CLEAVAGE OF THE PHOSPHATE BOND IN THE HYDROLYSIS OF NUCLEOTIDYL (5' → N) - AMINO ACIDS

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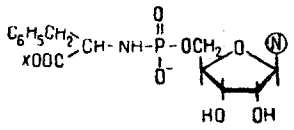
In an investigation of the hydrolytic stability of methyl esters of nucleotidyl (5' → N) amino acids [1, 2, 3] we showed that in an acid medium cleavage of the phosphate bond in these compounds takes place, while in an alkaline medium only the saponification of the ester grouping of the amino acid moiety occurs.

In the present paper we give the results of the hydrolysis of nucleotidyl (5' → N) phenylalanines with a free carboxy group. Such compounds are of interest in view of the fact that they can be considered as models of nucleotidyl peptides of the phosphoramide type in which the N end of the peptide is a dicarboxylic amino acid (these nucleotidyl peptides have been recently detected in natural materials [4]). It might have been expected that the presence of a free carboxy group in a phosphoramide molecule would have an influence on its behavior on hydrolysis. We have studied the acid hydrolysis of uridyl (5' → N) phenylalanine (I) and adenylyl (5' → N) phenylalanine (II). The synthesis of compounds (I) and (II) has been described in previous communications [1, 5], and their characteristics are given in the table.



I, N = uracil X = H;
II, N = adenine X = H;
III, N = uracil X = CH_3;
IV, N = adenine X = CH_3.

At a pH above 5, these compounds are stable (37° C, 1 hr) but in an acid medium they undergo decomposition. In contrast to the methyl esters of the nucleotidyl (5' → N) phenylalanines (III) and (IV), compounds (I) and (II) are hydrolyzed not only at the phosphoramide bond to the nucleotide (UMP-5' and AMP-5', respectively), but also at the phosphate bond to the nucleoside (uridine or adenosine). At pH 1 (37° C, 1 hr), compound (I) is 47% converted into uridine and compound (II) 49% converted into adenosine (Fig. 1).

Since neither the nucleotides themselves nor the esters of the nucleotidylamino acids are cleaved at the phosphate bond under the conditions that we used, it may be concluded that the formation of the nucleosides from compounds (I) and (II) is due to the simultaneous presence in the molecule of a phosphoramide group and a free carboxy group. The stability of the nucleotides in an acid medium permits the assumption that the primary processes in the action of an acid on the nucleotidylamino acids (I) and (II) is, on the one hand, the cleavage of the phosphoramide bond with the formation of the nucleotide and the amino acid and, on the other hand, the cleavage of the phosphate bond with the formation of the nucleoside and the N-phosphorylamino acid. A secondary process consists of the hydrolysis of the N-phosphorylphenylalanine to inorganic phosphate and amino acid, which takes place incompletely during the time of the experiment. The parallel cleavage of the phosphoramide and phosphate bonds greatly complicates the interpretation of the available data. However, the shape of the curves of the dependence of the hydrolysis of the phosphate bond on the pH (see Fig. 1) shows that the compound with an unionized carboxy group is apparently active in this reaction. In a strongly acid medium where the concentration of hydrogen ions is sufficiently high, for example in 1-6 N hydrochloric acid, protonation, not only of the amide nitrogen but also of the phosphoryl or ester oxygen, may apparently take place in a similar manner to the protonation of phosphoric esters described by Bunton et al., [6], which leads to the formation of so-called conjugate acids of type A or B. Such compounds are readily attacked by a molecule of water, in view of which not only the phosphoramide but also the phosphate bond is cleaved.

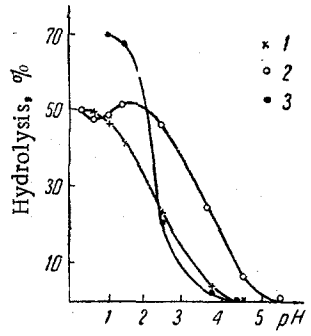
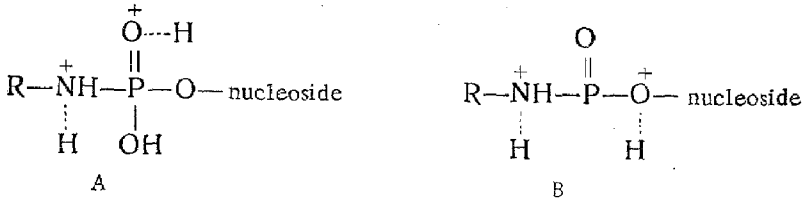


