A MODEL OF ENZYMATIC SYNTHESIS OF THE INTERNUCLEOTIDE BOND BETWEEN OLIGODEOXYNUCLEOTIDES

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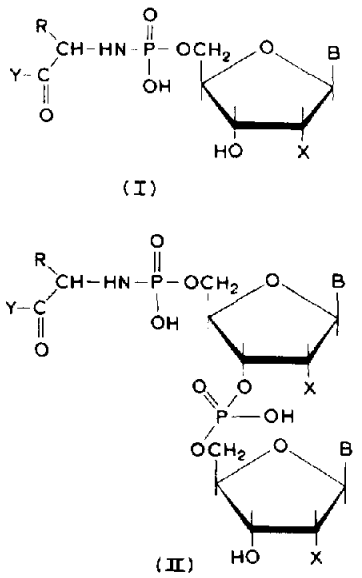
Progress in the field of chemical synthesis of the nucleic acids is hampered by the absence of an effective method of synthesis of internucleotide bonds. The usual methods of organic chemistry cannot be employed in elaborating a quantitative method of synthesis of oligo- and polynucleotides owing to the complexity of mononucleotide and, especially, oligonucleotide structure as well as the relative inertness of the hydroxyl and phosphate groups involved in the formation of the internucleotide bond. The principle of enzymatic catalysis, i.e. mutual activation of the reacting groups at the moment they are brought together in space, seems to be the most promising approach to the problem. We have, therefore, attempted to make a model of the enzymatic synthesis of the internucleotide bond, hoping to use this model for elaborating a procedure for chemical linking of oligonucleotide blocks. In this paper a new method of chemical synthesis of internucleotide bonds between oligodeoxynucleotide blocks based on the principle of enzymatic catalysis is suggested. Amino acid amidates of the monoesteric phosphate group of oligodeoxynucleotides have been used. In the presence of the complementary template, spatial proximity of the terminal phosphoramidate and hydroxy groups of oligonucleotides is achieved, resulting in their simultaneous activation. The hydroxyl is the proton donating group being converted into an alkoxy ion; the phosphoramidate group is the proton acceptor being converted into an "active" phosphate group able to undergo nucleophilic substitution. As a result, the internucleotide bond is formed.

We have studied [1] mono- (I) and oligo-nucleotide-(P–N)-amino acids (II), compounds which have some features of enzyme–substrate complexes (scheme 1)*.

The study of such compounds [1] has shown their nucleotide phosphorus to be in the potentially active state. These amidates are stable compounds. They can be stored for a long time and are sufficiently stable in



Scheme 1. B = pyrimidine or purine; X = H or OH; Y = H, OCH_3 or peptide.

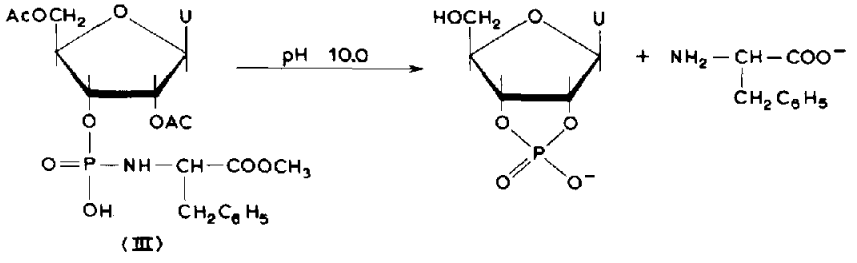
* The possibility of formation of intermediate complexes when nucleotides are carried by the respective enzymes in the biosyntheis of the internucleotide bond is discussed in [1].

aqueous solutions at neutral and alkaline pH values. But as soon as the phosphoramidate group is protonated they become active phosphorylating agents. It is very important that the activation of the phosphate group by protonation can occur not only inter- but also intramolecularly. This can be exemplified by conversion of ribonucleoside-3′-phosphoramidate into the corresponding cyclophosphate in aqueous solutions at high pH values [2] (scheme 2).

