Studies on Polynucleotides. CII.[1] The Use of Aromatic Isocyanates for Selective Blocking of the Terminal 3′-Hydroxyl Group in Protected Deoxyribooligonucleotides[2]

K. L. Agarwal and H. G. Khorana*

Contribution from the Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.
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Abstract: In the synthesis of deoxyribooligonucleotides by the stepwise addition of protected mono- or oligonucleotide blocks to the 3′-hydroxyl end of the growing polynucleotide chains, the yields at the individual condensation steps are not quantitative. Consequently, polynucleotide synthesis cannot usefully be carried out on polymer supports; furthermore, in the standard solution phase approach, time-consuming separation of the required product is necessary. An important advantage would accrue if the 3′-hydroxyl end groups of the unreacted oligonucleotides could be specifically and quantitatively blocked. Phenyl (and naphthyl) isocyanate adds quantitatively in dry pyridine at room temperature to the 3′- and 5′-hydroxyl groups of deoxythymidine and, in addition, to the amino groups of deoxycytidine, deoxyadenosine, and deoxyguanosine. When *N*-benzoyldeoxyadenosine, *N*-anisoyldeoxycytidine, and *N*-isobutyryldeoxyguanosine are used, reaction occurs only with the 3′- and 5′-hydroxyl groups. With deoxynucleosides containing amino and 5′-hydroxyl groups blocked, reaction occurs specifically and quantitatively with the 3′-hydroxyl groups. Although the 5′-phosphomonoester group in thymidine 5′-phosphate also reacts to give a carbamoyl phosphate, the phosphodiester anion in protected dinucleoside phosphates and higher oligonucleotides is inert. Several suitably protected dinucleoside phosphates and a pentanucleotide were shown to undergo specific reaction with the 3′-hydroxyl end group. A practical application of the principle of blocking the 3′-hydroxyl group after the condensation reactions has been made in the synthesis of a pentanucleotide.

Current methodology for the synthesis of long-chain DNA duplexes of defined nucleotide sequences consists of the following three steps: (1) chemical synthesis of deoxyribopolynucleotide segments, usually 10–12 units long, corresponding to both strands of the desired DNA, (2) enzymatic phosphorylation of the 5′-hydroxyl end groups of the synthetic segments, and (3) ligase-catalyzed head-to-tail joining of the segments when appropriately aligned to form hydrogen-bonded bihelical complexes. Successful syntheses of the DNA corresponding to the major yeast alanine transfer RNA[3] and of a major part of the DNA corresponding to *Escherichia coli* tyrosine suppressor transfer RNA[4] have thus been accomplished. It is clear that total synthesis will continue to provide a definitive approach of wide scope in future studies of gene structure–function relationships. Therefore, it is important that attention be focused on achieving high efficiency at every one of the above three steps. Step 2 usually presents no problem in that polynucleotide kinase catalyzed phosphorylations proceed quantitatively. Step 3 involving the ligase-catalyzed joining has so far proceeded with a varying degree of success and further studies of the reaction are required. Undoubtedly, the single progress-determining factor in DNA synthesis is the chemical synthesis of the deoxyribopolynucleotide segments. Despite intensive efforts to date,[5] chemical synthesis continues to be time consuming and is lacking in efficiency.

For polynucleotide synthesis to be rapid and efficient, two requirements must be met. Firstly, the incoming protected mono- and oligonucleotide blocks should be readily available in quantity. This now is the case: protected mononucleotides are prepared rapidly and quantitatively on a 20–60-mmol scale routinely, while a rapid method, involving simple extraction procedures, for the preparation of protected dinucleotides has recently been developed.[6] The second requirement is that condensations between the 3′-hydroxyl end group of the growing chain and the 5′-phosphate group of the protected mono- or oligonucleotide blocks should proceed as efficiently as possible and, equally important, the required product should be rapidly separable from the unchanged starting material. So far, the yields in stepwise condensations with protected mononucleotides have been 40–80%, while with di- and trinucleotides they have been even lower (25–70%). Consequently, reaction products have had to be separated by elaborate anion exchange chromatography, this being laborious and time consuming.

It seems unlikely that quantitative yields will be obtained at the individual condensation steps using the existing procedures. Therefore, we have considered the alternative possibility of blocking quantitatively the 3′-hydroxyl group of the unreacted portion of the starting oligonucleotide after each condensation. Such a step would obviate the separation of the condensation product from the unreacted starting material. This

(1) Part CI: R. T. Walker and U. L. RajBhandary, *J. Mol. Biol.*, in press.

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(5) K. L. Agarwal, A. Yamazaki, P. J. Cashion, and H. G. Khorana, *Angew. Chem.*, in press.

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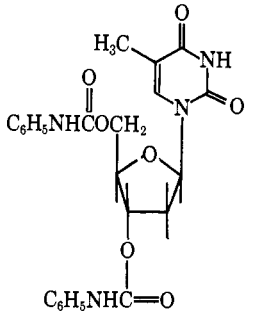
would result in some rapidity when the conventional solution phase approach is used. Further, it should be possible to carry out polynucleotide synthesis on polymer supports despite the fact that the yields during the individual condensations are far from quantitative (see Discussion).

The present paper describes a selective method for the quantitative derivatization of the 3′-hydroxyl groups in suitably protected deoxyribonucleotides and deoxyribooligonucleotides. Phenyl (and naphthyl) isocyanate add quantitatively in dry pyridine at room temperature to the 3′- and 5′-hydroxyl groups of deoxythymidine and, in addition, to the amino groups of deoxycytidine, deoxyadenosine, and deoxyguanosine. When *N*-benzoyldeoxyadenosine, *N*-anisoyldeoxycytidine, and *N*-isobutyryldeoxyguanosine are used, reaction occurs only with the 3′- and 5′-hydroxyl groups. With deoxynucleosides containing amino and 5′-hydroxyl groups, blocked reaction occurs specifically and quantitatively with the 3′-hydroxyl groups. Although the 5′-phosphomonoester group in thymidine 5′-phosphate also reacts to give a carbamoyl phosphate, the phosphodiester anion in protected dinucleoside phosphates and higher oligonucleotides is inert. Several suitably protected dinucleoside phosphates and a pentanucleotide were shown to undergo specific reaction with the 3′-hydroxyl end group. A practical application of the principle of blocking the 3′-hydroxyl group after the condensation reactions has been made in the synthesis of a pentanucleotide.

Reaction of Phenyl Isocyanate with Unprotected Deoxyribonucleosides. Isocyanates are useful reagents for forming adducts with alcohols and other reactive functions. In the present study, although no reaction occurred between methyl isocyanate and thymidine in dry pyridine, phenyl isocyanate gave a quantitative yield of a product which proved to be 3′,5′-*O*-diphenylcarbamoylthymidine (I). Thus, both elemental analysis



I

and the nmr spectrum indicated the presence of two phenylcarbamoyl groups. Like thymidine, the compound showed hypochromicity (22%) at 267 mμ at pH 14.0, thus indicating that no addition of phenyl isocyanate group to the thymine ring had occurred. Furthermore, treatment of 3′,5′-di-*O*-acetylthymidine with phenyl isocyanate in dry pyridine gave only the unchanged diacetyl nucleoside. It is therefore concluded that the thymine ring is inert to an addition reaction with the phenyl isocyanate under the conditions used.

