SYNTHESIS AND PROPERTIES OF THE METHYL ESTER OF POLYURIDYLYL-(5'→N)-PHENYLALANINE

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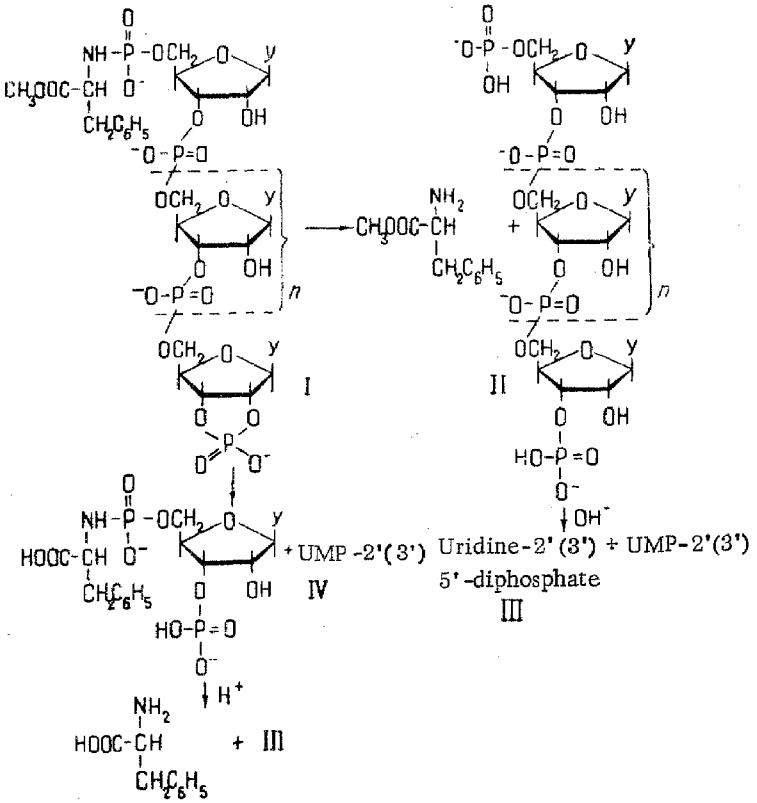
Hitherto, we have made a systematic study of nucleotidopeptides of the phosphoric amide type only on the basis of model structures in which the nucleotide moiety has been a monomeric compound (adenosine-5'-phosphate, guanosine-5'-phosphate, and uridine-5'- and -3'-phosphates). However, the nucleotidopeptides isolated from natural materials are generally oligo- and polynucleotide structures.

To investigate the properties of such polynucleotidopeptides we have carried out the synthesis of the phenylalanine derivative of polyuridylic acid (I). The formation of the phosphoric amide bond was carried out by the "carbodiimide" method [1].

The initial polyuridylic acid with terminal-3'- and -5'-phosphate groups ["poly-U-5'-phosphate" (II)] was obtained by the copolymerization of uridine-2'(3')-phosphate and uridine-2'(3'), 5'-diphosphate (III) under the action of diphenyl phosphorochloridate. This acid consists of a polymer with arbitrarily recurring $C_{2'} \rightarrow C_{5'}$ and $C_{3'} \rightarrow C_{5'}$, internucleotide bonds [2]. Uridine-2'(3')-phosphate and compound (III) were reacted in the form of the tri-n-octylammonium salts (5:1). The low-molecular-weight products were eliminated by dialysis, and the polymer was isolated in the form of the calcium salt and was then converted into the free acid and condensed with the methyl ester of phenylalanine in the presence of an excess of N, N'-dicyclohexylcarbodiimide (DCC) in dimethylformamide together with triethylamine. The reaction was run for 5 days at 37°. The product was purified by reprecipitation with ether from dimethylformamide and by paper chromatography in the isopropanol—concentrated NH_3 — water (7:1:2) system.

The structure of substance (I) was established by hydrolysis, which was carried out under various conditions (scheme). At acid pH values, the methyl ester of phenylalanine was split off from compound (I). At alkaline pH values,



no rupture of the bond between the nucleotide and the amino acid was found. Under standard conditions for the hydrolysis of internucleotide phosphoric diester bonds (0.5 N KOH, 37°, 18 hrs), uridine-2'(3')-phosphate and 2'(3')-phosphouridylyl-(5'→N)-phenylalanine (IV) were found in the hydrolyzate. The mild acid hydrolysis of (IV) (pH ~ 3), as was to be expected, gave uridine-2'(3'), 5'-diphosphate (III) and phenylalanine.