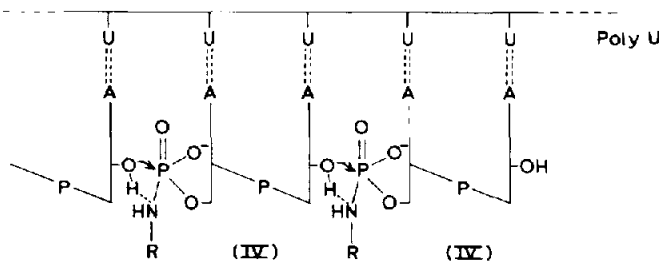
The alkali-resistant phosphoramidate bond in uridylyl-(3′ → *N*)-phenylalanine (III) cleaves readily. The new phosphoesteric bond thus formed is the result of general acidic intramolecular catalysis of nucleophilic substitution at the phosphorus atom. In the course of such catalysis both groups become activated: the phosphate group becomes a strong electrophilic agent, the hydroxy group a strong nucleophilic agent (an alkoxy ion). This and other [1] reactions suggest that the usually stable oligodeoxynucleoside-5′-phosphoramino acids as well as oligodeoxynucleoside-3′-phosphoramino acids [3] may be donors of terminal nucleotide phosphorus involved in the formation of the internucleotide bond in the aqueous media. This may be achieved when the terminal groups, i.e. the phosphoramidate group of one oligonucleotide and the hydroxyl group of the other, are brought together at a distance short enough to ensure formation of the internucleotide bond. In this case the phosphoramidate group of one oligonucleotide will be protonated by the hydroxyl of the other one, i.e. simultaneous "intramolecular" activation of both reacting groups will occur. Spatial proximity of the reactive centres of the two oligonucleotide blocks is best achieved by using complementary templates.

We have proved the feasibility of this approach by carrying out the reaction between deoxyadenylyl-(5′ → 3′)-deoxyadenylyl-(5′ → *N*)-phenylalanine (IV) [3] in aqueous solution in the presence of poly U (scheme 3).

The complex poly U:amidate IV has a melting temperature of 1° [4]. This means that at a temperature lower than 0° this complex exists as a double stranded structure. The reaction mixture containing 5 μmole IV, 5 μmole poly U, 1 M NaCl and 0.006 M $MgCl_2$ in 0.2 ml of phosphate buffer (0.002 M, pH 7.5) was left to stand at −7°. The reaction mixture was heated several times to +60°. After 10 days a portion of the solution was chromatographed in an ethanol–ammonium acetate (7:3) system (system A); the remaining solutions was passed through a DEAE-Sephadex A-25 column (CH_3COO^-). Paper chromatography was employed for the preparative isolation of the UV-absorbing substance with R_f 0.05, which was eluted with water. The eluate was analyzed in two ways: (1) it was hydrolyzed by 6 N HCl (105°, 2 hr) to yield uracil (R_f 0.76, in water, pH 10.0) and adenine (R_f 0.37); and (2) it was hydrolyzed with pancreatic ribonucleases (pH 7.5, 2 hr, 37°). The enzymatic hydrolysate was chromatographed in system A, and UMP (R_f 0.35) and a starting zone were observed. In the latter, adenine was detected after hydrolysis by 6 N HCl (105°, 2 hr). The reaction mixture was separated on DEAE-Sephadex A-25 with a linear gradient elution using 300 ml of 0.5 M NaCl, 7 M urea and 0.005 M sodium acetate at pH 5.5 in the reservoir and 300 ml 7 M urea and 0.05 M sodium acetate (pH 5.5) in the mixing vessel. The elution pattern obtained is shown in fig. 1. Fractions from the peaks were diluted to NaCl concentrations of 0.05 M, adjusted to pH 9.5 by conc. ammonia; the solutions were then passed



Scheme 2.



