# OLIGONUCLEOTIDES AND NUCLEOTIDYLPEPTIDES. XXXII. SYNTHESIS AND HYDROLYTIC STABILITY OF AMINO ACID DERIVATIVES OF ADENOSINE 5'-DI(TRI)PHOSPHATES

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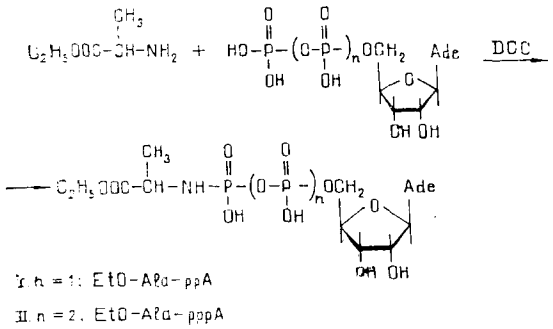
The synthesis has been effected of the ethyl esters of adenosine-5'-diphospho-($P_\beta \rightarrow N$)- and adenosine-5'-triphospho($P_\gamma \rightarrow N$)-alanine. It has been shown that under the conditions of synthesis the serine analogues of ATP and ADP decompose. An investigation of the hydrolytic stability of the compounds synthesized has shown that they are unstable in acid and alkaline media. In an acid medium the phosphoramide bond is cleaved more rapidly than the phosphoric anhydride bond (in the case of the ADP analogue), while in an alkaline medium the ester bond is saponified and the phosphoric anhydride bond is cleaved. The ATP analogue is more labile both in acid and in alkaline media.

Nucleic acids fulfill their biological functions in the form of complexes with proteins. All the nucleoprotein structures found in nature can be divided into noncovalent and covalent types [1-6]. Covalent nucleoprotein compounds in their turn, can be divided into those existing permanently in nature and those formed as intermediates of the action of topoisomerases on DNA [7]. Usually, in covalent nucleoprotein structures the protein is attached to a 5'- or, more rarely, to a 3'-terminal phosphate group of a nucleic acid [1-7]. It has recently been shown [1] that DNA contains protein inclusions. It is assumed [8] that one end of the protein is attached to a 5'-polyphosphate residue of a fragment of the DNA.

The aim of the present work was the synthesis of model nucleotidylpeptides in which amino acids are attached with the aid of a phosphoramide bond to the terminal phosphate of adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) and an investigation of their stability. Such compounds model a possible linkage unit in covalent nucleoprotein structures. On the other hand, various amides of nucleoside 5'-polyphosphates are substrate analogues or inhibitors of certain enzymes and can serve as affinity reagents [9]. Some properties of amino acid phosphoramides of nucleoside 5'-polyphosphates have been described previously [10].

Ethyl esters of adenosine 5'-diphospho-($P_\beta \rightarrow N$)- and adenosine-5'-triphospho-($P_\gamma \rightarrow N$)-alanine were synthesized by the dicyclohexylcarbodiimide (DCC) method. The reaction was performed in anhydrous dimethylformamide at 37°C. A tenfold excess of alanine ethyl ester and a fivefold excess of DCC with respect to the nucleosides were used:




I n = 1: EtO-Ala-ppA

II n = 2: EtO-Ala-pppA

The results of an investigation of the kinetics of the synthesis of compounds (I) and (II) showed that the optimum time of the reaction was 3 h. The yield of compound (I) was 75% and that of (II) 45%. It must be mentioned that in the synthesis of EtO-Ala-ppA about 5% of EtO-Ala-pA was formed, and in the EtO-Ala-pppA reaction mixture there were 8% of EtO-

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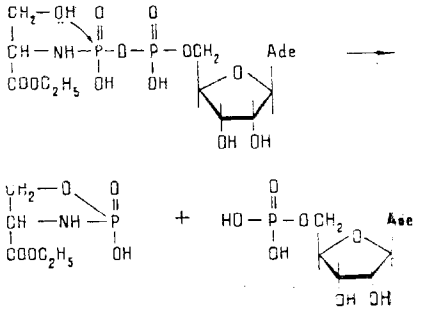
Ala-ppA and 3% of EtO-Ala-pA. These by-products were formed because in the preparation of ADP and ATP for the reaction (the conversion of these nucleotides into their ammonium salts and drying with organic solvents) partial dephosphorylation took place; the ADP contains AMP as impurity and the ATP contains impurities ADP and AMP.

