

THE INTERACTION OF THE Eco R1 RESTRICTION
ENZYME FROM *E.coli* WITH NUCLEOTIDES

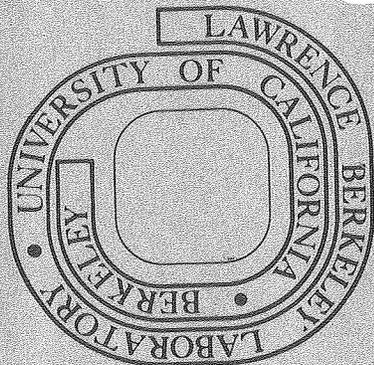
Donald F. Hollis
(Ph. D. thesis)

November 1978

Prepared for the U. S. Department of Energy
under Contract W-7405-ENG-48

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THE INTERACTION OF THE Eco R1 RESTRICTION
ENZYME FROM *E.coli* WITH NUCLEOTIDES

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ABSTRACT

The Eco R1 restriction enzyme can be shown to be inhibited by nucleotides which correspond to any part of its known site of phosphodiesterase activity.

A series of di-, tetra-, and hexa-nucleotide fragments were synthesized and their effect on the activity of the enzyme upon superhelical Col E1 DNA studied. The inhibition caused by the individual mononucleotides were also studied. In general all the nucleotide fragments showed some form of interaction with the enzyme system. Tetranucleotides were stronger inhibitors than dinucleotides, which in turn were stronger inhibitors than the mononucleotides. Within each category of inhibitors, those containing the phosphodiester bond which is acted upon by the enzyme were the strongest inhibitors. Only those fragments which were consistent with the enzymes' site of activity showed competitive inhibition kinetics. Nucleotides which do not fit within the site of phosphodiesterase activity show non-competitive inhibition kinetics.

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ACKNOWLEDGMENTS

The author wishes to thank J. Hirst for a valuable gift of the Col E1 DNA, and H. Gamper for his help in developing the gel electrophoresis techniques. Also a special thanks to M. Calvin for his advice and direction during this research study.

The author was supported by a graduate research assistantship from Lawrence Berkeley Laboratory. This work was done under the general auspices of the U. S. Department of Energy.

GENERAL INTRODUCTION

This study developed from our laboratory's ongoing research efforts into the mechanisms involved in viral and chemical carcinogenesis; a major consideration being how a virus system was able to insert itself into the host genome and cause physical and metabolic changes generally associated with cell transformation. The question of viral transformation as it applies to mammalian cell systems has become very complex, but if one assumes that the role of a virus in cell transformation is a reality, then understanding the enzyme systems associated with the action of this virus becomes extremely important.

The connection between a bacterial restriction enzyme and enzymes involved in mammalian cell transformation is not obvious until one examines the action and mechanism of the enzymes in question. Restriction enzymes have the function of opening a strand of DNA at a specific base sequence, often in an overlapping manner. A pair of "sticky ends" are generated which have the potential of being joined to any piece of DNA with similar ends by the action of an appropriate ligase. Thus the importance of restriction enzymes as models for any specific endonuclease which may be associated with cell transformation is evident. Whether the enzyme is associated with the virus protein or already present in the host cell is uncertain, but the existence of such a system is the probable explanation for the apparent ability of a virus to insert itself into a stable genome and "transform" it. Several reports of specific endonucleases associated with transforming virus' have appeared in the recent literature^{7,8,25,44} which strengthens the assumption of the part they play in the scheme of the viral transformation of a cell system.

The complex role of the restriction enzyme in bacteria is only now beginning to be understood. The known role of this enzyme system is that of degradation of foreign DNA within the bacterium. It is now suspected that they may have a dual role *in vivo*, catalyzing both degradation and synthesis of DNA.¹⁰ There is evidence that a variety of site-specific recombination events can be catalyzed by these enzymes in bacterial systems.⁴⁸ This new role of restriction enzymes *in vivo* strongly supports the hypothesis that restriction-like enzyme systems may be involved in the viral transformation of cells.

It must be stressed that the R1 restriction enzyme used in this study can only be considered a model, for any such system actually involved with *in vivo* transformation. The information derived from this investigation is of first importance in understanding the enzyme system as it relates to its role in bacterial restriction, which is apparently quite varied.

The thrust of this study was to study the specific inhibition of a restriction enzyme by short pieces of DNA which mimicked the known site of action of the enzyme. This required the chemical synthesis of short oligonucleotides of defined sequence and the *in vitro* study of the effects of these oligonucleotides on a restriction enzyme whose site of action was known. The choice of enzyme was already decided as there was only one such system when the study was undertaken. This was the R1 restriction enzyme from *E.coli*.¹⁸ The enzyme assay would require a valid enzyme substrate that had limited number of restriction sites and be obtainable in sufficient quantity to allow the large number of assays that would be necessary to investigate the potential inhibitors. The

material chosen was Col E₁ DNA derived from *E.coli* JC 411.⁵

Because of the varied aspects of this study it is divided into two major sections, the first being the chemical synthesis of potential inhibitors mimicking the site of restriction activity, and the second the kinetics of the enzyme and its inhibition.

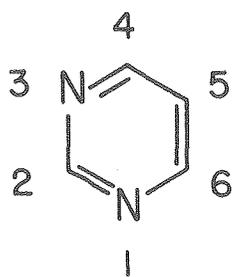
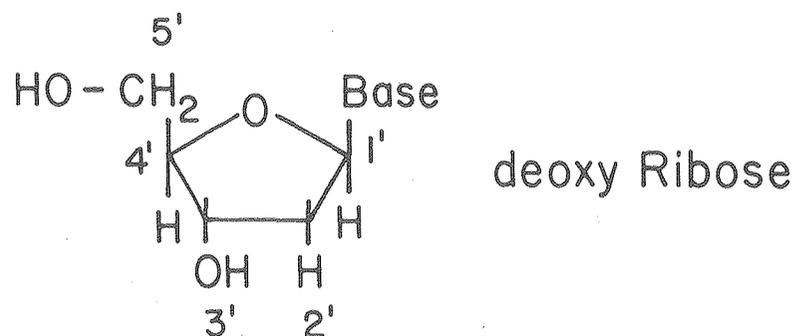
INTRODUCTION TO THE SYNTHESIS OF d-GAATTC

There have developed in the literature several conventions for naming the various mononucleotide and polynucleotide structures.²⁶ The first consideration is the numbering of the aromatic ring system and the ribose sugar residue (Fig. 1). The entire nucleoside, when one is not concerned with the various positions, can be abbreviated as a letter: A for adenosine, U for uridine, T for thymidine, G for guanosine, and C for cytidine. The prefix "d" is used to indicate the 2'-deoxynucleoside for example (d-A) (Fig. 2). In the case of thymidine, it is assumed that the deoxynucleoside is intended and the d prefix is omitted.

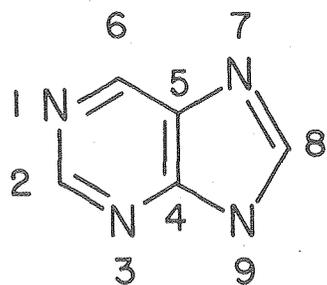
A phosphate is abbreviated with a "p" prefix or suffix, depending on whether it is a 5' or 3' linkage: d-pA, for example, is deoxyadenosine-5'-monophosphate.

The polymer was initially drawn as a series of vertical lines with the letter indicating the aromatic base at the top of each line. These lines were linked with -p- to indicate the phosphate linkage. The position of the linkage on the sugar was in turn indicated by its position on the vertical line (Fig. 3). This has been more recently shortened to just the letters and the p indicating a phosphate linkage, as in d-pApA indicating a 3'-5' dimer (Fig. 3). This is a great simplification for the fully drawn structure for this dinucleotide (Fig. 3).

There are still confusing ambiguities in shorthand notation for nucleotides when pyrophosphates are involved. The pyrophosphate of d-pA would be written d-AppA; this is confusing as it is not clear if it is a 3'-5' or a 5'-5' bond being discussed. This notation is only



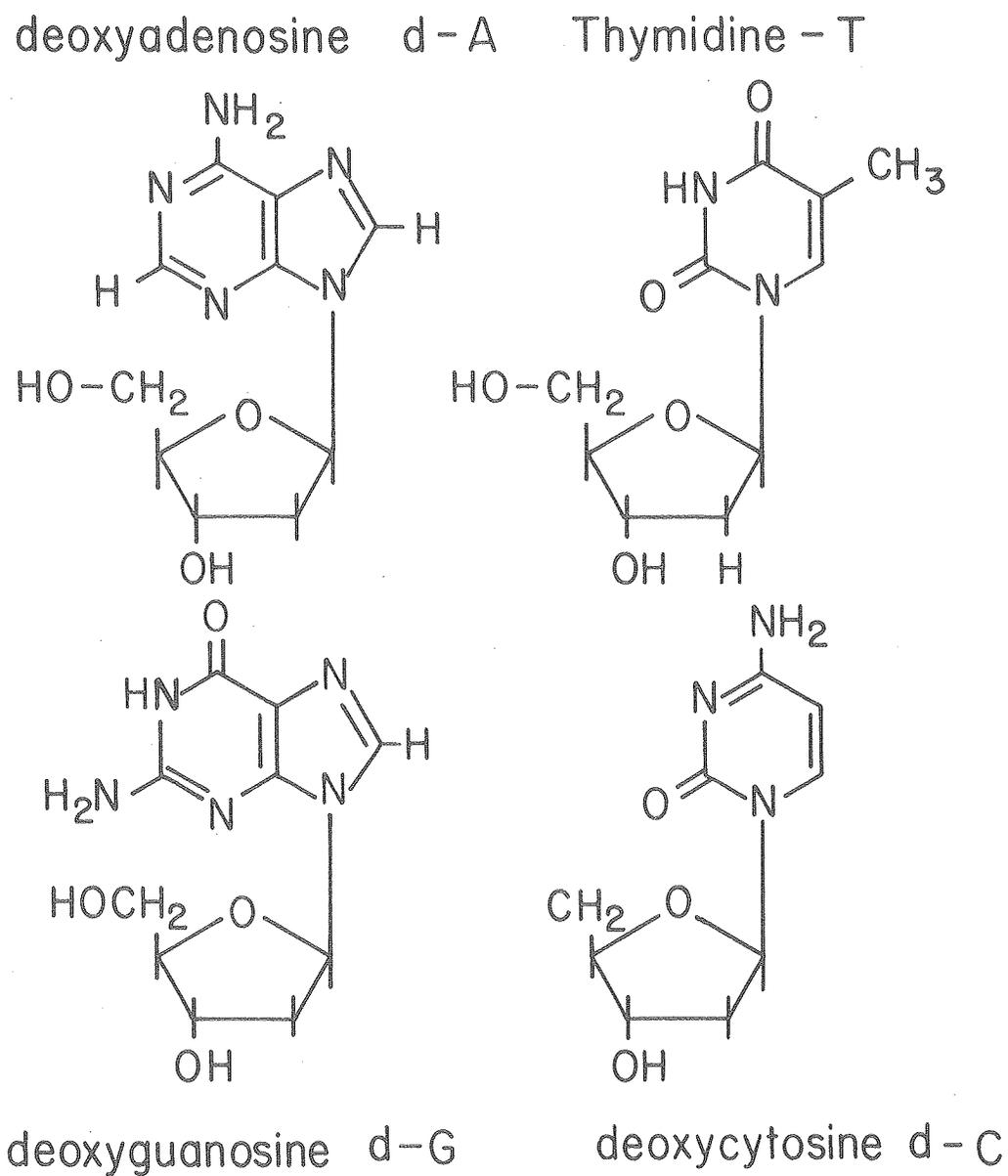
Pyrimidine



Purine

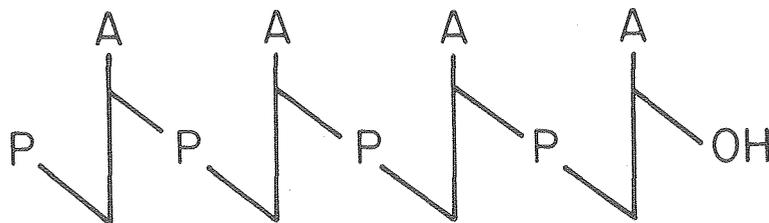
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Fig. 1. Numbering systems.



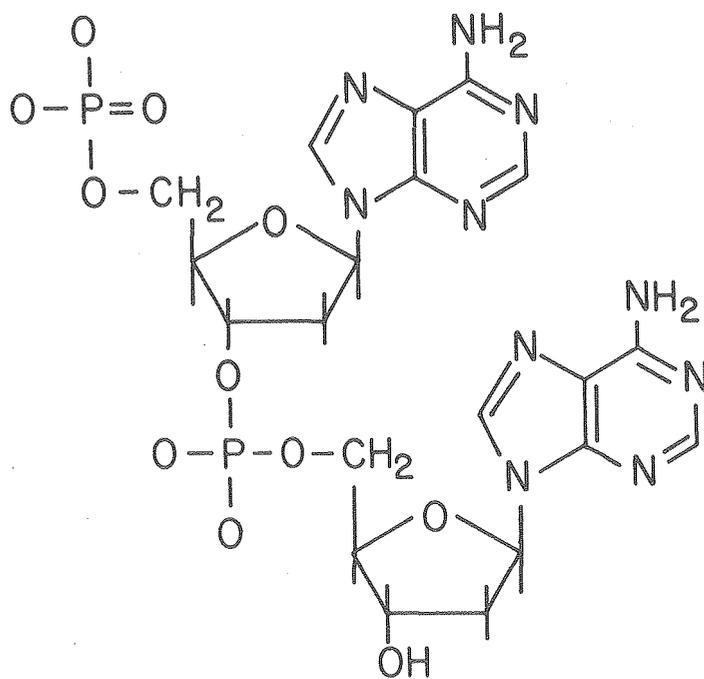
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Fig. 2. The four basic nucleotides.



d - pApApApA

d - (pA)₄



d - pApA

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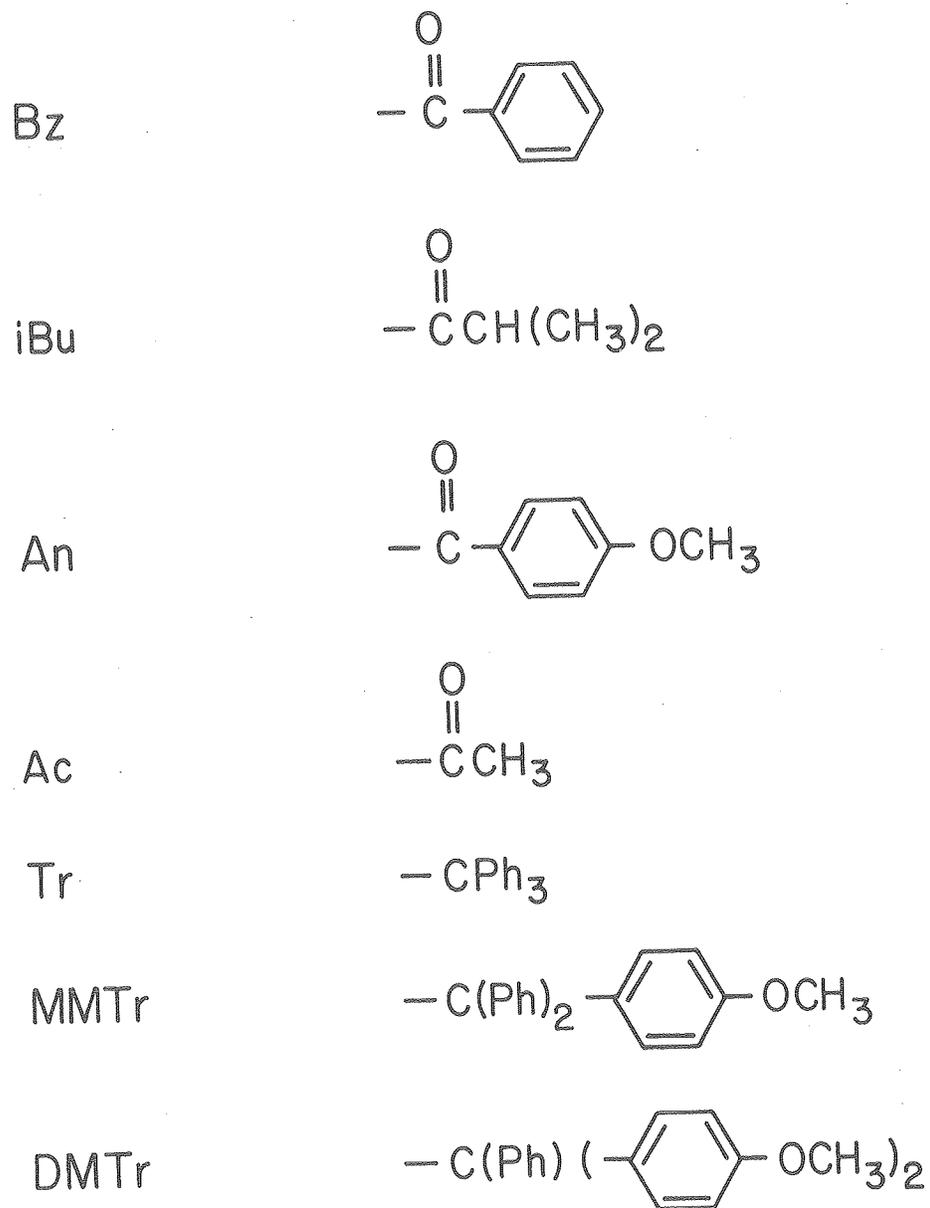
Figure 3.

Shorthand notation for polynucleotides.

used therefore for the 5'-5' situation and other linkages must be indicated by a longer form of notation. Diphosphates and triphosphates follow the prefix-suffix rules: the 5' diphosphate of d-A being d-ppA and the 3'-triphosphate being d-Appp.

Much of the chemistry involved in the synthesis of polynucleotides require protecting groups for the exocyclic amino groups on d-A, d-C, and d-G as well as the 3' or 5' ends of the nucleotide chain (Fig. 4). Some of the common condensation agents also have accepted abbreviations. If a protected nucleotide is indicated the abbreviation for the group attached to the exocyclic amine is written to the right and above the nucleotide as in d-pA^{Bz}. A blocking group on the 3'-OH is shown on the right preceded by a dash, i.e. d-pA^{Bz}-OAc. A group attached to the 5'-OH is written to the left, i.e. d-MMTr-A^{Bz}. To simplify the printing of protected nucleotides and oligonucleotides the amino protecting group has often appeared in the literature in parentheses to the right of the protected nucleotide, i.e. d-pA(Bz)-OAc. Groups attached to other than the exocyclic amine, or the 3' or 5' -OH are not indicated by these shorthand systems and must be shown by conventional chemical notation.

The first major consideration in forming a polynucleotide is whether to condense (i) a 5'-phosphate with a free 3'-OH, or (ii) a 3'-phosphate with a free 5'-OH (Fig. 4). This question was investigated by Khorana⁵⁵ and the results were contrary to expectations. The 5'-OH would be expected to be more reactive as it is less sterically hindered compared to the 3'-OH. The phosphate, which is enlarged upon formation of the mixed anhydride with the condensation agent, should react much faster with the relatively unhindered 5'-OH. The results, however, of Khorana's



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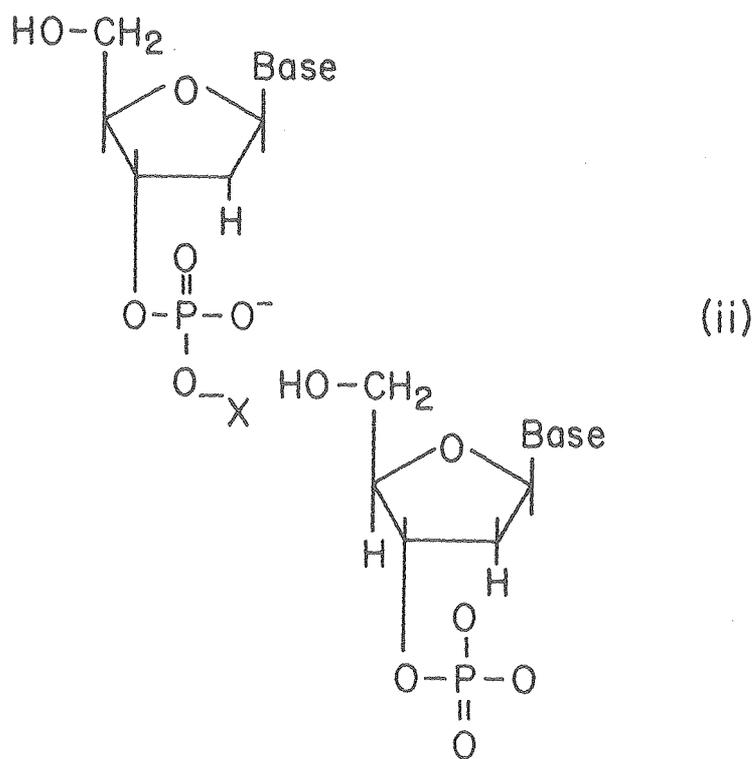
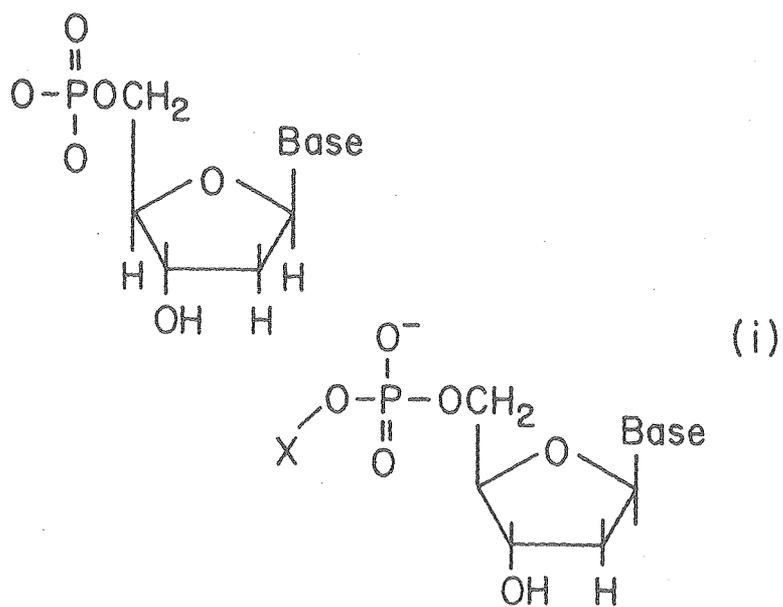
Figure 4.

Abbreviations for protecting group.

study indicates just the opposite. The reason postulated for this was the formation of a tricyclic anhydride intermediate (Fig. 6a) in the condensation reaction with DCC, the agent used in these early studies. The formation of this species, presumably rate determining, would be greatly impeded by steric interference such as that introduced by a 3'-phosphate. This tricyclic species is extremely reactive and is quickly quenched by H₂O. The reaction is therefore very sensitive to the presence of moisture. The more recent use of sulfonyl chlorides as condensation agents has somewhat clouded the picture as they do not seem to go through the same cyclic intermediate.⁵⁵ The active intermediate in this case appears to be a simple 1:1 mixed anhydride (Fig. 6b). Now the lesser steric hindrance of the 5'-OH would become important and indeed better yields have been reported with the use of 3'-phosphates (Fig. 7).

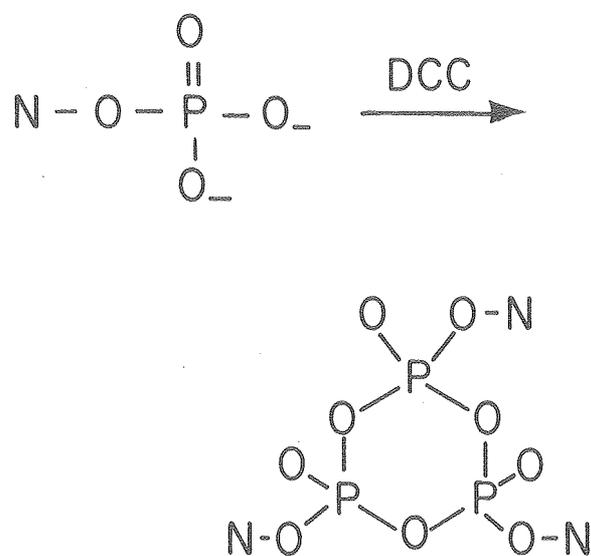
An important consideration is the availability of intermediates. The 3'-phosphates are relatively difficult to obtain and much more expensive than the 5'-variety. The protected intermediates which are available for the 5'-phosphates are totally unavailable for the 3'-phosphates. For these reasons most of the work in the literature to date has concerned the 5' moieties regardless of the condensation agent used.

Another important concept in polynucleotide synthesis is that of a "block synthesis"²⁸, more commonly termed convergent synthesis. Much better overall yields can be obtained by hooking together di- or trinucleotide blocks rather than adding one nucleotide at a time to an extending chain. Although the yields would be expected to increase even further with the use of even larger blocks, the advantage seems to



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Figure 5.
Condensation of 3' or 5' nucleotides.

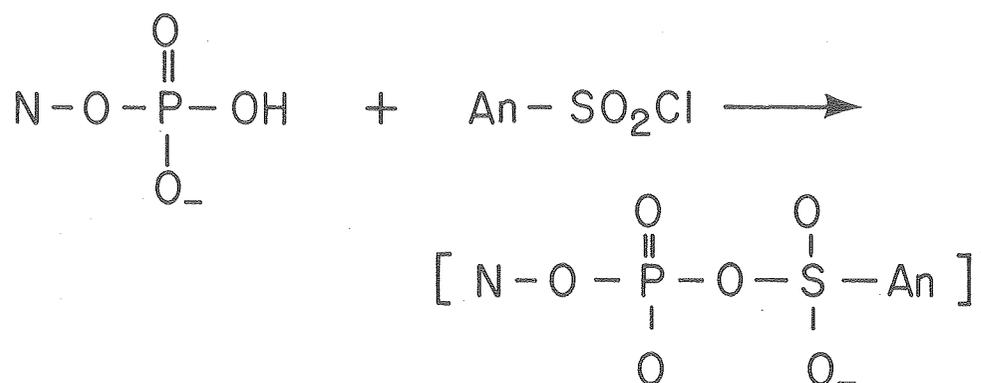


N=any nucleoside

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Figure 6a.

Intermediate in DCC condensation reaction.



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Figure 6b.
Mixed anhydride synthesis.

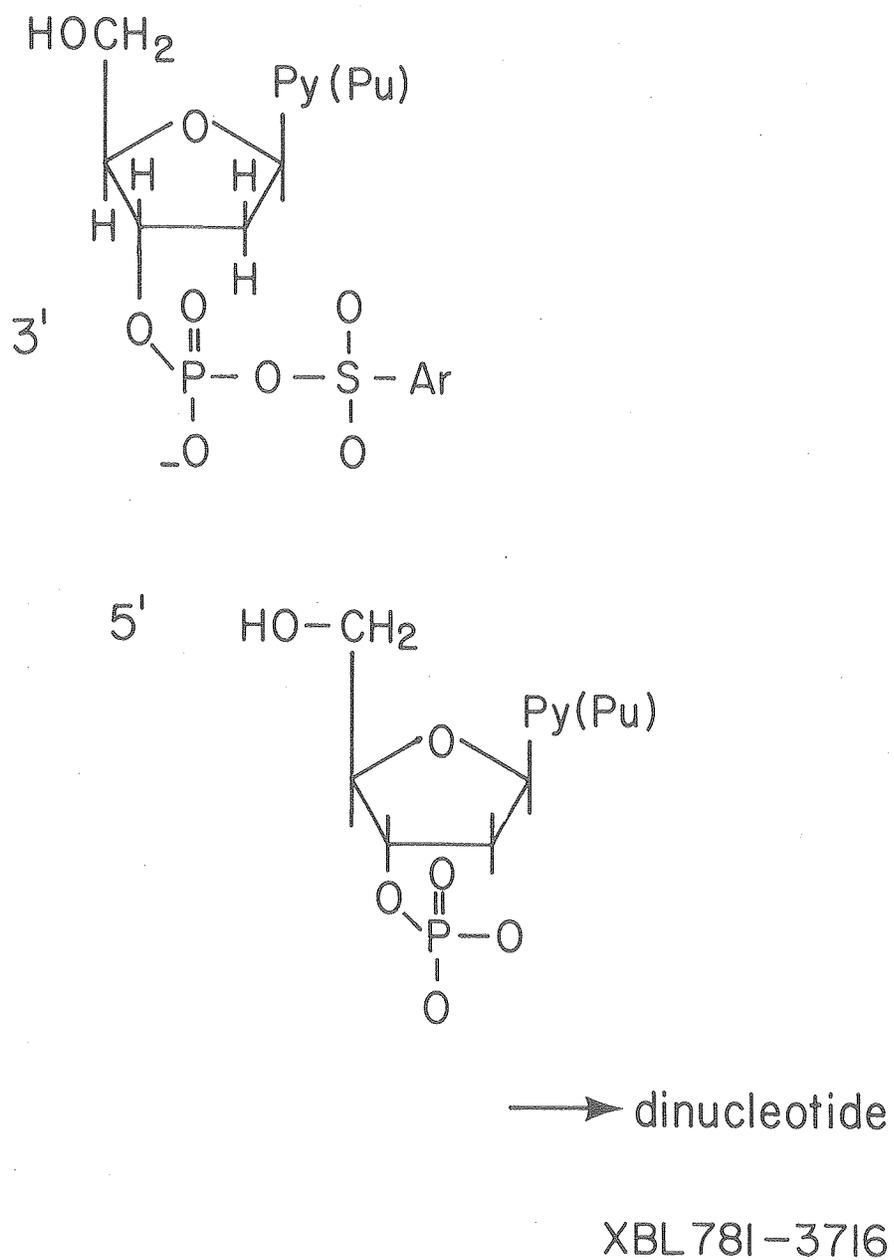


Figure 7.

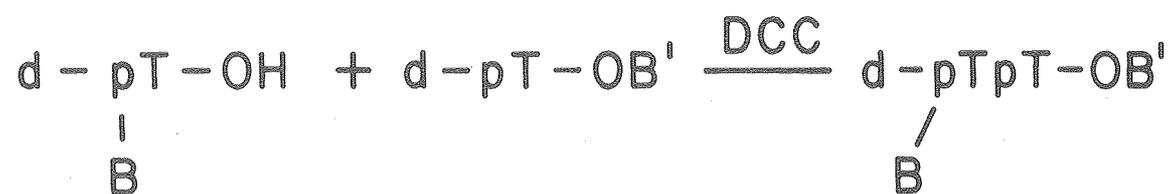
Reduced steric hindrance with 3' phosphates.

drop off rapidly at about four nucleotides due to low yields in the condensation step. Apparently steric interaction starts to become very large, making the condensation step very low in yield. In fact this steric effect can be felt if either the extending chain or the block to be added is too large. The largest oligonucleotide to be synthesized by purely chemical methods has been about 20 nucleotides in length.²⁷ These large oligonucleotides of defined sequence may also be used in a convergent synthesis but they must be enzymatically linked rather than chemically linked. In this combined chemical-enzymatic approach, polynucleotides with "defined sequences" as large as the gene for the yeast alanine tRNA have been synthesized and shown to be biologically active.³

The synthesis of small defined sequences as opposed to random polymers involves the problem of blocking groups. Both the 5-phosphate of the initiating block and the 3' end of the added block must be deactivated towards condensation to prevent the formation of extended random polymers (Fig. 8). Additionally, these two protecting groups must each be stable to the conditions for removal of the other, as well as the reaction conditions.

In early synthesis the phosphate protecting group of choice was the cyanoethyl group.²⁶ This group was added as $\text{Cl-CH}_2\text{CH}_2\text{OH}$ and could easily be removed later by base (Fig. 9). A variety of other phosphate protecting groups have been utilized for this purpose (Fig. 10) and are removed by a variety of methods.

The hindered N-arylphosphoramides are susceptible to nitrosation and are quantitatively decomposed under these conditions.²⁶ Of these

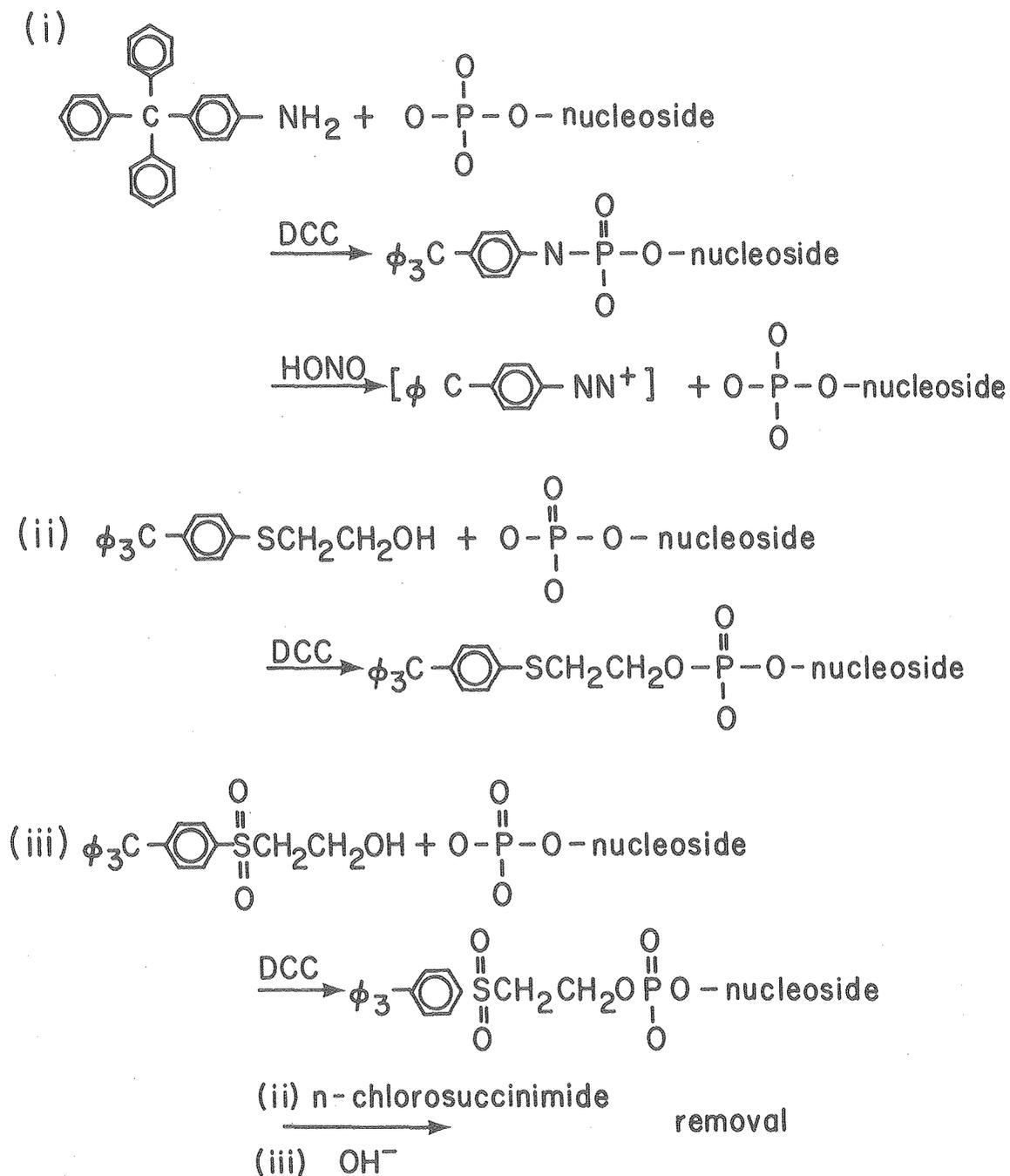


B = Blocking group

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Figure 8.

Forming dinucleotides with blocking groups.



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Figure 10.

Phosphate protecting groups.

triphenylmethane has been of particular interest as the solubility properties of the adducts formed allow the isolation of products by solvent extraction techniques rather than the more time consuming gradient ion exchange column purification methods.⁴

Recently 2-(p-trityl phenyl)thioethanol (TPTE) and 2-(p-tritylphenyl) sulfonylethanol (TPSE) (oxidized form) have been utilized as 5'-phosphate protecting groups.¹¹ They are removed selectively by N-chloro succinimide and OH^- respectively. These groups are reported to be superior to the TPM group in that there is little or no sulfonation products formed in the presence of TPS or MsCl . Thus higher yields of protected dinucleotides may be obtained through solvent extraction procedures than with the TPM derivatives.

In all syntheses it is necessary to protect the 3'-OH group of one of the nucleotides as well as the 5' end of the other nucleotide to prevent the formation of long random polymers. The acetyl group has been commonly used for this purpose as well as the isobutyryl in some cases. These groups are easily removed in base.^{27,46}

Condensation reactions involving nucleotides other than d-pT require an additional protecting group for the exocyclic amino group present on the aromatic base. This protecting group presents an additional problem as it must be stable to the reaction conditions through the entire synthesis for the formation of polymers. It must also be stable to the conditions of deprotection for both the 5' and 3' ends of the molecule.

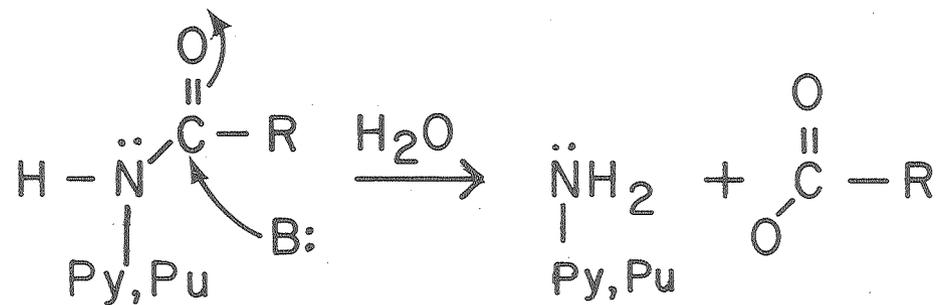
Because of the different electronic properties of each of the aromatic bases it was found that each base required a different protecting group^{4,27,30,45} (Fig. 5). The different groups used reflected the

ease with which each base could be deprotected. They were selected therefore to normalize the conditions for their removal to prevent (i) loss of protection during reaction, (ii) loss of protection during removal of 3'-protecting groups, and (iii) destruction of polymer during total deprotection reaction. Since these amine protecting groups are labile in weak base as are the 3' protecting groups, they would be expected to undergo decomposition upon treatment with base to remove the 3'-OAc group. For this reason the 3' end is deprotected with strong base (NaOH) to which the amino protecting groups are inert for short durations⁴⁵ (Fig. 11).

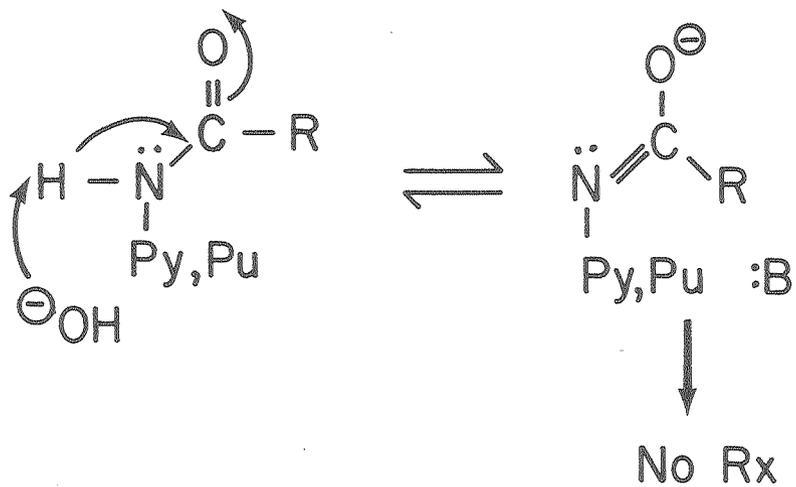
The 5'-end of the polymer may have either a protected phosphate or a protected 5'-OH group. Recent literature has shown syntheses where the 5'-OH has been protected with a bulky, lipophilic group such as monomethoxytrityl (MMTr), or dimethoxytrityl (DMTr), which are designed to interact with lipophilic column materials such as trityl or naphthyl cellulose.³⁴ This allows the products which are marked with the trityl derivatives to be quickly separated from the other intermediates by a one step elution from the tritylcellulose column material. The final purification on DEAE cellulose is greatly simplified by this procedure and the products identified more easily.

