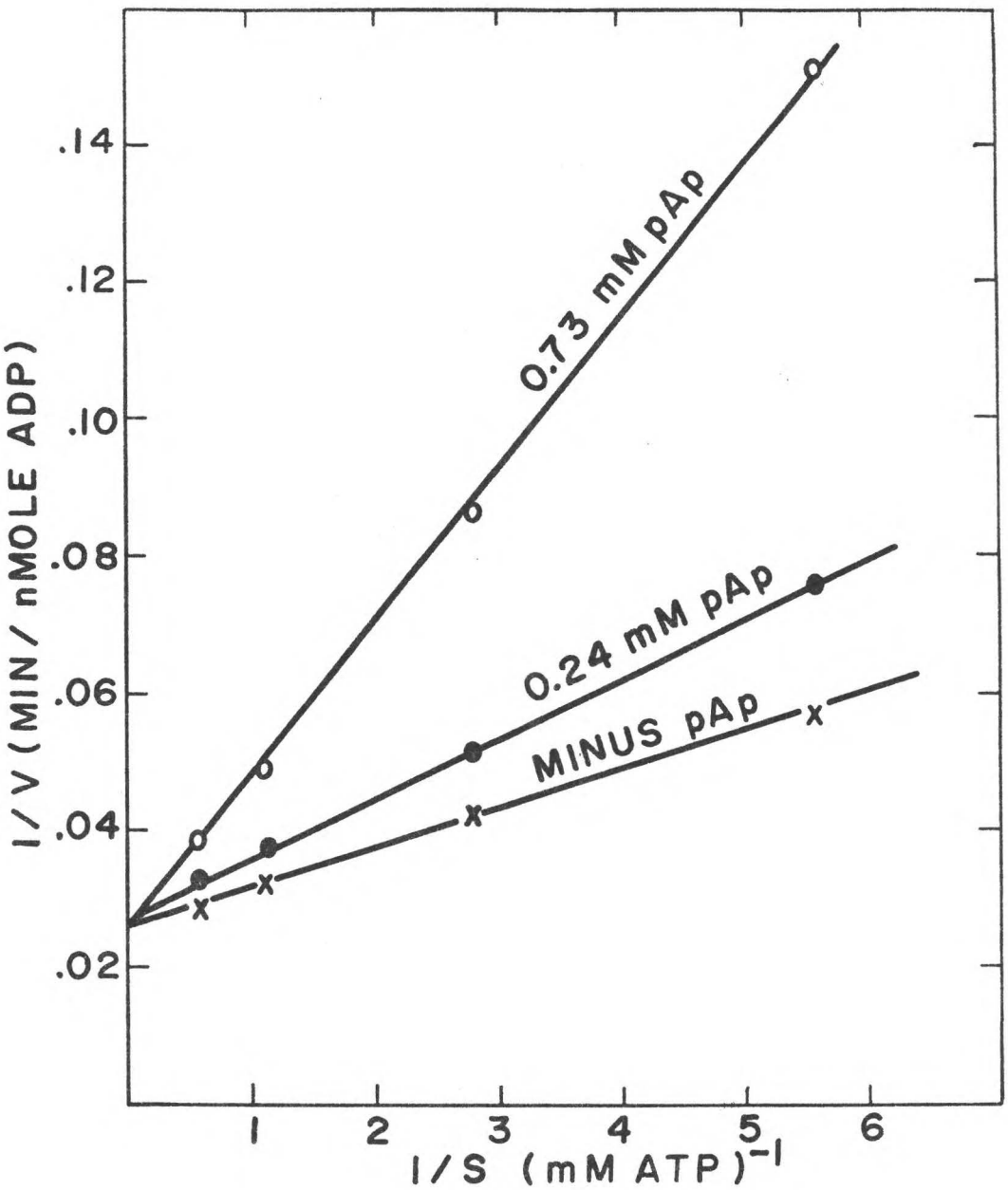
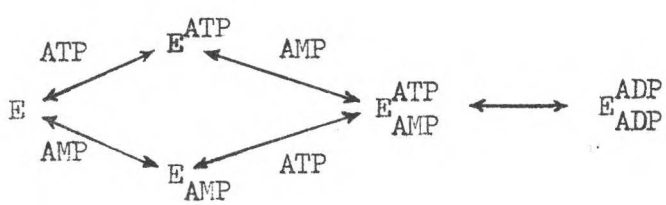


Figure 24.--Lineweaver-Burk plot of adenylate kinase with variable ATP.

reaction mechanism for adenylate kinase. The first involves an enzyme-phosphate intermediate. Such an intermediate would require an ordered uni uni uni uni mechanism. A second possibility involves a single-substrate, enzyme complex in which the other substrate forms a ternary complex without binding to the enzyme, but accepting or donating a phosphate. A third possibility involves a two site mechanism in which the substrates AMP and ATP have adjacent binding sites. This two site model could incorporate either an ordered or a random bi bi mechanism.