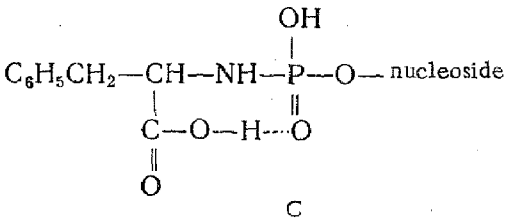
Fig. 1. Hydrolysis of the phosphate bond in uridylyl (5' → N) phenylalanine (1), adenylyl (5' → N) phenylalanine (2), and 3'(2')-phosphoryluridylyl (5' → → N) phenylalanine (3).



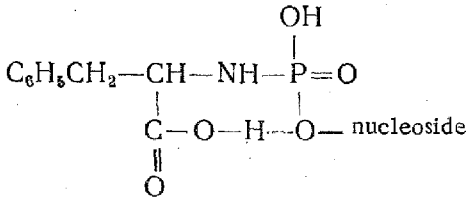
A B

It is natural that in a strongly acid medium the phosphate bond is labile even in the methyl esters of the nucleotidyl (5' → N) amino acids (Fig. 2, curve 2).

However, when a free carboxy group is present in the molecule of the nucleotidylamino acid, conjugate acids of type C or D may be formed in a weakly acid medium as a result of the intramolecular protonation of the phosphate group in which the role of proton donor is played by the unionized carboxy group of the same molecule.



C



D

In addition, it must be borne in mind that the carboxy group possesses higher I-effect than a methoxycarbonyl group. Consequently, in the nucleotidylamino acids the free pair of electrons of the phosphoramide nitrogen may be drawn in the direction of the carboxyl to some extent so that the capacity of the nitrogen for being protonated is diminished, resulting in the partial stabilization of the phosphoramide bond. At the same time, this leads to the situation that the protonation of the phosphoramide group is effected doubly: a proton is bound not only to the nitrogen but also to the oxygen. Accordingly, two competing processes take place, the cleavages of the phosphoramide bond and of the phosphate bond. In a strongly acid medium, obviously, protonation may take place with the participation of the protons both of the medium and of the carboxy group itself. Consequently, the cleavage of the phosphate bond in compounds (I) and (II) under these conditions takes place more intensively than that of their methyl esters (III) and (IV) (cf. Fig. 2, curves 1 and 2). Starting from the hypothesis put forward here that intramolecular protonation of the phosphate oxygen is possible, the greater lability of the phosphate bond in adenylyl (5' → N) phenylalanine (II) as compared with that in uridylyl (5' → N) phenylalanine (I) (cf. Fig. 1) may be explained as follows. The protonation of N_1 in the adenosine derivatives in an acid medium involves the appearance of a proton close to the phosphate oxygen which can form a hydrogen bond with this oxygen, resulting in the lability of the phosphate bond even in a weakly acid medium (cf. Fig. 1, curve 2). Taking account of this, it might be expected that the introduction of a nucleotidyl (5' → N) phenylalanine of a positive functional group capable of forming a hydrogen bond into the molecule, must increase the lability of the phosphate bond still further. In view of this, we have made an analogous study of 3'(2')-phosphoryluridylyl(5' → N)phenylalanine (V) obtained by the alkaline treatment of the methyl ester of 3'-phosphoryluridylyl (5' → N) phenylalanine (VI) or the methyl ester of 2', 3'-cyclophosphoryluridylyl (5' → N) phenylalanine (VII). The characteristics of compounds (V), (VI), and (VII) are given in the table.

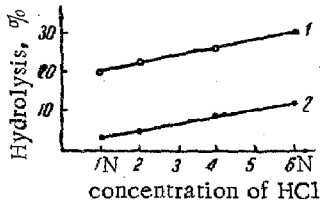
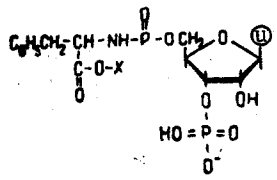


Fig. 2. Hydrolysis of the phosphate bond in adenylyl (5' → N) phenylalanine (1) and its methyl ester (2).