The hexanucleotide d-GaApApTpTpC was to be synthesized utilizing a convergent synthesis linking several preformed dinucleotide segments together forming first a tetranucleotide and then the hexanucleotide. This procedure was followed rather than one involving two trinucleotide blocks because it was felt that the intermediates produced in the process would be more useful in the enzyme inhibition study which was to follow.

(i) Weak base



(ii) Strong base



Py, Pu = Pyrimidine, Purine

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Figure 11. Protecting groups inert to strong base.

The 5' end of the polymer was to be protected by the MMTr group rather than the more easily removed DMTr group.⁵⁴ The individually protected dinucleotide blocks were to be produced by solvent extraction procedures which required first the formation of triphenylmethylanilidate derivatives of two nucleotides (d-pA and pT). The three protected dinucleotides as they were used in the synthesis are shown in the schemes for the synthesis of the various dinucleotide and mononucleotide intermediates and are detailed in the discussion (equations 2,3,4).

There are several considerations in deciding to use three dinucleotides rather than two trinucleotides. The apparent advantage to the trinucleotide procedure⁴¹ is the higher yield based on the amount of initial trinucleotide used. The problem in making a trinucleotide rather than a dinucleotide is in the isolation procedures. The dinucleotide may be isolated by solvent extraction in some quantity. The trimer must first be made from the dinucleotide and then the additional condensation completed to form the trimer. The trimer must then be isolated by gradient column chromatography on DEAE cellulose. This makes the amount of effort involved practically the same as if one had made a tetramer, i.e. the condensation of two dimers. The yields reported for the addition of trimers show lower yields than for the addition of a dimer to an extending chain. The third problem involves the stoichiometry and efficiency of the reaction which requires the use of a tenfold excess of the added nucleotide.²⁸ This means that the trinucleotide must be produced in quantity. The dinucleotides can be produced on a large scale much more easily than the trinucleotides.

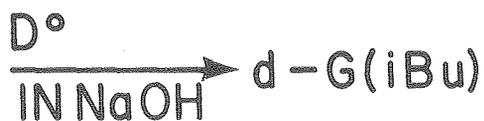
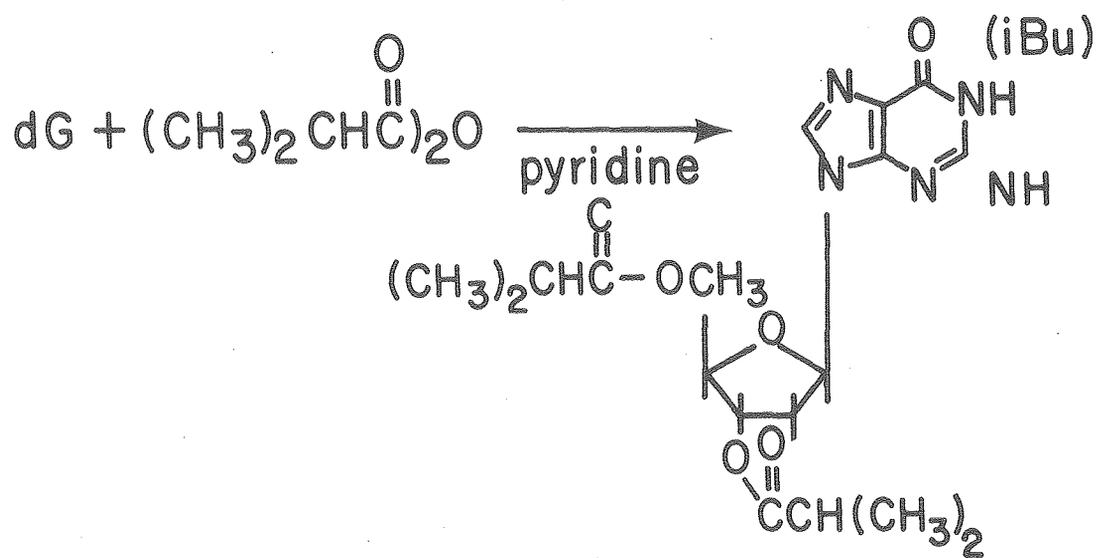
The final reason for choosing the dinucleotide procedure was that

the intermediates would be more useful in the enzyme inhibition study to follow. It was desirable to have a complete set of dinucleotides which conform to the restriction site of the enzyme as well as the tetranucleotide which also fit the site.

DISCUSSION OF THE SYNTHESIS

The protecting groups to be used to modify the various functional groups of the nucleotides were decided primarily from the literature. In the case of d-G, several possibilities were suggested for the deactivation of the exocyclic amino group. The properties of all these alternatives seemed to have some undesirable aspects. The blocking group must be stable to the conditions for removal of the 3'-OH protecting group as well as the 5'-OH or 5'-phosphate protecting group. Amongst the base labile groups, acetyl,⁴⁵ isobutyryl,² and benzoyl⁴⁵ were suggested. The benzoyl group was very stable towards base and the literature showed that acetate might be too easily removed during workup procedures. This left the isobutyryl moiety which could be added with isobutyryl anhydride under mild conditions. This group is still considerably more labile than the groups used on the other two nucleotides and did produce problems during the synthesis of the oligonucleotide. Attempts to acetylate d-G were initially unsuccessful due to the low solubility of d-G in the acetylating medium. It was found that MMTr-d-G (commercially obtainable) was easily acetylated due to its solubility and further attempts were made with d-G. Under conditions of large excess of isobutyryl anhydride and long reaction times, the tri-substituted isobutyryl adduct of d-G could be produced. This was easily converted to the mono substituted N-isobutyryl moiety upon treatment with NaOH. This was in turn converted to the 5'-MMTr derivative by reaction with MMTrCl which reacted exclusively with the free 5'-OH.²³ (Equation 1).

An additional difficulty plagues the synthesis due to the use of d-G as the initial nucleotide. There is an apparent drop in yield

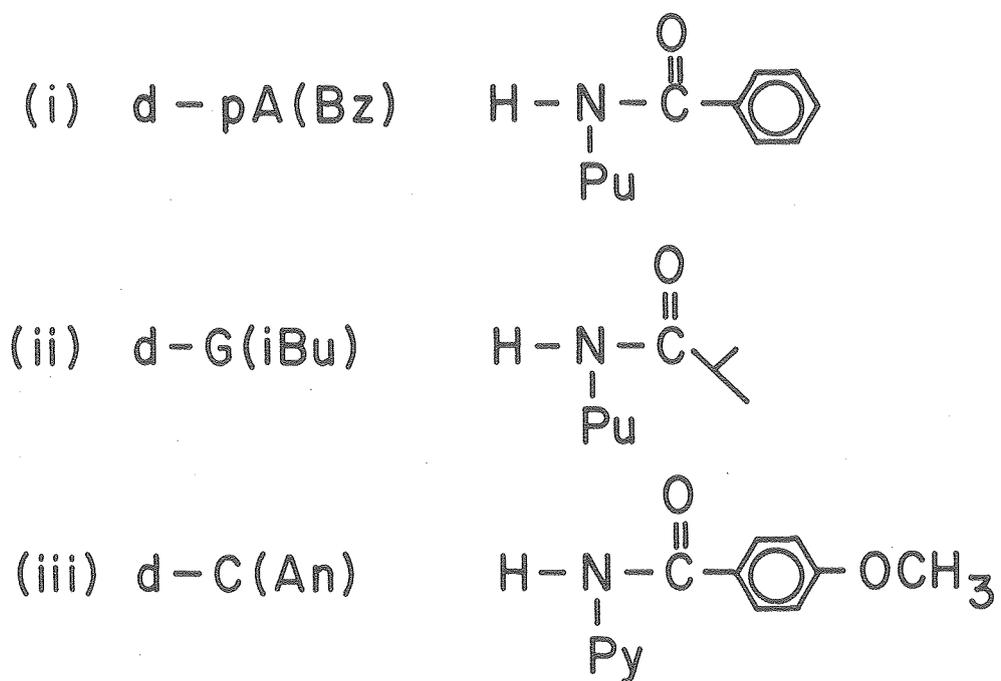


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Equation 1.
Synthesis of d-MMTr-G(iBu).

as one attempts to condense a purine nucleotide with a pyrimidine rather than combining two pyrimidines.¹ This observed decrease is intensified when one wishes to combine two purine residues. With d-G as the initial nucleotide the results are poor with the addition of any nucleotide. Apparently the presence of the MMTr group at the 5'-position brings reported yields to below 20%.¹ With the observed lability of the N-isobutyryl group the ramifications of starting with d-G are felt throughout the synthesis.

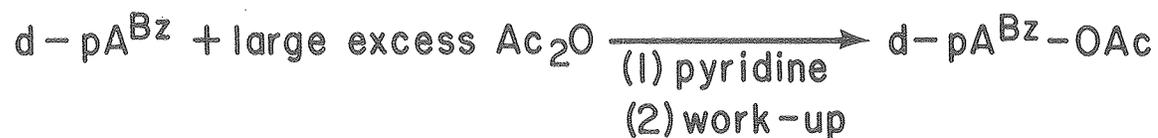
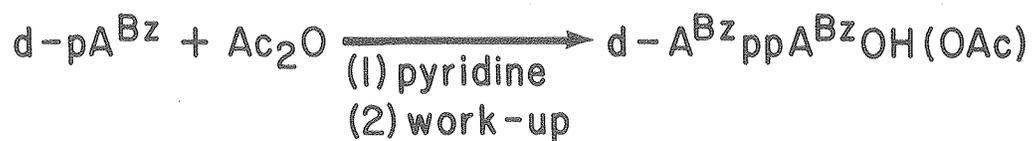
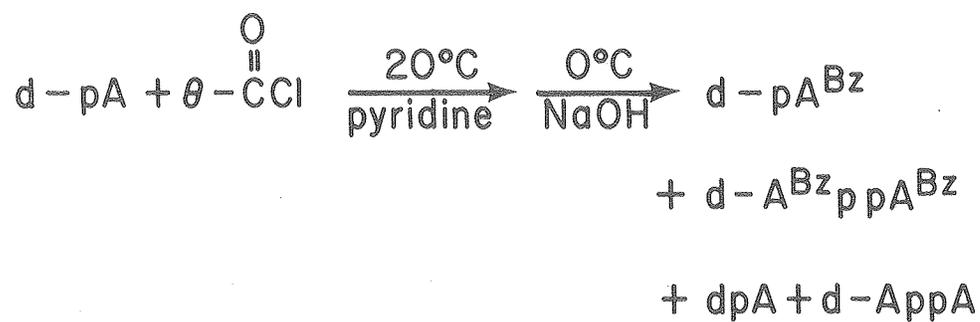
The exocyclic amino groups of d-G, d-p-A, and d-p-C were protected by different protecting groups: iBu, Bz, and An respectively (Fig. 13). The purpose of using different groups is to normalize the conditions of their removal and assure that the groups will have similar stabilities to the reaction conditions. The protection reaction invariably produces some pyrophosphate (except d-G) and the desired disubstituted intermediate which is easily converted to the N-substituted monoadduct (Eq. 2). The formation of the pyrophosphate, a side reaction, can be minimized by the use of a large excess of the acetylating agent.⁴⁶ This is particularly necessary in the protection of the 3'-OH group with acetic anhydride (Eq. 2), otherwise the major product will be the pyrophosphate. The isolation of the desired protected intermediates is done by solvent extraction which yields a relatively clean product. The reactions to form the protected mononucleotides proceeded generally with high yield and could be done in quantity (Table 1). The identity of the compounds was determined by (i) TLC with comparisons with authentic samples, and (ii) deprotection to the parent nucleotide and verification of its identity by TLC.



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Figure 13.

Protecting groups for exocyclic amines.



XBL 781-3761

Equation 2.

Formation of pyrophosphates.

TABLE 1. Protected mononucleotides.

Nucleotide	max	rf
TPM-pT	210, 252	*6.5, ^a 1.3, ^b 2.3 ^c
TPM-d-pA(Bz)	210, 256, 275	*6.0, ^a 1.8, ^b 2.1 ^c
MTr-d-G(iBu)	-	0.71 ^c
d-G(iBu)	259, 290	0.46 ^a
d-pA(Bz)-OAc	280	0.65 ^c
d-pA(Bz)	280	0.43 ^c
pT-OAc	264	0.52 ^c
d-pC(An)-OAc	-	0.73 ^c
d-pC(An)	302	0.32, ^c 0.48 ^a
d-G	253	0.40 ^c
d-pA	259	0.16, ^c 0.28 ^d
pT	267	0.36, ^c 0.14 ^a
d-pC	272	0.18 ^c

*Relative to pT.

^a isopropyl alcohol, NH₄OH, H₂O (7:1:2)

^b n-propyl alcohol, NH₄OH, H₂O (55:10:35)

^c EtOH, 1M NH₄OAc (7:3)

^d n-butyl alcohol, HOAc, H₂O (5:2:3)

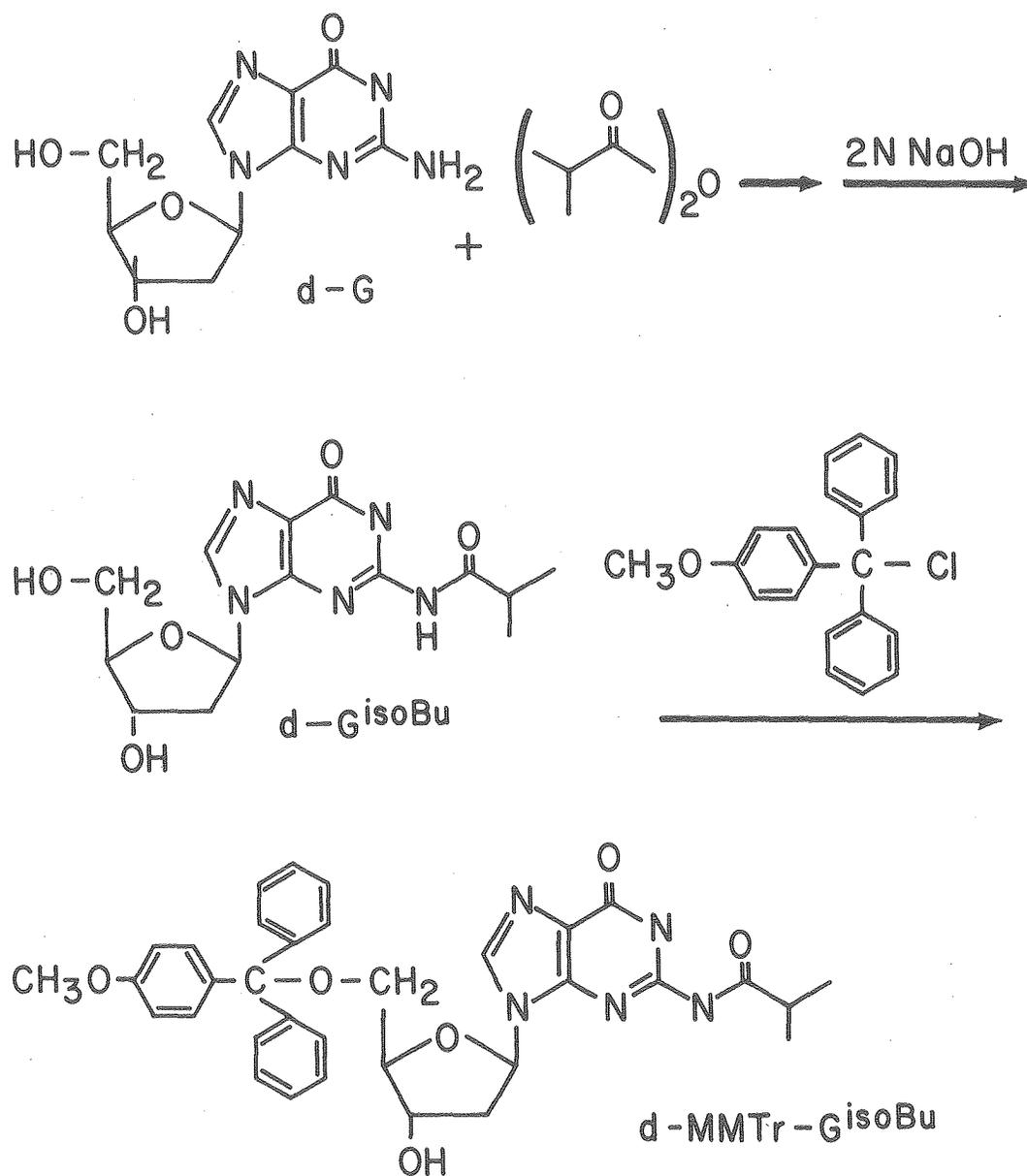
The dinucleotide d-MMTr-G(iBu)pA(Bz)-OH was synthesized [Eq. 3a,b)] and isolated by several different procedures:

- i) solvent extraction and precipitation,
- ii) purification on trityl cellulose column followed by purification by DEAE cellulose column,
- iii) purification by direct application to DEAE cellulose column,
- iv) application to QAE sephadex column,
- v) sizing with G-15 sephadex.

Other column materials such as PEI cellulose and silica gel were found to be unsuitable. The condensation reactions were done with MsCl, TPS, or DCC.

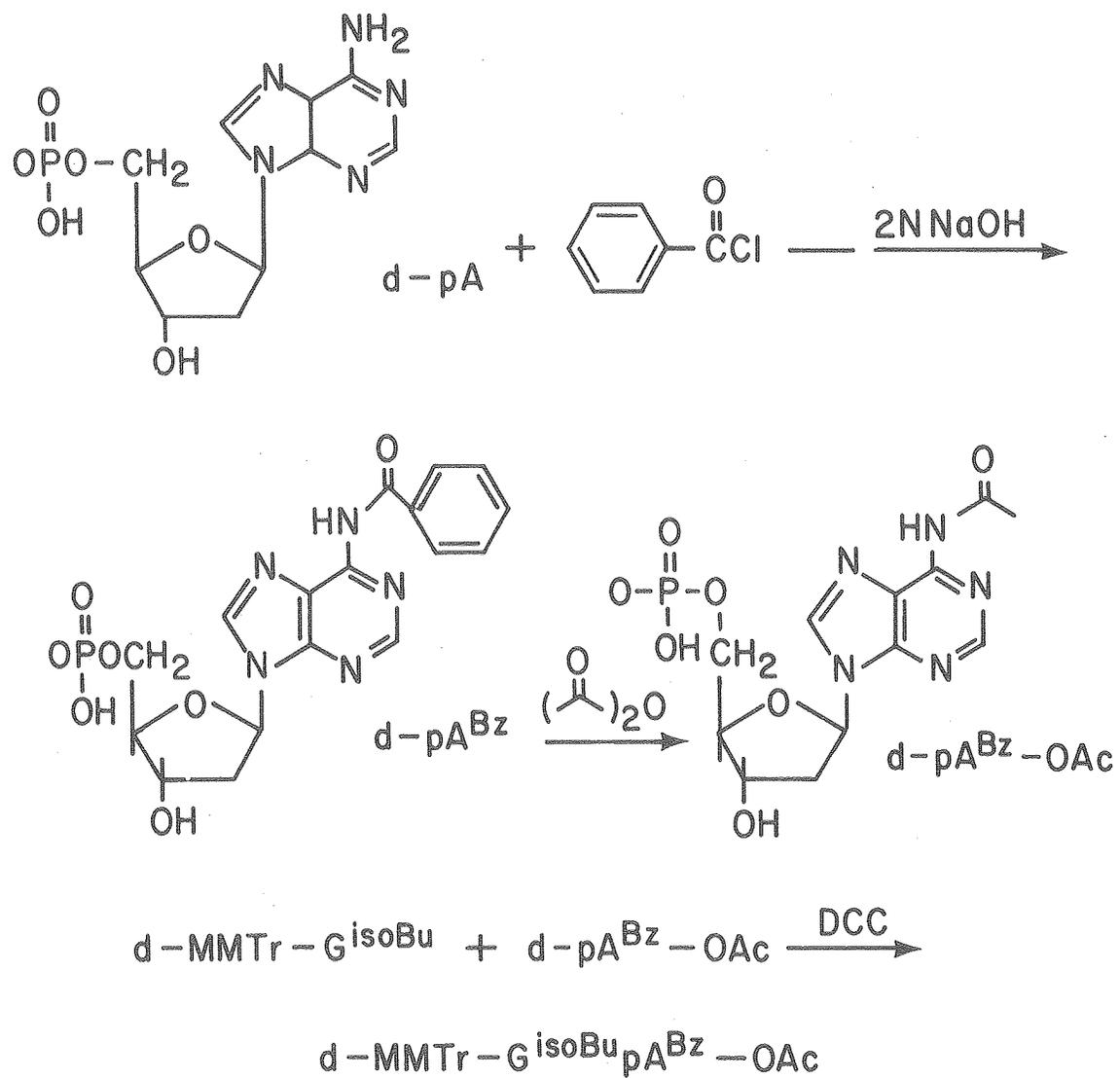
Trityl cellulose procedure. After condensation with either MsCl or TPS, the reaction mixture was treated with base to remove the 3'-OH protecting group. The neutralized mixture was pumped onto a trityl cellulose column in EtOH/0.05M TEAB buffer. Separation was achieved by increasing the EtOH content in a step elution process. The initial concentration of alcohol was either 20% or 40% and was increased to 70% to elute the trityl containing components (Fig. 14,15). A significant portion of trityl containing components were lost when the higher initial concentration of EtOH was used. Much of this was apparently dinucleotidic in character. When the lower concentration was used, however, the condensation agents (mesitylene sulfonic acid or triisopropyl benzene sulfonic acid) were also retained due to their large hydrophobic interactions. This proved to be a problem since they also showed elution properties similar to the desired initial dinucleotide on DEAE cellulose.

THE SYNTHESIS OF d-MMTr-G^{iso}Bu_pA^{Bz}-OAc



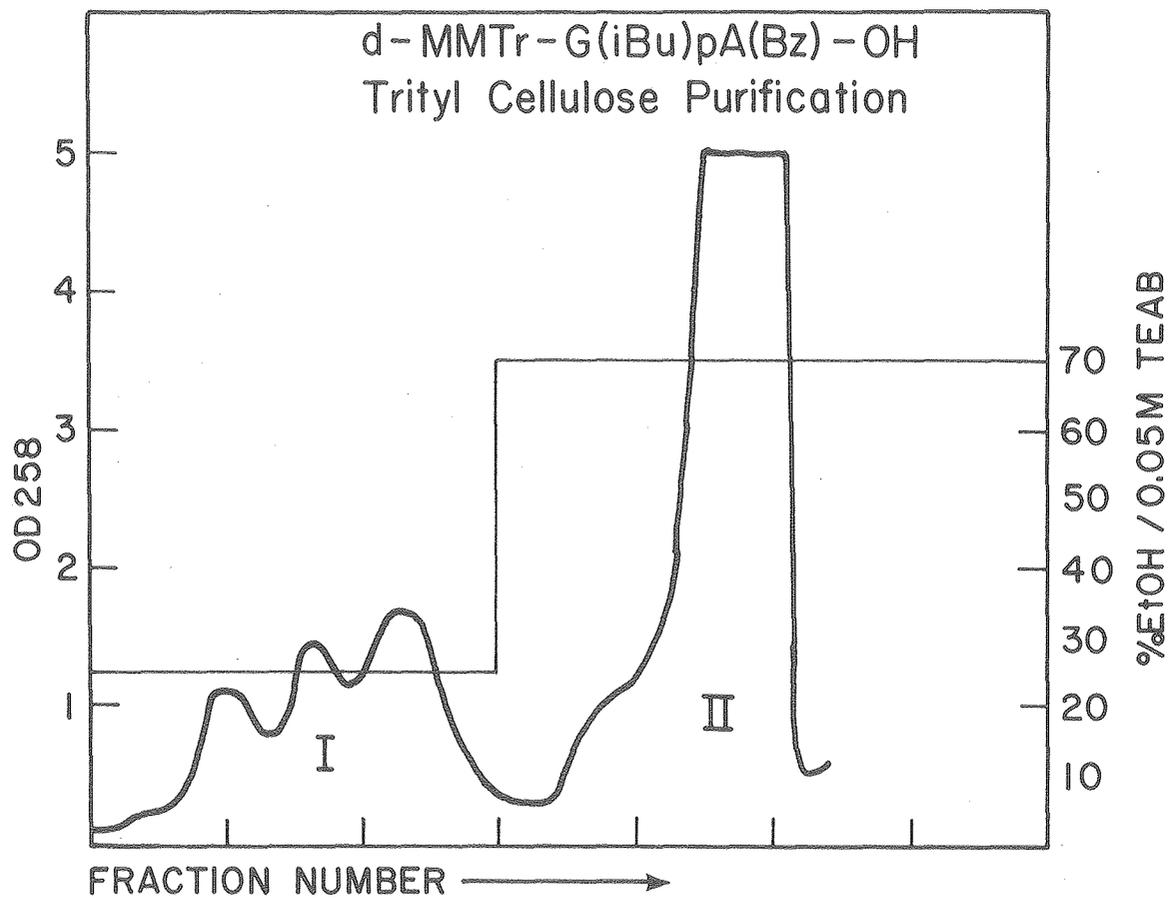
XBL 781-3767

Equation (3a).



XBL781-3766

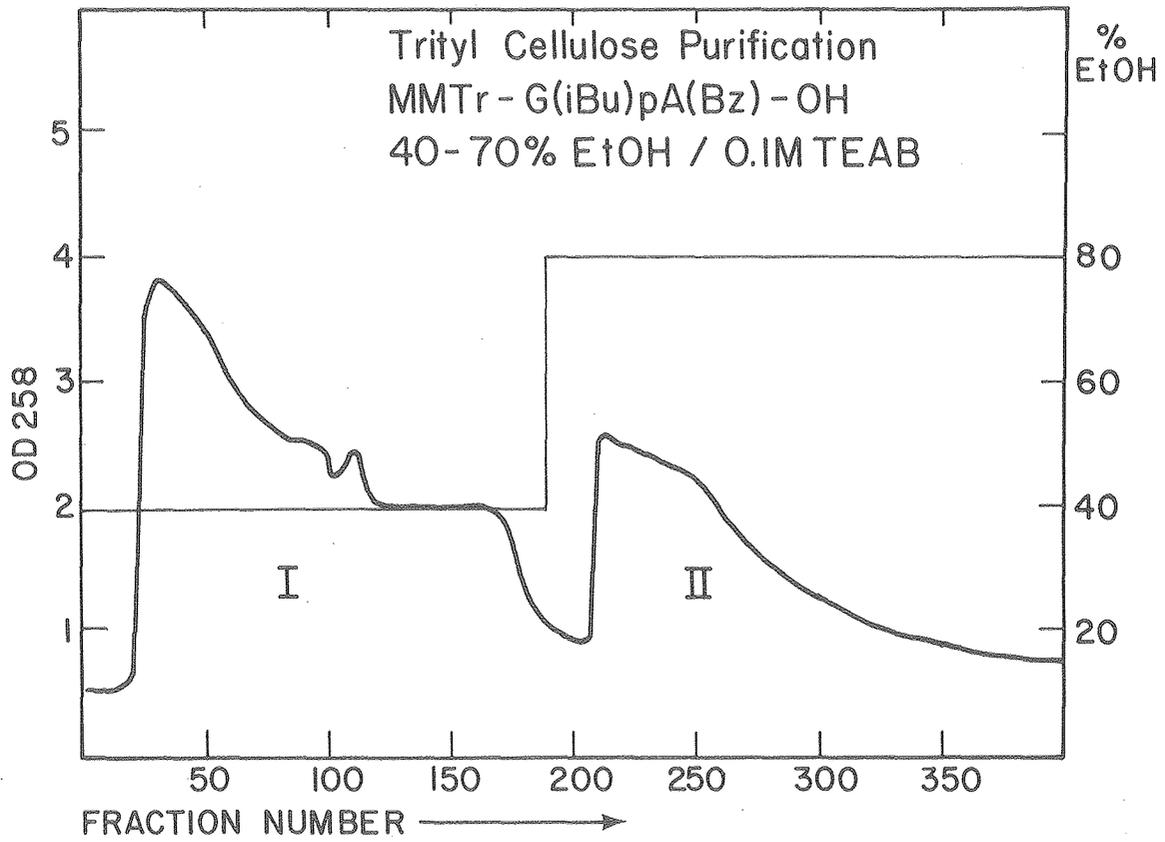
Equation (3b).



XBL 781-3729

Figure 14.

Trityl cellulose purification of d-MMTr-G(iBu)pA(Bz)-OH.



XBL 781-3727

Figure 15.

Trityl cellulose purification of d-MMTr-G(iBu)pA(Bz)-OH.

Separation on DEAE cellulose after the above procedure yielded dinucleotide contaminated with the sulfonic acid. Attempts to increase resolution by using a stronger cellulose ion exchanger such as PEI cellulose were unsuccessful due to the instability of the ion exchange resin in ethanol. Elutants tended to be severely contaminated with column material which was difficult to remove.

QAE Sephadex procedure. The use of a strong ion exchange sephadex seemed to override the gel filtration properties of the resin and provide a good resolution by ionic properties alone. The retention time was quite long for the dinucleotide d-MMTr-G(iBu)pA(Bz)-OH and required a TEAB buffer of high ionic strength to remove the product (Fig. 16). This column material is the material of choice for dinucleotides containing only one phosphate residue but was not used for the higher oligomers as extremely high ionic strength buffers would be necessary.

The dinucleotide was worked up as before with hydrolysis of the 3'-acetate blocking group and the mixture pumped on in high concentration in pyridine rather than dilute buffer which reduces the time that the product is on the column considerably.

Peak III (Fig. 16) contained the desired dinucleotide. The following peak (IV) is not a separate compound, but a mixture of peak V and III. Thus this did contain some of the desired dinucleotide but it also contained increasing amounts of the mononucleotide d-pA(Bz)-OH. There was also a small contamination of the trailing edge of peak III with material from V as indicated by the slight change in the 260/280 μ ratio in the absorbance in that region. The rest of the peak V was

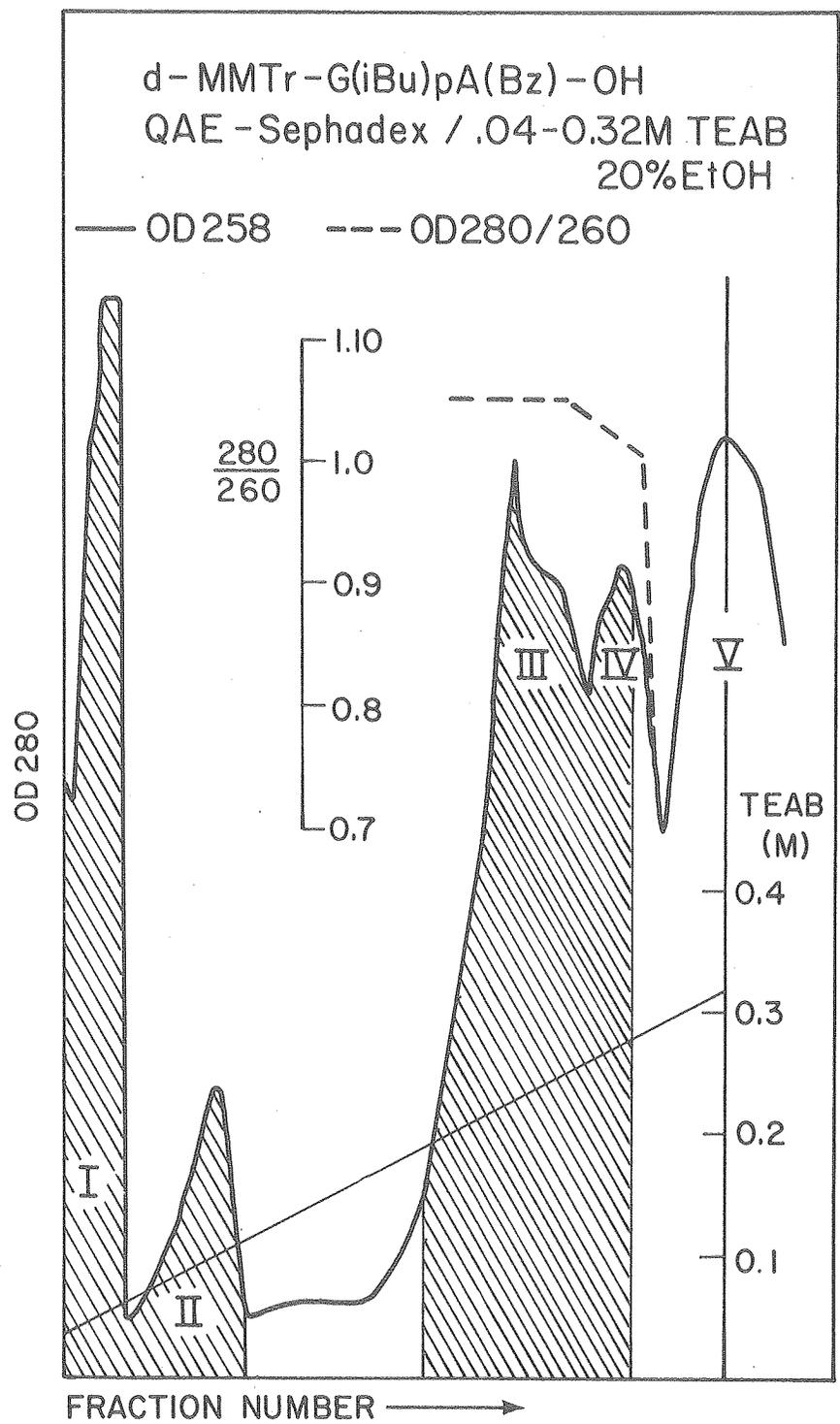


Figure 16.

QAE Sephadex purification of d-MMTr-G(iBu)pA(Bz)-OH.

chromatographically pure by TLC and was stored for use in anhydrous pyridine.

DEA Sephadex ion exchangers were also shown to be unacceptable as the two partitioning properties of the resin (ion exchange and gel filtration) were in opposition to one another. Resolution from non-trityl containing moieties was therefore poorer than with the cellulose derivatives (Fig. 17).

DEA Cellulose procedure. The use of Whatman preswelled ion exchange cellulose tended to alleviate some of the problems that had been previously associated with DEAE cellulose ion exchange mediums. In order to reduce losses associated with extended handling of the reaction products it was decided to place the reaction mixture on the column directly after base work up without utilizing the trityl cellulose procedure. Although this would complicate the separation, it seemed that the potential decrease in degradation would make the procedure desirable. In fact, it provided a very clean separation of the desired initial dinucleotide (peak II, Fig. 18) and was used in the purification of the tetra- and hexa-nucleotide reactions.

Solvent extraction procedure. This procedure²⁷ was tried as it promised rapid isolation of the reaction products and presumably the least loss due to prolonged handling of the mixture. The reaction mixture was partitioned between an aqueous phase and a chloroform (or other solvent) phase before removal of the 3'-OH protecting group so that the solubility difference between the desired product and the other components of the

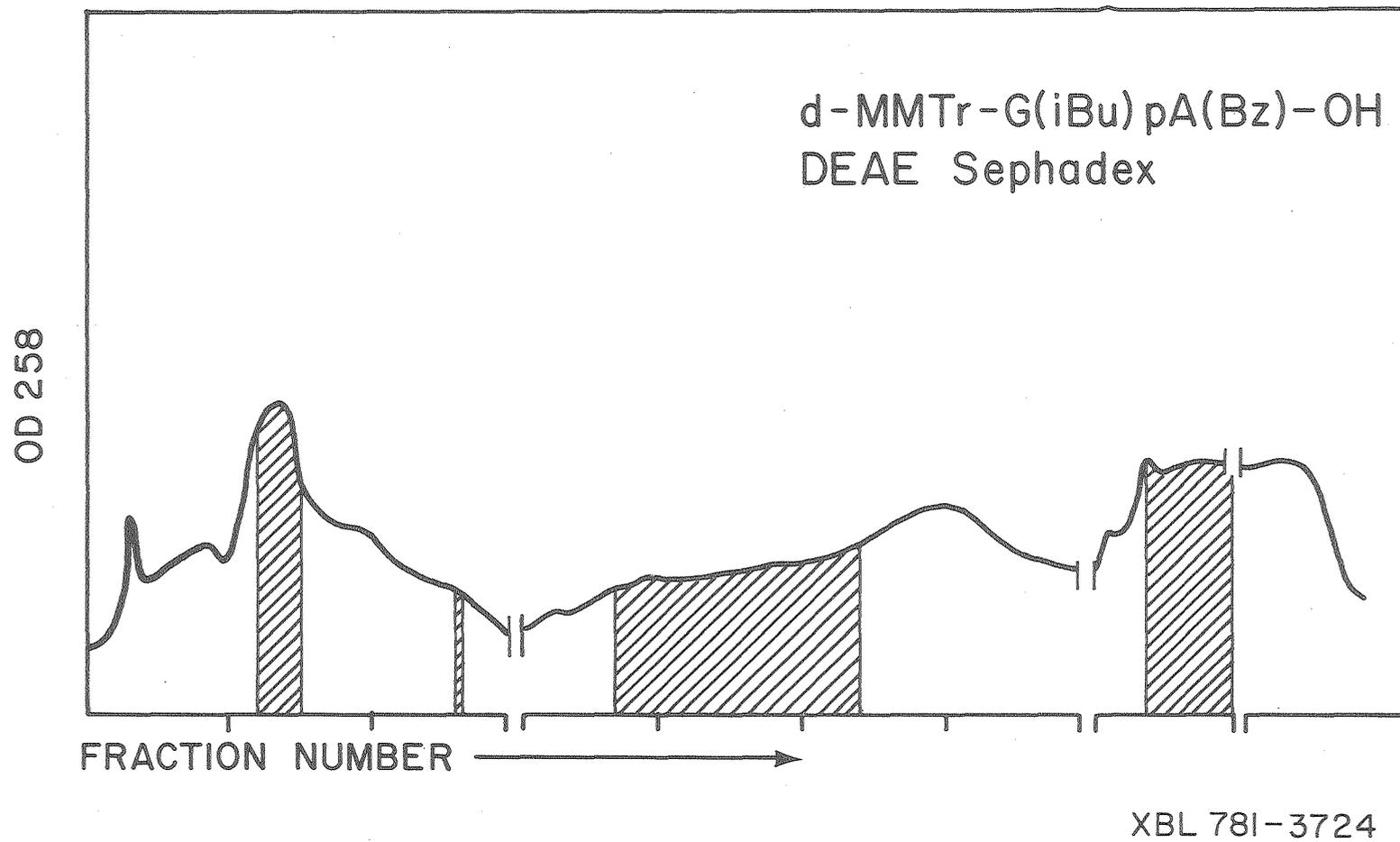


Figure 17. DEAE purification of d-MMTr-G(iBu)pA(Bz)-OH.