Scheme 3.

through DEAE cellulose (HCO_3^-) and eluted with 1 M triethylammonium bicarbonate buffer. The eluates were evaporated by adding water to remove the buffer; the remaining oil was analyzed. Chromatography of the fractions of peaks IV and V in system A revealed a substance with R_f 0.05. This substance was eluted, hydrolyzed by 6 N HCl (105°, 2 hr) and chromatographed in water, pH 10.0; uracil and adenine were found. The above experiment allows one to conclude that in the course of the reaction of dpApA-amidate (IV) in the presence of poly U at least two types of substance are formed: oligo dA (peak IV) and poly dA (peak V). The yield of these compounds is about 10%. We have not observed the formation of oligo dA and poly dA in a similar experiment with the unsubstituted dinucleotide d-pApA instead of amidate IV. In the elution pattern, after separation of this control reaction mixture, from DEAE-Sephadex A-25 peak IV is absent and in peak V adenine is also absent.

Similar results have been obtained with deoxyadenylyl-(3′ → 5′)-deoxyadenylyl-(3′ → *N*)-PheOCH$_3$ + poly U and deoxyadenylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-deoxyadenylyl-(5′ → *N*)-PheOCH$_3$ + poly U. The melting temperatures of these complexes are +3° and +5° respectively [4].

We hope that this synthesis of the internucleotide

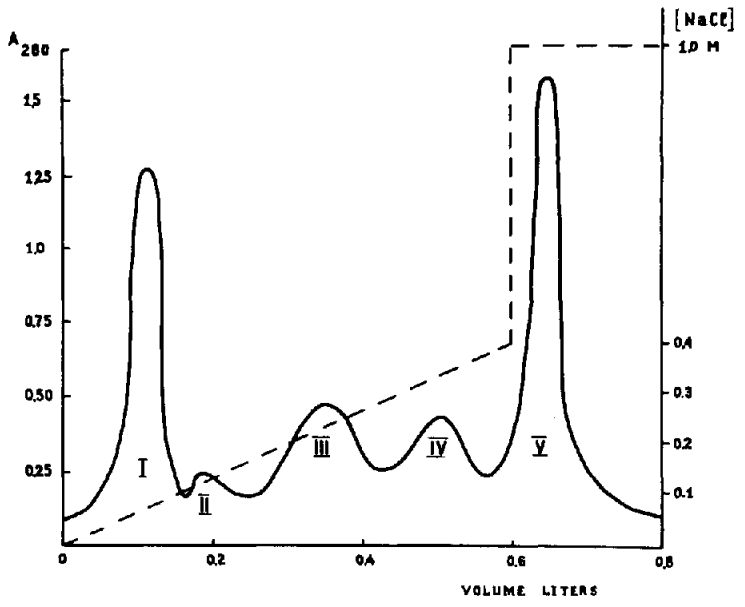


Fig. 1. Chromatography of the reaction mixture on DEAE-Sephadex A-25 (acetate) column (0.5 × 40 cm).

bond may become a preparative method of linking of oligonucleotide blocks, should the interaction between the template and oligonucleotide blocks be more specific than in the complexes we have studied. This method is being verified now with amidates of d-pTpTpTpGpG and the complementary template d-pCpCpApApApCpCpApApA, which have been synthesized by the usual method [5]. Specific A–T and G–C interactions should make this complex stable enough, i.e. it will have an ordered structure at temperatures above 0°. If we succeed in showing that the internucleotide bond between the pentanucleotide blocks in this system is formed in good yield, it will be possible to use this method for linking oligonucleotide blocks on the templates for the synthesis of DNA-like polynucleotides in aqueous solutions. This approach will be based on two principles: (1) the principle of the "sticky end" suggested by Khorana [6], when the spatial proximity of the reacting groups is provided for by the nucleotide sequence of the protruding single-stranded end of polynucleotide and (2) the principle of enzymatic catalysis when the closeness of the reactive groups in space results in their being activated. One may believe that the combination of these two principles will make possible self-assembly of oligonucleotide blocks. Then, instead of using a ligase, a chemical method will be available for linking oligonucleotide blocks; hence completely chemical synthesis of genetic material will become a reality.

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