Callaghan and Weber (1959) on the basis of differences in affinity between AMP and ATP for the enzyme, and the existence of only one binding constant for ADP, envisioned the enzyme as having a single site which binds specifically ATP. On contact with AMP (either by collision or a very weak binding) the transphorylation takes place.

Our results suggest that the adenylate kinase reaction proceeds via a random bi bi mechanism in which ATP and AMP have specific binding sites on the enzyme in random order. This mechanism may be outlined as follows:



The results of our inhibition studies with pAp (with respect to AMP) suggest that it combines with both the same form, and a different form of the enzyme than does AMP.

This may readily be explained by the random bi bi mechanism, with pAp binding at the ATP site of the free form of the enzyme (E, one of the forms with which AMP combines), but not with the form of the enzyme to

which ATP is already bound (E^{ATP}), the other form with which AMP combines. Stated differently, since AMP combines in a random bi bi mechanism, with both E and E^{ATP}, but of these two forms pAp can only combine with E, then the inhibitor combines with both the same form and also a different form of the enzyme than that with which the substrate AMP combines. This would produce the non-competitive inhibition pattern observed with AMP as the variable substrate.

Further evidence for this mechanism is provided by the competitive inhibition exhibited by pAp with respect to ATP. According to Cleland (1963) this indicates that pAp combines only with the same form of the enzyme as does ATP. Since substrate combination with the enzyme must be random to satisfy inhibition properties with respect to AMP, the competitive inhibition with respect to ATP requires that pAp combine both with free enzyme and the enzyme AMP complex (E and E_{AMP}), since these are the forms which combine with ATP. Such combinations with pAp would most likely occur only at the binding site for ATP.

Other possible mechanisms for the reaction of adenylate kinase and for the binding of pAp do not fit both inhibition patterns observed.

Results of experiments published simultaneously with our work (Su and Russell, 1968) indicate the homologous enzyme, adenylate kinase from baker's yeast (Su et al., 1967; Chiu et al., 1967;) also proceeds via a random mechanism. These results were obtained from experiments determining equilibrium exchange rates. The data also revealed that the rate limiting step of the reaction was the interconversion of the ternary complex and not the binding of substrates or the disassociation of products.

Subsequent work from the same laboratory on the baker's yeast

adenylate kinase (Khoo and Russell, 1970) presented initial velocity studies, substrate and product inhibition studies and substrate analogue inhibition studies which confirm the proposed mechanism. It is interesting to note that with this enzyme the 5'-AMP analogues 3'-AMP and 2'-AMP were inhibitors of the reaction, while 3',5'-cyclic AMP did not inhibit. With respect to both AMP and ATP, 2'-AMP was a noncompetitive inhibitor. These and other data were consistent with the addition of 2'-AMP at both the ATP and 5'-AMP sites. This is significant in light of our results with the inhibition of rabbit muscle adenylate kinase with pAp, which indicate that it binds only at the ATP site.

The results obtained earlier by Callaghan and Weber (1959) which indicated a one-site mechanism partially on the basis of only one binding constant for ADP may be rationalized on the grounds that ADP is only one of the substrates for the reaction, the other being MgADP as discussed previously.

Transphosphorylation at high enzyme concentrations.--As mentioned earlier, Dounce and Kay (1953) observed phosphorylation of RNA by adenylate kinase. Contrary to this report, Stahl and Ebel (1964) indicated that RNA could not be phosphorylated at the 5'-terminal phosphate by adenylate kinase. In better defined experiments, Klemperer and Harvey (1968) demonstrated that in the presence of large amounts of enzyme, short chain 5'-phosphorylated oligoadenylic acids incorporated ^{32}P from γ-labeled ATP. pA-A reacted most rapidly, the rate being only 0.01% that of the phosphorylation of AMP. The 5'-terminal phosphate was required for the reaction, and the reverse reaction occurred readily.