Compound I was completely stable to 1 *N* sodium hydroxide at room temperature for 0.5 hr, to concentrated ammonium hydroxide at 50° for 3 hr, and to 80% aqueous acetic acid at room temperature or at 50° for 1 hr. Further, no reaction occurred on treatment with mesitylenesulfonyl chloride or triisopropylbenzenesulfonyl chloride in dry pyridine. Following the above treatments, which are routinely used in polynucleotide synthesis, completely unchanged starting material was recovered (analysis by thin-layer chromatography and by melting point determination of the recovered product).

The unprotected deoxynucleosides, deoxycytidine, deoxyadenosine, and deoxyguanosine, all gave, in quantitative yields, products, which, from elemental analysis and nmr spectra, contained 3 mol of isocyanate/mol of the nucleoside. With these nucleosides, therefore, reaction had occurred also with the heterocyclic rings, presumably the amino groups. Furthermore, the shifts in the ultraviolet absorption maxima observed for every one of the products were suggestive of substitution on the amino groups of the heterocyclic rings. (A comparison of the ultraviolet absorption maxima of these products with those of the corresponding *N*-acyl deoxynucleosides is made in Table I.)

Table I. Ultraviolet Absorption Maxima of Tricarbamoyl Deoxynucleosides and *N*-Acyldeoxynucleosides

Compound	λ_{max}, nm
3′,5′-Di-*O*-(phenylcarbamoyl)-*N*-phenylureidodeoxyadenosine	278[a]
N-Benzoyldeoxyadenosine	280
3′,5′-Di-*O*-(phenylcarbamoyl)-*N*-phenylureidodeoxycytidine	295[a]
N-Anisoyldeoxycytidine	302
3′,5′-Di-*O*-(phenylcarbamoyl)-*N*-phenylureidodeoxyguanosine	272,[a] 260[a]
N-Isobutyryldeoxyguanosine	275, 260

[a] Determined in aqueous ethanol.

Reaction of Phenyl Isocyanate with Protected Deoxyribonucleosides. For use in specific blocking of the 3′-hydroxyl groups the isocyanates should be completely inert to the heterocyclic rings in the N-protected deoxyribonucleosides. *N*-Benzoyldeoxyadenosine, *N*-anisoyldeoxycytidine, and *N*-isobutyryldeoxyguanosine were each treated with phenyl isocyanate in dry pyridine. The products were treated with concentrated ammonia to remove the N-protective groups; chromatographically pure products (95% average yield) were thus obtained. These were all characterized as 3′,5′-di-*O*-phenylcarbamoyl derivatives on the basis of elemental analyses and nmr spectra. No evidence of the formation of a triphenylcarbamoyl derivative was obtained.

To confirm the absence of reaction with the heterocyclic rings, the two N-protected deoxynucleosides (d-C^{An}, d-A^{Bz})[7] were converted to the corresponding diacetyl derivatives by treatment with acetic anhydride in dry pyridine; in the case of deoxyguanosine, the protected derivative, 3′,5′-*N*-triisobutyryldeoxyguanosine was used. All the three fully protected nucleosides were then treated with phenyl isocyanate in dry pyridine. Analysis of the reaction mixtures on tlc in three different solvent systems indicated that the starting

(7) For the system of abbreviations used, see H. Schaller and H. G. Khorana, *J. Amer. Chem. Soc.*, 85, 3841 (1963). Additional abbreviations used in this paper and not defined in the text are: iB, the isobutyryl group on the guanine ring. MS stands for mesitylenesulfonyl chloride and TPS for triisopropylbenzenesulfonyl chloride.

materials remained unchanged after phenyl isocyanate treatment. This conclusion was further substantiated after isolation of the pure products and careful examination of their nmr spectra.

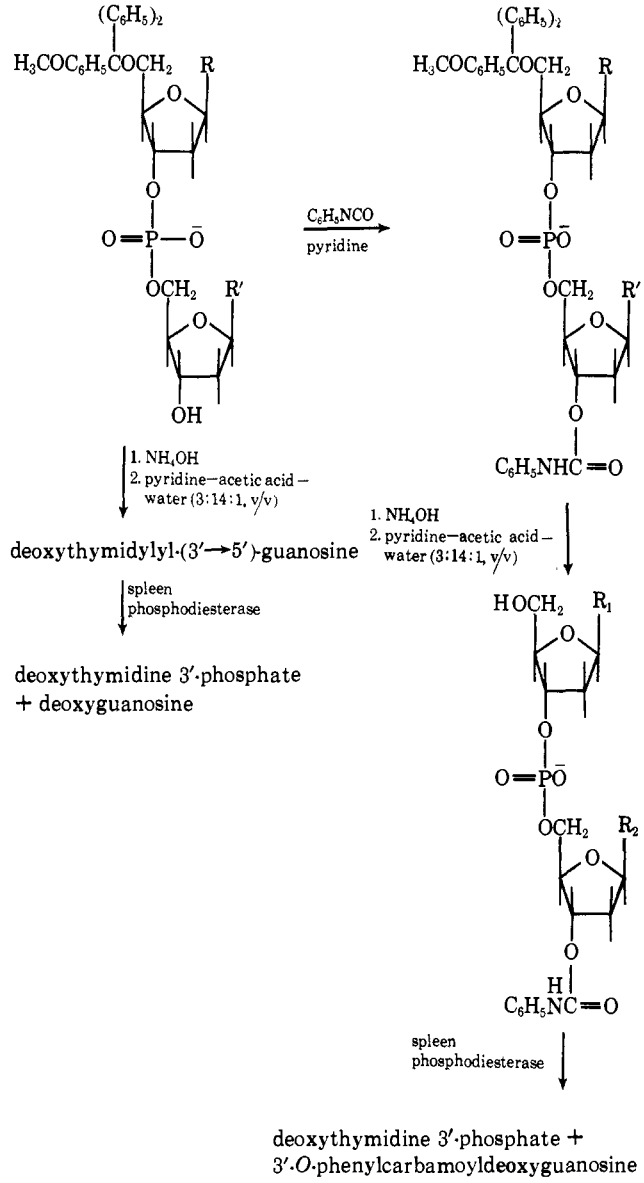
Protected deoxynucleosides bearing free 3′-hydroxyl groups and used commonly in synthetic work were next studied. 5′-*O*-Monomethoxytrityl-*N*-anisoyldeoxycytidine, 5′-*O*-monomethoxytrityl-*N*-benzoyldeoxyadenosine, 5′-*O*-monomethoxytrityl-*N*-isobutyryldeoxyguanosine, and 5′-*O*-monomethoxytritydeoxythymidine were each treated with phenyl isocyanate and the products obtained were treated directly with concentrated ammonia and buffered acetic acid to remove the protecting groups. The resulting unprotected phenylcarbamoyl deoxynucleosides, after extraction to remove nonnucleosidic material, were homogeneous on tlc in several solvent systems (Experimental Section). The elemental analyses and nmr spectra were as expected for the monophenylcarbamoyl derivatives. It should be emphasized that no free nucleosides were detected after removal of the N- and 5′-O-protecting groups in this experiment, showing that the reaction of phenyl isocyanate with the 3′-hydroxyl groups was complete and, further, that the carbamoyl group was stable to the treatments given to remove the protecting groups.