To determine the length of the polynucleotide chain in substance (I), the amino acid:nucleotide:phosphorus ratio

was determined (1 : 13.2 : 14.2). The alkaline hydrolysis of the poly-U-5'-phosphate (II) obtained by the acid hydrolysis of compound (I) gave uridylic acid and uridine-2'(3'), 5'-diphosphate (III) (11.2 : 1). The ratio of uridylic acid and compound (IV) in the alkaline hydrolyzate of substance (I) was found to be 10.2 : 1. However, this ratio is approximate, since in the determination of (IV) we used the conversion factor calculated for uridylic acid [3].

The results obtained show that substance (I) is a mixture of amino acid derivatives of polyuridylyl-5'-phosphate with a mean chain length of 13 units. The behavior of substance (I) under acid and alkaline conditions shows that the amino acid in it is attached to the 5'-end and not to the 3'-end or to an internucleotide phosphorus atom of the polynucleotide, since it has been shown with suitable model structures [4, 5] that the latter are readily hydrolyzed under alkaline conditions to the free amino acid and the initial nucleotide. Moreover, according to G. Khorana [6], internucleotide phosphorus is incapable of being activated in the presence of DCC.

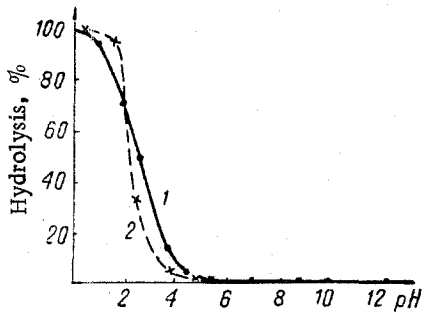


Fig. 1. Hydrolysis of the phosphoric amide bond in the methyl ester of polyuridylyl-(5'→N)-phenylalanine (I) and the methyl ester of uridylyl-(5'→N)-phenylalanine (II).

To elucidate the dependence of the strength of the bond between the nucleotide and the amino acid on the degree of polymerization of the nucleotide moiety, the hydrolysis of the methyl ester of polyuridylyl-(5'→N)-phenylalanine (I) was studied at various pH values (37°, 1 hr). The degree of hydrolysis of the phosphoric amide bond was determined from the amount of amino acid split off. Figure 1 gives the hydrolysis curve of compound (I) at various pH values and, for comparison, the hydrolysis curve (under the same conditions) of the methyl ester of uridylyl-(5'→N)-phenylalanine [8]. It can be seen from Fig. 1 that there is some difference between the stabilities of the phosphoric amide bonds in the polymeric compound and in the corresponding monomer derivative. In the strongly acid region, the polynucleotide derivative of phenylalanine is somewhat more stable than the phenylalanine derivative of UMP, and in the weakly acid region, on the contrary, it is more labile. This difference in the strength of the phosphoric amide bond is apparently due to the influence of the secondary structure of the polymer which, as has been observed in the case of peptide derivatives of mononucleotides [7] at pH < 2, as it were "screens" the amide nitrogen of the nucleotidopeptide bond, preventing its protonation, which determines the rate of hydrolysis [4]. This "screening" may take place both as the result of the delocalization of the positive charge of the protonated amide nitrogen and as a consequence of steric hindrance. At higher pH values, when the hydroxyl groups of the internucleotidic phosphate groups are completely dissociated, the strength of the electrostatic repulsion disturbs the secondary structure, uncovering the phosphoric amide bond. The latter also becomes accessible to intramolecular protonation, e.g., with the formation of hydrogen bonds with N_1 of the uracyl groups.

A further confirmation of the hypothesis of the influence of the secondary structure on the strength of the P→N bond in polynucleotidyl-(5'→N)-amino acids was obtained by studying the kinetics of the hydrolysis of substance (I) with a 0.05 N solution of hydrochloric acid at 40°. The hydrolysis was followed from the increase in the amount of amino acid determined by paper chromatography. From the results of the hydrolysis, the half-period of the reaction was found to be 20 min (Fig. 2). This value is considerably higher than those found for the methyl esters of adenylyl-(5'→N)-amino acids but of the same order as the figures for (5'→N)-peptide derivatives of AMP [7]. Thus, the degree of delocalization of the positive charge of the protonated amide nitrogen, which determines the rate of hydrolysis of the phosphoric amide bond, is comparable with the degree of delocalization in peptide derivatives owing to the closely adjacent peptide groups.