These results indicate that in the presence of large amounts

of enzyme it may be possible to phosphorylate a substrate which has at least a phosphate (and possibly a nucleotide) attached at the 3'-hydroxyl.

To test this possibility, reaction conditions employed by Klemperer and Harvey (1968) were simulated in a reaction replacing pA-A with pAp. The reaction mixture contained (in mM concentrations unless otherwise indicated): pAp, 0.73; ATP, 3; $MgCl_2$, 5; Tris-HCl (pH 7.8), 50; BSA 3 mg/ml; and 20 units of adenylate kinase in a 0.2 ml reaction mixture. The reaction mixture was incubated at 37° C for 24 hours and terminated and analyzed as described in methods and materials.

Whereas Klemperer and Harvey (1968) reported 20% of the ATP label incorporated into ppApA, there was no detectable ppAp formed in the above reaction. This is in contrast to the report of Klemperer and Harvey, but not surprising in relation to our results which indicate that pAp binds only at the ATP site, and therefore would have no opportunity to be phosphorylated.

CHAPTER VI

A POSSIBLE METHOD FOR THE PREPARATION OF PRIMER DEPENDENT POLYNUCLEOTIDE PHOSPHORYLASE

Introduction

It was mentioned in Chapter V that one advantage of using 3'-blocked nucleoside diphosphates in stepwise synthetic reactions catalyzed by polynucleotide phosphorylase, is that the substrate is not capable of participating in de novo synthesis of polymers, since a free 3'-hydroxyl is required for the addition of the nucleotide. However, in other methods for limiting addition to the primer, and in other reactions of polynucleotide phosphorylase, such as formation of a homopolymer at the 3'-end of a defined oligonucleotide, de novo polymerization of the nucleoside diphosphate becomes a problem. For this reason, it is desirable to study methods which may be used for the production of primer dependent preparations of polynucleotide phosphorylase.

Previous mention has been made of the fact that polynucleotide phosphorylase has been purified from a variety of bacterial sources. One of the most striking variables in the properties of these enzymes is related to the dependence of the polymerization reaction on the presence of oligonucleotide primers. Certain enzyme preparations are able to catalyze the polymerization of nucleoside diphosphates only after a lag period in the absence of primer. The lag period can be eliminated by the addition of oligonucleotides to the reaction mixture (Grunberg-Manago, 1963).

Singer et al., (1960) were the first to find that highly purified preparations of M. luteus polynucleotide phosphorylase had a nearly absolute requirement for oligonucleotide primer in the polymerization reaction. The primers acted as initiation points for synthesis, and were incorporated into the polynucleotide product. These enzyme preparations were incapable of initiating de novo synthesis. A highly purified preparation of the E. coli enzyme, on the other hand, catalyzed only de novo synthesis. Primers were not incorporated (Williams and Grunberg-Manago, 1964).

Various hypotheses have been presented in an attempt to explain why some preparations of polynucleotide phosphorylase are primer dependent, and others are capable of catalyzing de novo synthesis. The reasons suggested may be summarized as follows: (a) primer independent preparations are contaminated with sufficient oligonucleotide that addition of primer is not necessary, (b) there is a separate enzyme responsible for catalyzing the initiation of polymerization, (c) enzymes from various sources differ inherently in their ability to initiate polymerization, and (e) polynucleotide phosphorylase can exist in two forms, one which readily initiates polymerization, and one which does not (Grunberg-Manago, 1963).

The evidence available supports the last hypothesis. Whereas published procedures for preparation of M. luteus polynucleotide phosphorylase led to a primer dependent enzyme (Singer and Guss, 1962; Singer, 1963) more often than procedures for E. coli and A. vinelandii enzymes, Klee (1967) observed that original procedures no longer consistently led to a primer dependent enzyme. The form (primer dependent or primer independent) obtained in a given preparation appeared to

depend on the particular batch of cells used.

There have been many attempts to determine the exact nature of the difference between the primer dependent and primer independent forms of the enzyme. Those studies which are most successful in altering the primer dependence usually involve some type of treatment which could effect the conformation of the enzyme. Various reagents, such as urea, sodium chloride and guanidine hydrochloride were shown to be effective in altering the primer dependence of an enzyme preparation (Klee 1967; Harvey et al., 1967; Williams and Grunberg-Manago, 1964). Magnesium ion concentration also appears to have some effect on primer dependence (Thach and Doty, 1965).