Reaction of Phenyl Isocyanate with Nucleotides and Oligonucleotides. Thymidine 5′-phosphate reacted readily with phenyl isocyanate in anhydrous pyridine to give a product which had the following properties. It was insoluble in aqueous ammonium hydroxide but was soluble in methanol and ethanol. It had very high mobility on both paper and thin-layer chromatography but its mobility on electrophoresis was about one-half that of d-pT. From these properties it is concluded that the product is a diphenylcarbamoyl derivative, one carbamoyl group being on the 3′-hydroxyl group and the second being on the phosphate group. The result is not surprising in view of the high nucleophilicity of a phosphomonoester anion. Furthermore, the nmr spectrum clearly showed the presence of two aromatic groups per thymine residue. It is interesting that the carbamoyl phosphate group was stable to concentrated ammonium hydroxide at 50° for 3 hr and to 80% aqueous acetic acid for 1 hr at room temperature.

Protected dinucleoside phosphates bearing free 3′-hydroxyl groups, which would be the intermediates in polynucleotide synthesis, were next studied. Thus, d-MMTr-TpT-OH, d-MMTr-$A^{Bz}pA^{Bz}$-OH,[7] d-MMTr-$A^{Bz}pC^{An}$-OH,[7] and d-MMTr-TpG^{iB}-OH[7] were treated with phenyl or naphthyl (see below) isocyanates. Examination of the reaction products by tlc on silica using a variety of solvents showed the absence of starting materials. The crude reaction products without any purification were treated so as to remove the protecting groups. The deprotected products, after ether washings to remove nonnucleotidic material, showed in every case single uv-absorbing spots on both paper and thin-layer chromatography. No free dinucleoside phosphates were detected in the total reaction mixtures. Further analysis of the products was carried out by enzymatic hydrolysis as shown in Chart I. The derivatives obtained from all four dinucleoside phosphates were resistant to the snake venom phosphodiesterase, thereby suggesting substitution of the 3′-hydroxyl end groups. Spleen phosphodiesterase hydrolyzed the derivatives quantitatively to the corresponding deoxynucleoside 3′-phosphates and deoxynucleoside derivatives, presumably, the 3′-*O*-phenylcarbamoyl derivatives. Careful examination of the digests by thin-layer chromatography showed complete absence of free nucleosides and the 3′-O-substituted nucleosides formed were identical in chromatographic properties with the corresponding derivatives prepared by using 5′-*O*-monomethoxytrityl N-protected deoxynucleosides (see above).

As an example of a longer protected oligonucleotide, the pentanucleotide d-MMTr-$TpG^{iB}pA^{Bz}pC^{An}pG^{iB}$-OH was treated with phenyl isocyanate in anhydrous pyridine. After 4 hr at room temperature, no starting material was detected by tlc. The product without any purification was subjected to concentrated ammonia treatment followed by buffered acetic acid to remove the protecting groups. The crude product did not show a

Chart I. Reaction of the Protected Dinucleoside Phosphate, 5′-*O*-Monomethoxytrityldeoxythymidylyl-(3′→5′)-*N*-isobutyryldeoxyguanosine (d-MMTr-TpG^{iB}OH) with Phenyl Isocyanate and Enzymatic Characterization of the Product[a]



[a] R or R′ = thymine, *N*-benzoyladenine, *N*-anisoylcytosine, or *N*-isobutyrylguanine. R_1 or R_2 = thymine, adenine, cytosine, or guanine.

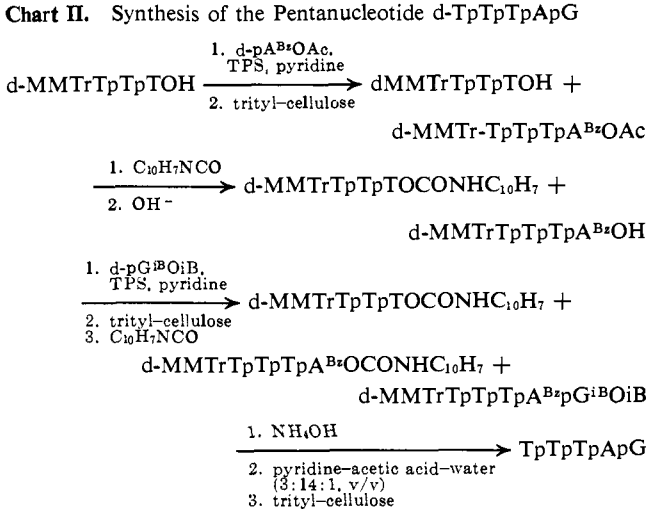
significant difference from the unprotected starting material in mobility on paper chromatography in solvents A and B, but degradation with spleen phosphodiesterase showed, without any doubt, that the derivatization of the 3′-hydroxyl end group was quantitative (Experimental Section).

Use of Naphthyl Isocyanates. As mentioned above, separations of reaction products in polynucleotide condensations are commonly carried out on DEAE–cellulose anion exchanger columns. The main aims are to separate, on the one hand, the excess of the mono- or oligonucleotide blocks and their pyrophosphates and, on the other hand, the unreacted trityl-containing oligonucleotide chain from the required elongated trityl-containing product. With a view to facilitating these separations, we have recently prepared two types of cellulose derivatives which bear large aromatic groups, namely, the naphthoyl[5,8] and trityl groups.[5] The use of such derivatives makes it possible to rapidly separate the total condensation products into nontrityl- and trityl-containing subgroups. The latter can then be further separated on DEAE–cellulose columns.

It would be a significant advantage if derivatization of the 3′-hydroxyl end group for a protected oligonucleotide (carrying 5′-*O*-methoxytrityl and N-protecting groups) would markedly enhance the affinity of the oligonucleotide for a trityl–cellulose column. In this way, it might be hoped that a facile separation of the required elongated deoxyribopolynucleotide from the unreacted material would be achieved. Aromatic isocyanates more lipophilic than phenyl isocyanate were therefore studied for reaction with the 3′-hydroxyl groups of protected deoxyribonucleosides. Naphthyl isocyanate reacted to give quantitative yields of the corresponding carbamoyl derivatives of the nucleosides, thymidine, and 5′-*O*-tritylthymidine, and the rate of reaction (complete in 20 min at room temperature) was almost the same as that of phenyl isocyanate. The much bulkier isocyanate, mono-*p*-isocyanatotetraphenylmethane, was also tried. It showed a slow rate of reaction, 12 hr being required for completion of reaction with thymidine. Nevertheless, the slowness may not be any impediment in the use of this reagent, particularly if the resulting derivatives show markedly increased affinity for the trityl–cellulose columns.