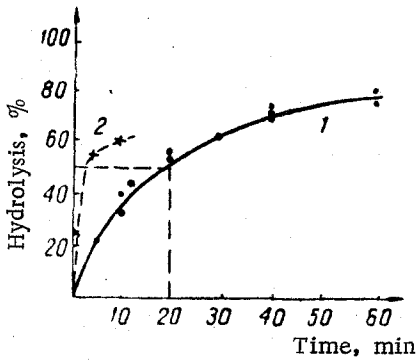


Fig. 2. Kinetics of the hydrolysis of the phosphoric amide bond in the methyl ester of polyuridylyl-(5'→N)-phenylalanine (1) and the methyl ester of uridylyl-(5'→N)-phenylalanine (2).

Experimental

Solvent systems: A) isopropanol — concentrated NH_3 — water (7 : 1 : 2); B) ethanol — 1 M ammonium acetate, pH 7.5 (7 : 3). The mobility U_{rel} was calculated with reference to the mobility of uridine-2'(3')-phosphate. The nucleotides were detected on the chromatograms and electrophoregrams by UV light absorption. The numerical coefficients found for uridylic acid were used for the quantitative determination of the nucleotides [3]. The amino acids were detected and quantitatively determined by the ninhydrin method [10].

The synthesis of uridine-2'(3'), 5'-diphosphate (III). Polyphosphoric acid was reacted with uridine [9]. Compound (III) was isolated in the form of the dibarium salt, R_f 0.03 (system A); 0.13 (system B); U_{rel} 1.30 at pH 4.5; 1.20 at pH 8.0.

Synthesis of polyuridylyl-5'-phosphate [2]. Seventy-seven mg (0.17 mmole) of the barium salt of uridine-2'(3')-phosphate and 22.5 mg (0.035 mmole) of the dibarium salt of compound (III) were converted into the mono-(tri-n-octylammonium) salts, and these were dissolved in 1 ml of absolute dioxane, and treated with 0.07 ml of diphenyl phosphorochloridate and 0.14 ml of tri-n-butylamine. After 1 hr, a further 0.07 ml of diphenyl phosphorochloridate and 0.14 ml of tri-n-butylamine were added to the reaction mixture, which was left for 3 hr for polymerization. After the elimination of the solvent, the residue was dissolved in water, and the aqueous solution was brought to pH 8.7 with ammonia and was extracted with ether. The aqueous layer was evaporated to small bulk and the polymer was precipitated in the form of the calcium salt (44 mg) by the addition of an ethanolic solution of calcium chloride. The polymer was dissolved in water and dialyzed against distilled water for 20 hrs. The residue was evaporated under reduced pressure to small bulk and precipitated with an alcoholic solution of calcium chloride. This gave 19 mg of substance (II), R_f 0.03 (system A); U_{rel} 0.96 at pH 8.0.

Synthesis of the methyl ester of polyuridylyl-(5'→N)-phenylalanine (I). Sixty-six mg of the calcium salt of (II) was passed through a column of "Dowex-50" (H^+), the eluate was evaporated to dryness, and the residue was treated with an ethereal solution of the freshly-prepared methyl ester of phenylalanine (from 70 mg of the hydrochloride). The solvent was distilled off, and the residue was dried by the repeated distillation of absolute dioxane and benzene from it and then dissolved in 1 ml of absolute dimethylformamide with the addition of a small amount of triethylamine. The solution was treated with 20 mg of DCC, and the reaction mixture was left protected from moisture at 37° for 5 days. The dicyclohexylurea which precipitated was separated off, and the solution was poured into 30 ml of absolute ether. The precipitate was carefully washed with absolute ether and dried in the air. Yield 72 mg.

The reaction product was dissolved in aqueous methanol, transferred to a sheet of Whatman (3 mm) paper and chromatographed in system A for 3 days. The new UV-absorbing substances (R_f 0.13, 0.31, and 0.39) were eluted together using 50% methanol. The solvent was distilled off to dryness, and the residue was dissolved in absolute dimethylformamide and precipitated with absolute ether. After careful washing with absolute ether, the substance was dried in a vacuum desiccator over P_2O_5. Yield 22 mg. UV spectrum: at pH 6 λ_{max} 260 mμ, $\varepsilon_{(P)}$ 8900; λ_{min} 230 mμ, $\varepsilon_{(P)}$ 2700; at pH 12 λ_{max} 258 mμ, $\varepsilon_{(P)}$ 6700; λ_{min} 245 mμ, $\varepsilon_{(P)}$ 5700.