The EtO-Ala-ppA and EtO-Ala-pppA were isolated from the reaction mixture with the aid of paper chromatography in solvent system 2. Compounds (I) and (II) were homogeneous on chromatography in solvent systems 1-4 and also in paper electrophoresis at pH 5.0 and 7.5. The structures of the compounds synthesized were established by determining the adenine: phosphorus:amino acid ratio after acid hydrolysis (6 N HCl, 100°C, 24 h).

To determine the influence of functional groups on the reactivity of the amino acid derivatives of the nucleoside 5'-polyphosphates we attempted to synthesize serine phosphoramides of ADP (EtO-Ser-ppA) and of ATP (EtO-Ser-pppA). The reaction was carried out in the same way as in the case of compounds (I) and (II). However, it was impossible to obtain EtO-Ser-ppA and EtO-Ser-pppA. An investigation of the kinetics of the synthesis of EtO-Ser-ppA and EtO-Ser-pppA showed that only AMP and EtO-Ser-pA accumulated in the reaction mixture. We have shown previously [1, 3, 6] that the hydroxy group of serine, being located adjacent to the phosphoramide center in nucleotidyl-(5'→N)-serine esters, is responsible for the cleavage of the phosphoester bond, i.e., for the appearance of the nucleoside. We assume that on the incubation of ADP or ATP with an excess of serine ethyl ester and DCC, EtO-Ser-ppA and EtO-Ser-pppA, respectively, are formed in the reaction mixture. However, these compounds are unstable: under the reaction conditions intramolecular nucleophilic catalysis takes place at the β- (in the case of EtO-Ser-ppA) or γ- (in the case of EtO-Ser-pppA) phosphorus atoms with the participation of the serine hydroxy group (the scheme shows the hypothetical mechanism of the cleavage of EtO-Ser-ppA):



The result of such an intramolecular nucleophilic reaction is the formation of AMP, which then reacts with serine ethyl ester, and a cyclic phosphoserine derivative. When $^{14}C$-labeled serine ethyl ester was used it was found that the labeled EtO-[$^{14}C$]-Ser-pA and a derivative of labeled phosphoserine ($R_f$ 0.14 in solvent system 2) were formed in the reaction mixture. The acid hydrolysis of the latter (6 N HCl, 24 h, 100°C) led to the formation of inorganic phosphate and [$^{14}C$] serine. These experiments confirmed the mechanism for the cleavage of EtO-Ser-ppA proposed above.

Thus, we have been unable to synthesize serine phosphoramides of ADP and ATP.

In the determination of the type of interbiopolymer bond in covalent nucleoprotein structures, use is frequently made of their chemical hydrolysis [1, 2]. Some information on the behavior of amino acid derivatives of nucleoside 5'-polyphosphates in an aqueous medium is given in the literature [10]. The detection in various organisms of a large amount of covalent nucleoprotein structures has led to the necessity of a more detailed study of this question.

An investigation of the hydrolytic stability of EtO-Ala-ppA showed (Fig. 1) that this compound is cleaved both in acid and in alkaline media. In the acid hydrolysates AMP, ADP, alanine ethyl ester, and inorganic phosphates were detected. Thus, in an acid medium EtO-Ala-ppA is cleaved in two directions: 1) with the formation of ADP and alanine ethyl ester; and 2) with the formation of AMP and the ethyl ester of N-phosphoalanine, which in an acid medium then breaks down into alanine ethyl ester and inorganic phosphate.