Facilitated Synthesis of a Pentanucleotide Using Naphthyl Isocyanate. The potential of the concept of blocking the terminal 3′-hydroxyl group in unreacted oligonucleotides especially by using the very aromatic naphthyl isocyanate has been demonstrated by the following accelerated synthesis of the pentanucleotide, d-TpTpTpApG, as shown in Chart II. The trinucleotide d-MMTrTpTpT-OH was synthesized by the standard extraction procedure. It was coupled with d-pA^{Bz}-OAc, using triisopropylbenzene–sulfonyl chloride (TPS) as the coupling agent. The reaction mixture was diluted with 0.05 *M* triethylammonium bicarbonate (TEAB) to a pyridine concentration of 5% and the solution was applied to a trityl–cellulose column which was washed with 0.05 *M* TEAB until no uv absorbing material was eluted from the column. Finally, the column was washed with 90% ethyl alcohol–0.05 *M* TEAB. This eluted only the monomethoxytrityl-containing oligonucleotides as shown by paper chromatography in solvent B. This mixture, after being rendered anhydrous, was treated with naphthyl isocyanate in anhydrous pyridine. The total product was next treated with alkali to remove the acetyl group and, after the usual work-up, was further condensed with d-pG^{iB}-OiB using TPS as the condensing agent. The reaction mixture was freed from nontritylated products by passing through trityl–cellulose. The monomethoxy-tritylated mixture of products was treated with naphthyl isocyanate in dry pyridine. The crude reaction mixture was subjected to both amino and 5′-O deprotection and then applied on trityl–cellulose and washed with 0.1 *M* TEAB. The TEAB wash contained a product which was about 95% pure on paper chromatography in solvents C and D. The base ratios after enzymatic hydrolysis were in agreement with the pentanucleotide structure.

Chart II. Synthesis of the Pentanucleotide d-TpTpTpApG



Discussion

A method has been described for the selective and quantitative blocking of the 3′-hydroxyl group of otherwise fully protected deoxyribooligonucleotides. It is hoped that this will facilitate the synthesis of polynucleotides, both by the conventional solution phase approach and by using insoluble polymer supports. A major obstacle in the use of the latter has been the fact that the yields during the condensation of 3′-O-acetylated mono- or oligonucleotides with the 3′-hydroxyl end group of the mono- or oligonucleotides bound to the polymers have been far less than quantitative. By using the present method, the 3′-hydroxyl group in the unreacted portion could be blocked and chain elongation could continue.

The formation of sequential isomers would thus be avoided and the products at the end of synthesis would consist of the desired polynucleotide, which would be the longest, and the successively shorter intermediates. Separation by anion exchange chromatography after removal of the protecting groups should be straightforward. In the solution phase approach, the method could be used in two ways. In the first, after each condensation step, the trityl-containing oligonucleotides (unreacted starting material and the reacted product) could be separated from the nontrityl components on a trityl–cellulose column; the trityl-containing portion would be treated with an isocyanate and the condensa-

(8) R. von Tigerstrom and M. Smith, *Science*, **167**, 1266 (1970).

tion repeated. At the end of the synthesis, the product would, again, consist of the required polynucleotide, the longest in size, and the shorter intermediates. A modification of this approach has been used in the synthesis. An example has been given in the present work in the synthesis of a pentanucleotide. In this, naphthyl isocyanate would be the reagent used and after successive condensations and work-up as described above, the trityl-containing products finally obtained would be treated to remove the protecting groups. The mixture would now contain the required polynucleotide, containing no protecting group, and the shorter oligonucleotides containing naphthylcarbamoyl groups at 3′-hydroxyl ends. The latter could all be removed by passage through a trityl–cellulose column. The unabsorbed desired polynucleotide could then be further purified by anion exchange chromatography.

Work is in active progress to integrate the isocyanate step into the general methodology for polynucleotide synthesis.

Experimental Section

Materials and General Methods. TPS, phenyl isocyanate, and naphthyl isocyanate were commercial products (Aldrich Chemicals). TPS was freshly crystallized from dry *n*-pentane before use. Phenyl isocyanate and naphthyl isocyanate were distilled and kept under dry nitrogen. Pyridine was treated with a small amount of chlorosulfonic acid before distillation, redistilled from potassium hydroxide, and stored over molecular sieves. All the condensation reactions were carried out by repeated evaporation *in vacuo* of a solution of the components with added dry pyridine (at least four times) and the reaction flask was opened into a drybox which had a positive pressure of dry nitrogen.

Paper chromatography was performed by the descending technique using Whatman no. 1 or no. 40 paper. The solvent systems used were: solvent A, 2-propanol–concentrated ammonia–water (7:1:2, v/v); solvent B, ethanol–ammonium acetate (1 *M*, pH 7.5, 7:3, v/v); solvent C, 1-propanol–concentrated ammonia–water (55:10:35, v/v); solvent D, isobutyric acid–concentrated ammonia–water (66:1:33, v/v).

Thin-layer chromatography (tlc) was carried out on precoated silica gel plates containing fluorescence indicator (Brinkmann F_{254}). The solvent systems used for tlc were: solvent E, chloroform–methanol (95:5, v/v); solvent F, chloroform–methanol (90:10, v/v); solvent G, acetonitrile–water (80:20, v/v); solvent H, acetonitrile–water (85:15, v/v); solvent J, acetonitrile–water (92:8, v/v).

Trityl–cellulose columns were run at 4°. Melting points were determined by capillary melting point apparatus and are not corrected. Nuclear magnetic resonance spectra were determined on a Varian T-60 spectrophotometer.

3′,5′-Di-*O*-phenylcarbamoyldeoxythymidine. Dry deoxythymidine (121 mg, 0.5 mmol) was suspended in anhydrous pyridine (5.0 ml) and treated with phenyl isocyanate (0.54 ml, 5 mmol). The reaction was shaken at room temperature for 1 hr and then concentrated to a thick gum under high vacuum. The gum was dissolved in chloroform (20 ml) and added dropwise to a large excess of ether (2 l.). The resulting precipitate was collected by filtration and washed with ether (three 100-ml portions). The crude product (237 mg, 97%) was homogeneous as judged by thin-layer chromatography in solvents E and J. Crystallization from hot aqueous methanol provided an analytical sample which had mp 195–197°, λ_{max}^{EtOH} 267 mμ. Its nmr spectrum in DMSO-d_6 showed a three-proton singlet (CCH_3) at 104 and ten aromatic protons and one proton $-C(=O)-N-CH=C(CH_3)-$ from the thymine ring between 456 and 416 Hz together with other protons. *Anal.* Calcd for $C_{24}H_{24}N_4O_7$: C, 60.00; H, 5.00; N, 11.66. Found: C, 60.12; H, 5.03; N, 11.66.