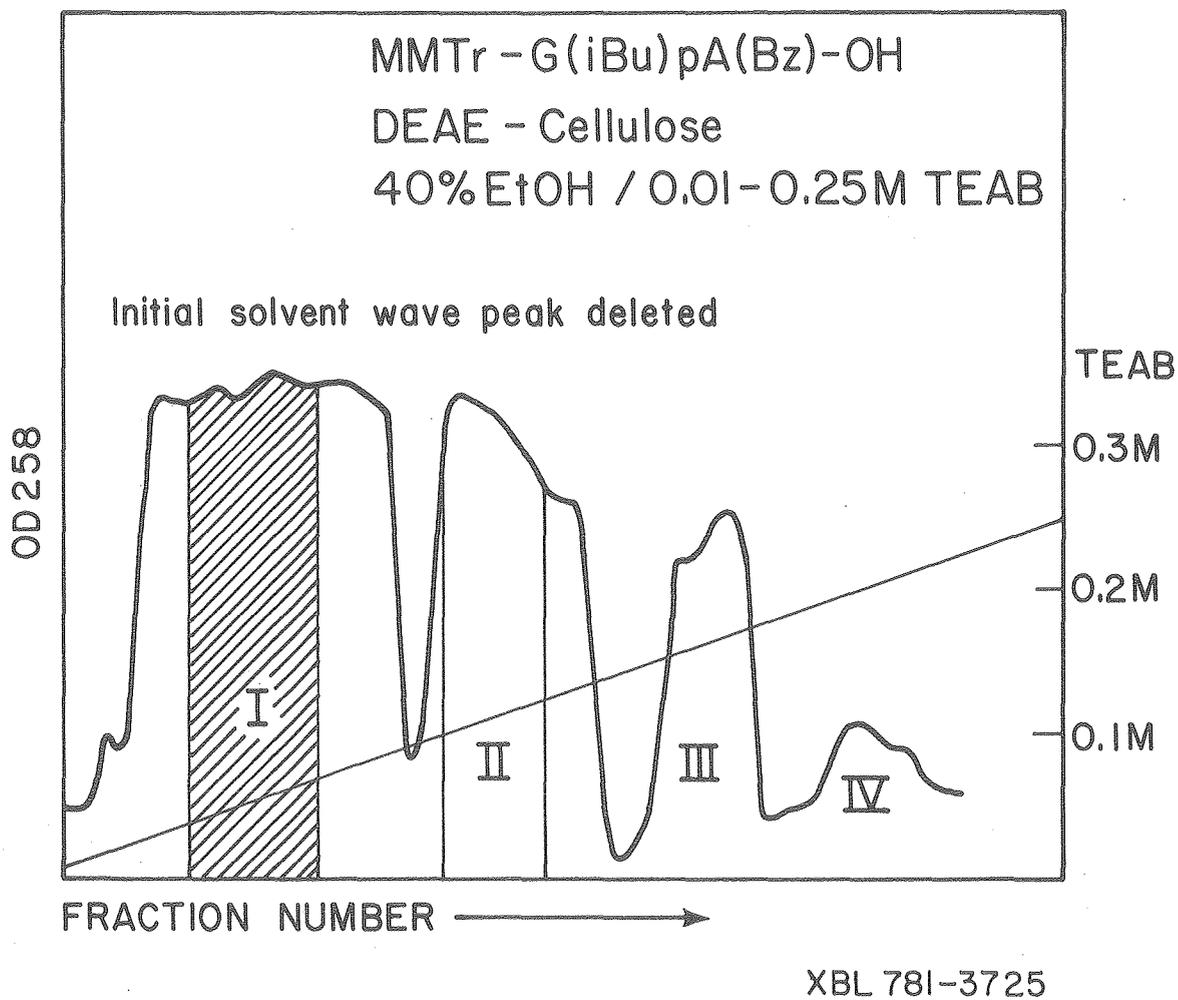


Figure 18.

DEAE cellulose purification of d-MMTr-G(iBu)pA(Bz)-OH.

mixture would be maximized. TLC analysis of the phases consistently revealed a complex mixture of components and a large amount of degradation of the desired product in chloroform. This procedure was abandoned in favor of column chromatography procedure.

Sizing with G-15 Sephadex. It was possible to purify the DEAE cellulose dinucleotide elutants with G-15 sephadex. This eluted the higher molecular weight materials first and the lower molecular weight sulfonic acid later.

The contaminated fraction was evaporated and dissolved in buffer and applied in minimum volume to the column. The resulting peak from the column did not show good resolution but trityl-containing elutants came off together and could be separated from the non-trityl-containing elutants by combining the appropriate fractions. This procedure was not used extensively as it required the products to be on a column for several different time periods, all of which caused degradation of the desired products.

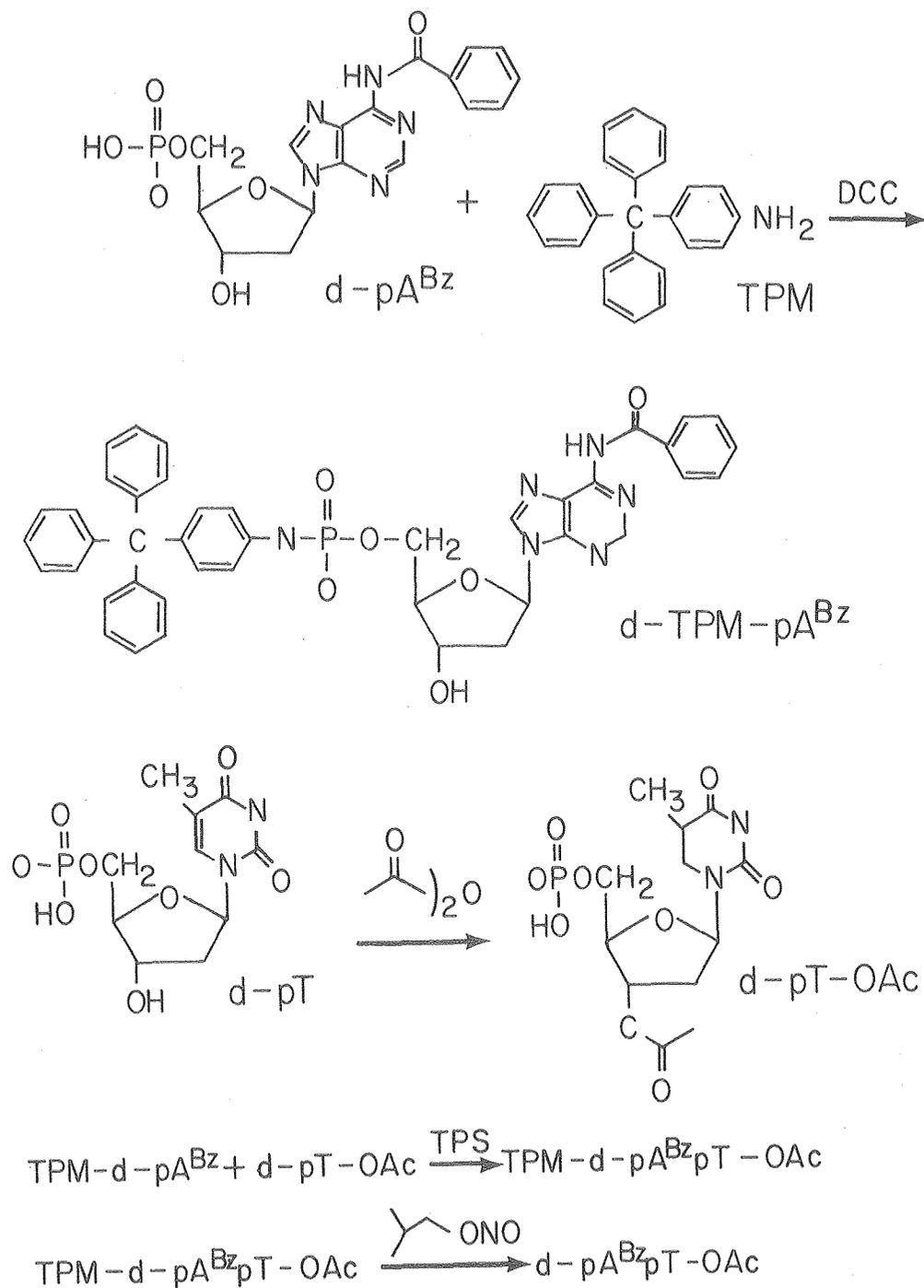
The remaining two protected dinucleotides, pA(Bz)pT-OAc and d-pTpC(An)-OAc, were made through a different process because of the terminal phosphate at the 5' end. A process that allowed isolation of the products by solvent extraction was chosen. The 5'-phosphate was to be protected by the triphenylmethyl anilidate group (TPM).⁴ This group was added to the mononucleotide phosphate residue by condensation of triphenylmethyl chloride with DCC (Eq. 4a,b). The fully protected mononucleotide with a free 3'-OH was then condensed with the appropriate

protected mononucleotide with a free 5'-phosphate.

The condensation of the TPM protected mononucleotides to form the dinucleotide posed certain difficulties. Yields prior to removal of the phosphoramidate protecting group seemed to be reasonable (approximately 60%), but dropped considerably upon isolation of the desired protected dinucleotide after removal of the TPM group. It was initially thought that the deprotection was merely incomplete due to various factors, but prolonged reaction times and variations in the stoichiometry used failed to improve yields. Since deprotection of the mononucleotides had been shown to be quantitative,⁴ something had to be different for the dinucleotide case. Apparently another reaction involving the phosphoramidate moiety was taking place with the condensation agent triisopropylbenzenesulfonyl chloride (TPS). It had been shown that if the TPM protected dinucleotide is formed utilizing MsCl as the condensation agent rather than TPS, a considerable amount of side reaction occurs⁴ (Eq. 5). This side reaction was reported to be negligible with the use of TPS.⁴ This is apparently in error, at least for the experiments carried out in this laboratory. Since the sulfonated intermediate (Eq. 5) would not be expected to undergo the deprotection reaction it would definitely account for the drop in yield experienced during deprotection.

Indeed, recent publications have suggested alternative lipophilic agents for the protection of the 5'-phosphate group which do not undergo undesirable side reactions.⁴³ The TPM group, however, does allow the synthesis of dinucleotides although the yields are much lower than previously reported, as these yields are based on the protected dinucleotide before the deprotection reaction is undertaken.

THE SYNTHESIS OF d-pA^{Bz}pT-OAc

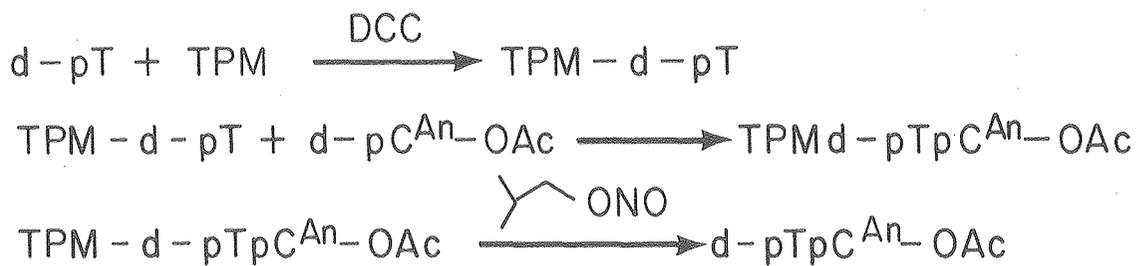
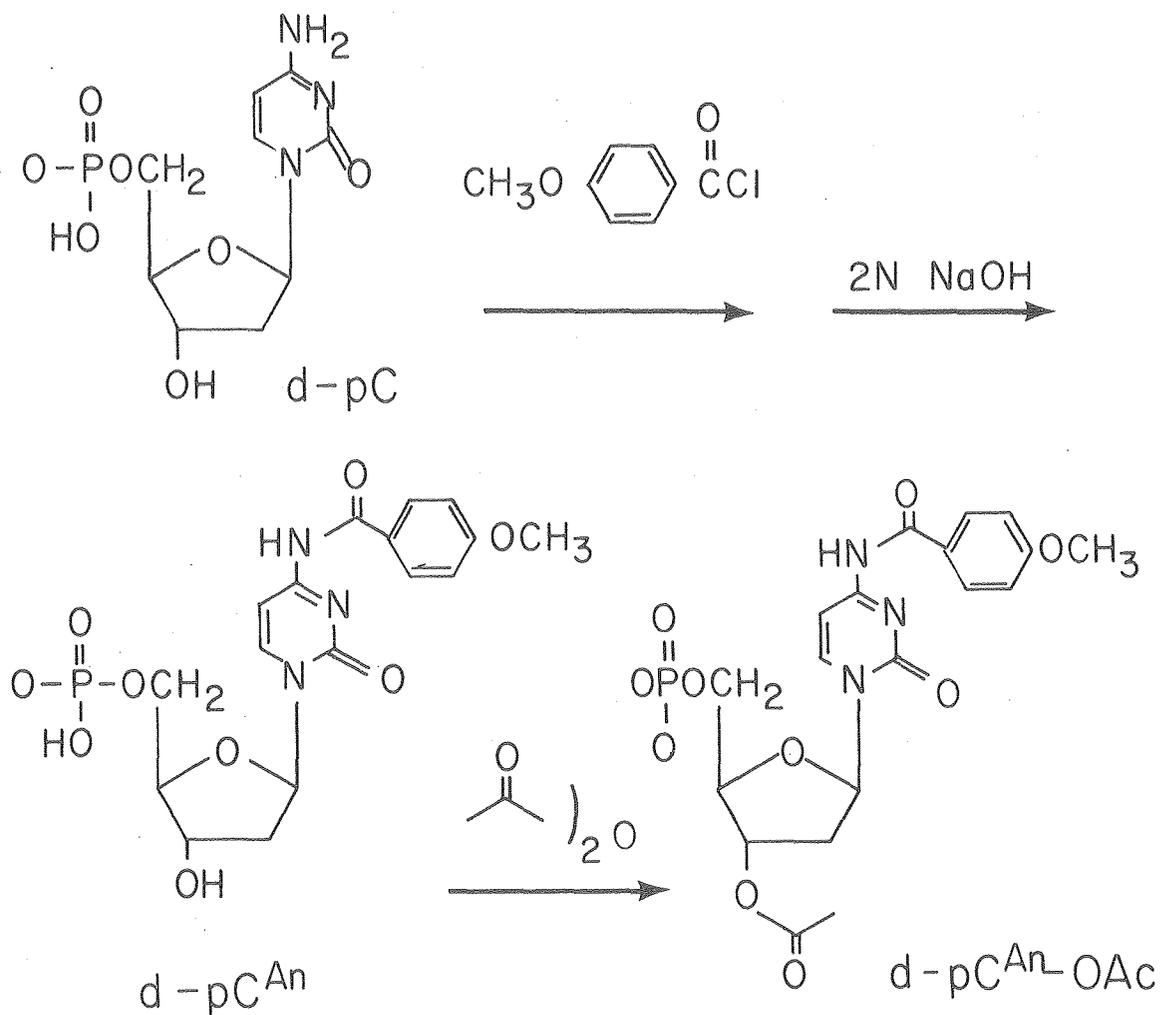


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Equation 4a.

Synthesis of d-pA^{Bz}pT-OAc.

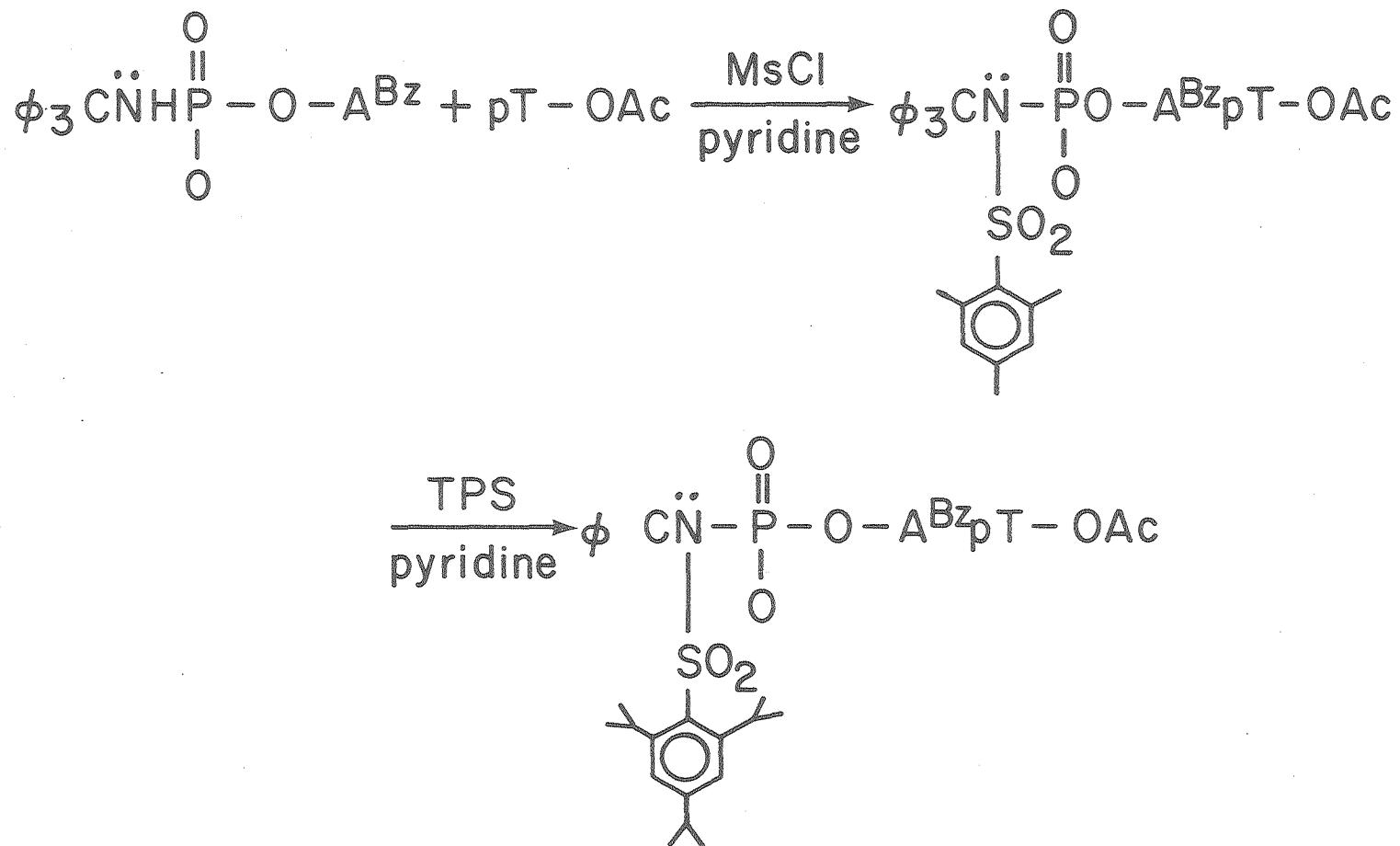
THE SYNTHESIS OF d-pTpC^{An}-OAc



XBL 786 - 4000

Equation 4b.

Synthesis of d-pTpC(An)-OAc.



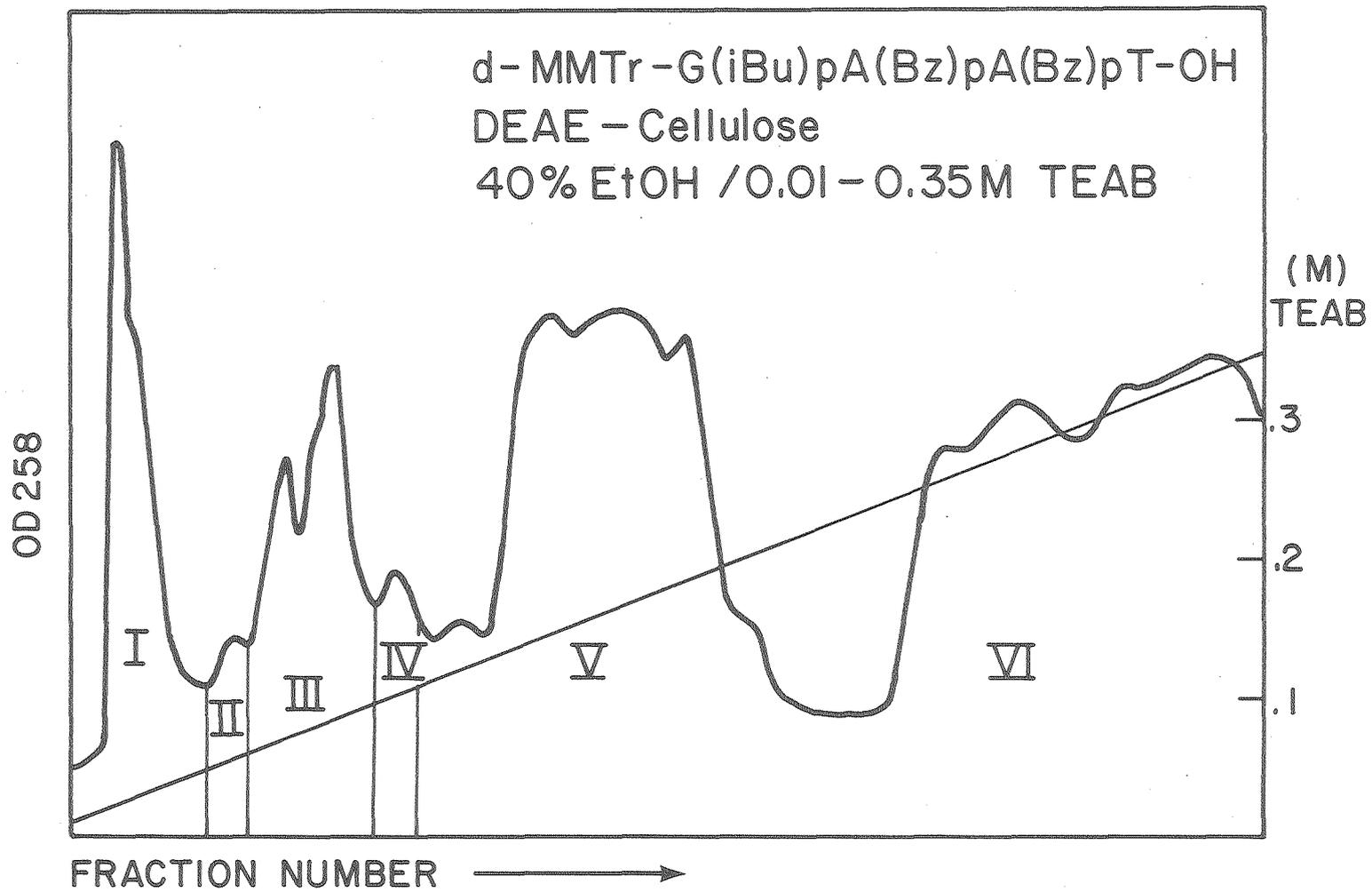
Equation 5. Formation of adducts with condensation agents.

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Preparation of the tetra nucleotide. Both MsCl and TPS were used in preparations of the tetra nucleotide MMTr-G(iBu)pA(Bz)pA(Bz)pT. This reaction required large molar excesses of the protected dinucleotide pA(Bz)pT-OAc on the order of 10 or 20 to 1.⁴¹ This made the reaction suitable only for the production of small quantities at any one time and increased the problems with isolation of the products.

The major preparation was done using MsCl. Column chromatography of the reaction mixture is shown in Fig. 19. The peaks containing MMTr adducts were identified initially by placing a few drops of column elutant in concentrated perchloric acid and observing the formation of a yellow color. This color is due to the formation of the MMTr ion which is stable under these conditions. The method may be used quantitatively³⁴ if absorption is measured at 380 nm.

The structure proof for the tetranucleotide involved initial deprotection in NH_4OH followed by treatment with acetic acid/pyridine to remove the MMTr group (Table 2). The totally deprotected tetranucleotide was treated (i) with venom phosphodiesterase⁴⁵ and (ii) with spleen phosphodiesterase.⁴⁵ The two sets of nucleoside, nucleotide mixtures were separated by TLC and the spots eluted with distilled water and absorbance determined for each spot (Table 2). The remainder of the deprotected material was retained for later studies. The results of the enzymolysis agreed reasonably well with the theoretical distribution of nucleotides expected from the tetranucleotide. The region about peak IV (Fig. 19) contained the desired tetranucleotide. The unreacted dinucleotide, d-pA(Bz)pT-OAc, and degradation products could be found in region V and the pyrophosphates in VI along with the higher polymers.



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Figure 19. Isolation of tetramer.

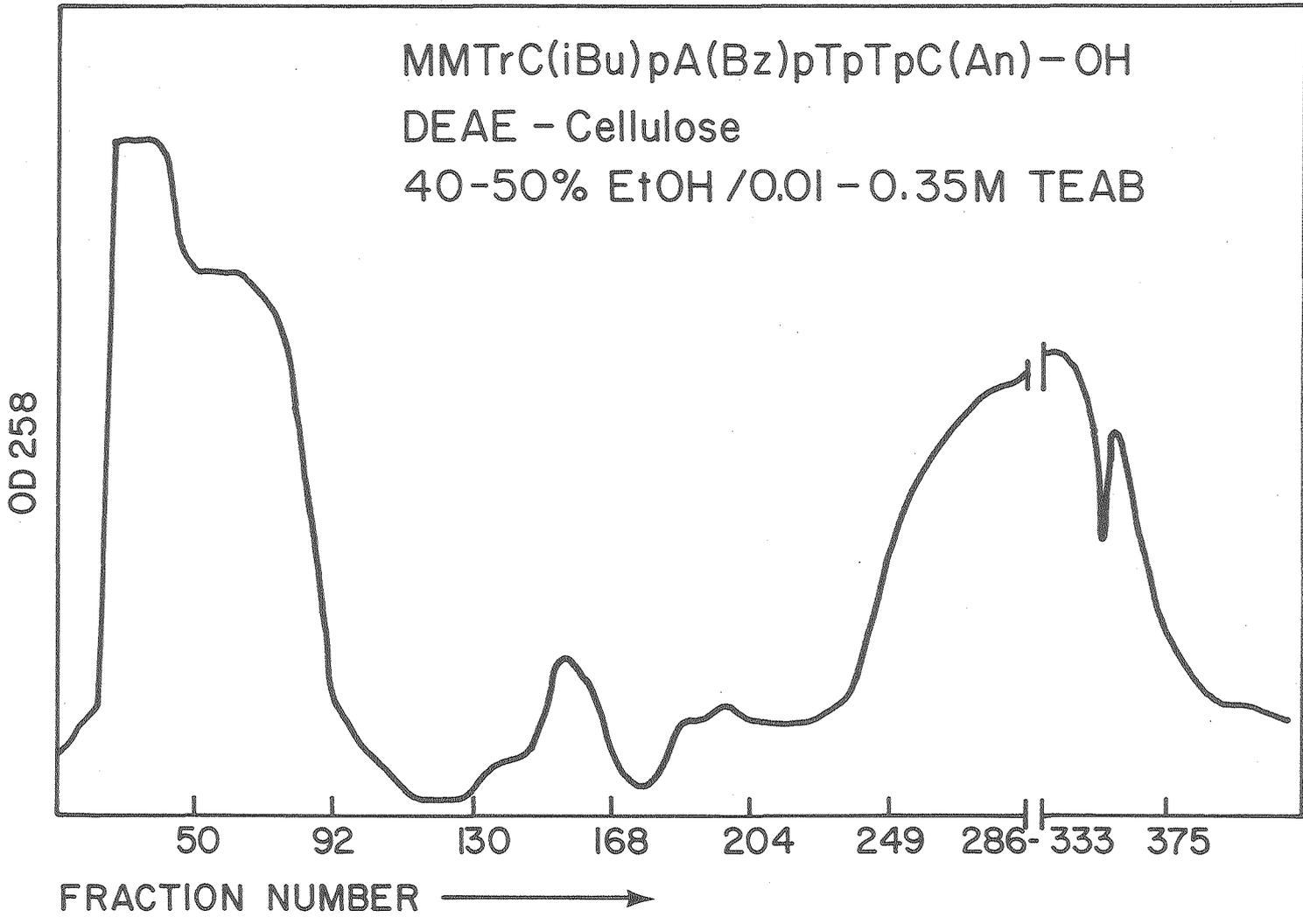
TABLE 2. Deprotection and enzymolysis of d-GAAT.

<i>Compound</i>	$E_{280/260}$	<i>rf</i>
MMTr-G(iBu)pA(Bz)pA(Bz)pT	1.21	0.7(B)
MMTr-GpApApT	0.48	0.68(C), 0.15(A)
d-GpApApT	0.45	0.28(C)

<i>Enzymatic Degradation</i>			
<i>Residue</i>	<i>Theoretical</i>	<i>Found</i>	$E_{260} \times 10^{-3}$
d-Gp	1	0.9	1.7
d-Ap	2	2	15.4
T	1	0.8	9.6
d-G	1	0.7	1.7
d-pA	2	1.8	15.4
pT	1	1	9.6

Synthesis of the hexanucleotide d-GpApApTpTpC. The small quantities of protected tetranucleotide available made it necessary to use the entire stock of intermediates in one reaction rather than doing an exploratory run to determine reaction conditions and purification procedures. It was decided to purify the hexanucleotide by applying it directly to an DEAE cellulose column after reaction and before basic workup. This would presumably lead to the least loss of product due to handling. It would, however, complicate the isolation procedure by introducing several products into the separation procedure that would not have been there after deprotection. These were the hexanucleotide in various stages of deprotection such as loss of OAc, iBu, MMtr, etc. These species, due to their low concentration, could easily be lost in the separation procedure.

A variety of fractions (Fig. 20) were found to show trityl positive reactions when tested with perchloric acid. Fractions in the range of 170 to 210 were combined and reduced in volume. Analysis of the mixture by TLC showed a mixture of many components. The entire mixture was deprotected in ammonium hydroxide to remove all base labile protecting groups. The resulting mixture was shown by chromatography to contain only one major trityl positive spot. The mixture was purified by preparative thin-layer chromatography on Whatman cellulose. The MMtr group was removed in HOAc/pyridine (48 hrs). The deprotection mixture was shown to contain only one nucleotidic fraction. This fraction was isolated by preparative thin-layer chromatography on cellulose. The result was a homogeneous nucleotidic material. In retrospect this procedure of thin-layer preparative chromatography to purify the various



XBL 781-3726

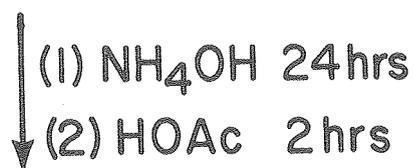
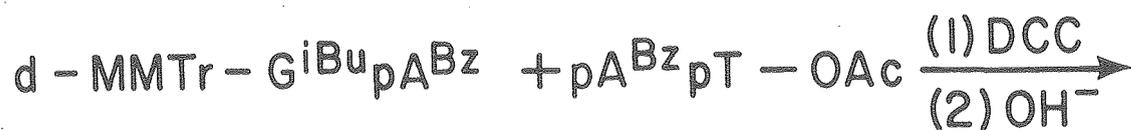
Figure 20. DEAE cellulose purification of hexanucleotide.

intermediates could have been used to great advantage in the earlier stages of the synthesis. It is conceivable that much of the column chromatography could either be replaced by preparative thin layer or a minimum of additional purification could have been carried out; the major advantage being the very short time that the intermediates would be subjected to potential hydrolysis on the chromatography medium.

The yield of the hexanucleotide was quite low overall and only one attempt was made to enzymatically degrade the product to verify its structure. The results of the degradation and subsequent chromatography (Table 2) clearly show the presence of the required nucleotides and nucleoside. The deviation of the ratios of the bases to the theoretical ratios is most likely due to the extremely small amount of hexanucleotide available for degradation. This is complicated by the great solubility difference between the nucleosides and nucleotides in H_2O or buffer systems as well as the affinity of nucleotides for cellulose and, of course, the large differences in extinction coefficients. These problems combined with the problems of handling very small quantities of nucleotidic materials under sterile conditions make the enzymatic approach to structure proof quite difficult.

Clearly there is much room for improvement in the chemical synthesis of polynucleotides. The thrust of this research was to produce the required hexanucleotide and utilize this substrate in the following restriction enzyme study. This forced the direction of the study away from the area of new methods of polynucleotide synthesis.

It is evident that a number of research areas would quickly yield large gains in the case of producing polynucleotides of defined sequence.



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Figure 21.

Synthetic scheme for syntheses of hexanucleotide.

One would be the use of a protecting group for the exocyclic amine which is consistent for the various bases and is not affected by the conditions of removal for the group protecting the 5'-OH. Possibilities include groups which are substrates for known enzyme systems, groups which are labile through photolysis, and groups labile to free radical processes.

More efficient methods of synthesizing phosphodiesteres would greatly simplify the procedures involved by reducing the amounts of unreacted materials in the reaction mixture. The obvious extension of this would be enzymatic procedures which might remove the need for some protecting groups entirely.

EXPERIMENTAL

General Methods

Thin-layer chromatography analysis was carried out on Kodak cellulose plates with fluorescence indicator. The solvents^{27,33,50} used routinely were: isopropyl alcohol-concentrated ammonia-water (7:1:2) [solvent A]; n-propyl alcohol-concentrated ammonia-water (55:10:35) [solvent B]; ethyl alcohol-1M ammonium acetate (7:3) [solvent C]; n-butyl alcohol-acetic acid-water (5:2:3) [solvent D]. The *rf*'s of the various protected mononucleotides are listed in Table 1 (pg.29).

All condensation and derivatization reactions were carried out in anhydrous pyridine under dry nitrogen in a positive pressure dry box. The lyophilized products were stored either as anhydrous powders under nitrogen at -20°C or in anhydrous pyridine at -20°C.

Mononucleotides were purchased from Sigma, analyzed by TLC and used without further purification. Small samples of available protected mononucleotides were purchased from Collaborative Laboratories and used for chromatographic and spectroscopic references. DCC and TPS (Aldrich) were used without further purification; p-tritylaniline was recrystallized from toluene (m.p. 252.5 - 253.5); acetic anhydride, benzoil chloride, anisoyl chloride, and isobutyric anhydride (Aldrich) were all distilled before use; pyridine was dried by distillation from KOH, redistilled fractionally and stored over molecular sieves in darkness.^{2,27,28,41}

The unprotected mononucleotides were converted to the pyridinium form before subsequent reaction. This was accomplished by placing an aqueous solution (1-2 mmole, 2ml) on a pyridinium ion exchange column. The elutant was lyophilized to a light yellow powder. The pyridinium

resin was prepared by passing an aqueous pyridine solution (20%) through the resin (Biorad H⁺ exchange resin 50W, 20-50 mesh) and by washing the column (2 × 30 cm) with distilled water until the column was used (1-2 hr).

A small amount of MMTr-G(iBu) was synthesized in this laboratory but the major amount was commercially obtained from Collaborative Laboratories.

General Conditions for Phosphodiesterase Treatment

Venom Phosphodiesterase.

Compounds to be digested were first totally deprotected and then lyophilized from aqueous solution (10 O.D. units). The lyophilized material was then dissolved in stock buffer (1M NH₄OAc, 10 ul, pH 4.5; 0.3M Mg OAc₂, pH 8.8; 1M tris-acetate, pH 8; H₂O; mixed 10:3:10:6). The commercially obtained enzyme (Worthington) was diluted from stock vial to H₂O (1 ml) and again into H₂O (0.1 ml to 10 ml). The diluted enzyme (40 ul) was added to the nucleotidic material (total volume 50 ul). Complete digestion required approximately six hours at 37°C. Resolution of mononucleotide 5'-phosphates was achieved on cellulose TLC plates with solvent A (isobutyric acid, conc. NH₄OH, H₂O; 66:1:33). The rf values given are with reference to pT used as a standard for most chromatography.

Spleen Phosphodiesterase.

Nucleotidic material was first totally deprotected and then lyophilized from aqueous solution in a small tube. The lyophilized powder was dissolved in stock buffer (1M NH₄OAc, pH 6.5, 20 ul) and

H₂O (50 ul). The enzyme stock (40 ul) was added and reaction allowed to proceed at 37°C for more than 5 hrs. Stock enzyme was prepared from Worthington spleen phosphodiesterase diluted with H₂O (to 1 ml) and diluted again in H₂O (0.2 ml to 1 ml). Resolution of mononucleotide 3'-phosphates was achieved on cellulose TLC plates in solvent B (EtOH, 1M NH₄OH; 7:3) or solvent C (isopropyl alcohol, conc. NH₄OH, H₂O; 7:1:2).

d-G(iBu)-OH.

Deoxyguanosine (0.5 g) was dried under vacuum and dissolved in isobutyric anhydride (10 ml) and anhydrous pyridine (20 ml) and allowed to react for 20 hrs at 50°C under dry N₂. The reaction was quenched in ice and extracted with CHCl₃ (3 × 30 ml). The CHCl₃ fractions were evaporated to a gum and the gum taken up in EtOH (85%) at reflux and recrystallized at 10°C. The washed crystals were dissolved in pyridine and treated with 1N NaOH at 0°C for 10 minutes and the reaction quenched with the addition of pyridinium ion exchange resin. The pyridine solution was lyophilized to yield a white powder (0.12 g). A small sample of d-G(iBu) was hydrolyzed in 1N NH₄OH to yield d-G as indicated by the chromatography.

N-Benzoyldeoxyadenosine-5'-monophosphate (d-pA^{Bz}).⁴⁶

Finely divided pyridinium deoxyadenosine-5'-phosphate (1 mmole) prepared by lyophilization of an aqueous solution was dissolved in a solution of dry pyridine (20 ml) and benzoyl chloride (2.5 ml, 20 mmole) under dry nitrogen. The orange mixture was reacted in the dark with occasional stirring for 1 hr. The reaction was stopped by pouring the

mixture into a separatory funnel containing chloroform (50 ml) and water (50 ml) chilled in an ice bath. The aqueous layer was extracted with chloroform (2 × 30 ml). The chloroform layers were combined and evaporated to a syrup at 20 C under vacuum and dissolved in a cold mixture of pyridine (20 ml) and water (10 ml). To the solution was added 2N sodium hydroxide (30 ml) in an ice bath. The reaction was allowed to proceed for 9 min when excess pyridinium ion exchange resin was added to neutralize the basic solution. The liquid and washings were placed on a chilled (15°C) pyridinium ion exchange column (2 × 30 cm) and eluted with distilled water. The yellow column elutant was rotary evaporated (20°C) to a volume of 20 ml. Benzoic acid, precipitated during the evaporation, was filtered out and the filtrate was extracted with ether (2 × 30 ml). The aqueous layer was lyophilized to a yellow powder (0.385 g).

Chromatography of the product in solvent C showed the absence of either benzoic acid or unreacted deoxyadenosine monophosphate. The product was homogeneous and identical with an authentic sample of N-benzoyldeoxyadenosine-5'-monophosphate.

Hydrolysis of a small sample of synthesized N-benzoyldeoxyadenosine-monophosphate in concentrated ammonium hydroxide yielded a product identical chromatographically to deoxyadenosine-5'-phosphate.

N-Benzoyl-3'-O-acetyldeoxyadenosine-5'-monophosphate (d-pA^{Bz}-OAc). ³¹

Pyridinium N-benzoyldeoxyadenosine-5'-monophosphate (1 mmole) was rendered anhydrous by repeated evaporation of dry pyridine solution. The resulting gum was dissolved in anhydrous pyridine and acetic anhydride (3 ml) added. A clear solution resulted which was allowed to react for

4 hrs in the dark. Methanol (7 ml) was added and allowed to react for an additional 4 hrs. The resulting solution was evaporated to 4 ml. Ether (50 ml) and water (2.5 ml) were added. The aqueous layer was extracted with ether (2 × 20 ml) diluted with water (200 ml) and lyophilized.

The product was homogeneous chromatographically in solvent "C". Hydrolysis in 2 N sodium hydroxide yielded N-benzoyldeoxyadenosine-5'-phosphate and some d-pA.

N⁶-Benzoyldeoxyadenosine-5'-phosphoro-(p-triphenylmethyl)-anilidate
(TPM-p-dA^{Bz})⁴

Anhydrous d-pA^{Bz} (1 mmole) was dissolved in a solution of DCC (1 g) in anhydrous pyridine (6 ml). The resulting mixture was allowed to incubate 10 min whereupon p-tritylaniline (2 mmole) in pyridine (10 ml) was added. The total volume was reduced to 6 ml and the fluorescent pink liquid was allowed to stand in the dark under dry nitrogen overnight. Water (3 ml) was added and after 6 hrs at ambient temperature the mixture was taken up in ethyl acetate. After filtration the ethyl acetate was washed with 0.2 M TEAB, and concentrated to 5 ml. The concentrate was dropped into pet. ether (200 ml) and isolated by centrifugation at low temperature.