Other treatments which were effective in altering the primer dependence of a preparation included aging (Gajda et al., 1970), and the now very popular proteolytic treatment (Gajda et al., 1970; Klee, 1967; Klee and Singer, 1968; Fitt and Fitt, 1967). Klee and Singer (1970) found trypsin treatment on a preparative scale to be successful for the preparation of a primer dependent polynucleotide phosphorylase.

Klee (1969) compared the properties of a primer independent preparation of M. luteus polynucleotide phosphorylase, with the properties of a primer dependent preparation produced by limited proteolysis with trypsin. The results indicate that the treatment breaks five or six peptide bonds per molecule, and results in the removal of a small portion of the enzyme (about 10%). He concluded that the change is not directly related to a change in primary structure, but is due to an indirect effect of proteolysis on the conformation of the enzyme. This agrees with the studies showing alteration of primer dependence in the presence of urea, sodium chloride, and guanidine hydrochloride.

Thang et al., (1971) have recently demonstrated and discussed the proteolytic degradation of polynucleotide phosphorylase by endogenous proteases during isolation procedures. Such enzyme preparations were compared with trypsin degraded preparations and found to be similar. Therefore, endogenous proteolysis may account for the different properties of different enzyme preparations.

Since it has been observed that the primer dependent properties of polynucleotide phosphorylase preparations vary with the lot of cells used, or the method of purification as discussed previously, one can imagine that there exists a control mechanism which regulates the primer dependence of the enzyme in its ill-defined function in vivo. Such a mechanism could easily be rationalized with the observed effects of the conformation changing reagents, proteolysis, and batch of cells.

Thang and Meyer (1971) have recently postulated such a mechanism in the form of a phosphorylation-dephosphorylation mediated control. The basis of this model is their observation that rabbit skeletal muscle protein kinase treatment of polynucleotide phosphorylase can replace the priming action of oligonucleotides in removing the lag phase of primer dependent preparations from E.coli and M. luteus. They postulate that phosphorylation of polynucleotide phosphorylase renders the enzyme completely primer independent, and that removal of the phosphate group from a fraction of the enzyme molecules during purification, or the state of the enzyme in the cells used, may result in the variations in primer dependence observed.

With the goal of exploiting the postulated phosphorylation-dephosphorylation control mechanism as an easy method for the preparation of a completely primer dependent polynucleotide phosphorylase, we have

purified beef spleen protein phosphatase (Revel, 1963) and the protein kinase used by Thang and Meyers (1971) (Walsh et al., 1968). The spleen protein phosphatase was chosen because it has several substrates in common with the protein kinase; since the protein kinase apparently phosphorylates polynucleotide phosphorylase, the phosphatase may dephosphorylate it. The results of enzymatic treatments will be discussed.

Materials and Methods

Nucleotides used in enzyme assays were obtained as follows: γ-^{32}P-ATP was purchased from New England Nuclear, 8-^{14}C-ADP from Cal Atomic, 3',5'-cyclic AMP from Sigma, and triadenylic acid primer was a gift from B. Hughes.

Beef spleen protein phosphatase was prepared and assayed by the method of Revel (1963) and stored frozen at -20° C. The procedure resulted in a 100 fold purification, giving a preparation which catalyzed the release of inorganic phosphate from casein at the rate of 1.05 µmoles/min/ml under the standard assay conditions.

Rabbit skeletal muscle protein kinase was prepared according to the procedure of Walsh et al., (1968) up to the second DEAE-cellulose chromatography step. The preparation catalyzed the covalent binding of 200 nmoles of phosphate to casein per hour/ml. Casein used in the assay had previously been incubated with protein phophatase to allow maximum phosphorylation with ^{32}P during the protein kinase assay.

The protein kinase involved the phosphorylation of casein with γ-^{32}P-ATP as described by Walsh et al., (1968). The reaction (1 ml) was terminated immediately after the addition of one volume of 0.2% BSA by precipitation with 1.0 ml of 10% trichloroacetic acid. After

standing at 0° C for 15 minutes the precipitate was collected by centrifugation and dissolved in 1.0 ml of 0.1 N NaOH. The precipitation and solubilization was repeated, after which the protein was precipitated with a 3.0 ml of 5% trichloroacetic acid and collected on a millipore filter. The radioactivity was determined with a Nuclear Chicago planchet counter.