3′,5′-Di-*O*-(1-naphthylcarbamoyl)deoxythymidine. To a solution of dry deoxythymidine (61 mg, 0.25 mmol) in dry pyridine (3 ml) was added 1-naphthyl isocyanate (0.35 ml, 2.5 mmol) and the reaction mixture was shaken at room temperature. After 1 hr of shaking, the reaction mixture was concentrated under a high vacuum. The residual gum was worked up to give a solid material (140 mg, 90%) by the procedure described above. The crude product was crystallized from hot aqueous ethanol. It showed mp 208–209° and λ_{max}^{EtOH} 267 mμ. Its nmr spectrum in DMSO-d_6 showed a three-proton singlet (CCH_3) at 100 Hz and 14 aromatic protons and one proton from the thymine ring between 480 and 440 Hz together with other protons. *Anal.* Calcd for $C_{32}H_{28}N_4O_7$: C, 66.20; H, 4.82; N, 9.65. Found: C, 66.28; H, 5.26; N, 9.59.

3′,5′-Di-*O*-(phenylcarbamoyl-N^6-phenylureidodeoxyadenosine. To a suspension of dry deoxyadenosine (63 mg, 0.25 mmol) in anhydrous pyridine (5.0 ml) was added phenyl isocyanate (0.43 ml, 4.0 mmol). The yellow solution was allowed to stand at room temperature for 1 hr and thereafter concentrated under high vacuum to give a thick gum. The gum was dissolved in hot aqueous methanol (80:20 v/v) to give fine white needles (140 mg, 92%). It had mp 194–195° and λ_{max}^{EtOH} 278 mμ. Its nmr spectrum in DMSO-d_6 showed a two-proton singlet (CCH_2O) at 266 Hz and 15 aromatic protons between 400 and 460 Hz together with other protons. *Anal.* Calcd for $C_{31}H_{28}N_8O_6$: C, 61.18; H, 4.64; N, 18.42. Found: C, 61.20; H, 4.56; N, 18.35.

3′,5′-Di-*O*-(phenylcarbamoyl)-*N*-phenylureidodeoxycytidine. Dry deoxycytidine (57 mg, 0.25 mmol) was shaken at room temperature with phenyl isocyanate (0.43 ml, 4.0 mmol) in anhydrous pyridine (5 ml). After 1 hr the reaction mixture was concentrated to a gum. The gum was dissolved in hot methanol. On cooling, fine white needles separated (135 mg, 93%). It showed mp 215–216° and λ_{max}^{EtOH} 295, 283, and 275 mμ. Its nmr spectrum in DMSO-d_6 showed a two-proton singlet (CCH_2O) at 266 Hz and 15 aromatic protons between 410 and 480 Hz together with other protons. *Anal.* Calcd for $C_{30}H_{29}N_6O_7$: C, 61.53; H, 4.95; N, 14.35. Found: C, 61.54; H, 4.71; N, 14.01.

3′,5′-Di-*O*-(phenylcarbamoyl)-*N*-phenylureidodeoxyguanosine. Deoxyguanosine (67 mg, 0.25 mmol) was dried by repeated evaporation (two times) of anhydrous pyridine. The dry powder was then suspended in anhydrous pyridine (5 ml) and treated with phenyl isocyanate (0.43 ml, 4.0 mmol). The reaction mixture was shaken for 1 hr at room temperature and then evaporated to a gum *in vacuo*. The gum was dissolved in hot 90% aqueous ethanol (50 ml) and the solution on cooling deposited fine white needles (140 mg, 90%). The product had a very small impurity (<5%) running ahead of the major product and could not be removed even after two crystallizations. This impurity presumably arose from the contamination of deoxyguanosine with riboguanosine. It had mp 220° with decomposition and λ_{max}^{EtOH} 272 and 262 mμ. Its nmr spectrum in DMSO-d_6 showed a two-proton singlet (CCH_2O) at 263 Hz and 15 aromatic protons between 410 and 465 Hz together with other protons. *Anal.* Calcd for $C_{31}H_{28}N_8O_7$: C, 59.60; H, 4.48; N, 17.94. Found: C, 58.80; H, 4.51; N, 17.44.

3′,5′-Di-*O*-(phenylcarbamoyl)deoxycytidine. *N*-Anisoyldeoxycytidine (90.5 mg, 0.25 mmol) was dried by repeated evaporation of dry pyridine and finally was treated with phenyl isocyanate (0.25 ml, 2.5 mmol) in anhydrous pyridine (4.0 ml). The reaction mixture was kept for 1 hr at room temperature and then evaporated to a thick gum under vacuum. The gum was dissolved in a mixture of pyridine and concentrated ammonium hydroxide (25 ml, 1:1 v/v) and was kept at 50° for 3 hr in a carefully sealed flask. Pyridine (30 ml) was added and the solution dried by repeated evaporation with pyridine. The product was finally dissolved in pyridine (10 ml) and precipitated into ether (250 ml). The precipitate was collected by centrifugation and washed thoroughly with ether and dried (106 mg, 92%). The product was crystallized from aqueous methanol. It had λ_{max}^{EtOH} 272 mμ. Its nmr spectrum in DMSO-d_6 showed a two-proton singlet (CCH_2O) at 260 Hz and ten aromatic protons and one proton (>N—C*H*=C(CH_3)) from the cytosine ring between 470 and 412 Hz together with other protons. *Anal.* Calcd for $C_{23}H_{24}N_5O_6$: C, 60.52; H, 5.26; N, 15.34. Found: C, 60.16; H, 5.03; N, 15.23.

3,′5′-Di-*O*-(phenylcarbamoyl)deoxyadenosine. An anhydrous solution of d-A^{Bz} (89 mg, 0.25 mmol) and phenyl isocyanate (0.25 ml, 2.5 mmol) in pyridine (4.0 ml) was allowed to stand at room temperature for 1 hr with exclusion of moisture. After following the work-up procedure described above, the gum was treated with concentrated ammonium hydroxide in pyridine (30 ml, 1:1 v/v) at 50° for 3 hr. Pyridine (20 ml) was added and the solution dried as described above. The dry and concentrated solution (5 ml) was added dropwise to anhydrous ether (300 ml) to give a nice heavy white precipitate which was collected by centrifugation and washed with ether. Long white needles were obtained on crystallization from chloroform (110 mg, 90%). It had mp 220–224°

dec and λ_{max}^{EtOH} 262 mμ. *Anal.* Calcd for $C_{24}H_{23}N_7O_5$: C, 58.89; H, 4.70; N, 20.04. Found: C, 58.61; H, 4.36; N, 19.86.