The product was dissolved in pyridine (2 ml) and a portion (100 ul) was treated with conc. NH₄OH/MeOH (1:1) to remove the base labile protecting group (Bz). The solution was evaporated with the addition of pyridine and lyophilized. The dried material was shown to be homogeneous by TLC (Table 2). The product was dissolved in pyridine (0.5 ml) and acetic

acid (0.5 ml). Isoamyl nitrite (20 ul) was added and the mixture allowed to sit at room temperature for two hours. The solvent was removed by repeated evaporation with addition of pyridine and the residue dissolved in chloroform (5 ml). The chloroform solution was extracted with 0.1 M TEAB (3 1 ml). TLC of the aqueous solution revealed one spot (rf 0.28 B) identical with authentic d-pA. Yield of d-TPM-pA(Bz) was 13,000 OD 267 units or 85% based on d-pA.

3'-O-Acetylthymidine-5'-phosphate (d-pT-OAc) ³²

Pyridinium thymidine-5'-monophosphate (d-pT) (0.5 g, 1.7 mmole) was dissolved in a solution of anhydrous pyridine (16 ml) and acetic anhydride (5 ml). After 18 hrs in darkness under dry nitrogen, water (50 ml) was added in an ice bath. About 2 hrs was necessary to assure that the anhydride was completely reacted, at which time the mixture was evaporated to a syrup. Water (50 ml) was again added and the mixture evaporated to a syrup to remove pyridinium acetate. The final gum was diluted with water (200 ml) and lyophilized. Yield was 0.5055 g. Chromatography and hydrolysis showed the product to be homogeneous d-pT-OAc.

Thymidine-5'-phosphoro-(p-triphenylmethyl)-anilidate (TPM-p-dT) ⁴

Dicyclohexylcarbodiimide (DCC) (4 g) in pyridine (20 ml) was added to dry pyridinium d-pT (1 g, 2 mmole). The mixture was allowed to react at room temperature for 5 min before p-tritylaniline (1.35 g) in pyridine (16 ml) was added and the total volume reduced to 20 ml. The reaction continued overnight, after which water (10 ml) was added. Hydrolysis was allowed to continue overnight; then DCU was filtered out of the

reaction mixture and the solid washed with 50% aqueous pyridine (2 × 40 ml). The filtrate and washings were evaporated to a gum and taken up in CHCl_3 (100 ml). The CHCl_3 was washed with 0.2 M TEAB (2 × 40 ml). The CHCl_3 layer was concentrated and triturated with toluene-pet. ether (1:1, 20 ml). The resulting precipitate was washed with the same solvent and dried under vacuum.

A portion of the synthesized d-T M-pT (10 mg) was dissolved in pyridine (0.5 ml) and glacial acetic acid (0.5 ml) added followed by distilled isoamyl nitrite (20 μl). The reaction was allowed to proceed for two hours at room temperature. The solvent was removed by repeated evaporation with addition of pyridine at room temperature under reduced pressure. The gum was taken up in chloroform and extracted with TEAB (0.1 M, 3 × 1 ml). The TEAB solution was evaporated to dryness and diluted with fresh TEAB (5 ml). UV showed OD 267 to be 9 units and TLC showed only the presence of d-pT homogeneous with authentic d-pT.

N^2 -Anisoyldeoxycytidine-5'-monophosphate ⁴⁹

A solution of d-pC (1 mmole) in dry pyridine (30 ml) and anisoyl chloride (3 ml) was allowed to sit at room temperature for 1 hr under nitrogen. Water (100 ml) was added in an ice bath and the resulting mixture was extracted with chloroform (3 × 150 ml). The chloroform layers were combined and extracted with water (3 × 50 ml) and the organic layer evaporated to a gum. Pyridine (20 ml) and water (10 ml) were added to the solidified gum and 2 N sodium hydroxide (30 ml) was quickly added in an ice bath. Excess pyridinium ion exchange resin stopped the reaction after 20 min at room temperature. The neutralized solution was passed through a pyridinium ion exchange column (2 × 30 cm). The elutant was

concentrated to 40 ml and precipitated anisic acid was filtered out. The concentration process was repeated with the filtrate washing, and the final solution extracted with ether (3 × 50 ml). Pyridine (30 ml) was added and the solution evaporated to dryness, then redissolved in anhydrous pyridine.

Chromatography of the final product showed it to be homogeneous d-pC^{An}. Hydrolysis in concentrated NH₄OH yielded d-pC.

N⁶-Anisoyl-3'-O-acetyldeoxycytidine-5'-phosphate (d-pC^{An}-OAc) ⁴

An anhydrous solution of d-pC^{An} in pyridine (25 ml) was reacted with acetic anhydride (2.5 ml). After 1 hr, methanol (6.5 ml) was added and the mixture allowed to stand at room temperature for 4 hrs. The solution was concentrated to 5 ml and water (10 ml) added. The mixture was extracted with ether (60 ml, 20 ml), the aqueous layer diluted with water (200 ml) and lyophilized.

The final product was determined chromatographically to be d-pC^{An}-OAc. Partial hydrolysis with NH₄OH yielded d-pC and d-pC^{An}; complete hydrolysis yielded d-pC as the only nucleotidic material.

The dinucleotide MMTr-G^{iBu}_{pA}^{Bz}OH

The protected mononucleotides d-pA^{Bz}OA (1.5 g, 4 mmole) and MMTr-G^{iBu}OH (300 mg, 1 mmole) were combined and dried by repeated evaporation from anhydrous pyridine (3 × 10 ml). The flask was placed in a dry box under dry nitrogen and tri-isopropyl benzenesulfonylchloride (TPS) (0.6 g, 1.8 mmole) was added. The mixture was agitated until a clear solution was obtained and allowed to stand for 2.5 hrs. The flask was cooled to -20°C and diisopropylethylamine (1 M in pyridine, 3 ml) was added, followed by

H₂O (3 ml). After 16 hrs at room temperature, the mixture was diluted (200 ml, 45% EtOH/0.05 M triethylammonium bicarbonate, TEAB) and pumped onto a trityl cellulose column (5 × 6 cm, 0°C). Step elution with (1) 45% EtOH/0.05 M TEAB, and (2) 75% EtOH/0.05 M TEAB allowed the separation of trityl and nontrityl containing elutants. Pooled fractions were roto-evaporated and partitioned with TEAB/CHCl₃. The CHCl₃ residue, after evaporation, was dissolved in pyridine (20 ml) and H₂O (10 ml) and 2 N NaOH (30 ml) and allowed to react for 10 minutes. The solution was neutralized with ion exchange resin (100 ml pyridine form) and passed through an ion exchange column (pyridine form). The column effluent was redissolved again by roto-evaporation, dissolved in a minimum volume of 0.01 M TEAB, and pumped onto a DEAE cellulose column at 0°C. The column was eluted with a gradient (0.01 M TEAB/20% EtOH: 0.3 M TEAB/40% EtOH). Fractions containing the dinucleotide were identified as follows: a small sample was hydrolyzed in concentrated NH₄OH (48 hrs) followed by treatment with HOAc/pyridine/H₂O (48 hrs) to remove all protecting groups. The sample was then digested by venom phosphodiesterase.⁴⁵ TLC showed the presence of dG and dpA in equal amounts, confirming the presence of d-GpA. Total yield of dinucleotide was 0.7 g of white powder after lyophilization.

Trityl cellulose⁹

Dried cellulose powder (30 g, 50°C in vacuum) and recrystallized trityl chloride (30.5 g, from petroleum ether) were reacted in pyridine (250 ml) under reflux (3.5 hrs). Ethanol/H₂O (90%, 250 ml) was added and the solution cooled. The thick syrup was washed with ethanol (200 ml)

and benzene (200 ml), then again with ethanol (0.5 M) and dried under vacuum. Yield was 40 g. The trityl content was determined by suspending a small sample of trityl cellulose (10 mg) in 10 ml of 60% perchloric acid and determining the absorption at 430 nm. Lit. $\epsilon_{430} = 35,200$.

The dinucleotide, pA^{Bz}pT-OAc ⁴

Dry TPM-pA^{Bz}OH (0.6 g) and pT-OAc (0.9 g) were combined in anhydrous pyridine (10 ml) and dry TPS (1.2 g) introduced into the flask in a dry box. After 3 hrs the solution was cooled (-20°C dry ice/alcohol) and di-isopropyl amine (1 M in pyridine, 6 ml) added, followed by H₂O (6 ml). The solution was left overnight (16 hrs) and then concentrated (>10 ml). H₂O was added (10 ml) and the solution extracted with CHCl₂/n-Bu-OH (7:3, 3 × 40 ml) and the organic phase washed with TEAB (0.2 M, 2 × 50 ml). The organic layer was concentrated (<5 ml) and precipitated into ethyl acetate/ether (50/50, 200 ml) and additional ether added until precipitation was complete. The solid was collected by filtration through "M" sintered glass and washed with ether. Total yield was 486 mg of brown powder.

The brown powder (above) was dissolved in pyridine/HOAc and distilled isoamyl nitrate (1 ml) added. The mixture was allowed to react for 24 hrs. The hydrolysis was not complete as shown by TLC and longer reaction times yielded negligible results. The hydrolysis mixture was evaporated at low temperature at reduced pressure with the addition of pyridine. The resulting gum was taken up in chloroform/n-butyl alcohol (7:3) and washed with TEAB (0.2 M, 3 × 10 ml). Examination of the aqueous TEAB solution revealed only one major component. Pyridine was added and the solution lyophilized. The lyophilized material was stored at -70° under anhydrous conditions. Yield based on absorbance of TEAB solution was 7600 OD units.

A portion of the TEAB solution (10%) was lyophilized separately and completely deprotected by reaction with $\text{NH}_4\text{OH}/\text{MeOH}$. The deprotected material was lyophilized, dissolved in buffer and digested by venom phosphodiesterase according to standard procedure. The resulting mixture was shown by TLC (Table 3) to contain d-pA and d-pT in equal amounts.

The dinucleotide, pTpC^{An}OAc⁴

Dry TPS (0.6 g) was added to an anhydrous solution of d-pC^{An}OAc (1.3 g) in pyridine. After 5 min, TPM-pT-OH (0.5 g, pyridine, 2 ml) was added and the mixture was concentrated under vacuum at 20 C. The fluorescent red solution (<10 ml) was placed in a dry box and allowed to react for 3 hrs. The solution was cooled (-20°C) and di-isopropylethyl amine (1 M in pyridine, 4 ml) was added followed by H₂O (4 ml). The mixture was allowed to stand overnight at room temperature. The solution was extracted with CHCl_3 (2 × 60 ml) and then by $\text{CHCl}_3/\text{n-butanol}$ (9:1, 2 × 60 ml). The combined organic extracts were washed with 0.2 M TEAB (2 × 20 ml). The organic fraction was condensed (<4 ml) with the addition of pyridine and precipitated onto ethyl acetate (200 ml), washed with ether, and isolated by filtration through an "F" sintered glass funnel; yield: 0.42 g.

The solid material was dissolved in pyridine/HoAc (1:1, 20 ml) and isoamyl nitrite (1 ml) added and allowed to react overnight at room temperature (reaction was most efficient in the presence of light). The dark solution was evaporated at room temperature to a gum with the addition of pyridine. This was dissolved in chloroform/n-butanol (7:3, 100 ml) and extracted with TEAB buffer (0.2 M, 3 × 1 ml). TLC showed the presence of one major spot (Table 3). A sample (100 ul) was removed and deprotected

TABLE 3. Dinucleotides

Dinucleotide	Rf in various solvents
TPM-d-pTpC(An)-OAc	1.32(B), 1.92(C)
d-pTpC(An)-OAc	1.45(A), 1.16(B), 1.39(C)
d-pTpC	0.26(A), 0.81(C)
TPM-d-pA(Bz)pT-OAc	6.32(A), 1.32(B), 2.06(C)
d-pA(Bz)pT-OAc	1.5(A), 1.36(B), 1.42(C)
d-pApT	0.75(B), 0.82(C)

totally in $\text{NH}_4\text{OH}/\text{MeOH}$ (1:1, 24 hrs) and lyophilized. The sample was digested by venom phosphodiesterase under standard conditions. TLC revealed equal quantities of d-pT and d-pC. Yield of product after deprotection was 5200 OD units (0.2 mmole).

The protected dinucleotides d-pA(Bz)pT-OAc and d-pTpC(An)-OAc used in the synthesis of the oligonucleotide were a combination of dinucleotides prepared in this laboratory by solvent extraction and dinucleotides obtained commercially. Because of the long procedures involved in the synthesis of the dinucleotides and their inherent instability, the intermediates invariably suffered considerable degradation before they could be used, even when stored at -70°C . Therefore they required purification just before use. They were combined with the commercially prepared material before it had undergone acetylation to protect the 3'-OH group. The TLC of either material before purification invariably showed several spots of significant intensity, even in the case of the commercial material, upon receipt. Often the major impurity upon storage was loss of the 3'-OAc protecting group, which is apparently quite labile.

The initial protected dinucleotide MMTr-d-G(iBu)pA(Bz)-OH was synthesized entirely in this laboratory as it was not available commercially.

The yields of the protected di-, tetra-, and hexanucleotides were estimated spectrophotometrically after total deprotection of a small sample. The amount of the first protected dinucleotide could be determined gravimetrically as the compound was not extremely hygroscopic and tended to be isolatable as a powder rather than a solvent-occluded gum or flocculant.

The tetranucleotide, d-G^{iBu}_{pA}^{Bz}_{pA}^{Bz}_{pT}-OH

The protected dinucleotide, d-pA(Bz)pT-OH (200 mg, pyridine + form), was dried by evaporation from anhydrous pyridine (3 × 5 ml) and reacted with a large excess of acetic anhydride (0.5 ml). After five hours at room temperature in the dark, under dry nitrogen, all solids had dissolved and the solution was clear. The mixture was chilled to 0°C and water (5 ml) was added. The resulting solution was allowed to reach room temperature and then roto-evaporated with the addition of H₂O at 20° or lower. The gum was dissolved in pyridine (5 ml) and H₂O (250 ml) and lyophilized.

A quantity of the protected dinucleotide, MMTr-d-G(iBu)pA(Bz) (100 mg, 0.1 mmole), was dried by evaporation from anhydrous pyridine (3 × 5 ml). The acetylated dinucleotide d-pA(Bz)pT-OAc (prepared above) was dried by evaporation from anhydrous pyridine (3 × 5 ml) and reacted with MsCl (0.065 g, 3 ml) for five minutes. MMTr-d-G(iBu)pA(Bz)-OH in pyridine (5 ml) was added and the mixture shaken and allowed to react under dry nitrogen at room temperature in the dark. After 2.5 hrs diisopropylethyl amine (1M in pyridine, 5 ml) was added (0°C) followed by H₂O (5 ml), and the mixture allowed to react in the dark (24 hrs, 10°C). The dark solution was chilled (0°C) and reacted with NaOH (2 N, 20 ml) and H₂O/pyridine (1:1, 10 ml) for 10 min. The hydrolysis was quenched by the addition of pyridinium ion exchange resin (100 ml wet). The ion exchange resin was filtered out and the resulting solution and washings were roto-evaporated under reduced pressure and temperature (10°C). The residue was dissolved in buffer (0.01 M TEAB/EtOH, 20%). This solution was pumped onto DEAE cellulose ion exchange column and eluted with a TEAB

gradient (0.01 M: 0.35M, 20-40% EtOH). Fractions 165-190 (see Discussion, Fig. 19, pg.45) contained the desired tetranucleotide (UV λ max 270, λ min 240, sh. 255; trityl positive, TLC, Table 4).

The fractions were combined and roto-evaporated at reduced pressure and temperature. The solids were dissolved in dilute aqueous pyridine (20 ml) and passed through a pyridinium ion exchange column to remove all traces of triethylamine. The solution and washings were lyophilized with addition of pyridine. The solid was dissolved in anhydrous pyridine (2 ml).

A portion of the tetranucleotide (100 μ l) was deprotected in ammonium hydroxide/pyridine (1:1, 48 hrs). The solution was evaporated from aqueous pyridine at reduced pressure and further deprotected by reaction with acetic acid/pyridine (1 ml, 80%, 2 hrs). The resulting solution was evaporated at reduced pressure and dissolved in chloroform (1 ml). The chloroform solution was extracted with TEAB (4 \times 1 ml) and lyophilized. The resulting solid was dissolved in standard venom phosphodiesterase buffer and digested. TLC of the resulting products yielded d-G, d-pA, and d-pT (Table 4).

The hexanucleotide, d-GpApApTpTpC

Lyophilized d-pTpC(An)-OH (200 mg) was dried in pyridine by evaporation at reduced pressure (3 \times 10 ml) and an excess of Ac₂O added. The mixture was allowed to react in the dark for 24 hrs, by which time all solids had dissolved and a clear solution remained. The reaction was quenched with H₂O (5 ml) at 0°C (ice bath) and the cloudy solution evaporated under reduced pressure at 20°C with the addition of H₂O (4 \times 50 ml). The resulting aqueous solution was passed through a pyridinium

TABLE 4. Synthesis of MMTr-d-GpApApT

Compound	Rf cellulose	Theoretical ratio	Ratio found
MMTr-d-G(iBu)pA(Bz) -pA(Bz)pT	0.62(B)	-	-
MMTr-d-GpApApT	0.15(A)	-	-
d-GpApApT	0.32(C)	-	-
d-G	-	1	0.8
d-pA	-	2	2.1
d-pT	-	1	0.9

ion exchange column and the elutant and washings reduced in volume by evaporation with the addition of pyridine and the resulting solution (250 ml) lyophilized.

The tetranucleotide, MMTr-d-G(iBu)pA(Vz)pA(Bz)pTpOH (~30 mg), was passed through a pyridinium ion exchange column to remove all traces of Et₃NH⁺ and lyophilized. The resulting material was dissolved in anhydrous pyridine and dried from anhydrous pyridine (3 × 5 ml) by evaporation at low pressure.

The lyophilized pyridinium-d-pTpC(An)-OAc was dissolved in anhydrous pyridine and dried from anhydrous pyridine (3 × 5 ml) at reduced pressure. MsCl (0.078 g) was added to the anhydrous solution and allowed to react for five minutes, by which time all solids had dissolved. The solution of MMTr-d-G(iBu)pA(Bz)pA(Bz)pT-OH was added (5 ml total volume) and the reaction allowed to proceed for 2 hrs. The mixture (dark red) was cooled in a dry ice bath (-20°) and diisopropylamine (1 M in pyridine, 5 ml) was added, followed by H₂O (5 ml). The mixture was allowed to react in the dark (10°C, 24 hrs). The resulting solution was evaporated at reduced pressure (20°) to a gum. The gum was dissolved in buffer solution (TEAB 0.01 M, EtOH 20%, 100 ml) and pumped onto a DEAE cellulose column. A gradient was run from 0.01 M to 0.3 M TEAB, the samples assayed and the trityl positive fractions were collected. All but early trityl-positive fractions (unreacted dinucleotide) were combined and deprotected in base (conc. NH₄OH/pyridine, 1:1). Chromatography in solvent B on cellulose indicated a single trityl-positive spot of rf. 0.49. The entire mixture was evaporated and purified by preparative thin layer chromatography on cellulose. The material was further treated with acetic acid (HOAc,

pyridine, H₂O; 14:3:1) for 48 hrs to remove the MMTr group. The material was evaporated with the addition of water and concentrated to a small volume where it was applied to a cellulose preparative thin layer plate. The plate was eluted with solvent B (NH₄OAc, EtOH). The nucleotidic material was removed from the plate, eluted with dilute ammonium acetate and lyophilized. The yield was approximately 2 mg of chromatographically pure hexanucleotide ammonium salt/buffer. This material was dissolved in 500 ul of standard buffer and a sample (200 ul) removed for enzymatic degradation.

Degradation by diesterase yielded the expected three nucleotides and one nucleoside. The ratios of G:A:T:C were determined spectrophotometrically based on the extinction coefficients on the bases at neutral pH. The experimental ratios are found in Table 5. The yield as estimated spectroscopically was 7.0 OD 280, or approximately 0.1 umole.

TABLE 5. The synthesis of d-GpApApTpTpC

Compound	Rf cellulose	Theoretical	Ratio found
d-MMTr-G(iBu)pA(Bz)pA(Bz)pTpTpC(An)	-	-	-
d-MMTr-GpApApTpTpC	0.49(B)	-	-
d-GpApApTpTpC	0.41(C), *0.21(D)	-	-
d-G	-	1	0.7
d-pA	-	2	2.1
d-pT	-	2	1.8
d-pC	-	1	1.2

*Relative to p-T.

INTRODUCTION TO ENZYME STUDY

This study was designed to measure the interaction of the Eco R1 restriction enzyme with oligonucleotides which approximate its natural site of action on a DNA substrate. The enzyme is categorized as a specific endonuclease which is capable of binding an endonuclease activity on any duplex DNA containing the symmetrical hexanucleotide d-GpApApTpTpC.¹⁸

The rate of reaction of the enzyme was to be approximated utilizing reciprocal plots of the velocity of the reaction versus the concentration of substrate either in the presence or absence of the potential nucleotide inhibitors described above. From this data and data obtained by other researchers, it was hoped to calculate (a) the E activation for the enzyme, (b) V_{\max} and K_M , (c) K_I for any potential nucleotide inhibitors and the type of inhibition exhibited, and (d) postulate an acceptable mechanism for this type of restriction enzyme system.

Methods of Assay for Restriction Enzyme Activity

The assay for restriction enzyme activity must be able to discern between DNA which is continuous and that which has become nicked or cut by the endonuclease. The kinetics observed by this assay would be greatly simplified by utilizing a substrate which can interact only once with the restriction enzyme.

The substrate of choice was a plasmid DNA from *E coli*, Col E₁.⁵ This is a tightly wound circular duplex DNA containing one site of restriction activity for the Eco R1 enzyme system. The plasmid could also be harvested in quantity from *E coli* JC 411⁵ by amplification of

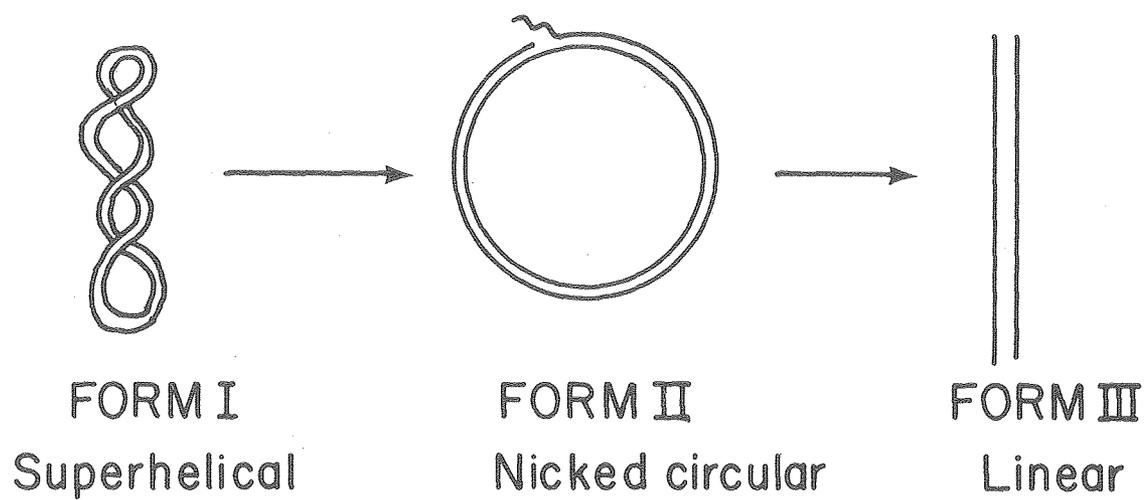
the plasmid with addition of chloramphenicol to the culture medium.²² This substrate, when acted upon by the Eco R1 restriction enzyme, will produce three distinct forms of DNA: [i] tightly wound circular DNA (unaffected by the enzyme), [ii] nicked circular DNA (acted upon once by the enzyme), and [iii] linear DNA (acted upon twice by the enzyme) (Fig. 22).³⁹

There are several assay systems which would be capable of measuring the various amount of each form of DNA produced by the enzyme action. The most traditional assay would be a CsCl/ethidium bromide gradient centrifugation. Each species of DNA would show up as a distinct band and could be quantized by fluorescence or counting (labeled substrate). The drawback to this assay is the long time periods required by the centrifugation itself and the general difficulties involved in quantizing the small amounts of material in each centrifugation tube. As a last resort this method of course be used to produce the desired data.

A more recent assay was developed based on the difference in fluorescence exhibited by each DNA species in the presence of ethidium bromide.²⁰ The tightly wound species would allow the least amount of ethidium bromide to intercalate in the DNA helix, thereby forming the least amount of DNA-EtBr complex. Both the relaxed circular DNA (nicked) and the linear DNA (cut) would exhibit similar fluorescence and the assay would therefore distinguish between substrate not acted upon and substrate acted upon one or more times by the enzyme. Although this would not be ideal, the information derived from this assay would be sufficient to determine the Michealis-Menten kinetic parameters.

The assay proved better on paper than in practice and was finally

Three Forms of ColE₁ DNA



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Figure 22.

Three forms of Col E1 plasmid DNA.

abandoned in favor of an electrophoresis procedure which was far more sensitive than the direct fluorescence assay and was, in addition, capable of distinguishing between the three forms of DNA formed during the assay.

The assay adopted for the study utilized an agarose gel electrophoresis system to resolve the three forms of DNA. The three separate forms were then quantized by measuring the fluorescence produced by complexing the DNA with ethidium bromide while the substrate was still suspended in the electrophoresis gel. The fluorescence was measured by a gel-scanning apparatus. The gels could also be photographed under fluorescing conditions and the resulting negative scanned with a densitometer to obtain similar results.¹²

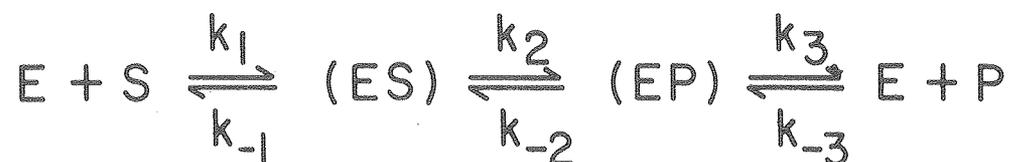
Enzyme Kinetics

The basic assumption when considering an enzyme reaction is the intermediate formation of an enzyme-substrate complex that is somewhat stable and whose decomposition into unbound enzyme and products is the slowest (rate determining) step in the overall reaction (Eq. 6). If this equation is valid then the rate of reaction (v) may be expressed as $v = d(P)/dt = k_2(ES)$ and the equilibrium concentration of the enzyme-substrate complex is $K = k_{-1}/k_1 = (E)(S)/(ES)$. The total enzyme concentration (E_t) can be defined as $(E_t) = (E) + (ES)$ and the enzyme-substrate complex concentration expressed in terms of the Michealis constant as $(ES) = (E_t)(S)/(S) + K$. The velocity of the reaction may also be expressed in similar terms as $v = k_2(E_t)(S)/(S) + K$ and if conditions are such that the reaction is done under conditions of high substrate concentration

Simple Enzyme Complex Formation



Stable Enzyme - Product Complex



Equilibrium for Overall Reaction $S \rightleftharpoons P$

$$K_0 = \frac{(S)}{(P)} = \frac{k_{-1}k_{-2}k_{-3}}{k_1k_2k_3} = \frac{K_S}{K_P} K_{SP}$$

$$v = \frac{V_m(S)K_p - V_p(P)K_m}{K_mK_p + (S)K_p + (P)K_m}$$

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Equation 6.

Enzyme-substrate interactions.

(enzyme saturation), the maximum rate V_m is limited to $k_2(E_t)$ and the equation may be written in the traditional form $v = V_m(S)/(S)+K$ where V_m is the maximum rate under conditions of enzyme saturation, and where K (the Michaelis constant) is the dissociation constant for the enzyme-substrate complex and an inverse measure of the affinity of the enzyme for a substrate.

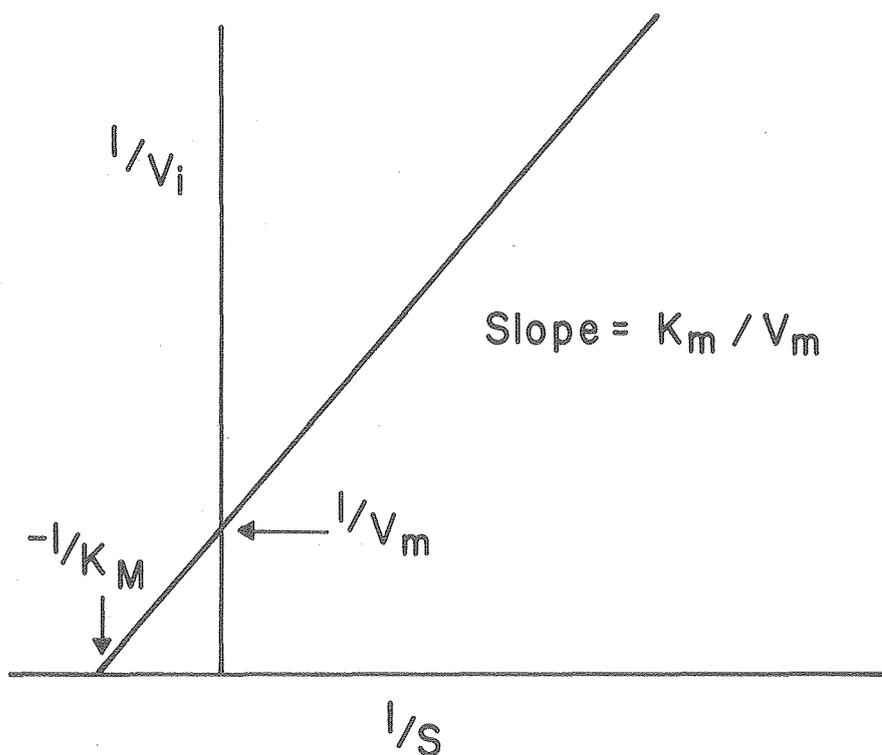
Thus, Michealis-Menton kinetic analysis is valid only under certain conditions: (i) the rate determining step of the reaction being dissociation of the enzyme substrate complex, and (ii) that the reaction be carried out under conditions of high (enzyme saturating) substrate concentration. If these prerequisites are met then the various parameters of the reaction can be experimentally determined by the application of the reciprocal form of the Michaelis-Menton equation (Eq. 1, Fig. 23). From this V_m and K_M may be determined graphically. It is clear that under certain conditions Michaelis-Menton kinetics will describe the action of an enzyme system. However, in many situations they will not adequately interpret the enzyme kinetics. Each of the following assumptions must be considered before attempting to analyze a particular enzyme:

- (i) Is K actually the dissociation constant of the enzyme complex?

This can be more adequately determined if one considers a steady state analysis of an enzyme reaction where k_1 , k_{-1} , and k_2 are considered to be of equal value and the concentration of the enzyme substrate complex is constant. The general form for the change in complex is $d(ES)/dt = k_1(E)(S) - k_{-1}(ES) - k_2(ES) = 0$ and the rate of the enzyme reaction is defined as $v = V_m(S)/(S) + (k_{-1} + k_2)/k_1 = V_m(S)/(S) + K_m$. Thus the

Reciprocal Plot of Michealis - Menton Kinetics

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K}{V_m(S)}$$



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Figure 23.
Reciprocal plot.

Michaelis constant may have a variety of meanings depending on the various values of k_1 , k_{-1} , and k_2 . Only under conditions where k_{-1} is much larger than k_2 is the Michaelis constant actually the dissociation constant for the Es complex.

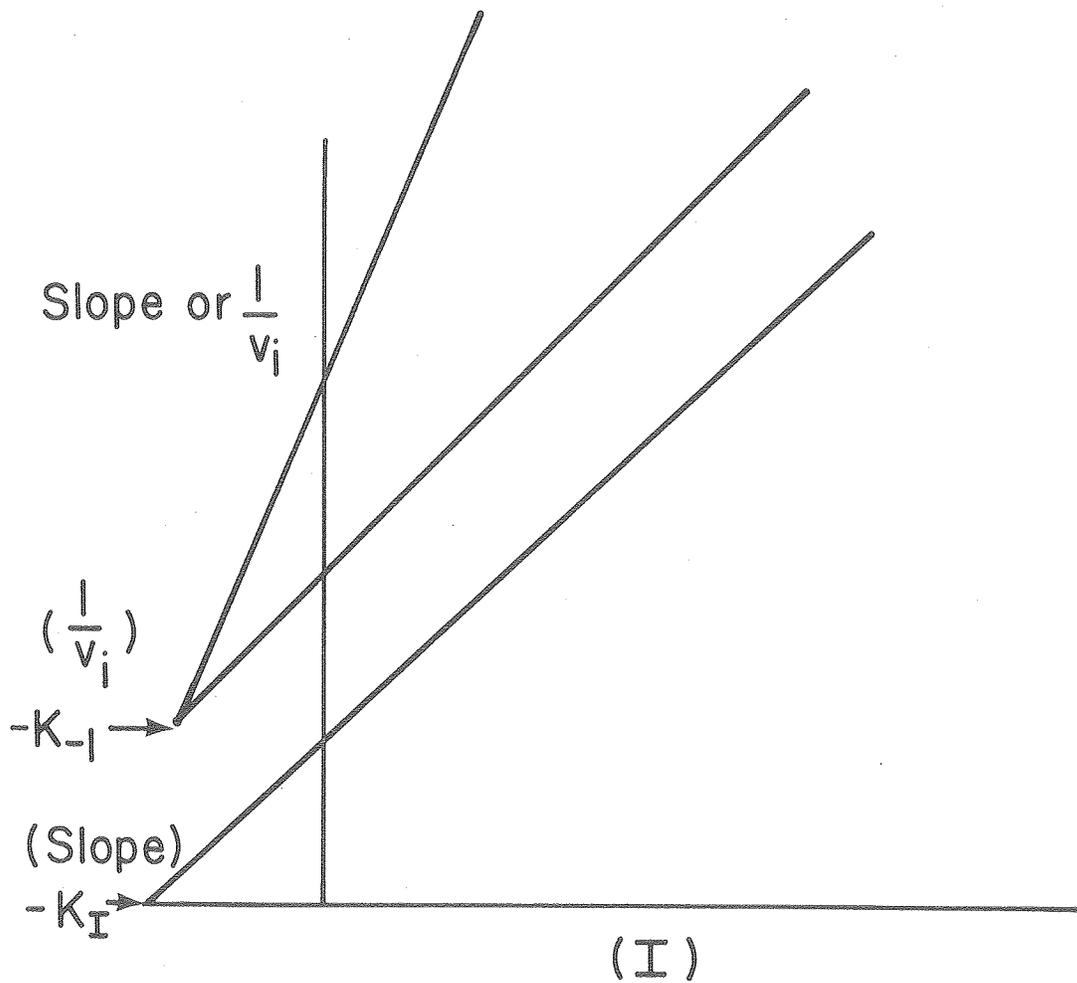
- (ii) The concentration of substrate must be greater than the concentration of enzyme and it is assumed that the concentration of substrate is not depleted by the formation of enzyme-substrate complex. This assumption is generally valid if a large excess of substrate is used and initial velocities are measured. This removes the possibility that sufficient substrate will be used up in the reaction to invalidate this prerequisite. It should be noted that *in vivo* reactions would not be expected to adhere to these conditions and therefore parameters derived from Michaelis-Menton assumptions or steady state analysis would not necessarily describe *in vivo* reactions.
- (iii) A major assumption is that products are released rapidly from the enzyme-substrate complex. It is reasonable to assume that in many cases there is a stable enzyme-product complex and if k_3 (Eq. 6) is slow then it will be the rate determining step in the reaction and K_m now takes on an entirely different meaning and the general form $K_m = (k_{-1}k_2 + k_{-1}k_3 + k_2k_3)/k_1(k_2 + k_{-2} + k_3)$. This cannot be readily distinguished from Michaelis-Menton kinetics and may be important in the consideration of restriction enzyme kinetics. If k_3 is large then the formation of the (EP) complex is of little consequence as K_m approaches $(k_{-1} + k_2)/k_1$ under these conditions.

(iv) Another consideration involving the formation of products through an (EP) complex is whether this is reversible. If this is the case, the formation of products will heavily effect the formation of (ES) complexes and an equilibrium will be maintained. This would have profound effects in the presence of an inhibitor which approximates the product of an enzyme reaction (as well as the substrate) as this would also change the concentration of (ES) through the equilibrium constant $K_o = (K_s/K_p)/K_{sp}$, where K_{sp} is the equilibrium (ES)/(EP), and K_o for the overall reaction (Eq. 6). This effect is possibly involved in the inhibition of restriction enzymes by nucleotides and therefore may play a role in the inability of restriction enzymes to turn over at low temperatures in certain buffer systems.

There are a variety of data replots that may be employed to determine mechanisms and various constants of inhibition. The validity of these results, of course, must be linked to the basic assumptions for steady state or Michaelis-Menton kinetics. The Lineweaver-Burke ($1/V$ vs $1/S$) plot is the most familiar and one of the most useful methods (Fig. 23). This is the basic analysis to which all data in this work were fitted. From this analysis one can calculate directly K_m and V_{max} . This analysis can be applied to inhibitors as well if series of substrate concentrations are run with a constant amount of inhibitor. The type of plot that results will indicate if the inhibition may be considered to be (i) competitive, (ii) non-competitive), (iii) mixed), (iv) uncompetitive, or (v) inhibited of substrate (Fig. 25).

Another plot developed by Dixon allows one to directly calculate K_I and simultaneously differentiate between competitive and partially competitive as well as non-competitive and partially non-competitive inhibition (Fig. 24). This is a partially reciprocal plot ($1/v_i$ vs I) where experimentally a series of inhibitor concentrations with substrate held constant are plotted against the reciprocal of initial velocity. One may optionally simply plot the slope of the inhibited reaction (any inhibitor concentration) and the slope for the uninhibited reaction (inhibitor concentration = 0) to obtain K_I . The slopes of additional inhibitor concentrations must be plotted if information about partially competitive or partially uncompetitive reactions is to be obtained (Fig. 26). Both the Dixon and Lineweave-Burke plots were utilized in this study to determine various constants.

Dixon Plot of Inhibition



$$\frac{1}{v_i} = \frac{1}{V_m} \left[1 + \frac{K_m}{S} \right] + \frac{K_m (I)}{V_m (S)_i K_i}$$

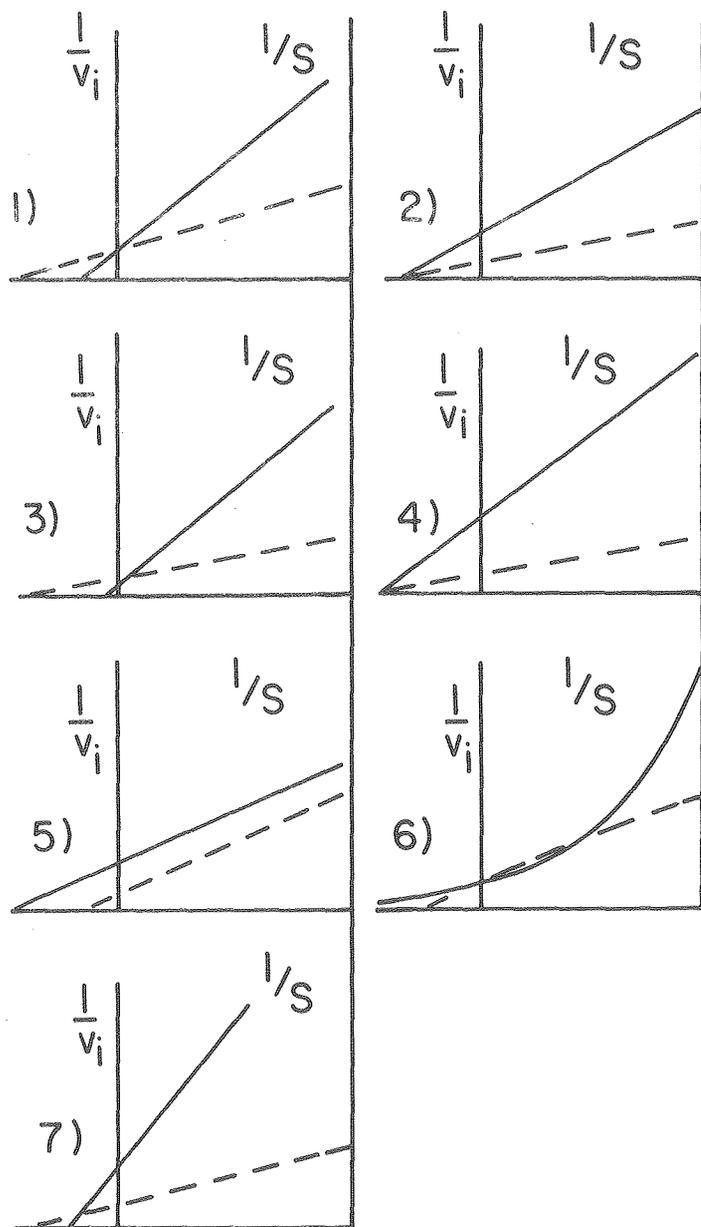
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Figure 24.