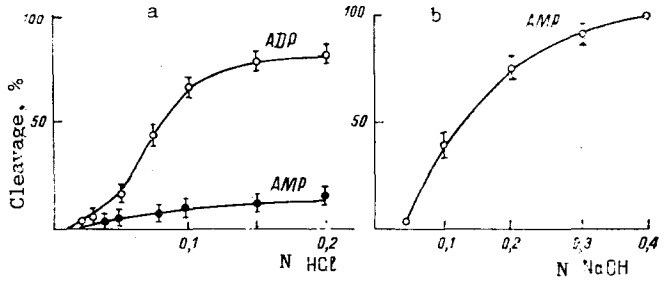



Fig. 1. Accumulation of the products of the cleavage of the ethyl ester of adenosine-5'-diphospho-($P_\beta \rightarrow N$)-alanine as a function of the concentration of acid (a) and of alkali (b) (37°C, 1 h).

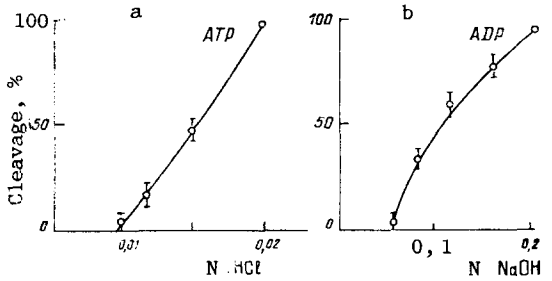
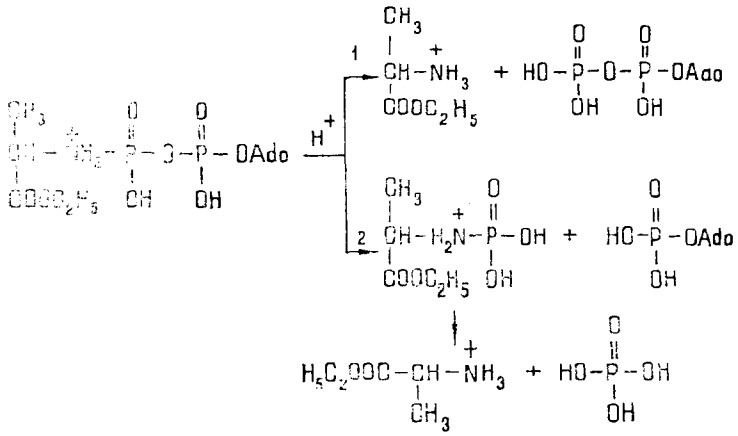



Fig. 2. Accumulation of the products of the cleavage of the ethyl ester of adenosine-5'-triphospho-($P_\gamma \rightarrow N$)-alanine as a function of the concentration of acid (a) and of alkali (b) (37°C, 1h).




It was established that in an acid medium the cleavage of the phosphoramide bond (direction 1) predominated; in 0.1 N HCl (37°C) the rate constant for the hydrolysis of the phosphoramide bond ($k_1 = 12.2 \cdot 10^{-3}$ $sec^{-1}$) was four times greater than the rate constant of the hydrolysis of the phosphoric anhydride bond ($k_2 = 2.9 \cdot 10^{-3}$ $sec^{-1}$). It may be assumed that in an acid medium protonation of the phosphoramide nitrogen takes place, and the conjugation between the nitrogen and phosphorus atoms is disturbed, which facilitates the hydrolysis of the phosphoramide bond. Since ADP is stable under the acid conditions used, the cleavage of the phosphoric anhydride bond in EtO-Ala-ppA depends on the amino acid attached to the ADP. Apparently, the protonation of the phosphoramide nitrogen causes a redistribution of the electron density not only on the β- but also in the α-phosphorus atoms in EtO-Ala-ppA, which leads to the hydrolysis both of the phosphoramide bond and also, particularly, of the phosphoric anhydride bond.