3′-*O*-Phenylcarbamoyldeoxythymidine. To an anhydrous solution of 5′-monomethoxytrityldeoxythymidine (129 mg, 0.25 mmol) in pyridine (5 ml) was added phenyl isocyanate (0.25 ml, 2.5 mmol) and the reaction mixture was kept at room temperature with exclusion of moisture. After 1 hr, the reaction mixture was evaporated to a thick gum. The gum was dissolved in pyridine (4 ml) and precipitated into petroleum ether (400 ml). The precipitate was collected by centrifugation and washed thoroughly with petroleum ether. The white precipitate without any further purification was treated with 80% aqueous acetic acid (40 ml) at room temperature. Thin-layer chromatography in chloroform at different intervals showed that the deprotection was complete after 55 min. The reaction mixture was evaporated and dried by continuous evaporation with benzene. The gum on trituration with ether gave a white solid which crystallized from aqueous methanol (70%) as long needles (79 mg, 89%). It had mp 204–205° and λ_{max}^{EtOH} 267 mμ. Its nmr spectrum in DMSO-d_6 showed a two-proton singlet (CCH_2O) at 318 Hz and five aromatic protons and one proton (>N—C*H*=C(CH_3)) from the thymine ring between 470 and 406 Hz together with other protons. *Anal.* Calcd for $C_{17}H_{19}N_3O_6$: C, 56.50; H, 5.26; N, 11.63. Found: C, 56.10; H, 5.35; N, 11.98.

3′-*O*-Phenylcarbamoyldeoxyadenosine. An anhydrous solution of d-MMTr-A^{Bz}-OH (157 mg, 0.25 mmol) in pyridine (5 ml) was treated with phenyl isocyanate (0.25 ml, 2.5 mmol) for 1 hr at room temperature with the exclusion of moisture. The gum after usual work-up was dissolved in pyridine–concentrated ammonium hydroxide (20 ml, 3:5, v/v) and kept at 50° for 3 hr. Pyridine (30 ml) was added and the solution was dried by repeated evaporation with pyridine, and the dry solution (5 ml) so obtained was added dropwise to petroleum ether (400 ml). The white precipitate was collected by centrifugation washed thoroughly with petroleum ether. The dry powder was dissolved in pyridine–acetic acid–water (20 ml, 3:14:1, v/v) and kept at room temperature. After 3 days, deprotection of monomethoxytrityl was complete as judged by thin-layer chromatography using chloroform as the developing solvent. The reaction mixture was worked up as described above and the precipitate obtained was crystallized from aqueous methanol (80 mg, 87%). It had mp 250–251° and λ_{max}^{EtOH} 260 mμ. *Anal.* Calcd for $C_{17}H_{18}N_6O_4$: C, 55.13; H, 4.86; N, 22.70. Found: C, 54.82; H, 4.94; N, 22.62.

3′-*O*-Phenylcarbamoyldeoxycytidine. d-MMTr-C^{An}-OH (159 mg, 0.25 mmol) was treated with phenyl isocyanate (0.25 ml, 2.5 mmol) in dry pyridine (5 ml) for 1 hr at room temperature. The reaction mixture was concentrated to a gum and dissolved in pyridine–concentrated ammonium hydroxide (20 ml, 3:5, v/v) and allowed to stand at 50° for 3 hr before being evaporated to dryness. The residue was further dried by evaporation of benzene, and then its pyridine solution (5 ml) was precipitated into petroleum ether (200 ml). The precipitate was collected by centrifugation and washed with petroleum ether. The solid was treated with 80% aqueous acetic acid (20 ml) for 70 min at room temperature. The deblocking of the monomethoxytrityl group was complete as judged by thin-layer chromatography when developed in chloroform. After the usual drying and precipitation the product was crystallized from 50% aqueous methanol (75 mg, 85%). It had mp 210–212° and λ_{max}^{EtOH} 273 mμ. *Anal.* Calcd for $C_{16}H_{19}N_3O_5$: C, 55.33; H, 5.53; N, 16.14. Found: C, 55.10; H, 5.32; N, 15.98.

3′-*O*-Phenylcarbamoyldeoxyguanosine. d-MMTr-G^{iB}-OH (152 mg, 0.25 mmol) was treated with phenyl isocyanate (0.25 ml, 2.5 mmol) in dry pyridine (5 ml) at room temperature for 1 hr. The reaction mixture was worked up as described above and treated with concentrated ammonium hydroxide in pyridine (20 ml, 5:3, v/v) at 50° for 3 hr. After the usual work-up and precipitation into petroleum ether, the residue was treated with pyridine–acetic acid–water (20 ml; 3:14:1, v/v) for 2 days at room temperature. The reaction mixture was concentrated and dried by evaporation with benzene. The dry residue was dissolved in pyridine (5 ml) and precipitated into ether. The residue was crystallized from aqueous methanol to give a gel (80 mg, 83%). It had mp 260° dec and λ_{max}^{EtOH} 252 mμ. *Anal.* Calcd for $C_{17}H_{18}N_6O_5$: C, 52.84; H, 4.66; N, 21.75. Found: C, 52.53; H, 4.64; N, 20.32.

3′-*O*-Phenylcarbamoyldeoxythymidylyl-(3′→5′)-thymidine. d-MMTr-TpT-OH (0.1 mmol, 1920 OD_{267}) was treated with phenyl isocyanate (0.1 ml, 1.0 mmol) in dry pyridine (3 ml) for 2 hr at room temperature. Thin-layer chromatography in solvents F and H showed the completion of the reaction. The reaction mixture was cooled in Dry Ice–ethanol (−20°) and methanol (0.5 ml) was then added. After 20 min at room temperature, the reaction mixture was concentrated *in vacuo* (1 ml) and then triturated with ether to give a white solid. The solid was collected by centrifugation and washed with ether. This was then dissolved in 80% aqueous acetic acid (10 ml). Thin-layer chromatography in solvent H at different intervals of the reaction mixture showed that the deprotection required 70 min at room temperature. The reaction mixture was concentrated and dried by repeated evaporation of ethanol benzene. The dry residue on trituration with dry ether gave a white solid which was homogeneous by paper and thin-layer chromatography. The product was completely hyrolyzed by spleen phosphodiesterase to deoxythymidine 3′-phosphate and 3′-phenylcarbamoyldeoxythymidine. In the digest, no thymidine was detected on thin-layer chromatography in solvents E and H.

3′-*O*-Phenylcarbamoyldeoxyadenosyl-(3′→5′)-adenosine. d-MMTr-$A^{Bz}pA^{Bz}$-OH (0.1 mmol, 3660 OD_{280}) was treated with phenyl isocyanate (0.05 ml, 0.5 mmol) in dry pyridine (2 ml) for 3 hr at room temperature. Thin-layer chromatography showed the completion of the reaction. The reaction mixture was worked up as described above. The white solid obtained after ether precipitation was dissolved in concentrated ammonium hydroxide (20 ml) and the solution was kept at 50° for 3 hr. After the usual evaporation and drying the residual gum was triturated with ether to give a solid which was collected by centrifugation. The crude product was treated with pyridine–acetic acid–water (20 ml, 3:14:1, v/v) for 3 days at room temperature (tlc showed completion of demethoxytritylation) before being evaporated and dried by continuous evaporation of pyridine. Its pyridine solution (3 ml) was added dropwise to anhydrous ether (200 ml). The off-white precipitate was collected by centrifugation and washed thoroughly with ether. The product was homogeneous by both paper and thin-layer chromatography and was free from ApAOH. The product was completely hydrolyzed with spleen phosphodiesterase at pH 5.9 at 37° to d-Ap and d-A-$OCONHC_6H_5$. The digest did not contain any free nucleoside.