Determination of the composition of substance (I). A weighed amount of (I) was dissolved in 0.5 ml of distilled water. Two 0.1-ml samples were taken. One sample was subjected to combustion to determine the amount of phosphorus. The phosphorus content [11] was 2.13 μmole/mg. The other sample was hydrolyzed with a 1 N solution of hydrochloric acid for 1 hr at 37° and the hydrolyzate was chromatographed in system A. By the ninhydrin method [1] it was found to contain 0.15 μmole/mg of amino acid.

The UV-absorbing part of the hydrolyzate was eluted with water and the content of nucleotides was determined (1.98 μmole/mg). Thus the amino acid:nucleotide:phosphorus ratio was 1:13.2:14.2.

The eluate of the UV-absorbing fraction of the acid hydrolyzate was evaporated to dryness and was hydrolyzed with a 0.5 N solution of caustic potash for 18 hr at 37°. The hydrolyzate was neutralized with "Dowex-50" (H^+), the resin was filtered off, and the filtrate was subjected to electrophoresis at pH 8.0. Uridine-2'(3')-phosphate and compound (III) were detected. They were eluted and their ratio was determined (11.2:1).

Alkaline hydrolysis of substance (I). About 2 mg of (I) was hydrolyzed with 0.5 N caustic potash solution for 18 hr at 37°. After careful neutralization of the hydrolyzate with "Dowex-50" (H^+) it was subjected to electrophoresis. Two UV-absorbing compounds were obtained: one of them corresponded to uridine-2'(3')-phosphate, and the other had U_{rel} 1.20 at pH 4.5 and 1.05 at pH 8.0, R_f 0.12 (system A). Their ratio was 10.2:1. The more rapidly moving substance was eluted with water and was hydrolyzed at pH 3 (37°, 1 hr). The hydrolyzate was chromatographed in system A, phenylalanine and substance (III) (0.97:1) being found.

Study of the strength of the phosphoric amide bond in compound (I) at various pH values. About 25 mg of substance (I) was dissolved in 2 ml of distilled water and 0.1-ml portions of the solution were transferred to 16 test tubes. One sample was hydrolyzed with 1 N hydrochloric acid solution at 37° for 1 hr in order to rupture the phosphoric amide bond completely, and 0.1 ml of an appropriate buffer solution (pH 0.5-14) was added to each of the other samples. After the samples had been held for 1 hr at 37°, they were transferred quantitatively to a chromatogram and were chromatographed in system A. The amount of amino acid hydrolyzed off was determined for each sample and the degree of hydrolysis was calculated, taking as 100% the amount of amino acid formed in the hydrolysis of substance (I) with 1 N hydrochloric acid (see Fig. 1).

Study of the kinetics of the hydrolysis of the phosphoric amide bond in compound (I) with 0.05 N HCl. Approximately 10 mg of substance (I) was dissolved in 0.7 ml of distilled water and 0.1-ml portions were poured into six test

tubes. The test tubes were thermostatted at 40°, after which 0.1 ml of 0.1 N solution of hydrochloric acid was added to each of five test tubes, while 0.1 ml of 1 N hydrochloric acid was added to one test tube for the complete hydrolysis of the phosphoric amide bond. After predetermined intervals of time, the samples were neutralized with caustic soda and were transferred quantitatively to a chromatogram. The degree of hydrolysis of the phosphoric amide bond was determined as described above and a graph of the degree of hydrolysis as a function of the time was constructed (see Fig. 2). The half-period of reaction ($\tau_{1/2}$) found by the graphical method was 20 min.

The paper chromatography was carried out by the ascending method on type "B" Leningrad paper, and the preparative chromatography on Whatman (3 mm) paper. The electrophoresis was also carried out on type "B" Leningrad paper, for 4 hr in a field of 8 V/cm and at pH 4.5 (0.002 M KH_2PO_4) and pH 8.0 (triethylammonium bicarbonate).

Summary

1. The methyl ester of polyuridylyl-(5'→N)-phenylalanine containing an average of 13 nucleotide residues has been synthesized by the carbodiimide method.

2. The hydrolysis of the methyl ester of polyuridylyl-(5'→N)-phenylalanine has been studied as functions of pH and time. In this compound, the phosphoric amide bond is hydrolyzed in an acid medium and is stable at pH < 4, and the half-period of hydrolysis of the phosphoric amide bond in 0.05 N hydrochloric acid solution is 20 min.

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