Dixon plot.

Lineweaver-Burke Plots of Inhibition

— Inhibitor present
 --- No inhibitor

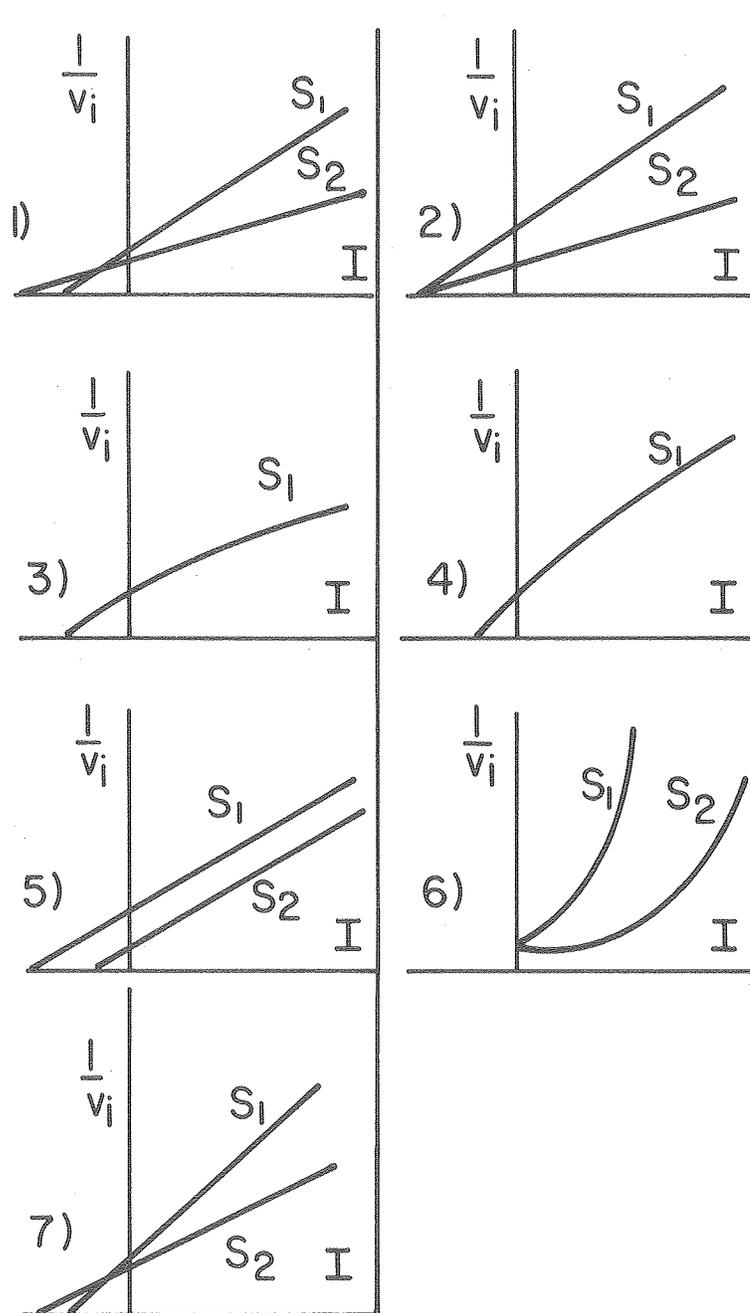


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Fig. 25. Lineweaver-Burke plots of various types of inhibition. 1) competitive; 2) non-competitive; 3) partially competitive; 4) partially non-competitive; 5) uncompetitive; 6) inhibitor-substrate interaction; 7) mixed inhibition.

Dixon Plots of Inhibition

$S_1 < S_2$



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Fig. 26. Dixon plots of various types of inhibition.
 1) competitive; 2) non-competitive; 3) partially competitive; 4) partially non-competitive; 5) uncompetitive; 6) inhibitor-substrate interaction; 7) mixed inhibition.

DISCUSSION OF E_A FOR THE Eco R1 RESTRICTION ENZYME

The Eco R1 enzyme has been shown to be a relatively slow enzyme based on the rate of turnover shown on several substrates.³⁹ From the rate of reaction determined at different temperatures the energy of activation for this range can be estimated and information about the mechanism of reaction can be suggested by the results. The rate limiting step for which the E_a can be estimated is probably the dissociation of the enzyme from the substrate or the dissociation of an enzyme-product complex (Eq. 6, p.76) and probably does not reflect the phosphodiesterase activity of the enzyme. The actual bond breaking steps of the enzyme action have been shown to be quite facile³⁹ (Table 7).

The information that can be gathered about the mechanism of the enzyme would be whether the enzyme must significantly distort the DNA molecule (or conversely, be distorted by the DNA) during reaction. This would have a strong bearing on the question of loop formation during the action of restriction enzymes or if the enzyme is more simply just fitting into the major groove of the DNA substrate. One must suggest a very high E_a of loop formation vs a lower E_a for the lesser distortion of fitting the enzyme into the helix. While loop formation has been described as unlikely because of the short length of restriction sites on DNA substrates, it should be noted that little is known about the actual extent of the sequence of the DNA to either side of the site of activity and whether it is also capable of self-pairing. It has been shown that DNA molecules have a number of long symmetrical sequences which are capable of forming stable loops.³⁸ In addition, many of these stable loops contain restriction sites and must of course always contain a point of symmetry analogous

to a restriction site at their apex (i.e., the center of the loop-forming segment).

The E_a for the Eco R1 enzyme can be estimated by comparing V_{max} for the enzyme at several different temperatures and plotting $\log V_m$ vs T . For the temperature range 15-37°C, E_a can be shown to be roughly 12 Kcal mole⁻¹. This is relatively high for an enzyme system and is probably indicative of distorting either the substrate or the enzyme itself. Since this figure may most likely be associated with dissociation of the enzyme substrate complex it can be inferred that while dissociation and concomitant reformation of DNA helix and free enzyme is thermodynamically advantageous, there is a large barrier to this process; formation of an unstabilized DNA, i.e., non-duplex molecule. This would be in agreement with the formation of some stabilized non-helical DNA enzyme structure, possibly a loop.

The nature of the active site of the enzyme may be further studied by considering the action of the enzyme on different substrates. There is a small difference in both the turnover and the K_m for the enzyme at 15°C for two similar substrates, Col E1 and SV 40 DNA¹³ (Table 7). SV 40 substrate tended to turn over twice as fast as the Col E1 substrate and to exhibit a K_D for dissociation of the enzyme substrate complex slightly smaller than that for the Col E1 system (Table 7). Thus the differences for very similar substrates are practically negligible. These two substrates can be categorized as similar in all aspects except molecular weight. Their degree of superhelicity is about the same and of course the site of restriction activity should be identical.

It can be shown that Col E1 DNA which is randomly nicked (relaxed),

TABLE 7. Kinetic parameters for Col E1 and SV-40 DNA.

Substrate	Eco R1 Constants		
	Temperature	V_{\max}	$K_M \times 10^8$
SV 40	15°	1.5	3.0
Col E1	37°	4.0	0.8
Col E1	15°	1.8	8.0
d-pTpGpApApTpTpC	15°	4.0	160

Rates of Phosphodiester Cleavage			
Col E1	30°	Step 1	40 min ⁻¹
		Step 2	14 min ⁻¹

yet contains the complete restriction site, shows qualitatively a much lower K_M than the superhelical moiety.²⁰ This would be indicative of less distortion in the enzyme DNA complex and therefore less thermodynamic drive towards dissociation of enzyme-substrate complex.

An interesting comparison can be drawn from the K_M for the reaction of the enzyme with the naked restriction site d-TpGpApApTpTpCpA studied by Goodman¹⁴ (Table 7). The kinetic analysis of endonuclease activity on this substrate at 15°C while clouded by the fact that the substrate was in equilibrium between dimer and monomer yielded some interesting information: (i) that the enzyme definitely did not cleave monomeric restriction sites, (ii) it did react with the restriction site outside of a large DNA environment (as a duplex) and, (iii) the turnover is about the same for that with either SV-40 or Col E1 at the same temperature, but a large difference in the K_M for the reaction is observed; $K_M = 1.6 \times 10^{-6}$. This could indicate that the dissociation of the enzyme substrate complex still exhibits an uncommonly high E_a and that a large change in the structure of the DNA duplex is occurring. The large K_M would be explained by the large change in duplex stability and therefore the reduced stability of the enzyme substrate complex. At this lowered temperature the enzyme should also have an increased affinity for products and for the dissociated (non-duplex) substrate.

The results of this research would clearly indicate that the synthetic duplex would have a strong inhibitory effect on the phosphodiesterase activity of the enzyme system. This would occur from the dissociation of the duplex into two entities which would not only reduce the amount of available duplex substrate but would provide a concentration of strong

inhibitor of restriction activity, the non-duplex restriction site itself.

The effect of the superhelicity of the substrate can be somewhat quantized by examining the kinetics of the enzyme at two different temperatures. Wang²¹ has shown that the decrease in superhelicity for superhelical SV-40 DNA is of the order of 10% for each 12 increase in temperature. Thus a change from 15 to 37°C will change the superhelicity of the substrate by nearly 20%. The change in the affinity of the enzyme for the substrate is increased by an order of magnitude. It is reasonable to infer that the decrease in superhelicity at the higher temperature is responsible for the increase in affinity.

The observed similarity of turnover rate between most substrates may be an important clue to the type of kinetic analysis which best describes the overall enzyme reaction. The enzyme may be considered in terms of the simple case (Eq. 6) where the (ES) complex dissociates irreversibly to enzyme and product (E + P). The more complex form (Eq. 6), however, is probably more valid allowing for the formation of a stable (EP) complex. There is no solid evidence that the enzyme is capable of the reverse reaction (k_{-2}), but all evidence points towards the possibility that k_{-3} is large making the net reaction K_3 slow. Thus the enzyme would be tied up considerably by product. At lower temperatures where these interactions would be expected to be larger the turnover rate would be reduced considerably. The binding constants for the various mono-, di-, tetra-, and hexanucleotide sequences indicate that these product interactions are strong.

The action of the Eco R1 enzyme system has been shown to be limited to duplex DNA and no action on single strand DNA has been observed.¹⁴ It

is implied that the enzyme therefore does not interact with single strands of DNA to any great extent. To clarify this point it was decided to investigate the inhibitory effects of short strands of deoxy-oligonucleotides on the normal action of the enzyme. The kinetics exhibited by the various inhibitors would then indicate whether the enzyme was specifically interacting with the inhibitors as though they were acceptable substrates. This would indicate the enzyme did indeed recognize these short single-stranded substrates and that some other reason besides lack of interaction would have to be put forth to explain the lack of phosphodiesterase action shown toward single-stranded DNA.

The initial studies were done with the smallest possible sequences, deoxymononucleotides. The results were somewhat surprising (Table 8). The mononucleotides all showed significant inhibitory action. The kinetic analysis of the interaction between the enzyme and inhibitor showed that the enzyme was being competitively inhibited by the mononucleotides. This presumably indicates that the active site was strongly binding the individual mononucleotides to the exclusion of its "normal" substrate and was therefore "interacting" with other than duplex DNA. An inspection of the individual inhibition constants for the various mononucleotides reveals that the mononucleotides which contain the phosphate linkage which is normally acted upon by the endonuclease (d-Gp and d-pA) are considerably stronger inhibitors than the other nucleotides. This fact strengthens the proposition that the inhibition is actually "competitive" and not "partially competitive" or perhaps allosteric; differences which are not totally ruled out by the kinetic analysis used.

The mononucleotides d-Gp and d-pA both contain the phosphoric acid in question and both display high inhibition constants. The nucleotide

TABLE 8. Effects of mononucleotide inhibitors.

Nucleotide	Concentration ($\mu\text{g ul}^{-1}$)	Inhibition type	K_I M
d-pG	1.0	C	1.9×10^{-2}
d-Gp	1.0	C	1.2×10^{-3}
d-pA	1.0	C	1.8×10^{-3}
d-Ap	1.0	C	1.8×10^{-3}
d-pT	1.0	C	2.3×10^{-3}
d-pC	1.0	C	1.1×10^{-2}

d-pG also inhibits the enzyme but to a much lower extent than either d-pA or d-Gp. Most interestingly, the nucleotide which actually contains the bonds which are broken during diesterase activity is the most strongly inhibiting by a significant amount. It is not clear from these data whether the enzyme is interacting with the phosphate in such a way that the phosphorus-oxygen bond might actually be broken or whether it is merely blocking that active site with respect to the normal substrate. If it is actually bound in a normal manner to the active site of the enzyme one must explain why no free phosphate is being formed. That fact cannot be verified from the available data as no sensitive assay for free phosphate was undertaken in these experiments; however, in the available literature where this type of monitoring was done (large non-duplex substrate), no activity was found.¹⁴

If phosphodiesterase activity is not indicated then the absence of such activity must be explained. One possibility is that it is no longer thermodynamically favorable for such a reaction to occur if there is no strain for such a reaction to relieve. This would strengthen the possibility that some form of deformation was produced during the binding of the enzyme to a large or perhaps small duplex DNA. Another possibility would be that the enzyme being made up of more than one sub-unit is capable of either recognition or activity in either sub-unit but not at the same time. The evidence for this type of activity is circumstantial but totally consistent. First the fact that the enzyme must under certain conditions dissociate from a DNA substrate in order to complete the clipping of both sides of the DNA duplex. This would indicate that binding and phosphodiesterase activity are occurring at different places

on the substrate. Under conditions where the enzyme is in significant equilibrium with a four sub-unit moiety no such dissociation is evident before both sides of the DNA duplex are acted upon.

To determine the specificity of binding between enzyme and inhibitor and to further approximate the actual site of restriction found on DNA, a series of dinucleotides were investigated. All the dinucleotides that could be accommodated in the restriction site were used plus a number of dinucleotides that could not be accommodated by the normal or extended recognition site. The extended site will contain all dinucleotides terminating in d-pG and all dinucleotides starting with d-pC; eight in all. The importance of including this extended sequence is to include all dinucleotides which might be able to fit within the active site of the restriction enzyme and bind in a "normal" manner. It was also important to investigate those dinucleotides which could not properly fit within the active site and determine whether the inhibition kinetics exhibited would reaffirm this hypothesis.

There were two major parameters of interest. The first was simply the amount of inhibition exhibited by each of the dinucleotides in the enzyme substrate mixture and the inhibition constant, K_I , if it could be calculated from the data. The second and perhaps most important parameter was the type of inhibition exhibited by the dinucleotide. This would be the only clue as to whether the dinucleotide was actually binding to the active site of the enzyme or simply interacting with the enzyme or substrate in some nonspecific manner.

The types of plots expected from the various types of interactions mentioned are shown in Figs. 25 and 26 (pp. 83,84). The equations shown

are for steady state treatments of the enzyme-substrate inhibitor system and would not be valid for other treatments of the enzyme system. Thus the results are restricted in application to the dissociation of the enzyme-substrate or enzyme-inhibitor complexes. This is suggested to be a valid interpretation for this enzyme system, as the slow step of the enzyme mechanism seems to be dissociation of the enzyme substrate complex under several conditions.

An inspection of Table 9 reveals that those dinucleotides which fit the restriction site all show competitive kinetics much like the mononucleotides did. The major difference between the dinucleotides and the mononucleotides is the strength of the inhibition. The dinucleotides were all two orders of magnitude stronger than the mononucleotides. This observation strengthens the evidence for competitive inhibition. If the observed inhibition had only been a factor of two, on a molar basis, then one would expect a more non-specific interaction, perhaps partially competitive interactions which might show reciprocal plots identical with the plot expected from competitive kinetics.

The case is strengthened when one examines the data for dinucleotides which in no way conform to the recognition site of the restriction enzyme. The kinetics exhibited appear to be totally noncompetitive and the dinucleotides are not binding to the site of enzyme activity. Again this strengthens the notion that the dinucleotides which are contained by the normal restriction site do bind specifically with the active site of the enzyme yet are apparently immune to its action.

Those dinucleotides which could only be accommodated by the extended restriction site also showed kinetics consistent with binding

TABLE 9. Interaction of dinucleotides with Eco R1 enzyme

Dinucleotide	Slope	Intercept V_{max}	Intercept (-ug/ml)	Coefficient
d-pApA	407	4	-0.01	0.998
d-pTpC	878	14	-0.02	0.87
d-pGpA	1466	2	-0.00024	0.99
d-pApG	1235	6	-0.005	0.94
d-pApT	1064	2	-0.0018	0.997
d-pTpA	exponential	18	--	0.97
d-pGpT	exponential	6	--	0.95
d-pCpT	899	9	-0.01	0.95

to the active site of the enzyme, but the strength of interaction (binding) was reduced.

The observation that the nucleotides containing the phosphate which undergoes diesterase activity had stronger inhibition constants than other inhibitors was also observed in the dinucleotides. The dinucleotide d-pGpA was the most powerful inhibitor, followed closely by d-pApT which also contains the phosphate group of interest.

The two available tetranucleotides d-pApApTpT and d-GpApApT were stronger inhibitors (Table 10) than the dinucleotides but not in orders of magnitude. The results indicate that after compensating for the increased molecular weight the tetranucleotides are only slightly more powerful inhibitors than the dinucleotides. It is not clear what this observation means in terms of the binding of the enzyme to inhibitor. This result may indicate that any inhibitor which binds to the enzyme's active site may totally deactivate that site towards binding the normal substrate and additional blocking of the complete site is unnecessary.

The interaction of the enzyme with the complete substrate d-GpApApTpTpC is less clear. Qualitatively it appears to be a strong inhibitor. The plots tend to show curvature and therefore no quantitative estimate of K_I can be calculated. Presumably this curvature is due to the equilibrium between monomer and dimer. This would further complicate the kinetics as the dimer may be acted on by the enzyme and thus the concentration of inhibitor would be non-constant.

It is clear from the data on nucleotide binding that the enzyme does interact strongly with DNA monomers as well as duplex DNA. A model must be proposed which accounts for this binding with lack of enzymatic activity with the monomer. The observation of two "identical" enzyme

TABLE 10. Inhibition of Eco R1 restriction enzyme by tetra- and hexanucleotides

Nucleotide ^a (50 ug/ml)	Slope ^b	Intercept (V _{max}) ^c	Intercept (ug/ml)	Coefficient
d-pApApTpT	exponential	17	--	0.94
--	1.7×10^3	6	0.004	0.985
d-GpApApT	1.8×10^3	4	0.002	0.987
d-GpApApTpTpC	1.2×10^3	2	0.002	0.965
--	exponential	15	--	0.95

^aHexanucleotide concentration 10 ug/ml.

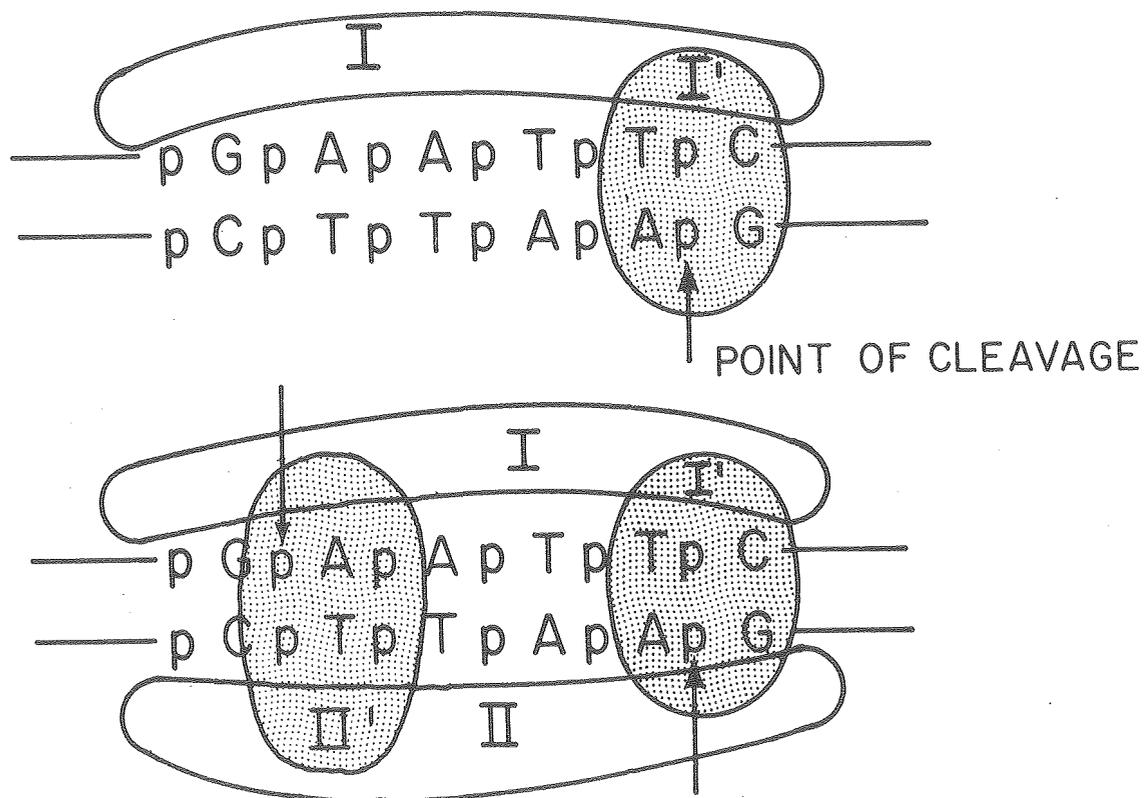
^bV_{max} in units of ug DNA/min.

^cExponential refers to curve fitting of ae^{-bx} (see Appendix).

sub-units in equilibrium with a tetramer form may be the clue.³⁹ A schematic such as Fig. 27a would also explain the situation where the enzyme system would be forced to dissociate from the substrate in order to complete the scission of the DNA duplex. The tetrameric form (Fig. 27b) would explain the more common situation at higher temperatures and probably *in vivo* situations where both parts of the DNA helix are acted upon without dissociation.

This schema (Fig. 27) would have to be somewhat more complex to satisfy the geometry of a helical DNA. That is the problem of fitting all four subunits into the major groove of the substrate DNA in such a manner as to bind to sub-units to each of the set of six nucleotides which comprise the recognition/action site for the restriction enzyme. This would of course be greatly simplified if the DNA were to be distorted into a loop formation of some sort (Fig. 28). The spatial requirements would be relaxed considerably. There is some evidence *vide supra* in the form of energies of activation that suggest that such a distortion is taking place, however, at this point this is still unknown.

Further studies are in order to verify the general applicability of these observations. The action of another restriction enzyme system should be investigated for competitive inhibition by the appropriate mono- and dinucleotide inhibitors. An appropriate system might be Hpa I which recognizes the hexanucleotide d-GTTAAC. With this system the mononucleotides of interest would be those containing the phosphodiester bond cleaved by the enzyme (Tp and d-pA). The dinucleotides of primary interest would be those which show other than competitive inhibition kinetics with the Eco R1 system, yet would be expected to show competitive inhibition with the Hpa I system. The dinucleotides d-pGpT and d-pTpA both fall into this category.



XBL 781-3731

Figure 27.

Enzyme interaction with substrate.

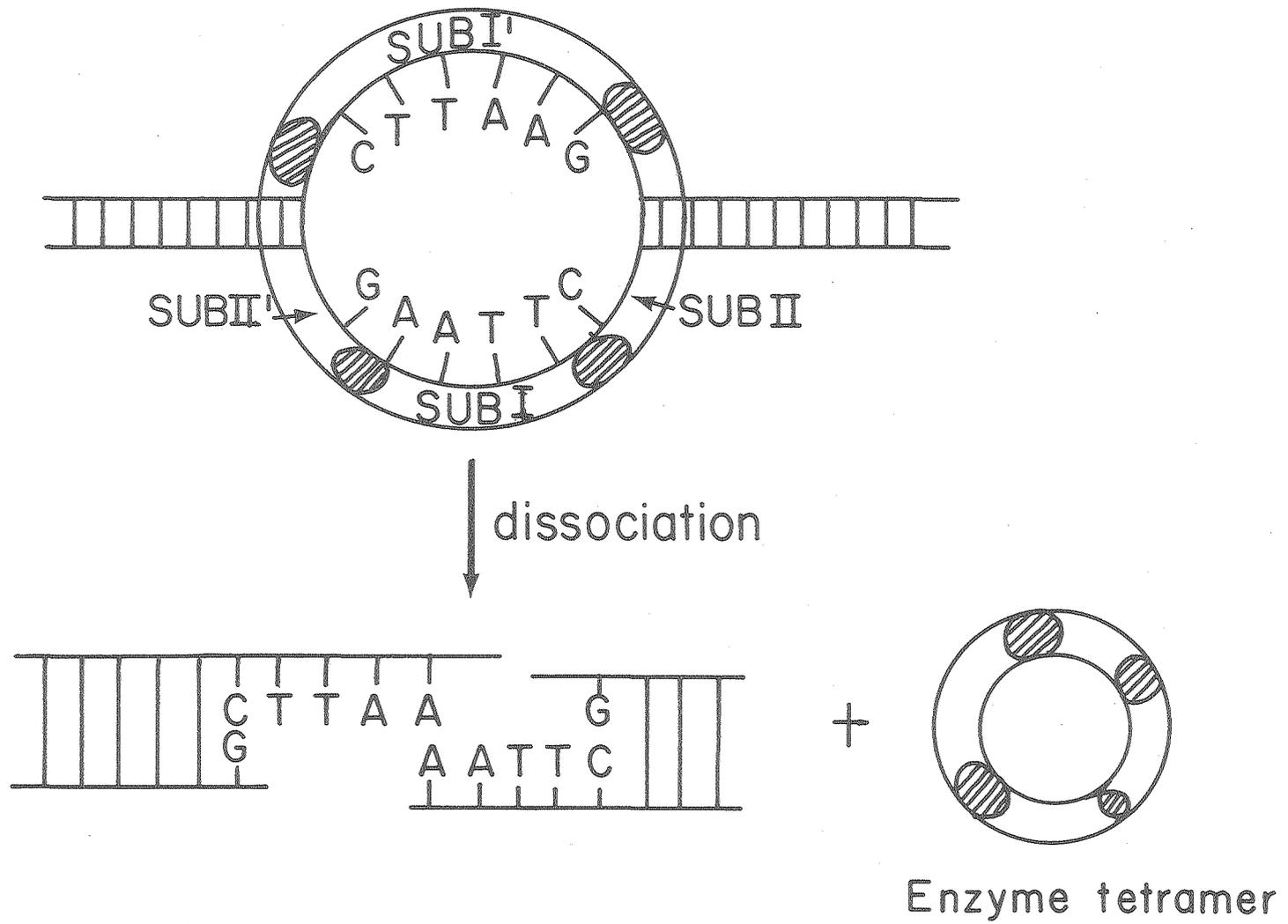


Figure 28. Distortion of DNA substrate during enzyme action.

XBL 784-395I

FUTURE RESEARCH

The obvious outgrowth of this research would be the isolation and purification of restriction enzymes by affinity chromatography. The long tedious procedures used for enzyme purification could be circumvented by the attachment of part of the restriction site to a convenient back-bond polymer and separating the enzyme by its binding to the non-duplex partial substrate. The affinity matrix could probably be something as simple as the dinucleotide which contains the phosphate acted on by the enzyme in question. Since there are only 16 possibilities, purification of any restriction enzyme might be possible.

The same procedures could be used to search for new restriction enzymes of unknown specificity. This would aid in the search for such enzyme systems in mammalian cell systems and virus-transformed cell systems.

It is possible that the inhibition of restriction activity by dinucleotides could be used to determine the binding sites of unknown restriction sites. A considerable amount of background work would have to be done with known enzymes to determine the reliability of such a procedure. The primary application of such a study would be to determine the sites of recognition for the $Ty\ 1^{73}$ restriction enzymes which apparently show diesterase activity at a site remote from site of recognition.

EXPERIMENTAL

General Assay Conditions

The temperature for all assays was controlled in an alcohol/water bath to $15^{\circ} \pm 0.1^{\circ}\text{C}$. The assay vials containing 60 μl of buffer/C01 E1 DNA/inhibitor were preincubated at 15° for a minimum of 10 minutes before introduction of enzyme/buffer mixture (10 μl). The enzyme stock was diluted at one time and divided into vials of dilute stock (200 μl , std. buffer). The vials were frozen at once and stored at -70°C . The enzyme vials were thawed immediately before use and stored no longer than five minutes at 0°C before start of assay. Enzyme was transferred into reaction vials by means of a fixed capacity pipette with disposable tip. Each reaction vial was removed from the temperature bath 20 seconds before addition of enzyme and replaced within 3 seconds after addition of enzyme. Upon termination of reaction the vial was removed 15 seconds before addition of SDS/Bromophenol blue/glycerol mixture (10 μl , termination buffer) and stored at 0°C until being placed in the gel electrophoresis apparatus. The sample was transferred into the gel well by disposable glass pipette (50 μl fixed) and the pipette was replaced between each sample to avoid contamination between samples of different concentration. The electrophoresis potential was applied immediately after the last sample was applied to the gel (40 v).

Gel electrophoresis was carried out on an apparatus described by Wang³⁸ and fabricated in this laboratory. The electrophoresis buffer (standard buffer) was maintained as a concentrated stock solution and diluted (10 \times) freshly before each run. The gels were made with the identical buffer but boiled shortly before use to concentrate the buffer

in the agarose gel. This would allow the gel to expand slightly in the apparatus and no leaks would occur during long electrophoresis runs.

Agarose (Biorad electrophoresis grade) was dissolved in buffer as 1.2% by weight gel. The hot solution was poured into the gel apparatus and allowed to cool to ambient temperature. The sample wells were formed by removal of a Lucite template upon cooling of the gel. This usually required cutting the gel away from the template with a stainless steel wire and pulling the template upward with a steady pressure. Immediately the gel reservoirs were filled with electrophoresis buffer and the apparatus stored at room temperature until use (2 hrs maximum).

Typical conditions for electrophoresis of Col E1 DNA and other superhelical DNA, such as SV-40, were voltages regulated at 40 volts at 20 ma for 20 hrs. A regulated power supply (Biorad model 400) attached to a timer was used to supply the electrophoresis current. The upper and lower buffer reservoirs were constantly exchanged to prevent the electrochemical decomposition of the buffer and consequent loss of electrophoresis current. The temperature of the electrophoresis apparatus was not controlled.

Actual enzyme assays varied in time between points over a range of 3 to 15 minutes; most assays were done with time points every 10 minutes. These points were taken in duplicate or triplicate and staggered at 30 second intervals. Each run represents four to six duplicate (or triplicate) time points. Because of the limited number of electrophoresis apparatus a maximum of two runs could be done at any one time.

The electrophoresis gels were generally made with spacers providing a 3 mm thickness. The dimensions of the gel were 10 × 10 mm of usable gel.

Standard Buffer Conditions

Enzyme buffer: 0.1 M Tris, 0.01 M MgCl_2 , 0.05 M NaCl.

Enzyme diluted 500 from stock buffer: 0.5 M KPO_4 , pH 7,
0.2 M NaCl, 7 mM 2-mercaptoethanol, 1 mM EDTA.

Stored at -70°C until use.

Reaction buffer: 0.1 M Tris, pH 7, 0.05 M MgCl_2 , 0.01 M NaCl.

Termination mixture: 1% SDS, glycerol.

Electrophoresis buffer: 0.04 M Tris, pH 7.9, 5 mM NaOAc,
1 mM Na_4EDTA .

Ethidium bromide buffer: EtBr 1 mg/ml, 0.04 M Tris, pH 7.9,
5 mM NaOAc, 1 mM Na_4EDTA .

The fluorescent yield of the EtBr/DNA complex has been shown to be quite different for the three different forms of the circular DNA.⁵³ This can either be accounted for mathematically while interpreting the data by a simple factor or can be eliminated by converting the tightly wound DNA form 1 to a relaxed species after electrophoresis has been done. This procedure is automatically taken care of in these experiments by the photographic process which irradiates the sample after electrophoresis for a minimum of three minutes before it is finally equilibrated with EtBr and scanned to extract the data.

To determine if the scanning procedure yielded quantitative results, two electrophoresis gels were run: the first contained a series of increasing amounts of DNA (form 1) in the same volume of buffer, the second contained a series of different volumes of the same concentration of DNA (form 1). The plots of fluorescence from each of the gels produced

linear results. Thus quantitative results could definitely be obtained if the data were to be normalized to the total amount of DNA known to be in the sample.

After electrophoresis the gel was removed from the apparatus and placed in an ethidium bromide-electrophoresis buffer (1 mg/ml) and allowed to equilibrate for 2 hrs at 0°C. The gel was removed from the bath and photographed in UV light (3 min short wave). Typical exposures ran 3 minutes after which the gel was placed back in the EtBr/buffer solution and allowed to equilibrate overnight before scanning. This procedure converted all the tightly wound circular DNA to nicked circular DNA, assuring that the quantum yield for all the fluorescing species was the same. The quantization of the DNA on the gel is done on a Schoeffel Gel Scanning apparatus, model SD-3000. Each data point was maximized by physically moving the gel back and forth to account for any lateral drift in the electrophoresis process.

The gel was scanned with the monochrometer set at 310 mu and maximized for each gel system. The fluorescence detector was set on reflectance mode and a Corning 3-69 filter placed between sample and detector.

Various controls were set as follows:

Function — positive

Ratio — numer

O.D. units — 0.1 to 4, as required.

Electronic filter — off

Gain — R, 6.5

Scan rate — typically 2

Time-dependent reaction of Col E1 DNA with Eco R1 enzyme at 37°C.

Preliminary run. DNA (20 ul, 0.268 ug) in standard buffer was reacted in time-dependent manner. Each sample was reacted five minutes longer than previous sample. The three forms of Col E1 DNA are evident in the gel photograph (Fig. 32). This data was not developed further as reaction was much too fast to obtain any rate. Reaction was complete in 10 minutes.

Time-dependent reaction of Col E1 DNA with Eco R1 enzyme at 19.5°C.

Preliminary run. Col E1 DNA (20 ul \times 12, 0.268 ug) in standard buffer was reacted in a time-dependent study. Samples were reacted in five minute time periods, each sample for five minutes longer than the previous sample. Enzyme (0.04 units) was introduced into reaction mixture at time zero; all samples were taken from the same reaction mixture. Gel photograph (Fig. 33) indicates reaction is complete in 50 minutes.

Time-independent reaction Col E1 DNA with Eco R1 enzyme at 15°C.

Preliminary run to determine Michealis-Menton conditions. Col E1 DNA (10 \times 42 ul) was reacted with a series of enzyme concentrations ranging from 10 \times to 0.3 \times enzyme concentrations used in actual assays. Time of each reaction was 15 minutes. Gel photograph (Fig. 35) indicates that most concentrations of enzyme under these conditions produce visible traces of all three expected forms of DNA. Data replot (Fig. 30)

Time-dependent reaction Col E1 DNA with Eco R1 enzyme at 15°C.

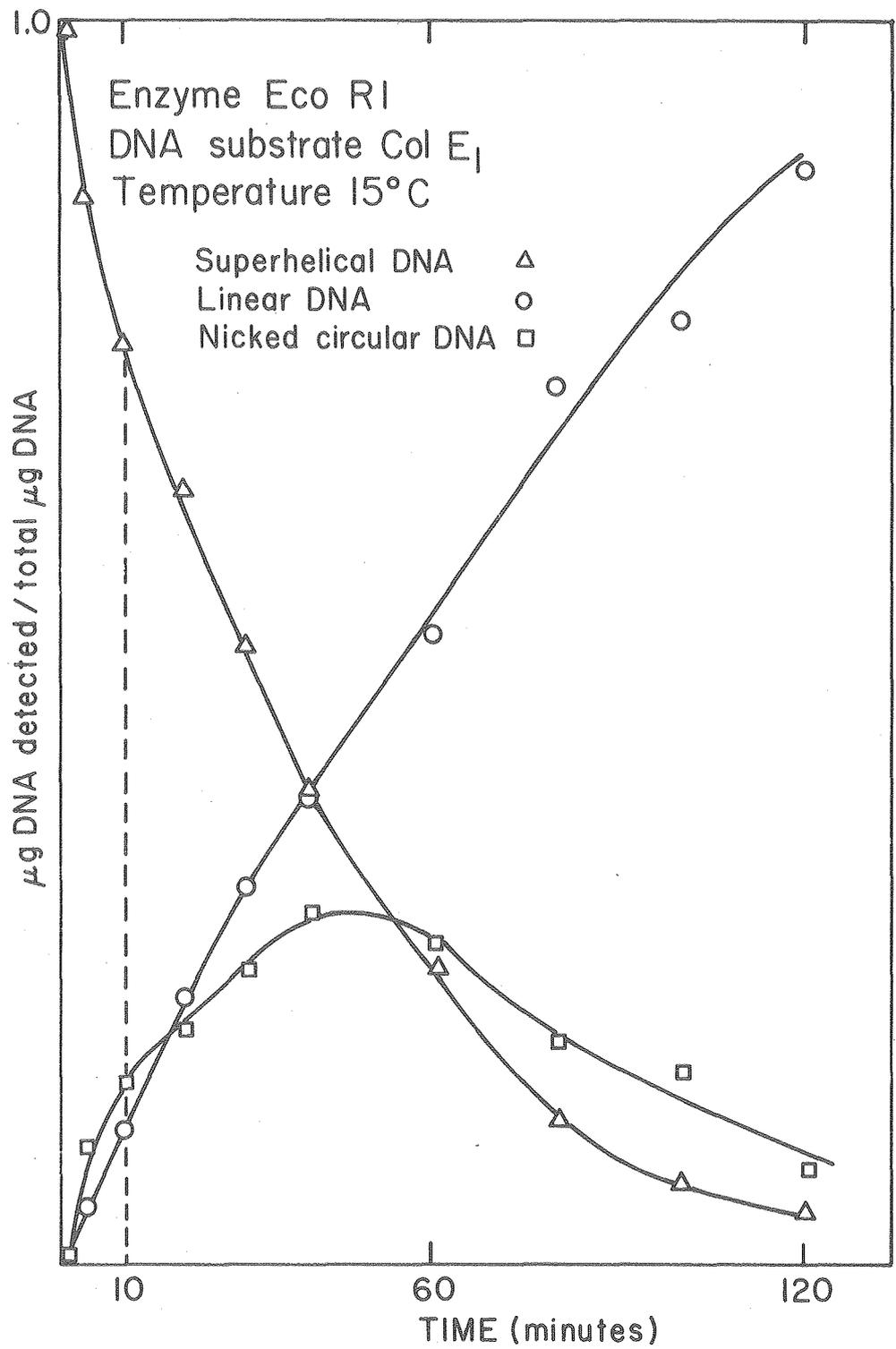
Preliminary run to determine Michealis-Menton conditions. Col E1 DNA (0.5 ml, 13 ug) in standard buffer and inoculated with Eco R1 enzyme (10 ul, 50 dilution). Reaction was run for 100 minutes and samples (20 ul) were removed at time 0, 2, 5, 10, 15, 20, 30, 40, 50, 60, 80, and 100. Replot (Fig. 29) shows slope of rate of loss of form I DNA to be nearly linear in region of 0 to 15 minutes. Electrophoresis and scanning done according to standard conditions. Gel photograph (Fig. 34).