3′-*O*-Phenylcarbamoyldeoxythymidylyl-(3′→5′)-cytidine. d-MMTr-TpC^{An}-OH (0.1 mmol, 2410 OD_{280}) and phenyl isocyanate (0.05 ml, 0.5 mmol) were treated in dry pyridine (2 ml) at room temperature. After 2 hr, the reaction mixture was worked up as described above and the product precipitated into ether. The white solid, collected after centrifugation, was dissolved in concentrated ammonium hydroxide (15 ml) and the solution was kept at 50° for 3 hr. After the usual work-up and trituration of the residue with ether, a white solid was obtained. The crude dinucleoside phosphate was further treated with pyridine–acetic acid–water (15 ml, 3:14:1, v/v) for 3 hr at 50°. Thin-layer chromatography in solvents F, G, and H showed a uv absorbing spot which was free of the monomethoxytrityl group. The reaction mixture was concentrated and dried by continuous evaporation of pyridine. The dry pyridine solution was then added dropwise to dry ether to give a heavy white precipitate. The product was collected by centrifugation and dried. The carbamoyl derivative of dinucleoside phosphate was homogeneous on paper and thin-layer chromatography (see Table II). The product was completely hydrolyzed with spleen phosphodiesterase to deoxythymidine 3′-phosphate and 3′-phenylcarbamoyldeoxycytidine. In the digest, no free deoxythymidine was detected by thin-layer chromatography.

3′-*O*-Phenylcarbamoyldeoxythymidylyl-(3′→5′)-guanosine. d-MMTr-G^{iB}-OH (0.1 mmol, 1790 OD_{280}) was treated with phenyl isocyanate (0.05 ml, 0.5 mmol) in dry pyridine (2 ml) at room temperature. After 2 hr, the reaction mixture was worked up as described above. The precipitate was further treated with concentrated ammonium hydroxide (15 ml) at 50° for 3 hr. The product obtained after usual work-up and precipitation was treated with pyridine–acetic acid–water (15 ml, 3:14:1, v/v) for 3 days at room temperature. The reaction mixture was then concentrated and dried by continuous evaporation with pyridine and the product was precipitated by adding to an excess of ether. The 3′-hydroxyl protected dinucleoside phosphate was homogeneous by paper (solvents A and B) and thin-layer chromatography (solvents G and H). The product was quantitatively digested with spleen phosphodiesterase to deoxythymidine 3′-phosphate and 3′-*O*-phenylcarbamoyldeoxyguanosine. No free deoxyguanosine was detected in the digest.

3′-*O*-Phenylcarbamoyl Pentanucleotide, d-TpGpApCpGOC(=O)NHC_6H_5. d-MMTr-$TpG^{iB}pA^{Bz}pC^{An}pG^{iB}$OH (10 μmol, 654 OD_{280}) was dried by evaporating with anhydrous pyridine (two 3-ml portions) and then treating with phenyl isocyanate (0.05 ml, 0.5 mmol) in dry pyridine (2 ml). After keeping the reaction mix-

Table II. R_f Values of Different Compounds on Paper and Thin-Layer Chromatography

Compound	Paper chromatography, R_f[a] values: Solvent A	Solvent B	Solvent C	Thin-layer chromatography, R_f values: Solvent E	Solvent F	Solvent G	Solvent H	Solvent J
3′,5′-Di-*O*-(phenylcarbamoyl)-deoxythymidine				0.69				0.89
3′,5′-Di-*O*-(1-naphthylcarbamoyl)-deoxythymidine				0.74				0.83
3′,5′-Di-*O*-(phenylcarbamoyl)-N^6-phenylureidodeoxyadenosine				0.75	0.89	0.91	0.93	0.90
3′,5′-Di-*O*-(phenylcarbamoyl)-*N*-phenylureidodeoxycytidine				0.70	0.84	0.87	0.89	0.86
3′,5′-Di-*O*-(phenylcarbamoyl)-*N*-phenylureidodeoxyguanosine				0.25	0.63	0.79	0.82	0.75
3′,5′-Di-*O*-(phenylcarbamoyl)-deoxycytidine					0.47			0.68
3′,5′-Di-*O*-(phenylcarbamoyl)-deoxyadenosine					0.73			0.82
3′-*O*-Phenylcarbamoyldeoxy-thymidine				0.43	0.78			0.82
3′-*O*-Phenylcarbamoyldeoxy-adenosine					0.38		0.70	0.66
3′-*O*-Phenylcarbamoyl-deoxycytidine					0.29		0.87	0.60
3′-*O*-Phenylcarbamoyl-deoxyguanosine	5.61				0.23		0.76	0.66
3′-*O*-Phenylcarbamoyldeoxy-thymidylyl-(3′→5′)-thymidine	3.39	2.51			0.16	0.52	0.43	
3′-*O*-Phenylcarbamoyldeoxy-adenosyl-(3′→5′)-adenosine	2.00	2.46				0.29	0.11	
3′-*O*-Phenylcarbamoyldeoxy-thymidylyl-(3′→5′)-cytidine	3.0	2.23				0.48	0.26	
3′-*O*-Phenylcarbamoyldeoxy-thymidylyl-(3′→5′)-guanosine	1.54[b]	1.11[b]				0.33	0.20	
3′-*O*-Phenylcarbamoyl penta-nucleotide, d-TpGpApCpG-OCONHC$_6$H$_5$		0.12	0.32					
d-MMTr-TpTpT-OCONHC$_6$H$_5$	2.3	1.90						
d-MMTr-TpTpTpA-OH	0.92	1.1						
d-TpTpTpApG-OH			0.26					

[a] R_f values were recorded relative to d-pT. [b] R_f values were recorded relative to d-TpG.

ture for 4 hr at room temperature, methanol (1 ml) was added to the precooled reaction mixture (−20°). This was further kept for 20 min at room temperature before being concentrated to 1 ml. Addition of anhydrous ether (10 ml) gave a white solid which was collected by centrifugation. The product was then subjected to concentrated ammonium hydroxide treatment and after the usual work-up, it was further treated with pyridine–acetic acid–water (5 ml, 3:14:1, v/v) for 3 days at room temperature. The product was isolated after the usual work-up and precipitation of its pyridine solution into a large amount of anhydrous ether. The crude material was homogeneous on paper chromatography. The product was quantitatively degraded with spleen phosphodiesterase to d-Tp, d-Gp, d-Ap, d-Cp, and 3′-phenylcarbamoyldeoxyguanosine. Paper chromatography of the digest in solvent A showed the presence of 3′-phenylcarbamoyldeoxyguanosine and no deoxyguanosine was detected.