EtO-Ala-ppA is unstable in an alkaline medium (Fig. 1b). AMP and N-phosphoalanine were detected in alkaline hydrolysates. This indicates that the cleavage of the phosphoric anhydride bond and the saponification of the ester bond take place. It is interesting to note that ADP is stable in the concentrations of alkali used, i.e., the cleavage of the phosphoric anhydride bond of EtO-Ala-ppA in an alkaline medium is also due to the amino acid attached

to the ADP. We assume that the addition of an amino acid of ADP lowers the total negative charge on the diphosphate fragment and facilitates the approach of a nucleophilic reagent.

An investigation of the reactivity of EtO-Ala-pppA showed that this compound is extremely labile, being cleaved in neutral, alkaline, and acid media (Fig. 2).

In an acid medium, EtO-Ala-pppA is cleaved at the phosphoramide bond with the formation of ATP and alanine ethyl ester. It must be mentioned that the phosphoramide bond in EtO-Ala-ppA is far more stable in an acid medium than that in EtO-Ala-pppA (see Fig. 1a, 2a). While EtO-Ala-ppA is stable in 0.02 N HCl (37°C, 1 h), EtO-Ala-pppA hydrolyzes completely under analogous conditions. In contrast to EtO-Ala-ppA, in EtO-Ala-pppA the phosphoric anhydride bonds are not hydrolyzed in an acid medium (0.02 N HCl, 37°C, 1 h). These facts do not agree with those published previously [10].

In an alkaline medium, EtO-Ala-pppA, like EtO-Ala-ppA is cleaved at the phosphoric anhydride bond (Fig. 2b). ADP and N-phosphoalanine were detected in the reaction mixture, and in this case EtO-Ala-pppA is far more labile than the ADP analogue.

In conclusion, it may be stated that in an acid medium the preferred position of rupture of ADP- and ATP-amino acids of the phosphoramide type is the phosphoramide bond, and in an alkaline medium it is the phosphoric anhydride bond. The results obtained on the hydrolytic stabilities of EtO-Ala-ppA and EtO-Ala-pppA indicate a considerable activating influence of the amino acid residue on the molecule of an adenosine 5'-polyphosphate. We assume that the activation of the pyrophosphate bond in an acid medium is connected with a shift of electron density along the phosphorus–oxygen–phosphorus–nitrogen chain of conjugation, while in an alkaline medium it is connected with a decrease in the total negative charge on the polyphosphate system.

The experimental results presented must be taken into account in a study of the type of the interbiological bond in natural covalent nucleoprotein structures.

## EXPERIMENTAL

We used the sodium salts of ADP, ATP, and L-alanine and L-serine from Reanal, N,N'-dicyclohexylcarbodiimide from Ferak, alkaline phosphatase from Worthington, 5'-nucleotidase from Sigma, and [3-$^{14}$C]-D-L-serine (1.3 GBq/mmole) and [2-$^{14}$C]-D-L-alanine (1.03 GBq/mmole) of domestic production. The ethyl esters of the amino acids were synthesized by a published method [11]. The hydrolytic stability of EtO-Ala-ppA and EtO-Ala-pppA was investigated as we have described previously [12].

The compounds synthesized were isolated with the aid of preparative chromatography on type SN-2 (fast) or FN-4 (medium) paper (GDR). (The chromatographic paper was previously washed with 2 N HCl and then with water to neutrality). TLC on Silufol plates was used for analytical purposes. The following solvent systems were employed: 1) propan-2-ol–concentrated ammonia–water (7:1:2); 2) isobutyric acid–concentrated ammonia–water (66:1:33); 3) butan-1-ol–acetone–5% ammonia–acetic acid–water (9:3:2:2:4); and 4) butan-1-ol–water–acetic acid (5:3:2). Paper electrophoresis was performed in 0.05 M triethylammonium bicarbonate buffer, pH 7.5, and in 0.05 M citrate buffer, pH 5.0. A horizontal high-voltage instrument (Labor) was used. UV spectra were recorded with the aid of a SF-26 spectrometer. The structures of the esters of the nucleotidyl-(P→N)-amino acids were shown by determining the base: phosphorus:amino acid ratio after complete hydrolysis (6 N HCl, 100°C, 24 h) and combustion (concentrated $HClO_4$, 180°C, 6 h) [12].