Determination of V_{max} for Eco R1 with Col E1 DNA at 15°C.

Preliminary run to determine V_{max} for very short time periods, $t = 5$ minutes. Col E1 DNA (conc. 10, 10, 20, 20, 30, 20, 30) was reacted with Eco R1 DNA (10 ul std. dilution) in a staggered series 30 seconds between points. The total time for each point was five minutes. Samples were maintained at 15° in a constant temperature bath and all conditions were standard conditions. Data is initially calculated in terms of ug/min and converted to pmoles/min. Replot of data is shown in Fig. 29 and gel photograph in Fig. 31.

Interaction of d-pG with Eco R1 restriction enzyme and Col E1 substrate.

Col E1 DNA was diluted in buffer (std. storage buffer) to the following concentrations (Table 11) providing duplicates of each concentration. Inhibitor (d-pG) was added (10 ul, 7 mg/ml) to provide a final concentration of 1 mg/ml after inclusion of enzyme. The DNA/inhibitor mixture was stored at 15° until addition of enzyme. Enzyme (10 ul, std.) was added according to general assay conditions and each sample was



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Figure 29.

Time-dependent reaction Eco RI with Col E₁ at 15°.

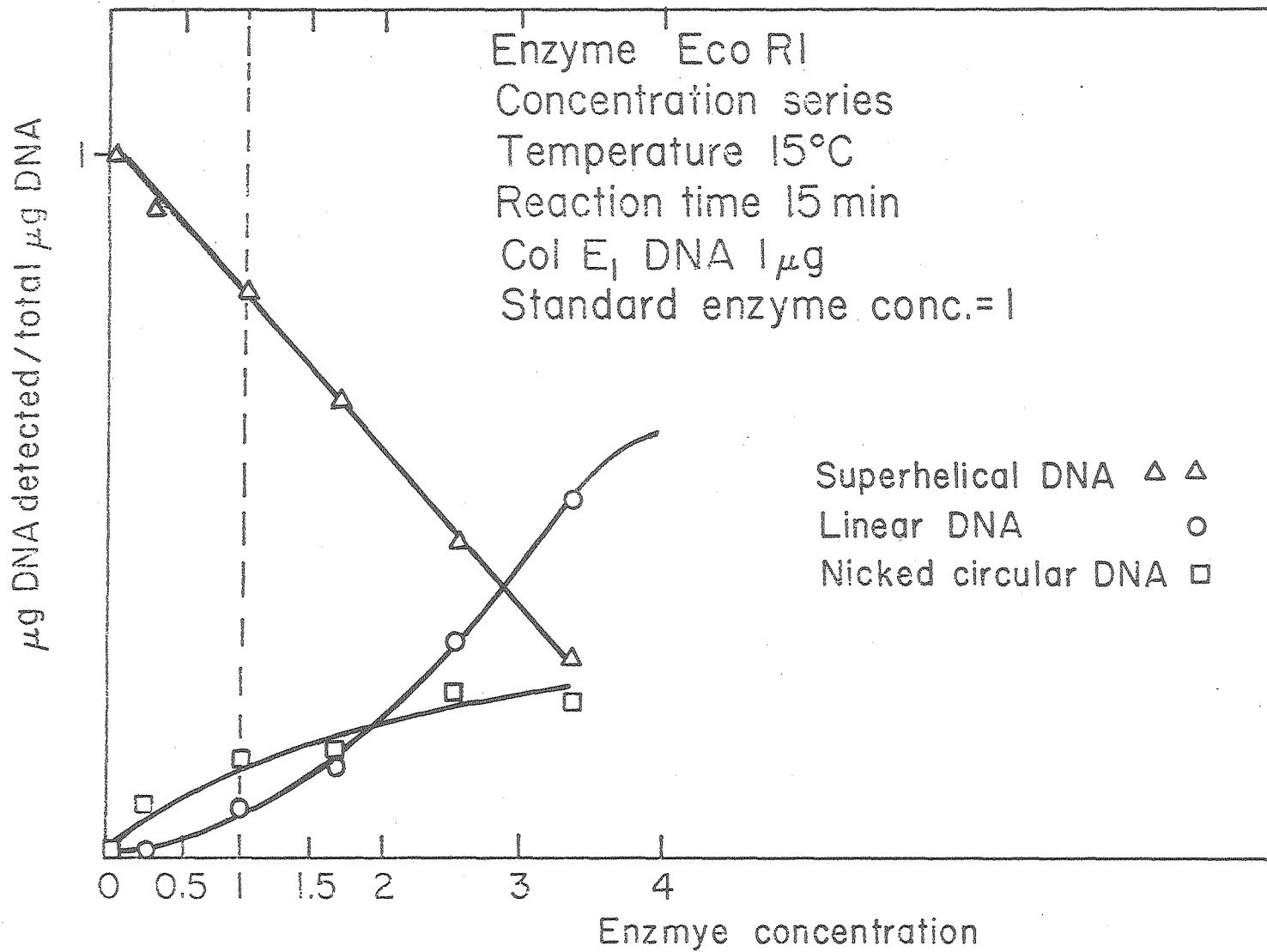
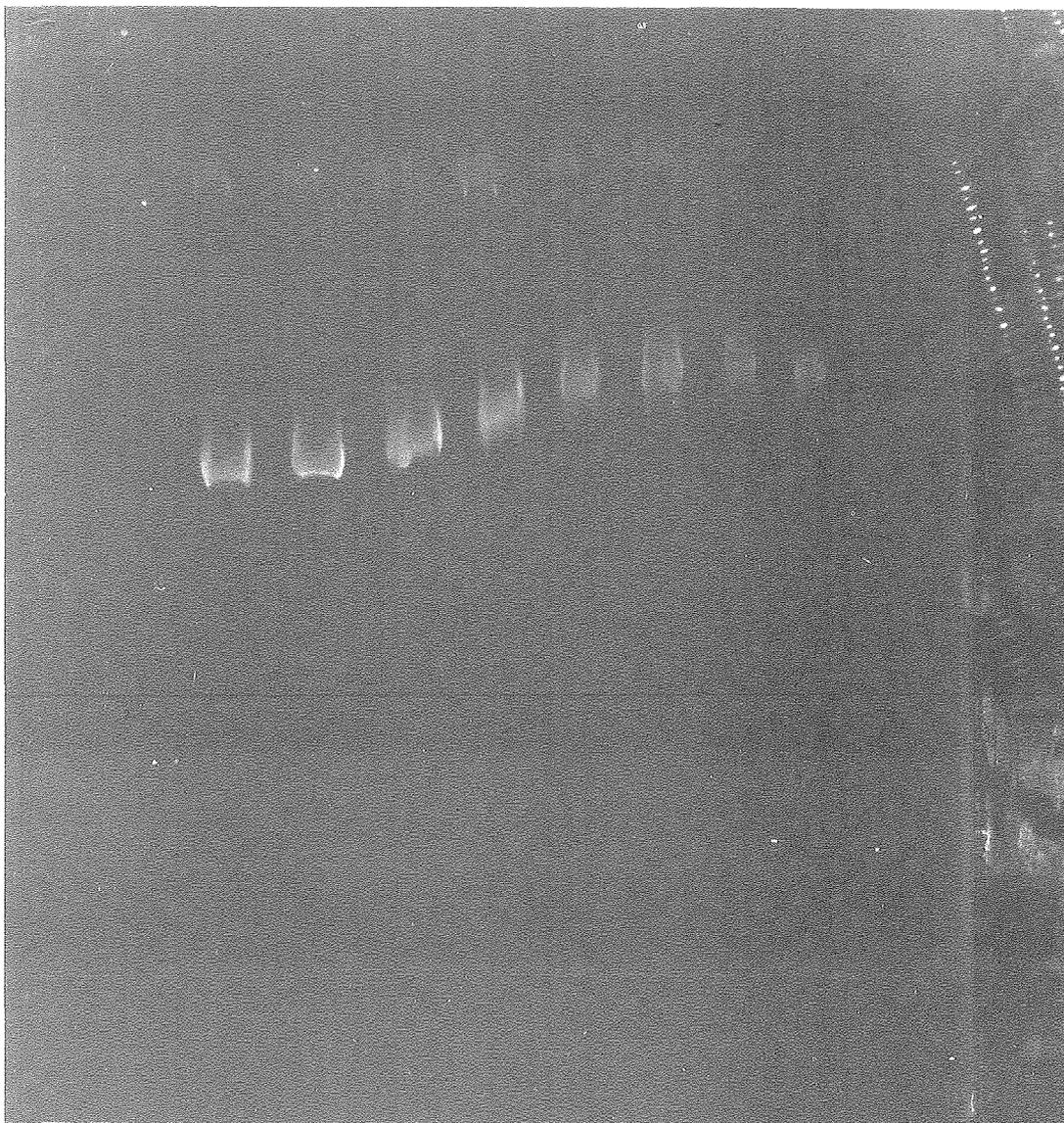


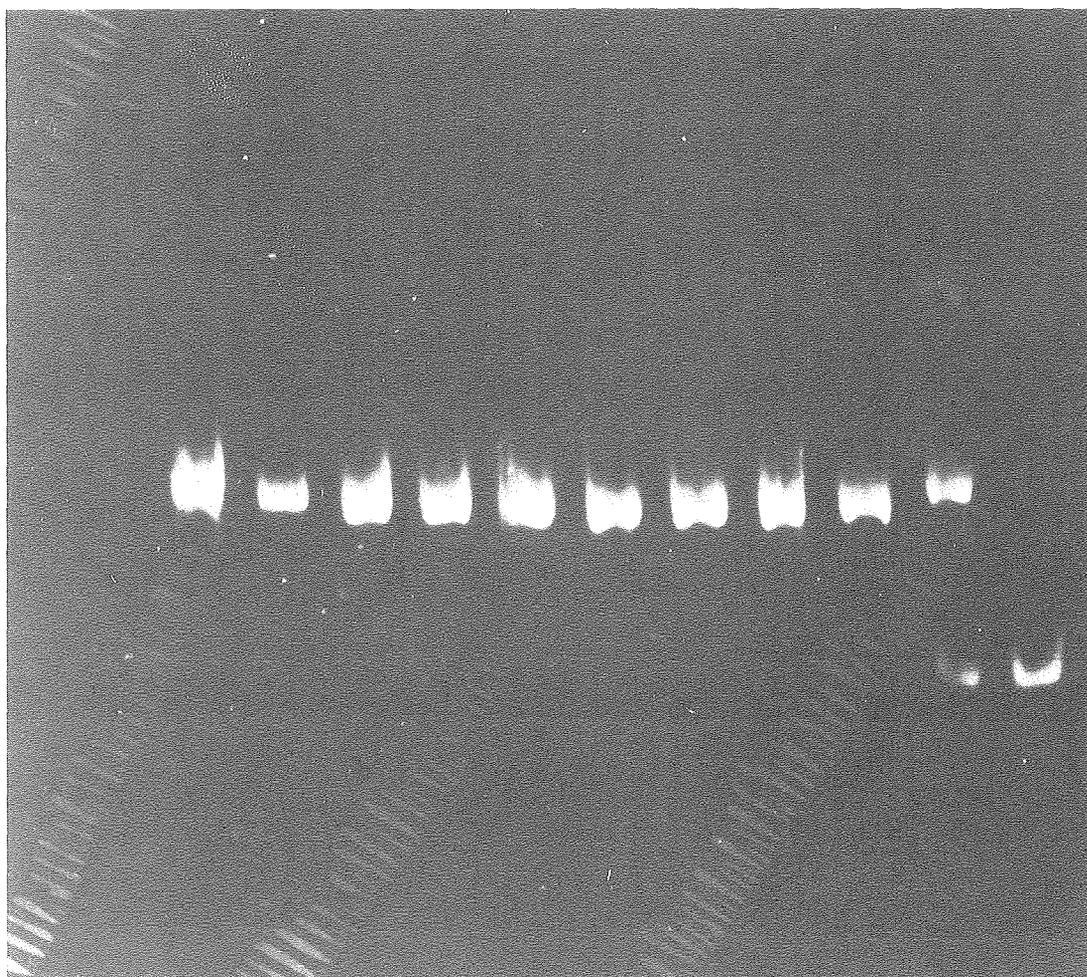
Figure 30. Enzyme concentration studies.



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Figure 31.

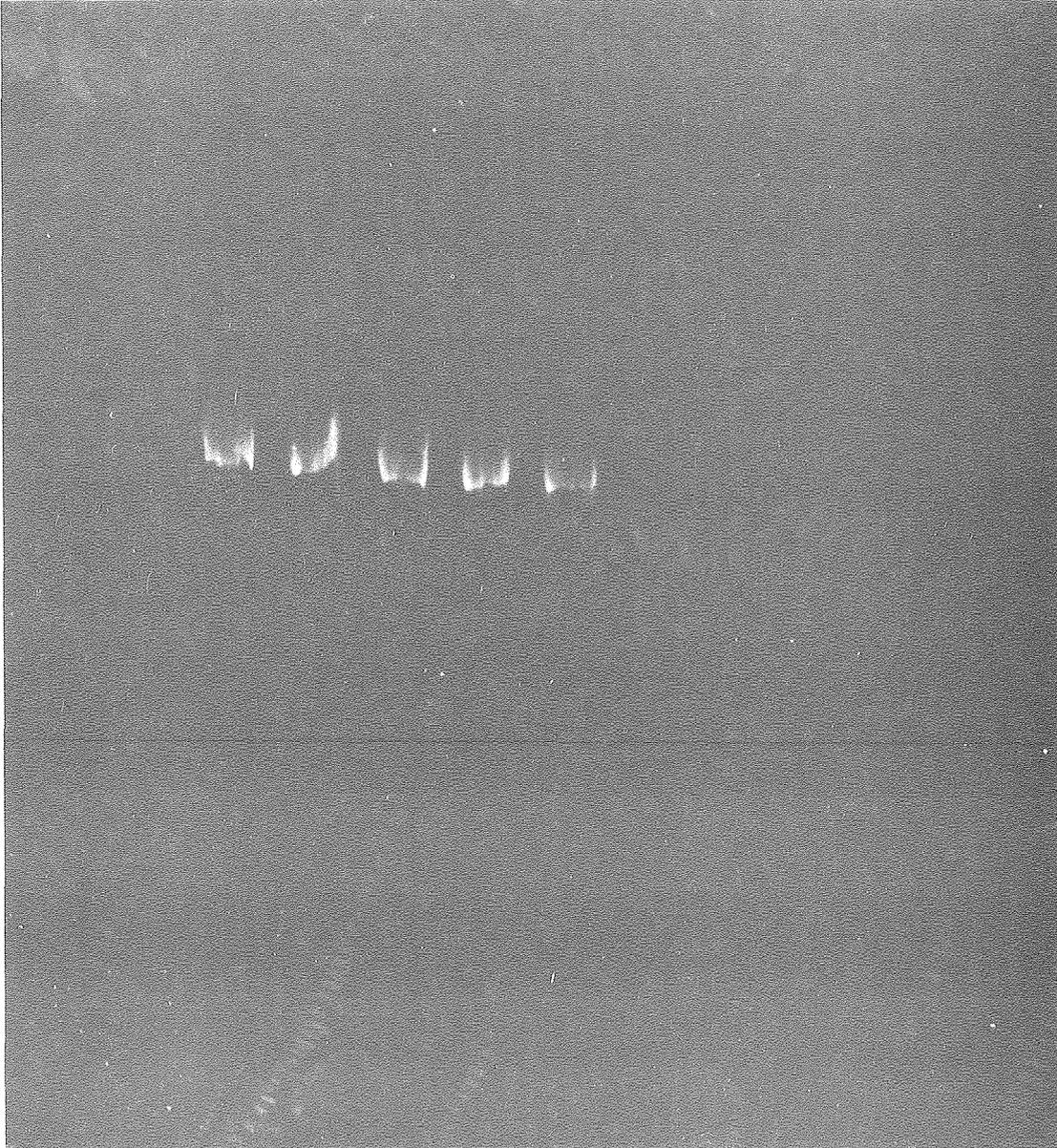
Gel photograph, determination of V_{\max} at 15° .



XBB 782-1846

Figure 32.

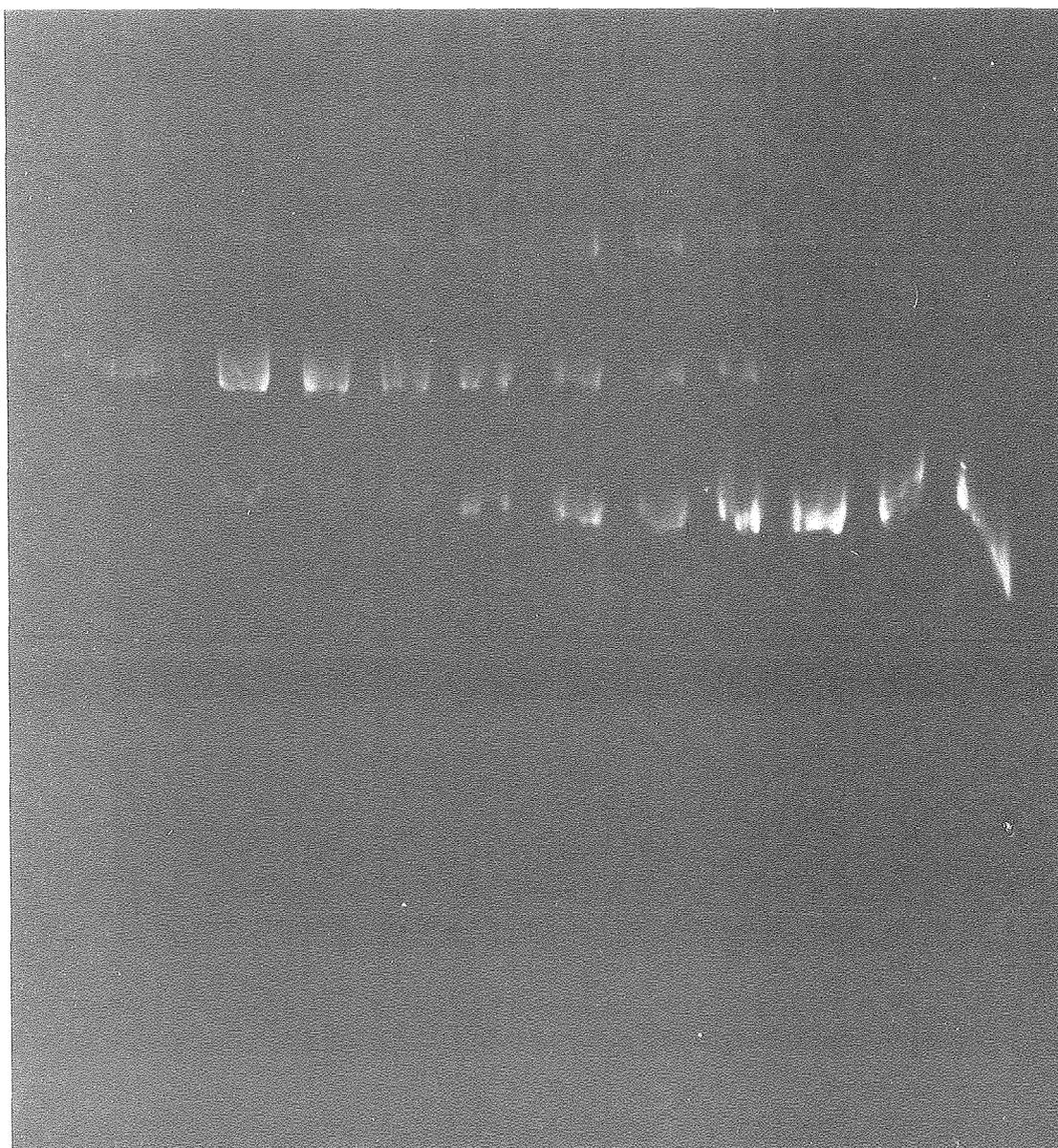
Gel photograph, enzyme reaction at 37°.



XBB 782-1847

Figure 33.

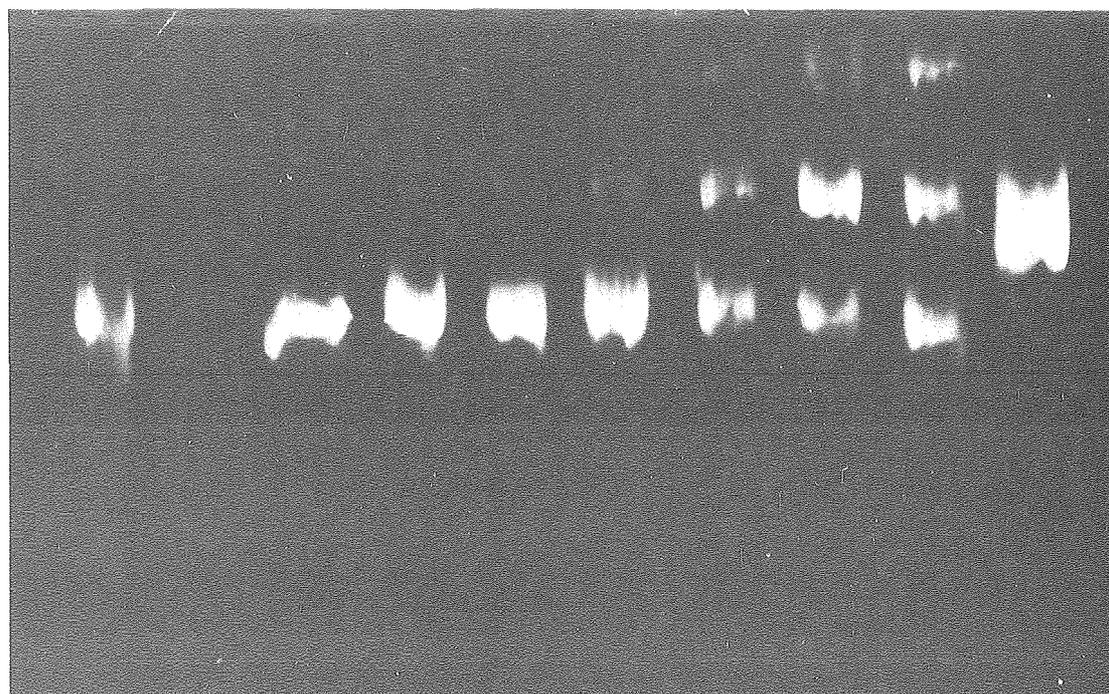
Gel photograph, enzyme reaction at 19.5°.



XBB 782-1848

Figure 34.

Gel photograph, enzyme reaction at 15°.



XBB 782-1858

Figure 35.

Gel photograph, enzyme concentration study at 15°.

stopped after ten minutes and placed in the electrophoresis apparatus. Electrophoresis was run for 20 hrs and the gel treated according to general scanning conditions. Results: gel photograph (Fig. 37) and replot (Fig. 36), amounts of each type of DNA formed shown in Table 11.

Interaction of d-Gp with Eco R1 restriction enzyme and Col E1 substrate.

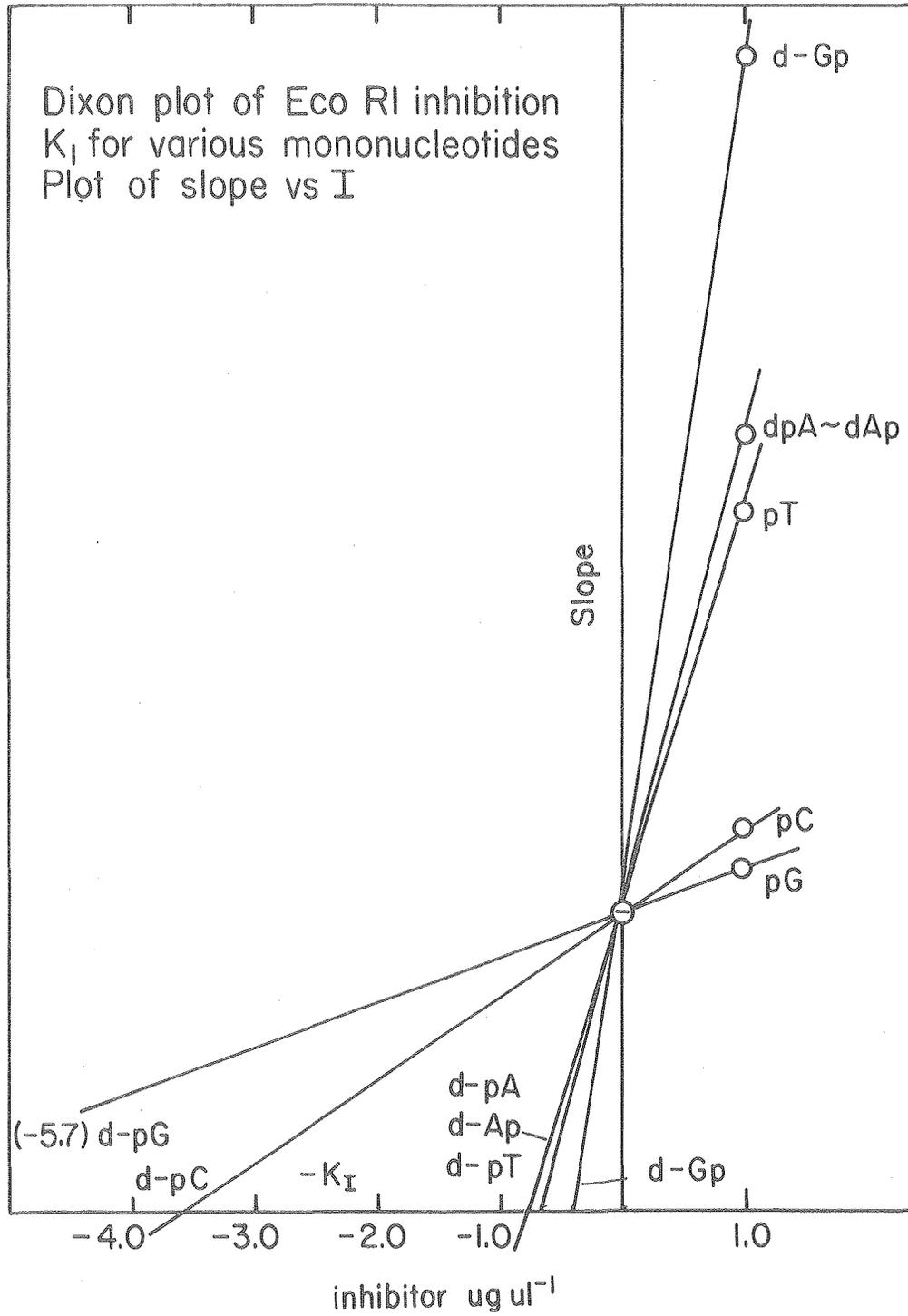
Col E1 DNA was diluted in standard buffer to the concentrations shown in Table 12 to provide duplicates of each concentration. Inhibitor (d-Gp) was added (10 ul, 7 mg/ml) to provide a final concentration of 1 mg/ml after inclusion of enzyme. The DNA inhibitor mixture was stored at 15°C until addition of enzyme.

Enzyme (10 ul, standard dilution) was added according to general assay conditions and each reaction was stopped after 5 minutes and placed in the electrophoresis apparatus. Electrophoresis was run for 25 hrs and the gel treated according to general scanning conditions. Results: gel photograph (Fig. 38), replot (Fig. 36), and data from gel scan (Table 12).

Interaction of d-pA with Eco R1 restriction enzyme and Col E1 DNA.

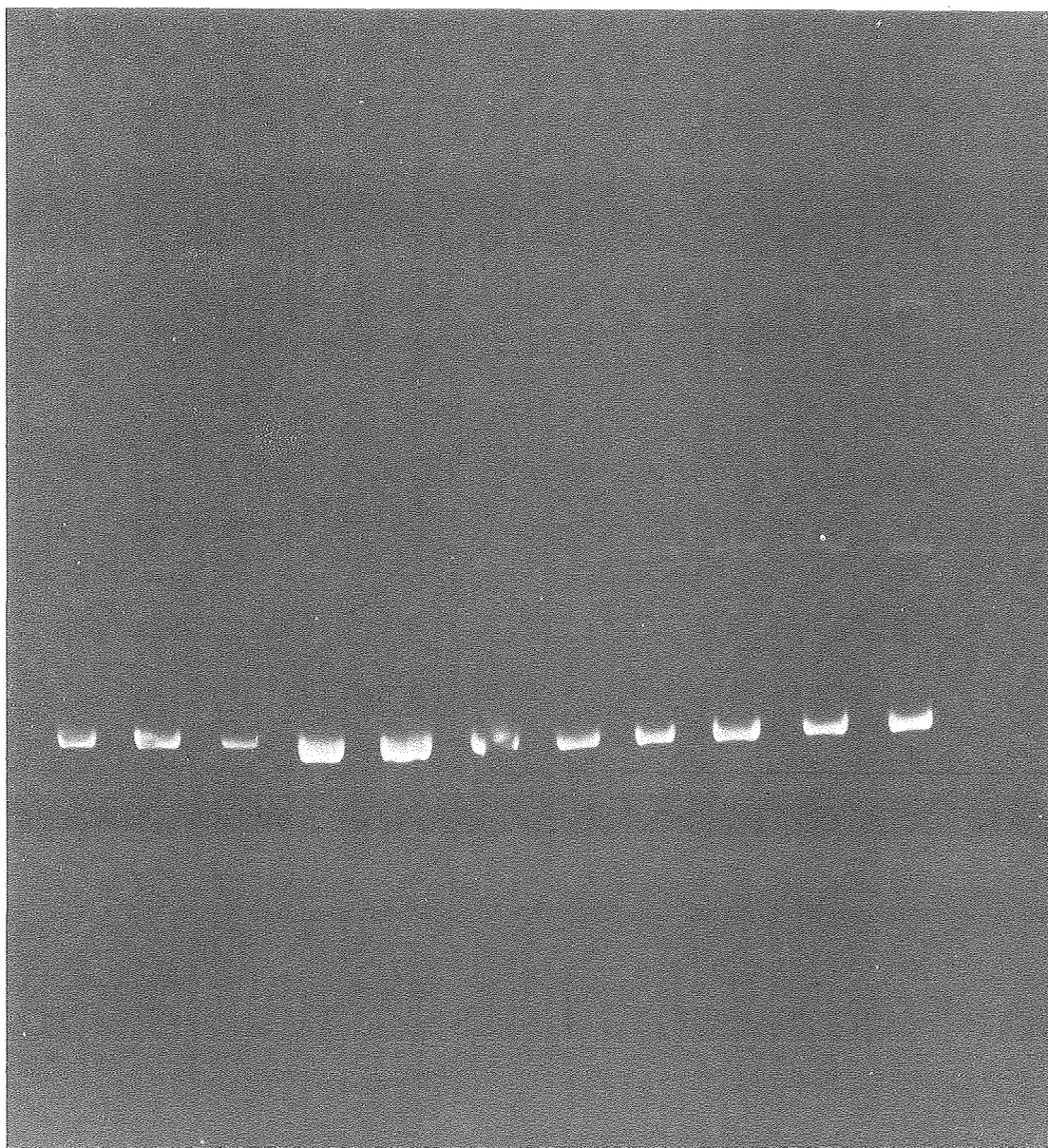
Col E1 DNA was diluted in standard buffer to the concentrations shown in Table 13 providing duplicates of each concentration. Inhibitor (d-pA) was added (10 ul, 7 mg/ml) to provide a final concentration of 1 mg/ml after addition of enzyme. The DNA inhibitor mixture was stored at 15°C until addition of enzyme.

Enzyme (10 ul, standard dilution) was added according to general assay conditions and each reaction was stopped after 10 minutes and placed in the electrophoresis apparatus. Electrophoresis was run according to standard scanning conditions. Results: gel photograph (Fig. 39) replot (Fig. 36), and data from gel scan (Table 13).



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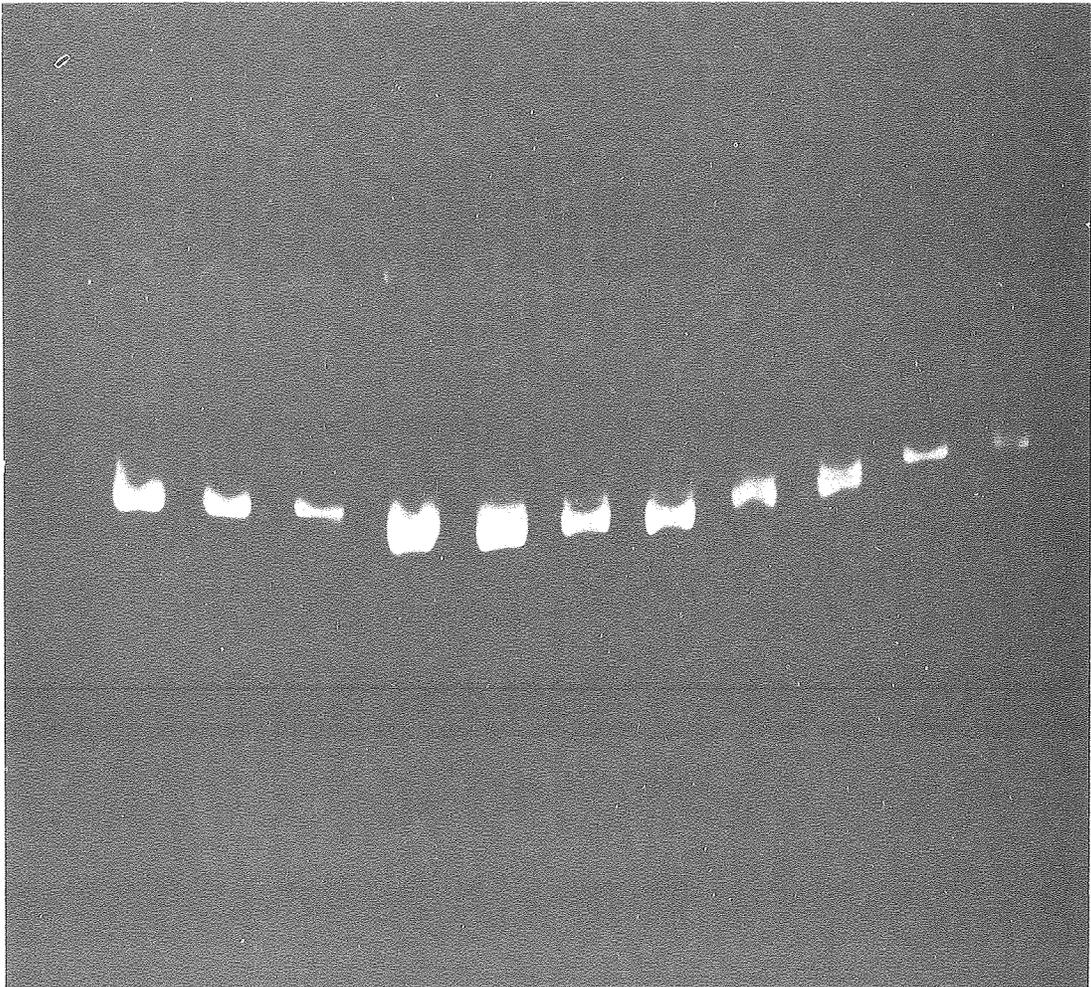
Figure 36. Inhibition of Eco RI by mononucleotides.

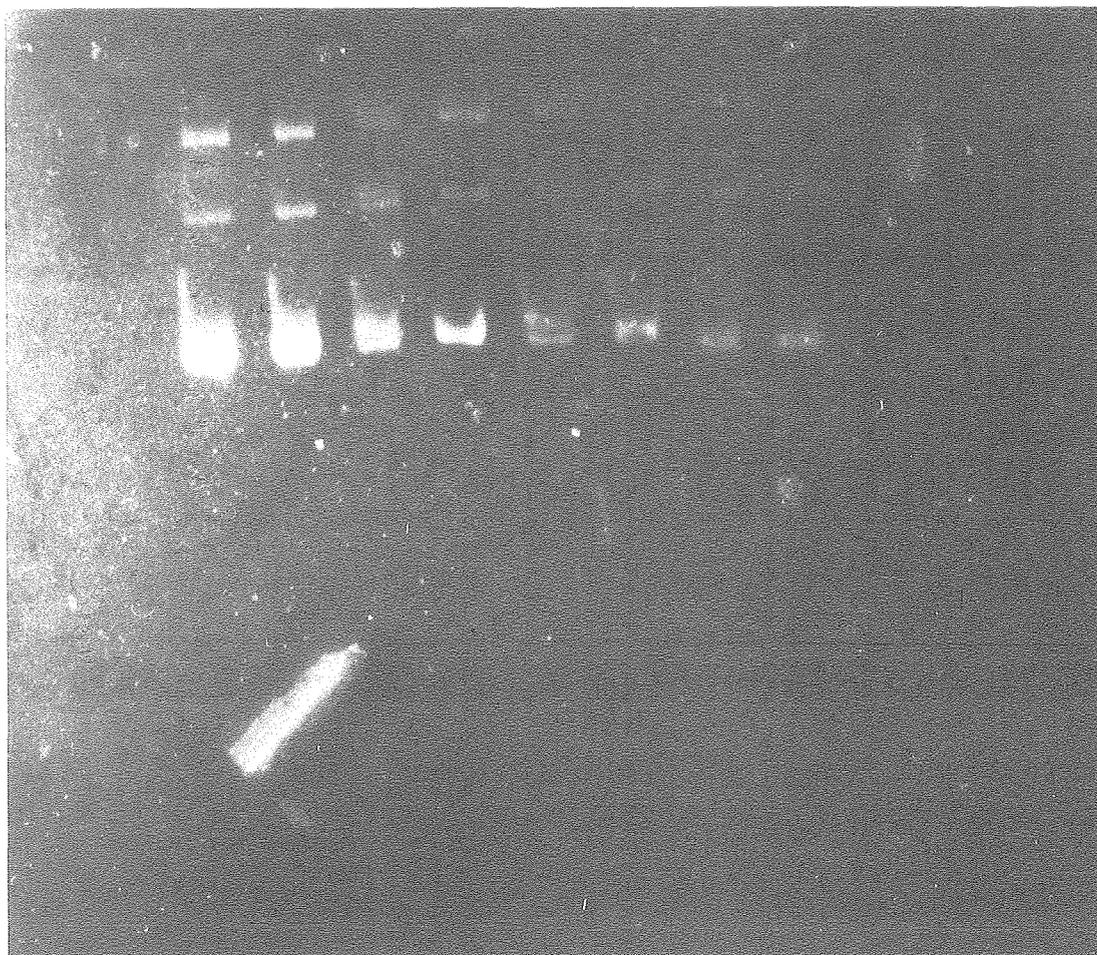


XBB 782-1834

Figure 37.

Inhibition of Eco R1 by d-pG.





XBB 782-1844

Figure 39.
Inhibition of Eco R1 by d-pA.

TABLE 11. Mononucleotide d-pG.

Sample #	Concentration DNA (ug/ml in 50 ul)	Total ug DNA	Amount Converted (form II + form III)
1	10	0.5	0.194
2	10	0.5	0.174
3	15	0.75	0.250
4	15	0.75	0.236
5	20	1.0	0.317
6	20	1.0	0.299
7	40	2.0	0.474
8	40	2.0	0.381

Concentration d-pG = 1 mg/ml.

Temperature 15°C, time of reaction 15 minutes.

TABLE 12. Mononucleotide d-Gp.