Synthesis of the Tetranucleotide, d-MMTr-TpTpTpA^{Bz}-OH. An anhydrous pyridine solution (3 ml) of d-MMTr-TpTpT-OH (110 μmol, 2880 OD_{267}) and d-pA^{Bz}-OAc (0.4 mmol, 7320 OD_{280}) was treated with TPS (1 mmol, 302 mg) for 3 hr at room temperature with exclusion of moisture. The reaction was terminated by cooling the reaction mixture in a Dry Ice–ethanol mixture and adding diisopropylethylamine solution in pyridine (1.5 ml of 1 *M*) followed by water (2 ml). After 24 hr of aqueous pyridine treatment, the reaction mixture was concentrated (1 ml) and diluted with 0.05 *M* TEAB (100 ml) and applied on a trityl–cellulose column (1.1 × 20 cm) preequilibrated with 0.05 *M* TEAB. The column was washed thoroughly with 0.05 *M* TEAB (2 l.) to remove the unreacted mononucleotide pA^{Bz}OAc, its symmetrical pyrophosphate, and the triisopropylbenzenesulfonic acid. The trityl–cellulose was then washed with 0.05 *M* TEAB containing 90% ethanol (1 l.). The eluate contained a mixture of tetranucleotide and trinucleotide. The solution was concentrated to 10 ml with repeated additions of pyridine. The residue was dissolved in anhydrous pyridine (3 ml) and treated with 1-naphthyl isocyanate (1 mmol, 0.14 ml) for 6 hr at room temperature. The reaction was terminated by cooling the reaction mixture (−20°) and adding methanol (1 ml). After 30 min at room temperature, the reaction mixture was concentrated (3 ml) and precipitated into anhydrous ether (200 ml). The precipitate was collected by centrifugation and washed with ether (three 50-ml portions). The mixture of tri- and tetranucleotides was then treated with sodium hydroxide solution (5 ml, 2 *N*) in 30% aqueous pyridine (5 ml) for 5 min at 0°. The excess of alkali was removed by the addition of pyridinium Dowex-50 ion exchange resin (10 ml); the resin was removed by filtration and washed with 20% aqueous pyridine. The combined filtrate and washings were concentrated under vacuum and the residue was taken up in dry pyridine (2 ml) and added to an excess of dry ether (200 ml). The R_f values of protected compounds are given in Table II. The mixture of tri- and tetranucleotides was used for the pentanucleotide synthesis without any further purification.

Synthesis of the Pentanucleotide, d-TpTpTpApG. An anhydrous pyridine solution (4 ml) of a mixture of d-MMTr-TpTpT-OCONHC$_{10}$H$_7$ and d-MMTr-TpTpTpA^{Bz}OH (3500 OD_{265}) obtained in the above experiment was condensed with d-pG^{iB}-OiB (0.4 mmol) using TPS (1.0 mmol, 302 mg) as a condensing agent for 3 hr at room temperature with the exclusion of moisture. After the usual work-up and aqueous pyridine treatment, the reaction mixture was concentrated (2 ml) and diluted with 0.05 *M* TEAB (200 ml). The solution was applied on a trityl–cellulose column (3 × 20 cm). The nontritylated components were simply washed off from the column with 0.05 *M* TEAB (1 l.). The tritylated products were eluted with 90% ethyl alcohol–0.05 *M* TEAB (200 ml). Paper chromatography in solvents A, B, and C of the ammonia-treated trityloligonucleotides was not quite clear. The reaction mixture was treated with naphthyl isocyanate (1 mmol, 0.14 ml) in anhy-

drous pyridine (3 ml) as described above. The mixture of products obtained after ether precipitation was first subjected to concentrated ammonium hydroxide treatment and then to pyridine–acetic acid–H_2O (5 ml, 3:14:1, v/v). The total mixture of products was then applied on the column of trityl–cellulose (2 × 10 cm) in 0.05 *M* TEAB containing 10% ethyl alcohol. The uv absorbing material eluted from the column contained 5% of impurities as judged by paper chromatography in solvent C. The product was completely digested with snake venom and had the constituent nucleotide ratio for d-pT:d-pA:d-pG of 1.96:0.96:0.93, respectively.

Nitrogen Isotope Effects on the Chymotrypsin-Catalyzed Hydrolysis of *N*-Acetyl-L-tryptophanamide

Marion H. O'Leary* and Michael D. Kluetz

Contribution from the Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received June 4, 1971*

Abstract: The hydrolysis of *N*-acetyl-L-tryptophanamide catalyzed by α-chymotrypsin at 25.0° is accompanied by a nitrogen isotope effect of 1.006 at pH 6.73, 1.010 at pH 8.00, and 1.006 at pH 9.43. The step in which the carbon–nitrogen bond is broken must be at least partially rate controlling in the enzymatic reaction. The variation of the isotope effect with pH requires that there be an intermediate in the acylation of the enzyme in addition to those which have been observed previously. The pH dependence of these reactions is more complex than has previously been believed.

A consistent and reasonable static picture of the structure of α-chymotrypsin has emerged from amino acid sequence studies[1-4] and X-ray structure studies,[5-10] but the dynamic picture of the active enzyme is much more poorly developed. Certain features of the dynamic structure of the enzyme are clear from mechanistic studies,[11-13] including the existence of an acyl enzyme, the existence of several important ionizations, the existence of more than one catalytically active conformation, and the existence of additional intermediates, but the precise mechanism of the enzymatic reaction remains unknown. Our present study delineates some additional features of this mechanism.

The existence of an acyl enzyme intermediate is now well established both for nonspecific[14-17] and for specific[12,18,19] substrates. The formation of this intermediate is rate determining for amide substrates, and its decomposition is rate determining for ester substrates.

The pH dependence of the activity of chymotrypsin indicates that two ionizable groups have primary control over the activity of the enzyme. The protonation of histidine-57, with a pK_a of about 7, causes loss of activity at low pH.[12,20-22] The ionization of the terminal amino group of isoleucine-16 at high pH induces a conformational change which results in the loss of activity. However, a more detailed study has revealed that the pH dependence of the activity of this enzyme is much more complex than this simple picture involving two ionizations. Thermodynamic studies of substrate and inhibitor binding[23,24] and careful studies of the temperature dependence of kinetic parameters[25,26] have shown that the enzyme can exist in any of several conformations, the exact state being governed by pH, temperature, and presence of substrates or inhibitors. Further, several of the conformations are catalytically active, and although there does not seem to be a large difference in rate constants among various forms, the enthalpies and entropies of activation for individual reaction steps differ among the various forms.

Rapid kinetic measurements on a number of substrates have been made.[27-30] Measurements of the

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