Two preparations of M. luteus polynucleotide phosphorylase were used. Both were obtained from P-L Biochemicals. One was used as purchased, its initial rate of polymerization being stimulated two fold in the presence of triadenylic acid. The other was further purified by Sephadex G-200 and DEAE-cellulose column chromatography (Hughes, 1970). This preparation was stimulated about eight fold by the presence of triadenylic acid.

Polynucleotide phosphorylase was assayed by following ^{14}C-ADP incorporation into acid insoluble polymer. The reaction mixture (135 µl) contained (in mM concentrations): Tris-HCl (pH 9.0), 150; magnesium acetate, 37; EDTA, 0.75; ^{14}C-ADP, 10; and 1.5 mg polynucleotide phosphorylase per ml. Triadenylic acid was 0.14 mM in reactions which contained primer. Reactions were terminated by the addition of 0.15 ml of 1.0 M $HClO_4$. After 5 minutes, 3.0 ml of cold water were added and the precipitated nucleic acid collected on a millipore filter, washed, dried, and the radioactivity determined in the Nuclear Chicago planchet counter. Under these conditions, incorporation of ^{14}C-ADP was linear for 40 minutes.

Treatment of polynucleotide phosphorylase with protein phosphatase was carried out in the reaction mixtures (150 ml) which contained (in mM concentrations): ascorbic acid (freshly prepared and neutralized

to pH 5.8), 3.3; sodium acetate buffer (pH 5.8), 67; polynucleotide phosphorylase, 2.7 mg/ml; and various concentrations of protein kinase. In some cases sodium chloride was added to eliminate formation of a precipitate. Incubations were at 37° C, various lengths of time being used. Aliquots were subsequently assayed for polynucleotide phosphorylase activity in the presence and absence of primer.

Treatment of polynucleotide phosphorylase with protein kinase was accomplished in reaction mixtures (120 µl) which contained (in mM concentrations): glycerophosphate buffer (pH 6.5) 10; $MgCl_2$, 10; EDTA, 1.0; ATP, 1.0; 3',5'-cyclic AMP, 0.01; polynucleotide phosphorylase, 1.7 mg/ml; and protein kinase in varying amounts. Reactions were incubated at both 26° C and 37° C. Aliquots of these reaction mixtures were assayed for polynucleotide phosphorylase activity in the presence and absence of primer.

Results and Discussion

Treatment of M. luteus polynucleotide phosphorylase with protein phosphatase under conditions which caused extensive dephosphorylation of casein, did not alter its primer dependent properties. This was observed with both preparations of polynucleotide phosphorylase mentioned in methods and materials.

If a phosphorylation-dephosphorylation control mechanism exists, it is possible that the polynucleotide phosphorylase preparations were completely dephosphorylated before incubation with protein phosphatase. Therefore, the protein kinase was prepared and used to treat both preparations of polynucleotide phosphorylase, in a repetition of the experiments of Thang and Meyer (1971). No alteration of the primer

dependence was observed in either preparation of polynucleotide phosphorylase.

At least two possibilities exist for the failure of the protein kinase and phosphatase to alter the primer dependence of polynucleotide phosphorylase in our reaction mixtures. First, as discussed by Thang et al., (1971), M. luteus polynucleotide phosphorylase is particularly susceptible to protease degradation during purification, more so than E. coli, polynucleotide phosphorylase. The degree of degradation varies with each preparation, and with the batch of cells used. Although E. coli polynucleotide phosphorylase is degraded during isolation, it is possible to restore it to complete primer dependence with protein kinase. This may not be possible with protease degraded M. luteus polynucleotide phosphorylase. The preparation of M. luteus polynucleotide phosphorylase shown by Thang and Meyer (1971) to be restored to primer independence by treatment with protein kinase may differ in degree of protease degradation from the P-L Biochemicals preparations used in our experiments.

Secondly, Thang (1972) mentions having observed other properties of his protein kinase preparation which may result in the stimulation of primer independence. In particular, that the stimulation effect could be related to a reaction (i.e., adenylation) catalyzed by a contaminant of his kinase preparation. If this were the case, we may have eliminated such a contaminant during preparation of our protein kinase.

With regard to the protein phosphatase, in addition to the possibilities mentioned above, it is also possible that a phosphorylated polynucleotide phosphorylase is not a substrate for the phosphatase. Although the kinase and phosphatase have several substrates in common, polynucleotide phosphorylase may not be one of them.