Sample #	Concentration DNA (ug/ml in 50 ul)	Total ug DNA	Amount Converted (form II + form III)
1	10	0.5	0.06
2	10	0.5	0.059
3	15	0.75	0.83
4	15	0.75	0.87
5	20	1.0	--
6	20	1.0	--
7	40	2.0	0.230
8	40	2.0	0.225
9*	10	0.5	0.146
10*	15	0.75	0.170
11*	20	1.0	0.40

Concentration d-Gp = 1 mg/ml.

Temperature = 15°C, time of reaction 15 minutes.

Interaction of d-Ap with Eco R1 restriction enzyme and Col E1 DNA.

Col E1 DNA was diluted in standard buffer to the concentrations shown in Table 14 providing duplicates of each concentration. Inhibitor (d-Ap) was added (10 ul, 7 mg/ml) to provide a final concentration of 1 mg/ml after addition of enzyme. The DNA-inhibitor mixture was stored at 15°C until addition of enzyme.

Enzyme (10 ul, standard dilution) was added according to general assay conditions and each reaction was stopped after 5 minutes. The samples (50 ul) were transferred to the electrophoresis apparatus where they were run for 20 hrs, and the gel removed and treated according to general scanning conditions. Results: gel photograph (Fig. 40), replot (Fig. 36), and data from gel scan (Table 14).

Interaction of d-pT and d-pC with Eco R1 restriction enzyme and Col E1 DNA.

Col E1 DNA was diluted in standard buffer to the concentrations in Tables 15 and 16 providing duplicates of each concentration. The inhibitor (d-pT or d-pC) was added (10 ul, 7 mg/ml) to provide a final inhibitor concentration of 1 mg/ml after the addition of enzyme. The DNA inhibitor mixture was stored at 0° until equilibration for enzyme reaction at 15°C.

Enzyme (10 ul, standard dilution) was added according to standard assay conditions and each reaction was stopped after 10 minutes \pm 5 seconds with addition of SDS/glycerol/bromophenol blue and chilling to 0°C. Samples (50 ul \pm 1 ul) were transferred to the electrophoresis gel and run for 20 hrs according to standard electrophoresis conditions. The gel was removed and treated according to standard scanning conditions. Results: gel photographs (Figs. 41,42) and replot (Fig. 36).

TABLE 13. Mononucleotide d-pA.

Sample #	Concentration DNA (ug/ml in 50 ul)	Total ug DNA	Amount converted (form I + form III)
1	--	--	--
2	--	--	--
3	4	0.2	0.03
4	4	0.2	0.045
5	6	0.3	--
6	6	0.3	--
7	10	0.5	0.085
8	10	0.5	0.086
9	20	1.0	0.155
10	20	1.0	0.140

Concentration d-pA = 1 mg/ml, temperature 15°C, time 15 minutes.

TABLE 14. Mononucleotide d-Ap.

Sample #	Concentration DNA (ug/ml in 50 ul)	Total ug DNA	Amount converted (form II + form III)
1	10	0.5	0.043
2	10	0.5	0.046
3	15	0.75	0.134
4	15	0.75	0.124
5	20	1.0	--
6	20	1.0	0.174
7	40	2.0	0.38
8	40	2.0	0.40
9*	10	0.5	0.147
10*	15	0.75	0.218
11*	20	1.0	0.260

Concentration d-Ap = 1 mg/ml, temperature 15°C, time 15 minutes.

TABLE 15. Mononucleotide d-pC.

Concentrations used: in duplicate, 6, 10, 20, 40 ug/ml.

Reference blank (no inhibitor), 6, 10, 20, 40 ug/ml.

Temperature = 15°C.

Time of reaction = 15 minutes.

Concentration d-pC = 1 mg/ml.

TABLE 16. Mononucleotide d-pT.

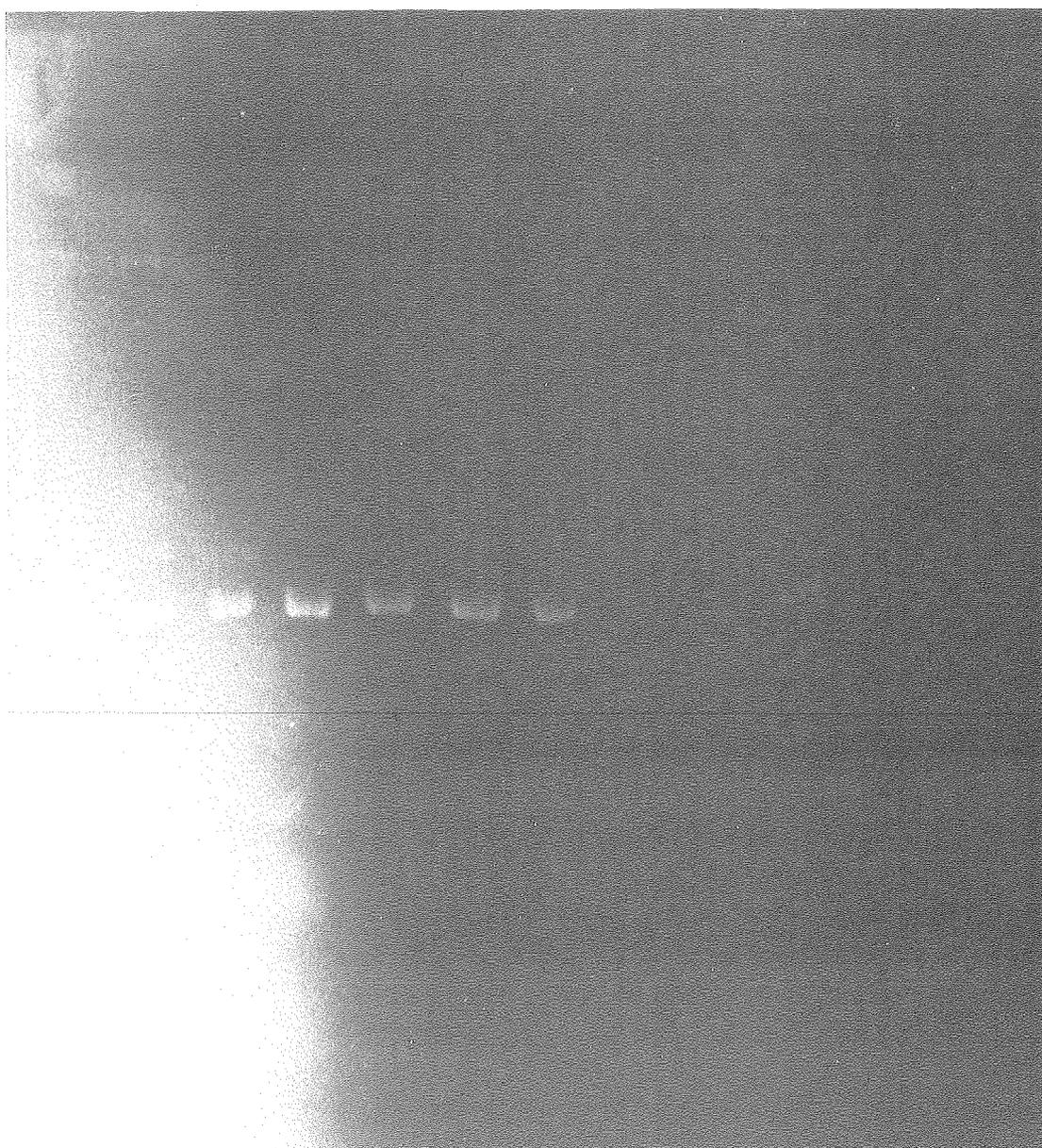
Concentrations used: in duplicate, 6, 10, 20, 40 ug/ml.

Reference blank (no inhibitor), 6, 10, 20, 40 ug/ml.

Concentration d-pT = 1 mg/ml.

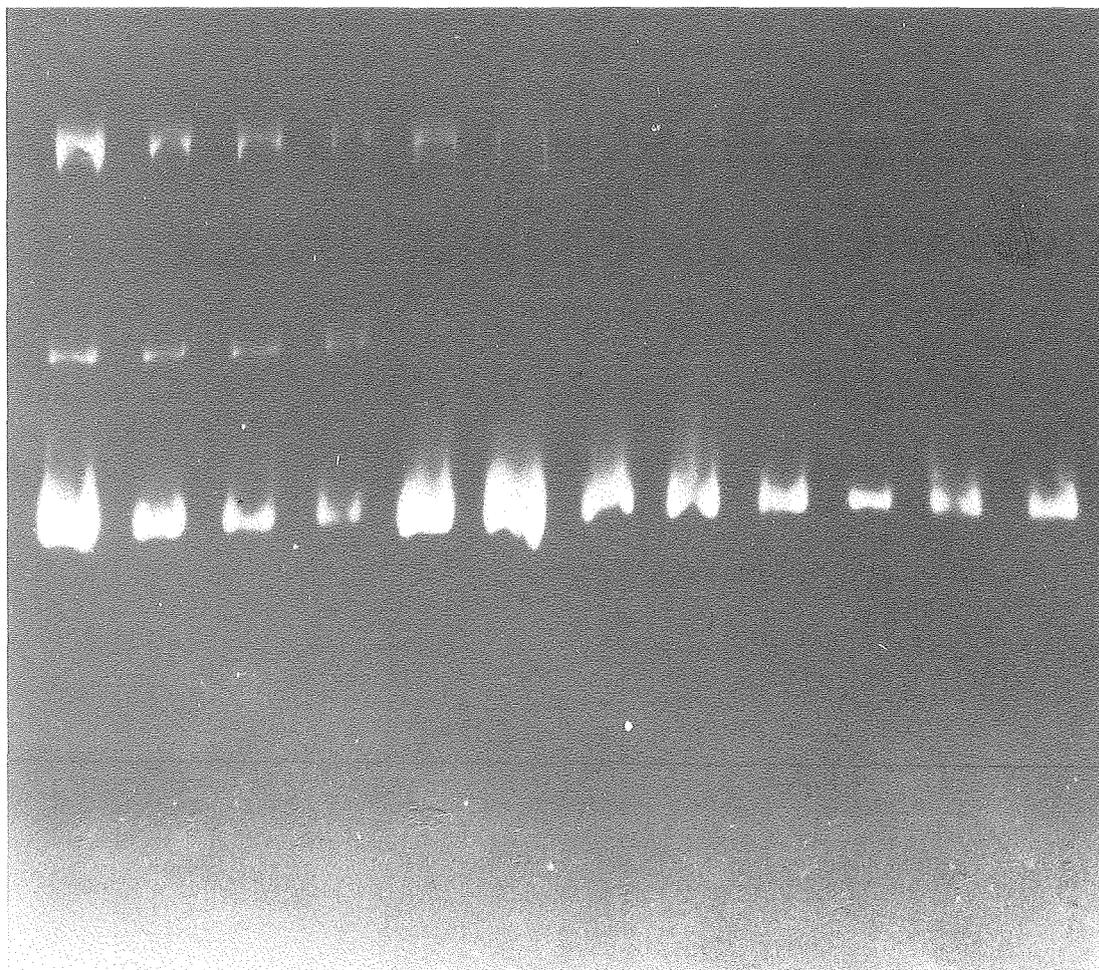
Time of reaction = 15 minutes.

Temperature = 15°C.



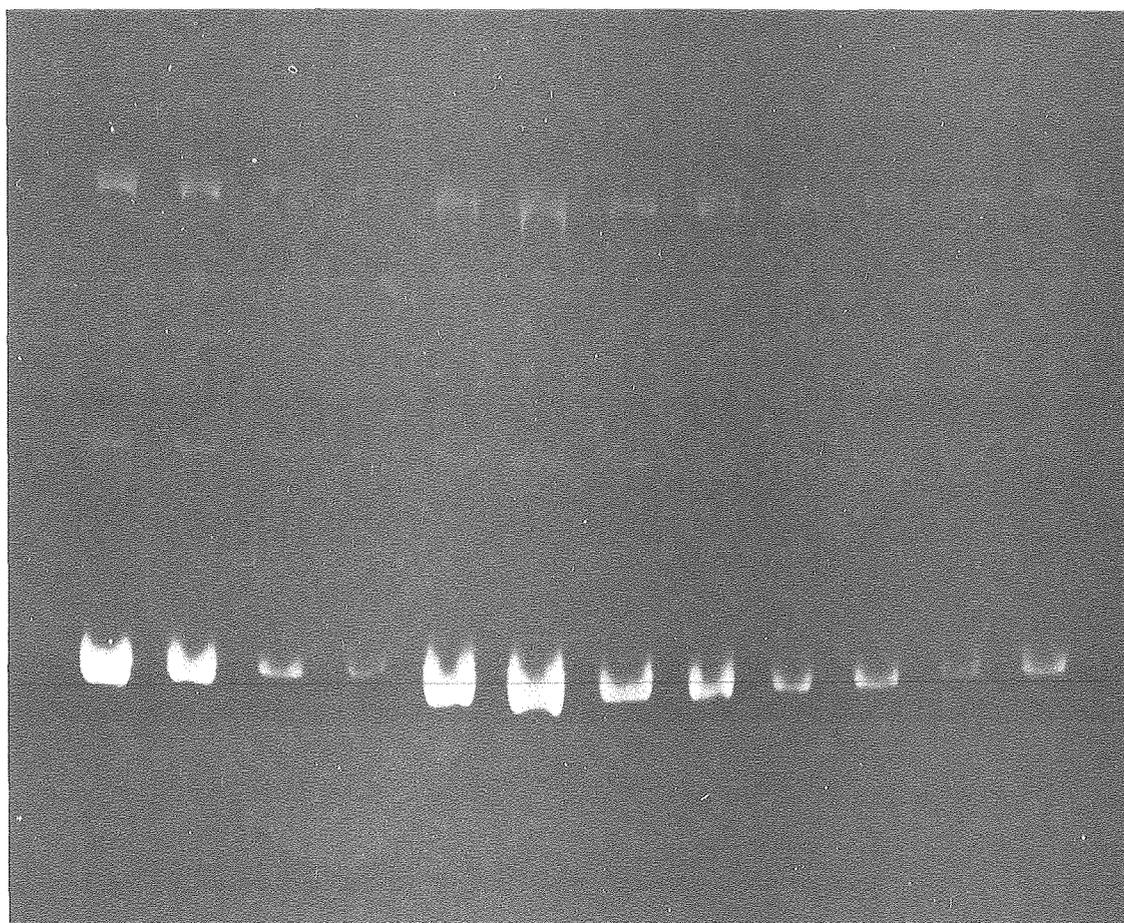
XBB 782-1827

Figure 40.
Inhibition of Eco R1 by d-Ap.



XBB 782-1838

Figure 41.
Inhibition of Eco RI by d-pT.



XBB 783-3193

Figure 42.

Inhibition of Eco R1 by d-pC.

Interaction of Eco R1 enzyme with d-pGpX dinucleotides and Col E1 substrate.

Preliminary runs. A series of dinucleotides: d-pGpA, d-pGpT, d-pGpC, and d-pGpG (0.5 mg/ml) were reacted in duplicate with Col E1 substrate and Eco R1 enzyme. DNA concentration was 20 ug/ml (50 ul) for each experiment. Each run was done under the same conditions (temperature was 15°C, time was 15 minutes). All procedures, conditions of electrophoresis, and gel scanning are described under standard conditions.

Results: d-pGpA under these conditions and concentrations totally inhibited the enzyme system as indicated by the gel photograph (Fig. 43). All the other dinucleotides inhibited the enzyme reaction to some extent. The calculation of inhibition constants could not be made from this data and that information is derived from later experiments (Table 17).

Interaction of Eco R1 enzyme with d-pCpX dinucleotides and Col E1 substrate.

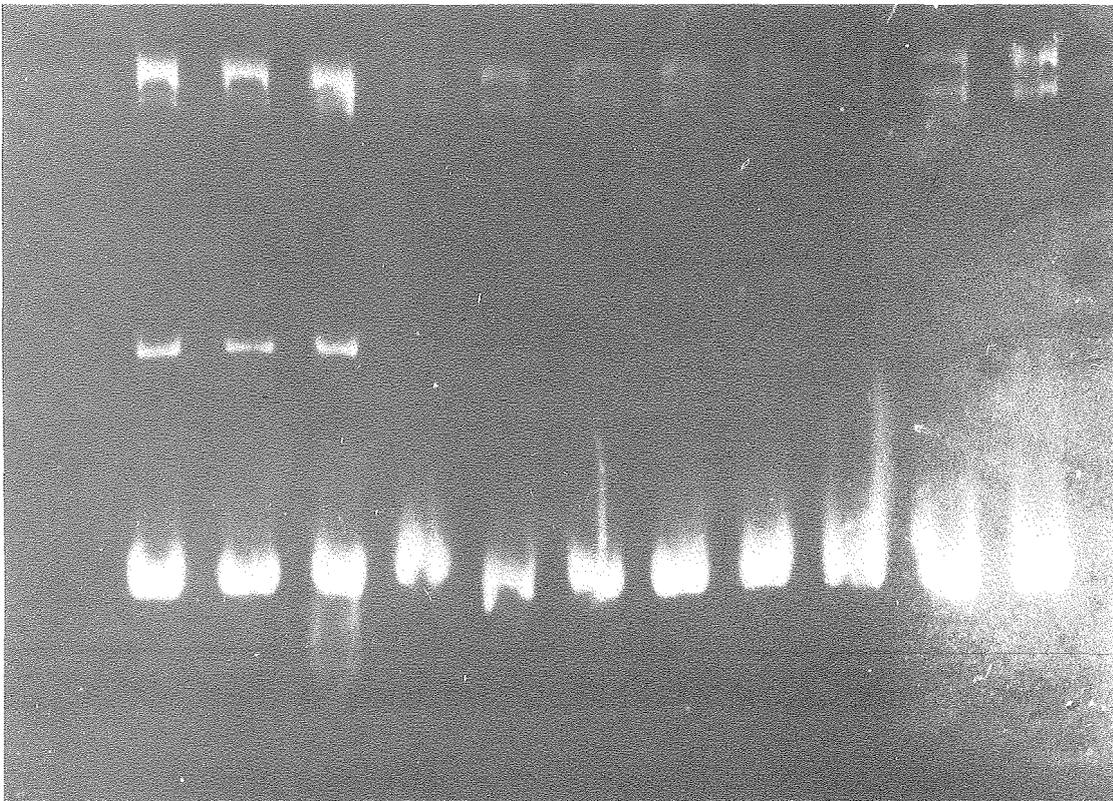
Preliminary runs. A series of dinucleotides: d-pCpA, d-pCpT, d-pCpG, and d-pCpC (1 mg/ml) were reacted with Col E1 DNA and Eco R1 enzyme. The DNA concentration was 15 ug/ml (50 ul) for each experiment. Each run was maintained for 15 minutes at 15°C. All procedures are described under standard conditions.

Results: All four dinucleotides inhibited the enzyme reaction under these conditions of temperature and concentration (Fig. 44). The one anomaly appears to be d-pCpG which exhibited moderate inhibition but seemed to specifically inhibit the formation of linear (form III) DNA during the reaction (Table 17).

TABLE 17. Inhibition of Eco R1 restriction enzyme by dinucleotides, preliminary studies.

Dinucleotide	Concentration (mg/ml)	Percent Inhibition	Substrate
d-pGpA	0.5	100	Co1 E1 DNA
d-pGpC	0.5	80	Co1 E1 DNA
d-pGpG	0.5	80	Co1 E1 DNA
d-pGpT	0.5	80	Co1 E1 DNA
d-pCpA	1.0	80	Co1 E1 DNA
d-pCpC	1.0	80	Co1 E1 DNA
d-pCpG	1.0	70 *	Co1 E1 DNA
d-pCpT	1.0	80	Co1 E1 DNA

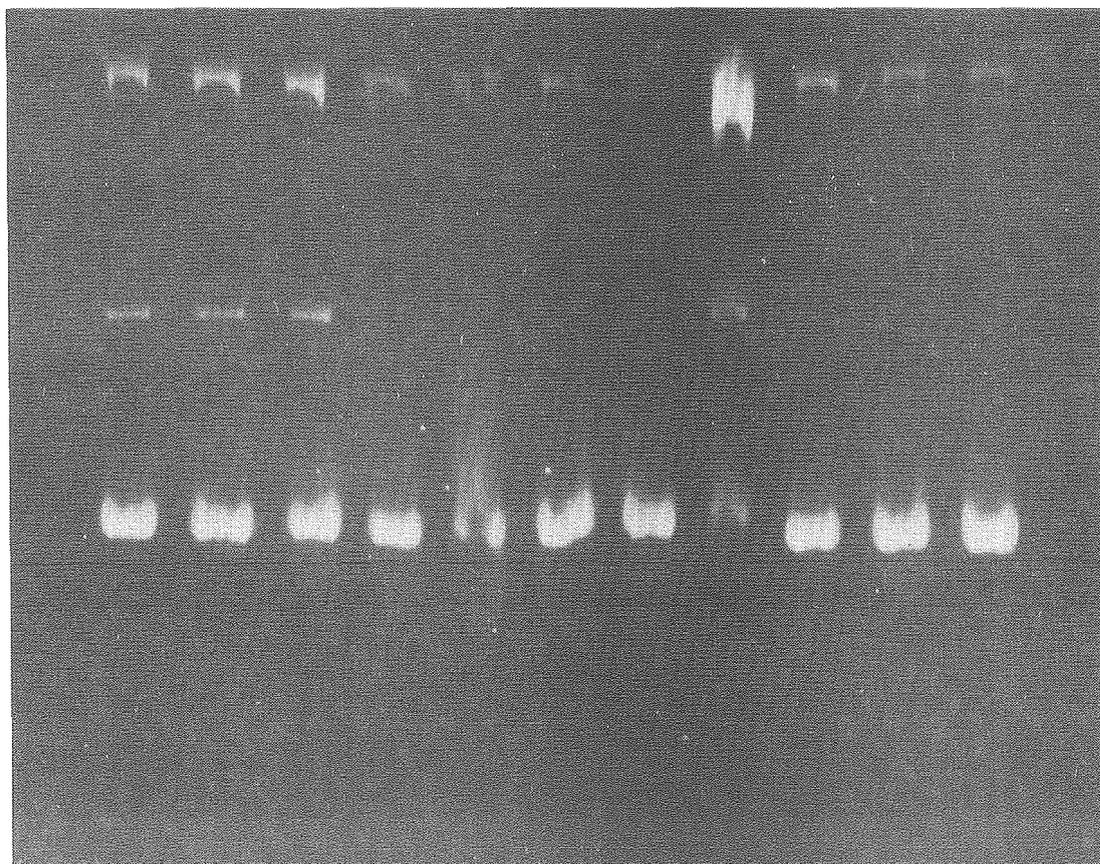
* Inhibits step II over step I, i.e., buildup of nicked circular DNA.



XBB 782-1856

Figure 43.

Gel photograph of d-pGpX dinucleotide interaction.



XBB 782-1857

Figure 44.

Gel photograph of d-pCpX dinucleotide interaction.

Interaction of dinucleotides with Eco R1 restriction enzyme and Col E1 DNA.

General assay conditions for all dinucleotide experiments. Col E1 DNA was diluted in standard buffer to the concentrations shown under each individual section. Runs were done in duplicate or triplicate as indicated individually. Duplicates or triplicates were run with 30-second staggering between reactions. Each individual reaction was run for 10 minutes \pm 5 seconds before termination with SDS/glycerol/bromophenol blue and chilled to 0°C.

Enzyme was added (10 ul, standard dilution) according to general assay conditions and the reaction terminated as above. Samples (5 ul) were transferred to the electrophoresis apparatus according to standard methods and run for 20 hrs under standard conditions.

Interaction of dinucleotide d-pGpA with Eco R1 restriction enzyme.

The dinucleotide d-pGpA (350 ug/ml, 10 ul) was diluted (70 ul) following concentrations of Col E1 DNA substrate: 10, 15, 20, 30 ug/ml. All samples were done in duplicate and two blanks were run at 15 ug/ml without any dinucleotide. Experiment was run according to standard conditions described earlier. Final concentration of d-pGpA was 50 ug/ml. Gel photograph (Fig. 46) shows results of inhibition study which is interpreted quantitatively in Table 12, the slope replot in Fig. 53.

Interaction of dinucleotide d-pApG with Eco R1 restriction enzyme.

The dinucleotide d-pApG (50 ug/ml) was diluted (60 ul) with the following concentrations of Col E1 DNA substrate: 10, 15, 20, and 30 ug/ml (50 ul in duplicate). The final concentration of dinucleotide was

50 ug/ml after addition of enzyme (10 ul). Three blanks were run with DNA concentrations of 10, 15, and 20 ug/ml with no inhibitor. Gel photograph (Fig. 47) shows results of inhibition study which is interpreted quantitatively in Table 18 and the slope replot in Fig. 53.

Interaction of dinucleotide d-pApT with Eco R1 restriction enzyme.

The dinucleotide d-pApT (10 ul, 350 ug/ml) was diluted (60 ul) with the following concentrations of Col E1 DNA substrate: 10, 15, 20, and 30 ug/ml (50 ul) in duplicate. The final concentration of inhibitor was 50 ug/ml after addition of enzyme (10 ul). Three blanks were run with DNA concentrations of 15, 20 and 30 ug/ml in the absence of inhibitor. Each reaction was stopped after 15 minutes. Gel photograph (Fig. 45) shows results of inhibition study, which is interpreted quantitatively in Table 18 and Fig. 53, which shows the slope replot.

Interaction of dinucleotide d-pTpA with Eco R1 restriction enzyme.

Dinucleotide d-pTpA (350 ug/ml, 10 ul) was diluted (60 ul) with the following concentrations of Col E1 DNA substrate: 10, 15, 20, and 30 ug/ml (50 ul) in duplicate. The final concentration of inhibitor was 50 ug/ml after addition of enzyme (10 ul). Three blanks were run with DNA concentrations of 10, 15, and 20 ug/ml in the absence of inhibitor. Each reaction was terminated after 15 minutes in the standard manner. Gel photograph (Fig. 48) shows the results of inhibition study, which is interpreted quantitatively in Table 18 and the slope replot in Fig. 53.

Interaction of dinucleotide d-pTpC with Eco R1 restriction enzyme.

Dinucleotide d-pTpC (350 ug/ml, 10 ul) was diluted (60 ul) with the following concentrations of Col E1 DNA substrate: 10, 15, 20, and 30 ug/ml (50 ul) in duplicate. The final concentration of inhibitor was 50 ug/ml after addition of enzyme (10 ul). Two blanks were run with Col E1 DNA (20 ug/ml) in the absence of inhibitor. Each reaction was terminated after 10 minutes with the standard procedure. Gel photograph (Fig. 49) gives the results of the inhibition study, which is interpreted quantitatively in Table 18 and in the slope replot (Fig. 53).

Interaction of dinucleotide d-pCpT with Eco R1 restriction enzyme.

Dinucleotide d-pCpT (350 ug/ml, 10 ul) was diluted (60 ul) with the following concentrations of Col E1 DNA substrate: 10, 15, 20 and 30 ug/ml (50 ul) in duplicate. The final concentration of inhibitor was 50 ug/ml after addition of enzyme (10 ul). Three blanks were run with Col E1 DNA in the absence of inhibitor. Each reaction was terminated after 10 minutes by the standard procedure. Gel photograph (Fig. 50) shows the results of the inhibition study, which is interpreted in Table 18 and in the slope replot (Fig. 53).

Interaction of dinucleotide d-pApA with Eco R1 restriction enzyme.

The dinucleotide d-pApA (350 ug/ml, 10 ul) was diluted (60 ul) with the following concentrations of Col E1 DNA substrate: 10, 15, 20 and 30 ug/ml (50 ul) in duplicate. The final concentration of inhibitor was 50 ug/ml after addition of enzyme (10 ul). Three blanks were run with DNA concentrations of 15, 20, and 30 ug/ml in the absence of inhibitor. Each

reaction was terminated after 10 minutes in the standard manner. Gel photograph (Fig. 51) shows the results of the inhibition study, which is interpreted quantitatively in Table 18 and in the slope replot (Fig.53).

Interaction of dinucleotide d-pGpT with Eco R1 restriction enzyme.

The dinucleotide d-pGpT (350 ug/ml, 10 ul) was diluted (60 ul) with the following concentrations of Col E1 DNA substrate: 15, 20, and 30 ug/ml (50 ul) in duplicate. The final concentration of inhibitor was 50 ug/ml after addition of enzyme (10 ul). Four blanks were run with DNA concentrations of 15 and 20 ug/ml in duplicate in the absence of dinucleotide. Each reaction was terminated after 15 minutes in the standard manner. Gel photograph (Fig. 52) shows the results of the inhibition study which is interpreted quantitatively in Table 18 and the slope replot of Fig. 53.

Interaction of tetranucleotide d-GpApApT with Eco R1 restriction enzyme.

The tetranucleotide, d-GpApApT (350 ug/ml, 10 ul), was diluted (60 ul) with the following concentrations of Col E1 DNA substrate: 10, 15, 20 and 30 ug/ml (50 ul) in duplicate. The final concentration of inhibitor was 50 ug/ml after addition of enzyme (10 ul). A duplicate blank of 20 ug/ml DNA was run in the absence of inhibitor. Each reaction was terminated after 10 minutes by the usual procedure. The gel photograph (Fig. 56) shows the results of the inhibition study which is interpreted quantitatively in Table 19 and the slope replot of Fig. 54.

TABLE 18. Dinucleotide inhibition.

Dinucleotide	Slope	Intercept (V_{max})	Intercept (-ug/ml)	Coefficient
d-pApA	407	4	-0.01	0.998
d-pTpC	878	14	-0.02	0.87
d-pGpA	1466	2	-0.00024	0.99
d-pApG	1235	6	-0.005	0.94
d-pApT	1064	2	-0.0018	0.997
d-pTpA	exponential	18	--	0.97
d-pGpT	exponential	6	--	0.95
d-pCpT	899	9	-0.01	0.95

Interaction of hexanucleotide d-GpApApTpTpC with Eco R1 restriction enzyme.

The hexanucleotide d-GpApApTpTpC (350 ug/ml, 10 ul) was diluted (60 ul) with the following concentrations of Col E1 DNA substrate: 10, 15, 20, and 30 ug/ml (50 ul) in duplicate. The final concentration of inhibitor was 50 ug/ml after addition of enzyme (10 ul). Three blanks were run with DNA concentrations of 10, 15 and 20 ug/ml in the absence of inhibitor. Each reaction was terminated in the standard manner. The gel photograph (Fig. 57) shows the results of the inhibition study which is interpreted quantitatively in Table 19 and the slope replot (Fig. 54).

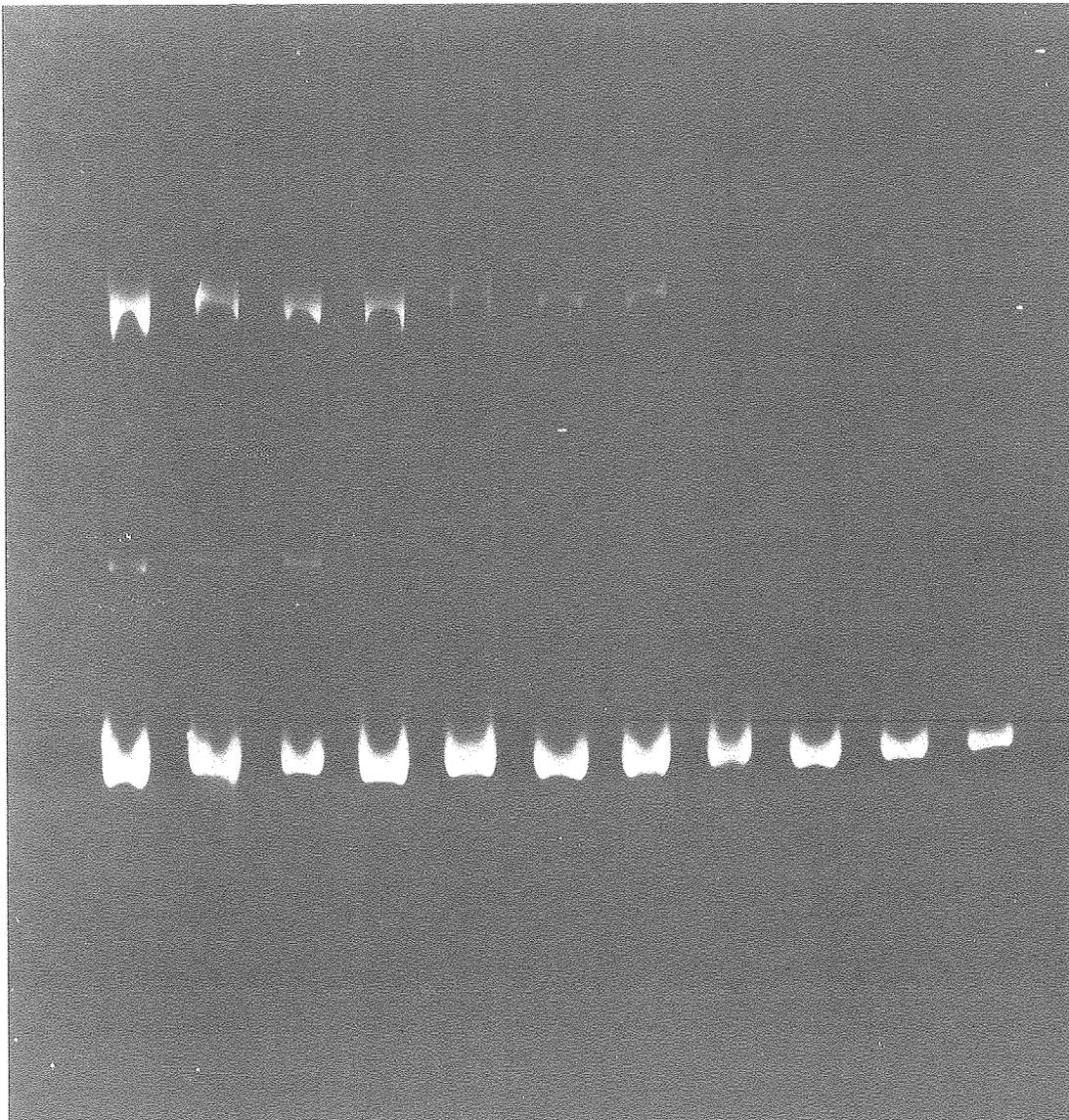
TABLE 19. Inhibition of Eco R1 restriction enzyme by tetra- and hexanucleotides.

Nucleotide (50 ug/ml) ^a	Slope ^b	Intercept (V _{max}) ^c	Intercept (ug/ml)	Coefficient
d-pApApTpT	exponential	17	--	0.94
--	1.7×10^3	6	0.004	0.985
d-GpApApT	1.8×10^3	4	0.002	0.987
d-GpApApTpTpC	1.2×10^3	2	0.002	0.090
--	exponential	15	--	0.95

^aHexanucleotide concentration = 10 ug/ml.

^bV_{max} in units of ug DNA/min.

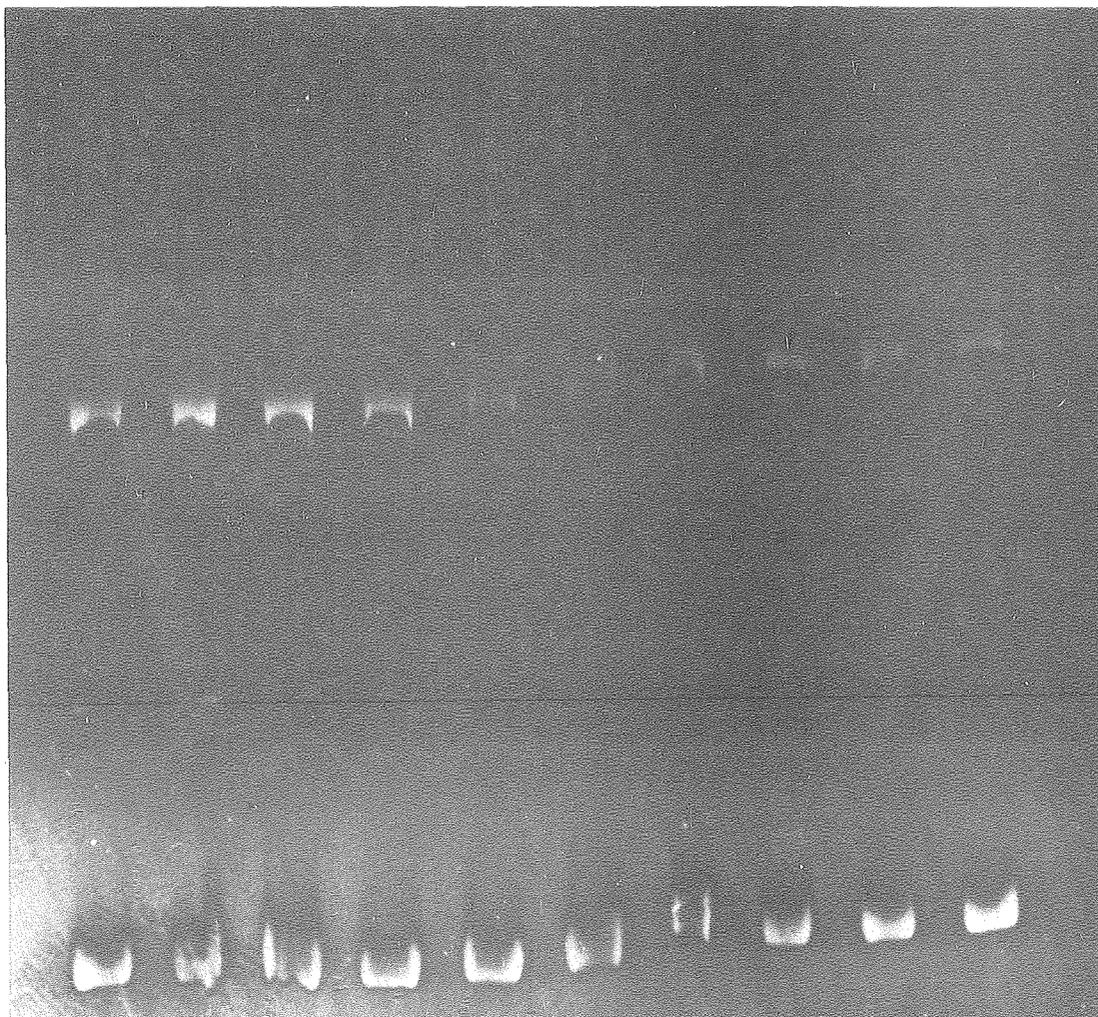
^cExponential refers to curve fitting of ae^{-bx} .



XBB 782-1837

Figure 45.