Synthesis of the Ethyl Esters of Adenosine-5'-diphospho($P_\beta$→N)-alanine and Adenosine 5'-triphospho-($P_\gamma$→N)-alanine. A solution of 0.1 mmole of a nucleotide in 1 ml of $H_2O$ was deposited on a column of Dowex-50 ($NH_4^+$ form) (10 × 50 mm) and elution was carried out with water. The concentrated eluate was treated with 150 µl (0.36 mmole) of tri-n-octylamine. The mixture was evaporated to dryness and the residue was dissolved in 1 ml of dimethylformamide. The trioctylammonium salt of the nucleotide was dried by azeotropic distillation with anhydrous benzene and dioxane, which were added to the dimethylformamide solution. To the solution were added 1 mmole of previously dried alanine ethyl ester in 1 ml of dimethylformamide and 103 mg (0.5 mmole) of DCC. The reaction mixture was kept in a thermostat at 37°C.

For the kinetic investigations, samples were taken for the reaction mixture after predetermined intervals of time (5, 10, 15, 30, and 60 min, etc.) and were deposited on

chromatographic paper, and after chromatography in solvent system 2 the yields of reaction products were determined spectrophotometrically [12].

The synthesis of the labeled ADP- and ATP-amino acids was performed similarly except that, in the synthesis of the amino acid esters, labeled amino – [$^{14}$C]alanine and [$^{14}$C]serine – were added to the reaction mixture. The specific activities of the amino acid esters were 30-40 MBq/mole.

The EtO-Ala-ppA and EtO-Ala-pppA were isolated with the aid of preparative paper chromatography in solvent system 2. The zones with $R_f$ 0.37 in the case of the synthesis of EtO-Ala-ppA and with $R_f$ 0.24 in the case of the synthesis of EtO-Ala-pppA were eluted with water at +4°C. Chromatographic characteristics of the compounds synthesized:

EtO-Ala-ppA – $R_f$ 0.34, in system 1, 0.43 in system 3;

EtO-Ala-pppA – $R_f$ 0.21, in system 1, 0.39 in system 3.

The electrophoretic mobilities of the compounds synthesized relative to AMP were:

EtO-Ala-ppA – $U_{rel.\,AMP}$ = 1.13 (pH 7.5) and 1.76 (pH 5.0);

EtO-Ala-pppA – $U_{rel.\,AMP}$ = 1.21 (pH 7.5) and 2.61 (pH 5.0).

In the case of EtO-Ala-ppA the adenine*:phosphorus:alanine ratio was 1:2.1:1.1, and in the case of EtO-Ala-pppA it was 1:3.03:1.18.

## CONCLUSION

1. The ethyl esters of adenosine-5'-diphospho-($P_\beta$→N)-alanine and adenosine-5'-triphospho-($P_\gamma$→N)-alanine have been synthesized.

2. The free hydroxy group of serine in EtO-Ser-ppA and EtO-Ser-pppA causes an extreme lability of these compounds, which excludes the possibility of preparing them.

3. The hydrolytic stabilities of the compounds synthesized were investigated, and it was shown that:

in an acid medium EtO-Ala-ppA is cleaved both at the phosphoramide and the phosphoric anhydride bonds. The phosphoramide bond is cleaved most effectively; and

in an acid medium EtO-Ala-pppA is cleaved at the phosphoramide bond. EtO-Ala-pppA is far more labile than the ADP analogue;

in an alkaline medium EtO-Ala-ppA and EtO-Ala-pppA are cleaved at the phosphoric anhydride bonds. EtO-Ala-pppA is more labile than the ADP analogue.

4. The results obtained on the hydrolytic stability of the amino acid derivatives of adenosine 5'-polyphosphates must be taken into account in determining the type of interbiopolymer bond in covalent nucleotido- and nucleoprotein structures.

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*Adenine partially decomposes under the conditions of acid hydrolysis.