SUMMARY AND CONCLUSIONS

The use of ribonuclease T_1 in synthetic reactions which have potential for use in the synthesis of oligoribonucleotides of defined base sequence has been studied and discussed. The study of this reaction was accomplished mainly with the use of a model reaction using guanosine 2',3'-cyclic phosphate and cytidine as substrates for the synthesis of guanylyl(3'-5') cytidine.

Using this model synthetic reaction, it has been shown that synthesis catalyzed by ribonuclease T_1 leads only to the natural 3'-5' phosphodiester linkage. The synthetic product contains no detectable unnatural 2'-5' phosphodiester linkages.

Physical reaction conditions which result in maximum synthesis have been studied. More important, it has been shown that the synthetic reaction can be driven toward good incorporation of guanosine 2',3'-cyclic phosphate (60%) at increased levels of cytidine (170 fold molar excess).

In addition, it has also been shown that the 5'-blocked substrate, guanosine 5'-phosphate, 2',3'-cyclic phosphate, does not participate in self-condensation reactions catalyzed by ribonuclease T_1, but otherwise acts as a substrate, and therefore may be used in large excesses to increase the percentage incorporation of the acceptor. Reaction conditions which result in maximum synthesis using this 5'-blocked substrate have been determined. With only a 4 fold molar excess of this blocked substrate 67% incorporation of cytidine into dinucleo-

tide was accomplished in this modification of the model synthetic system.

Oligocytidylic acids varying in chain-length from the monomer to the hexamer were also used as acceptors under conditions which permit good incorporation of acceptors into a guanosine containing oligonucleotide. The demonstration that good synthetic yields can be obtained in this type of reaction renders the ribonuclease T_1 reaction a valuable technique for the synthesis of oligonucleotides of defined sequence.

In addition to the synthetic reaction of ribonuclease T_1, two aspects of the use of polynucleotide phosphorylase for the synthesis of oligonucleotides are discussed. The first aspect concerns the use of 2',(3')-blocked nucleoside diphosphates as chain terminators in oligonucleotide synthesis catalyzed by polynucleotide phosphorylase. In this regard the synthesis of adenosine 2'(3')-phosphate, 5'-diphosphate was attempted by substituting adenosine 2'(3'),5'-diphosphate for AMP in a transphosphorylation reaction catalyzed by adenylate kinase. Adenosine 2'(3'),5'-diphosphate was not a substrate for adenylate kinase, but was found to be a non-competitive (or mixed) inhibitor with respect to AMP, and a competitive inhibitor with respect to ATP. The evidence indicating that this inhibition pattern suggests a random bi bi mechanism for adenylate kinase is discussed.

The second aspect of the synthetic reaction of polynucleotide phosphorylase discussed concerns a possible method for the production of a primer dependent preparation of that enzyme. Previous work has indicated that the primer dependence of polynucleotide phosphorylase may be regulated by a phosphorylation-dephosphorylation control mechanism. Assuming this to be the case, the mechanism may be exploited by enzymatically dephosphorylating polynucleotide phosphorylase for the

purpose of obtaining a primer dependent preparation.

A protein kinase and a protein phosphatase have been partially purified and individually incubated with polynucleotide phosphorylase with no consequential change in primer dependence. The possible reasons for the failure of any change to occur are discussed.

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ENZYMATIC SYNTHESIS OF OLIGORIBONUCLEOTIDES OF DEFINED BASE SEQUENCE

Mark J. Rowe

Graduate Section of Biochemistry

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ABSTRACT

The synthetic reaction of ribonuclease T_1 has been studied as to its potential for the synthesis of oligoribonucleotides of defined base sequence. Conditions have been determined for maximum synthesis with respect to percentage incorporation of both guanosine 2',3'-cyclic phosphate (donor), and cytidine (acceptor). With regard to maximum acceptor incorporation, a donor blocked at the 5'-hydroxyl has been used to drive the reaction. It has also been shown that acceptor incorporation decreases as the chain-length increases up to the hexanucleotide.

Synthesis of a 3'-blocked substrate for polynucleotide phosphorylase for use in chain termination studies was attempted using adenylate kinase. In this regard, adenosine 2'(3'),5'-diphosphate was shown to be a noncompetitive inhibitor with respect to AMP, and a competitive inhibitor with respect to ATP, of adenylate kinase.

Polynucleotide phosphorylase was incubated with a protein kinase and a protein phosphatase in an attempt to exploit a postulated phosphorylation-dephosphorylation control mechanism for the production of a primer dependent preparation. No reaction was observed.