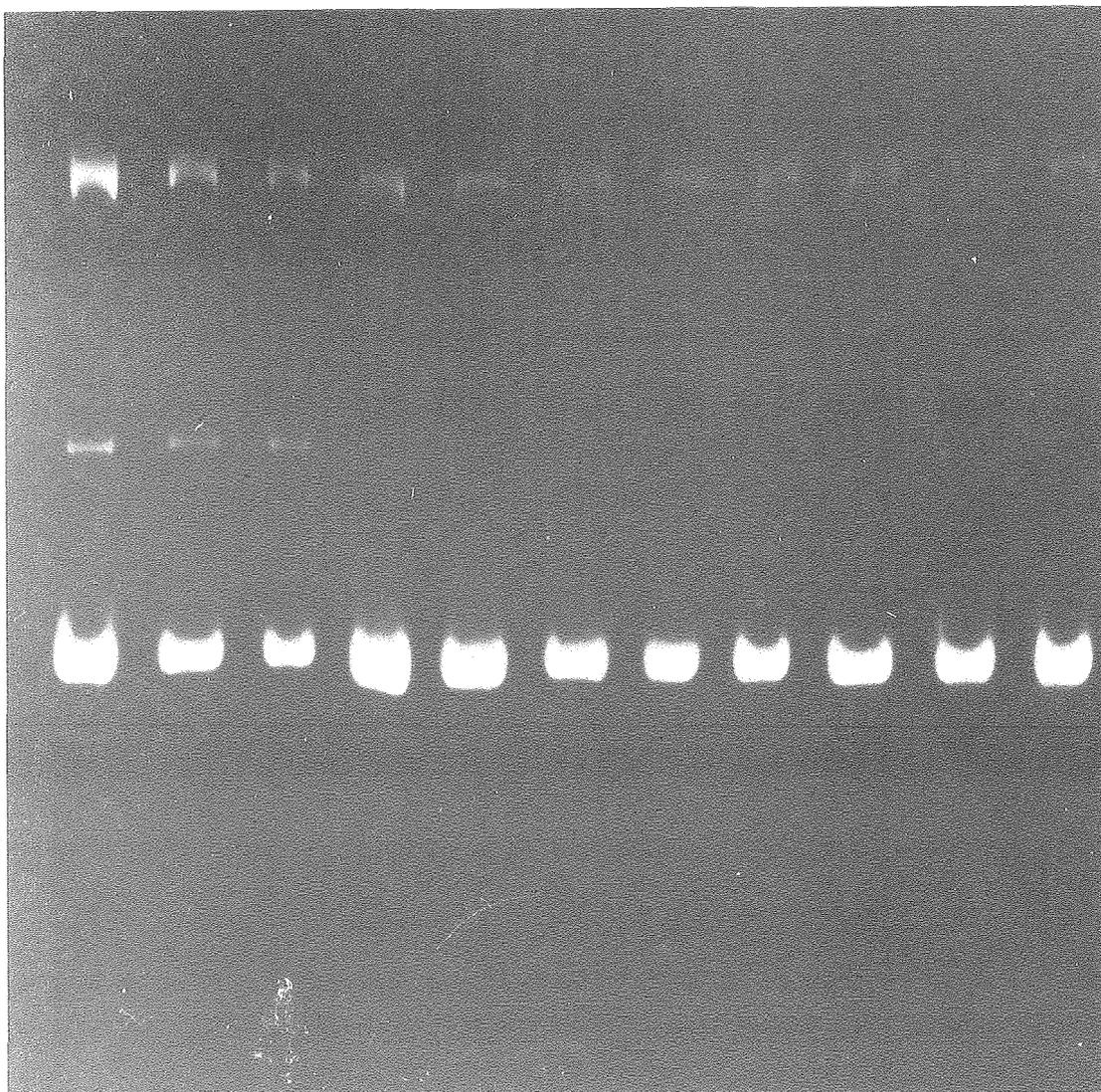
Gel photograph of enzyme inhibition by dinucleotide d-pApT.



XBB 782-1853

Figure 46.

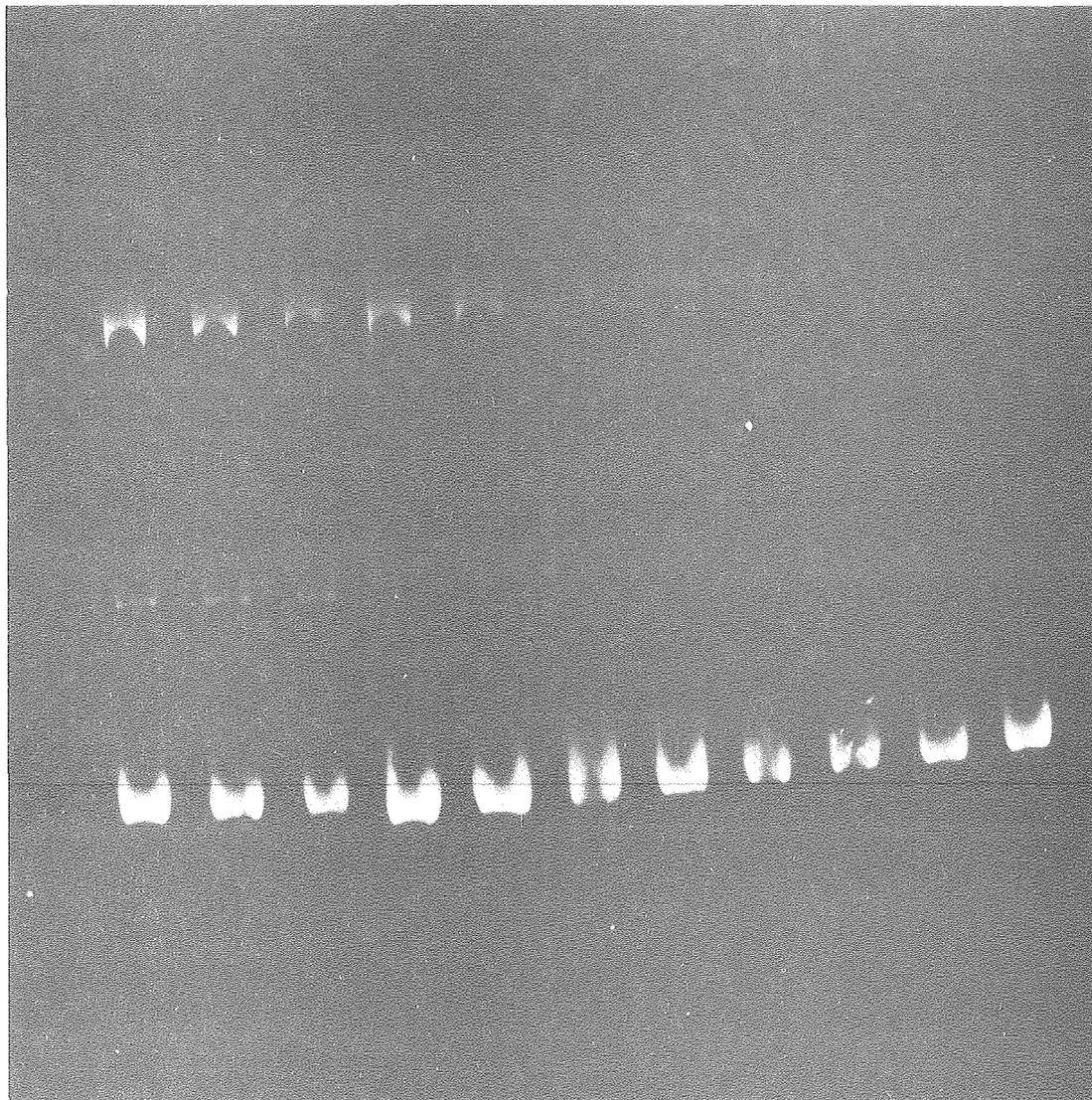
Gel photograph of enzyme inhibition by dinucleotide d-pGpA.



XBB 782-1854

Figure 47.

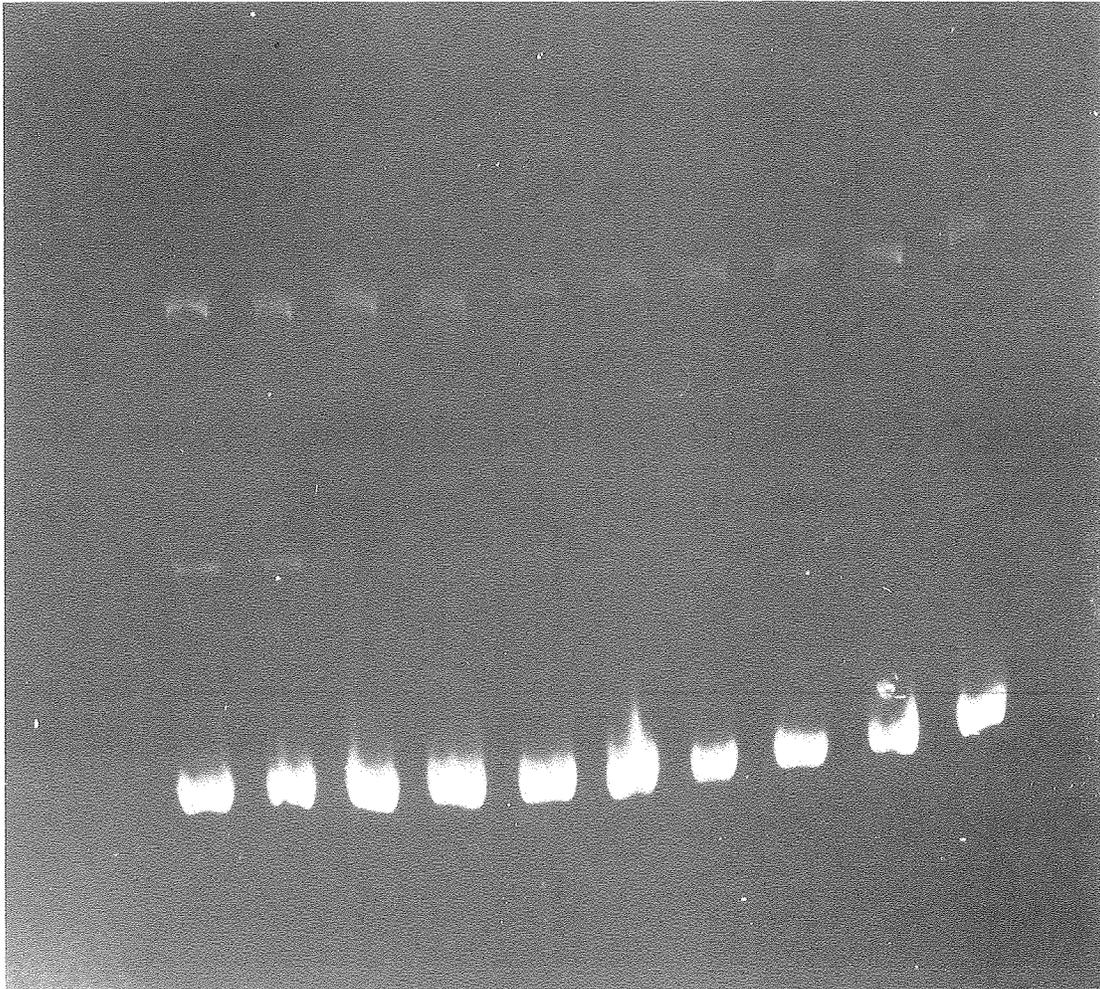
Gel photograph of enzyme inhibition by dinucleotide d-pApG.



XBB 782-1832

Figure 48.

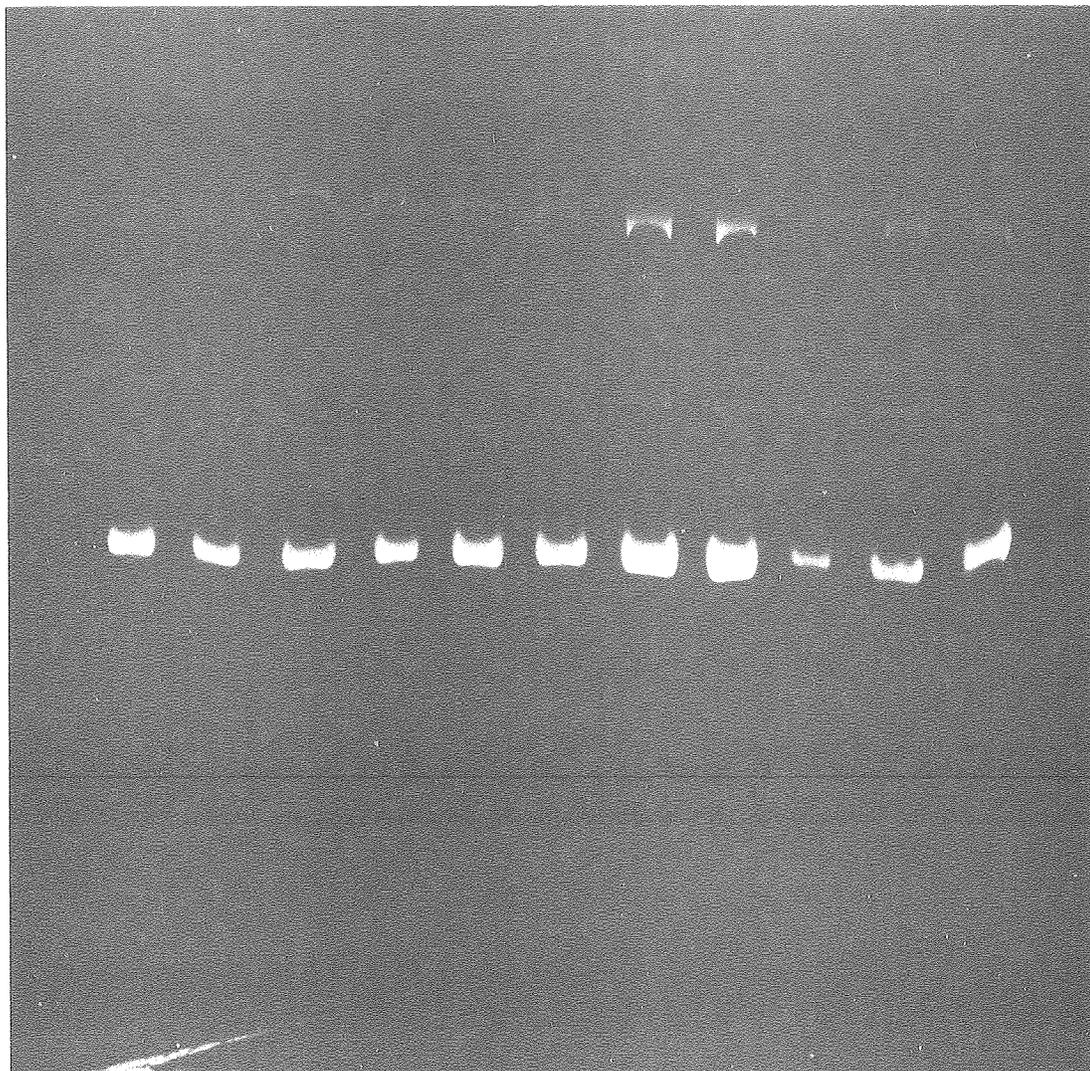
Gel photograph of enzyme inhibition by dinucleotide d-pTpA.



XBB 782-1842

Figure 49.

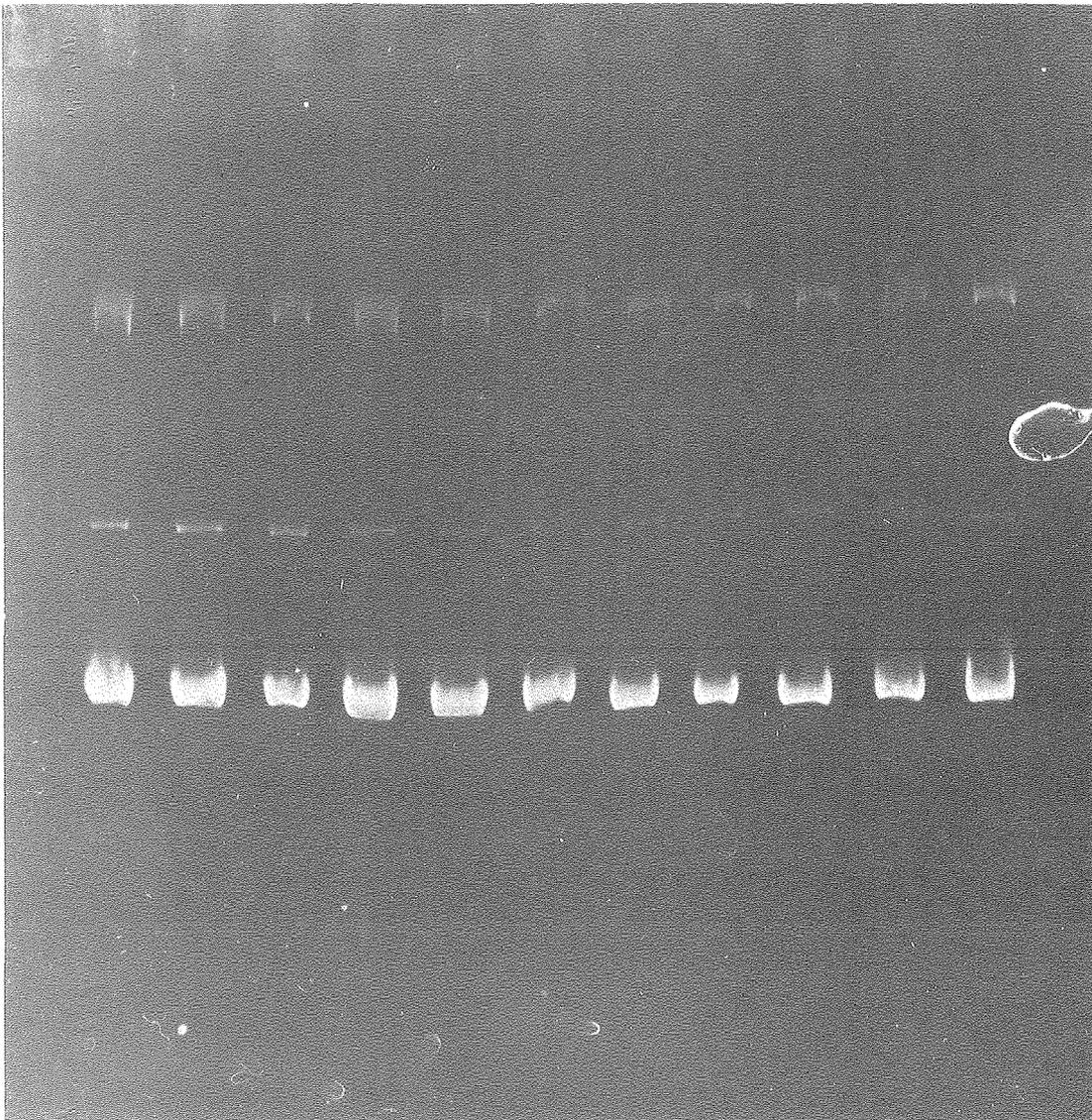
Gel photograph of enzyme inhibition by dinucleotide d-pTpC.



XBB 782-1836

Figure 50.

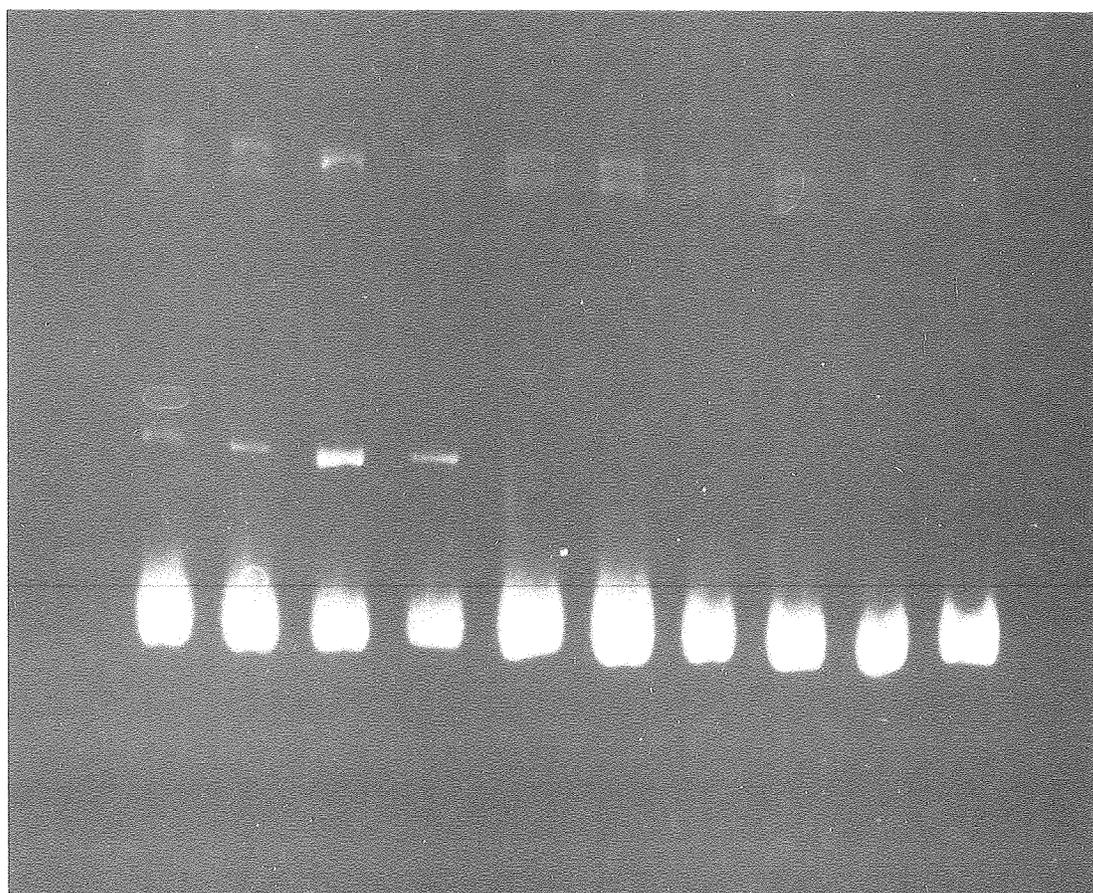
Gel photograph of enzyme inhibition by dinucleotide d-pCpT.



XBB 782-1840

Figure 51.

Gel photograph of enzyme inhibition by dinucleotide d-pApA.



XBB 782-1826

Figure 52.

Gel photograph of enzyme inhibition by dinucleotide d-pGpT.

Dixon Plot of Eco RI inhibition
 K_i for various mononucleotides
Plot of slope vs. I

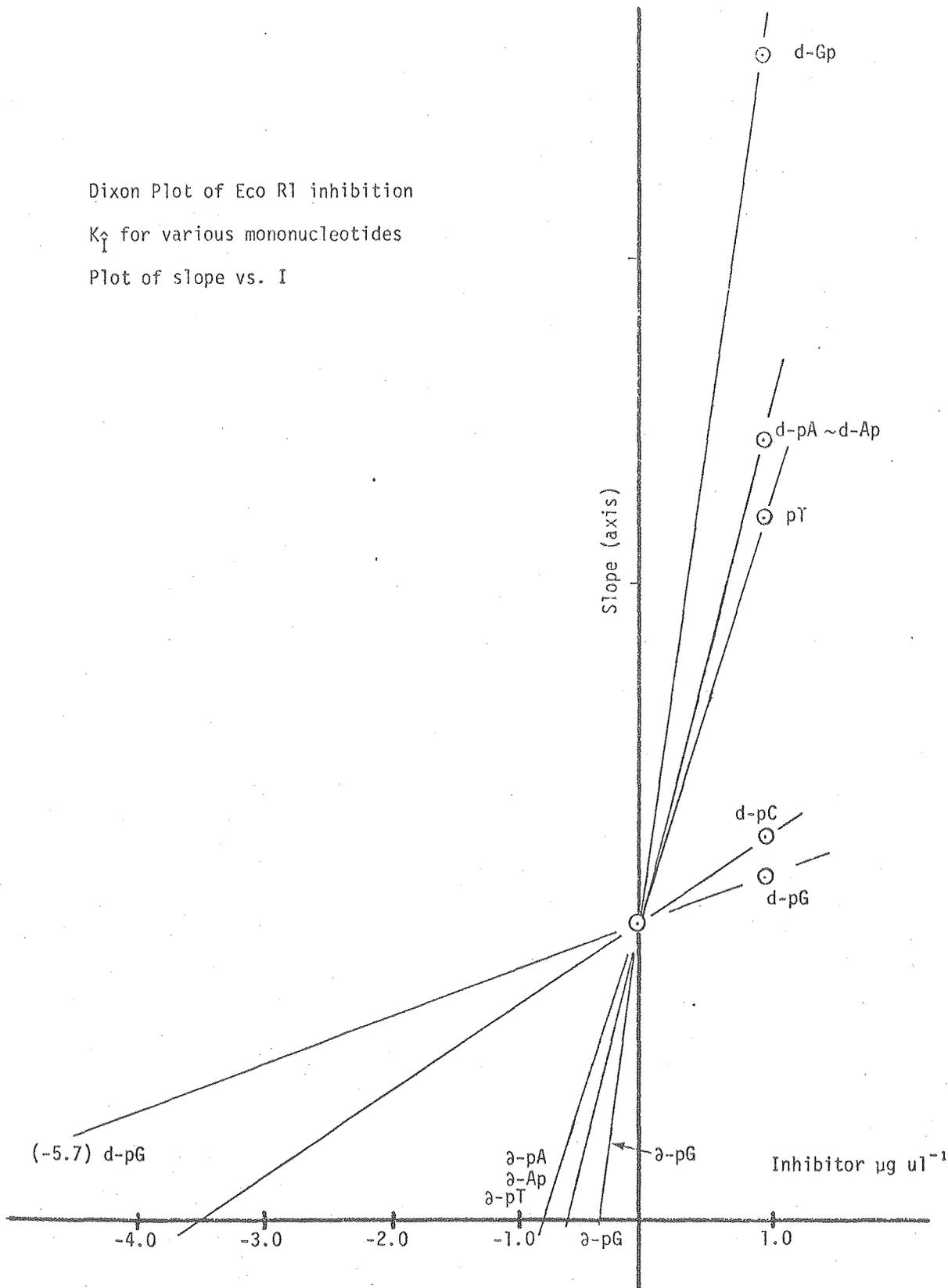
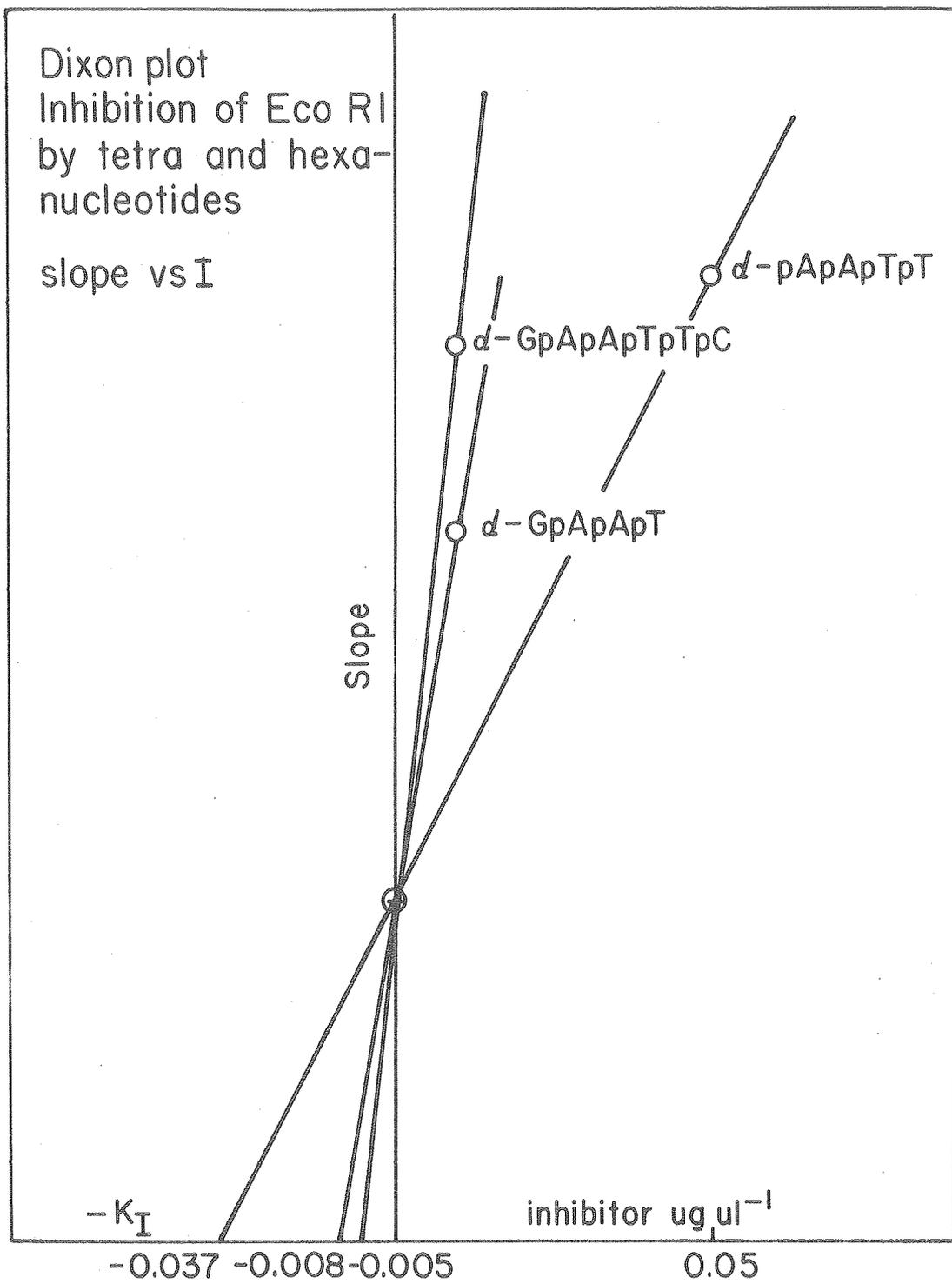


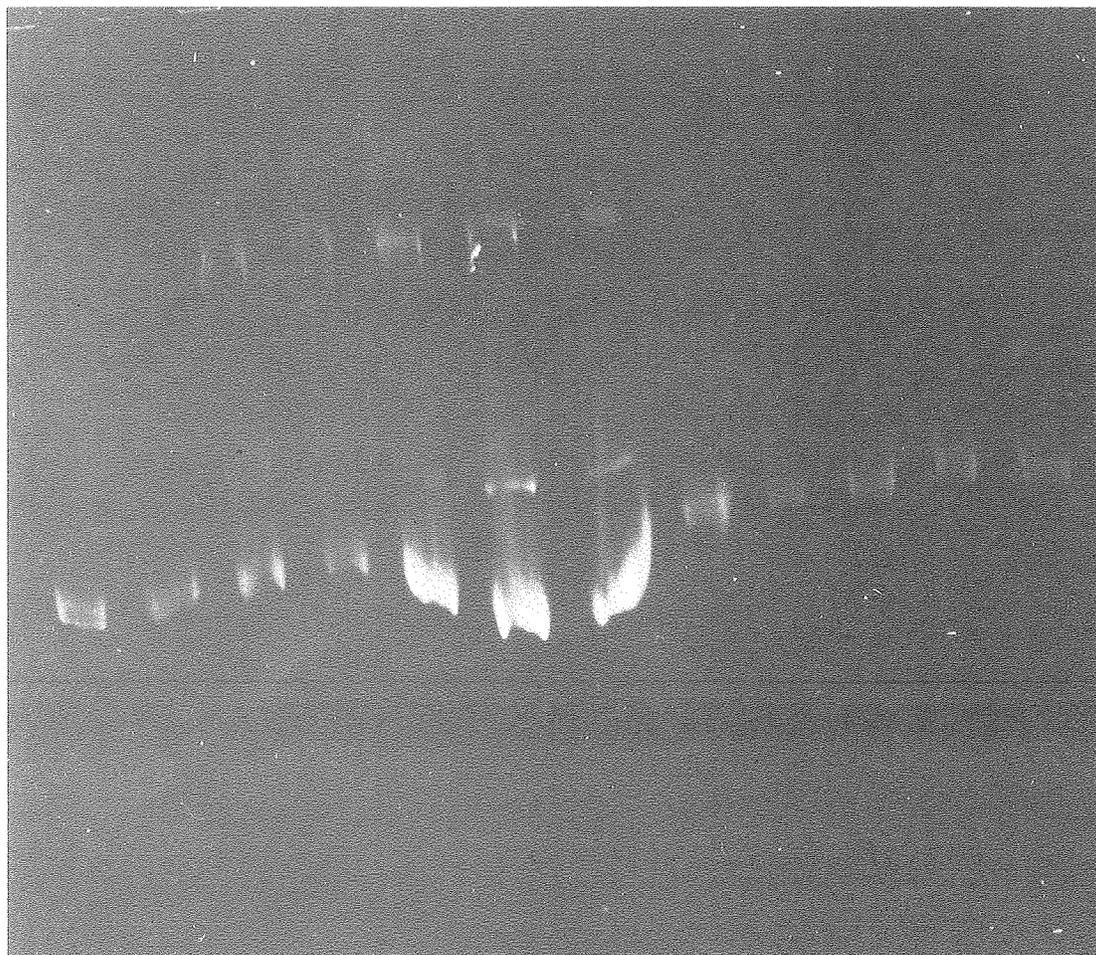
Figure 53. Slope replot of dinucleotides.



XBL 784-3918

Figure 54.

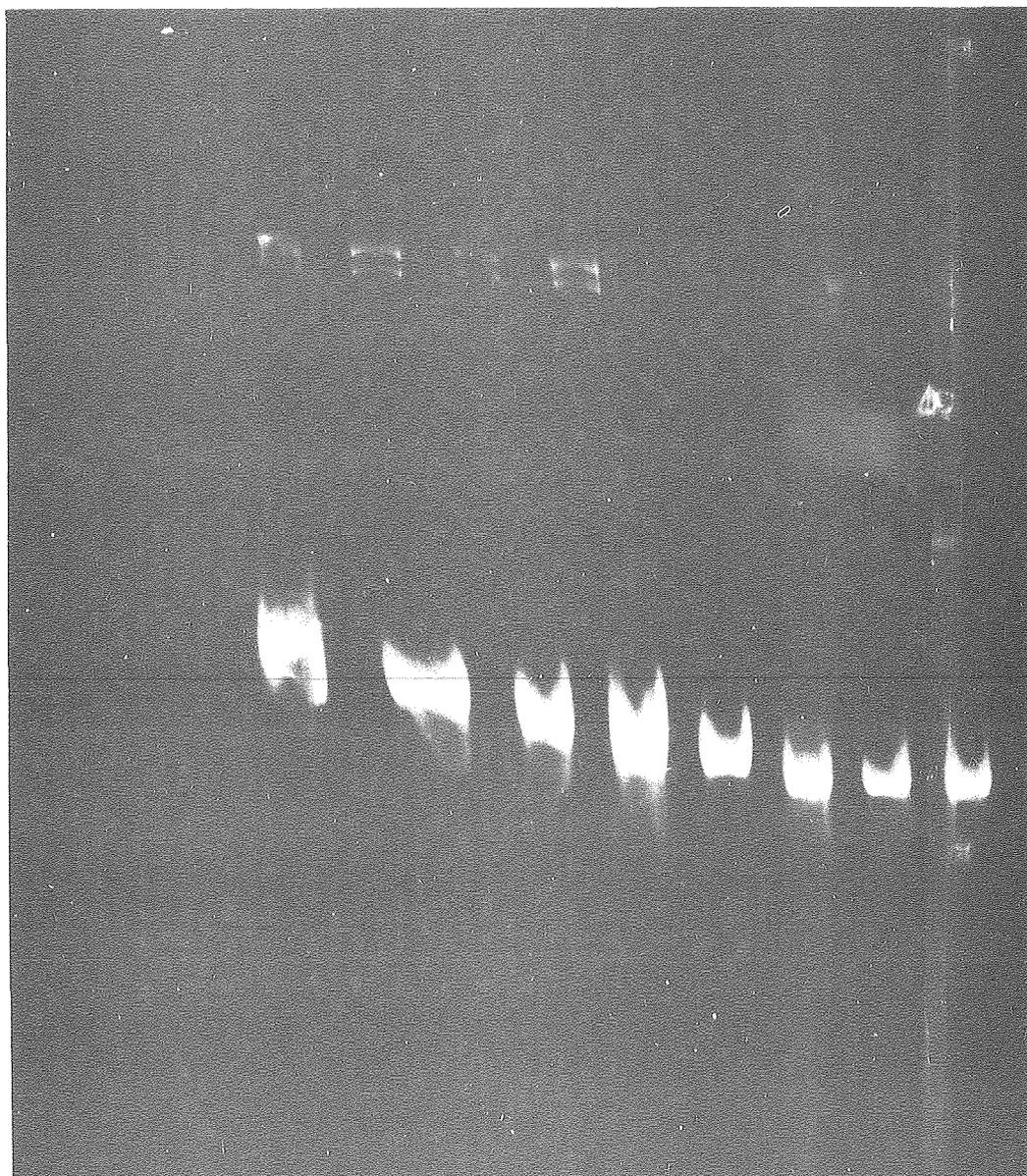
Dixon plots of Eco RI inhibition by oligonucleotides.



XBB 782-1325

Figure 55.

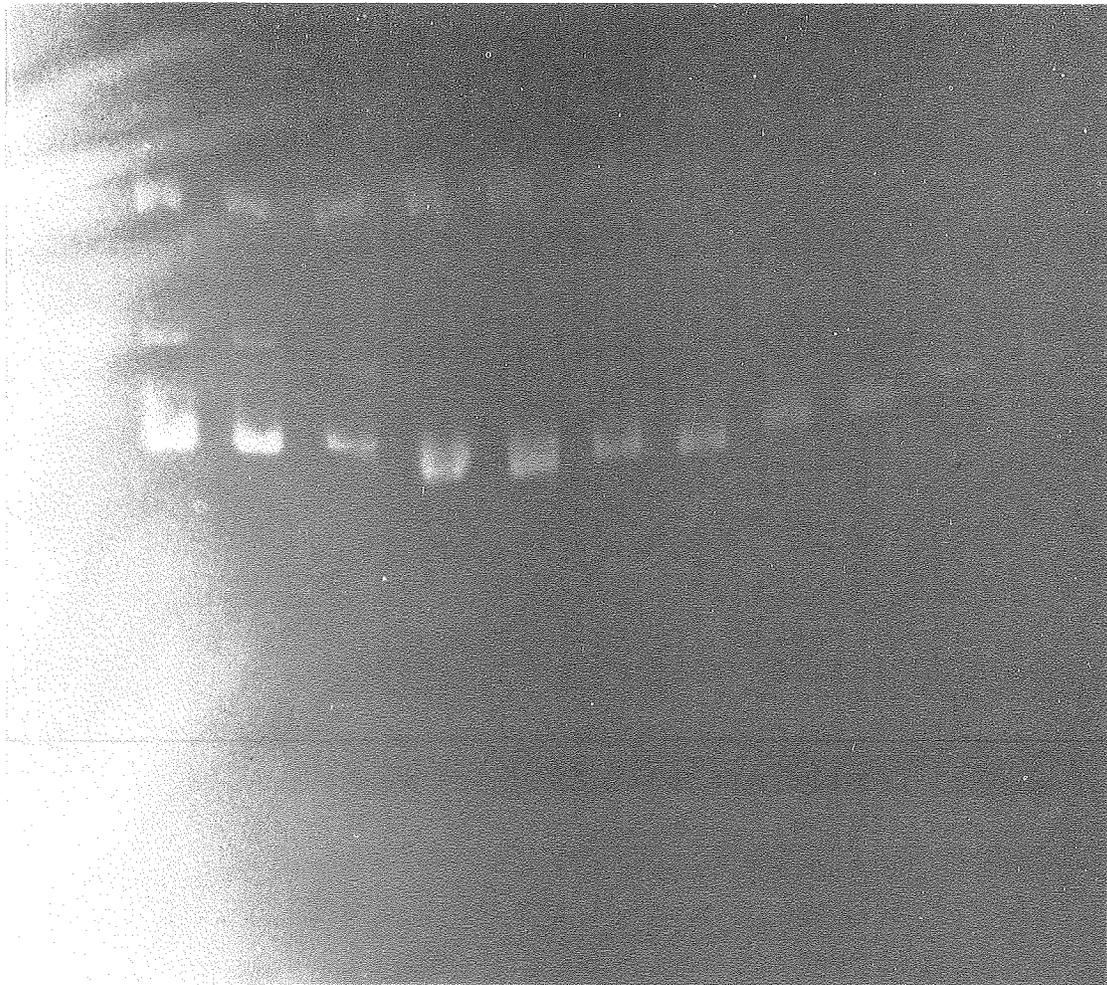
Gel photograph of enzyme inhibition by tetranucleotide d-pApApTpT.



XBB 782-1830

Figure 56.

Gel photograph of enzyme inhibition by tetranucleotide d-GpApApT.



XBB 782-1833

Figure 57.

Gel photograph of enzyme inhibition by hexanucleotide, d-GpApApTpTpC.

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This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

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