Actually, acid hydrolysates of compound (V) were found to contain uridine 3'(2'), 5'-diphosphate and uridine 3'(2')-phosphate, phenylalanine, N-phosphorylphenylalanine, and inorganic phosphate. The formation of uridine 3'(2')-phosphate (like the formation of uridine from compound (I) takes place to the extent of 70% at pH 1 (37° C, 1 hr). Thus, the introduction of an additional phosphate residue increases the lability of the phosphate bond still further. At a pH above 2.5, the curves of the cleavage of the phosphate bond in compounds (I) and (V) almost coincide (see Fig. 1, curves 1 and 3).



V, u = uracil X = H;
VI, u = uracil X = CH_3

This indicates that the 3'(2')-phosphate group, like the carboxy group, is active in this reaction only in the unionized form. However, compound (VI), which contains a phosphate group in position 3' but is esterified at the carboxy group, hydrolyzes only at the phosphoramide bond at pH 1, the phosphate bond undergoing no cleavage under these conditions (Fig. 3, curve 5). Consequently, the factor determining the direction of hydrolysis of the phosphoramide grouping is the state of the carboxy group.

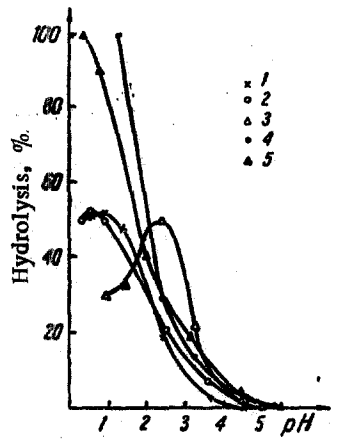


Fig. 3. Hydrolysis of the phosphoramide bond in uridylyl (5' → N) phenylalanine (1), adenylyl (5' → N) phenylalanine (2), 3'(2')-phosphoryluridylyl (5' → N) phenylalanine (3), the methyl esters of uridylyl (5' → N) phenylalanine and of adenylyl (5' → N) phenylalanine (4) [2], and the methyl ester of 3'-phosphoryluridylyl (5' → N) phenylalanine (5) [3].

The lability of the nucleoside – phosphorus bond in the structures investigated (I), (II), and (V) shows that this bond, which is stable in the free nucleotides, can be cleaved in some of their derivatives. This must evidently be taken into account in the analysis of nucleotide material isolated from biological samples since the existence of nucleotides in bound form in them is highly possible.

Experimental

The analytical methods used in the present investigation have been described previously [3].

The following systems of solvents were used in chromatography: 1) isopropyl alcohol – concentrated ammonia – water (7 : 1 : 2); 2) n-propyl alcohol – water (11 : 7) – concentrated ammonia to pH 7.5; 3) ethyl alcohol – 1 M ammonium acetate, pH 7.5 (7 : 3), and 4) isopropyl alcohol – concentrated ammonia – 1 M boric acid (7 : 1 : 2).

Buffers for electrophoresis: pH 4.5 – 0.02 M potassium dihydrogen phosphate; pH 8.0 – 0.05 M triethylammonium hydrogen carbonate.

Methyl ester of 2', 3'-cyclophosphoryluridyl (5' → N) phenylalanine (VII). Compound (VII) was obtained by the reaction of the methyl ester of phenylalanine with uridine 3'(2'), 5'-diphosphate after the latter had been activated with diphenyl phosphorochloridate by the method described previously [3] for the methyl ester of uridylyl (5' → N) phenylalanine (III). Compound (VII) was isolated by preparative paper chromatography in system 2.

Methyl ester of 3'-phosphoryluridylyl (5' → N) phenylalanine (VI). Compound (VI) was obtained from the methyl ester of 2', 3'-cyclophosphoryluridyl (5' → N) phenylalanine (VII) by the action of pancreatic ribonuclease (pH 7.5, 37° C, 5 hr) and was isolated by preparative paper chromatography in system 1.

3'(2')-Phosphoryluridylyl (5' → N) phenylalanine (V). Compound (V) was obtained by the alkaline hydrolysis of compound (VI) or compound (VII) (0.5 N KOH, 37° C, 1 hr), and after neutralization of the hydrolyzate with Dowex 50 resin (H^+ form), it was isolated by preparative paper chromatography in system 1.

Hydrolysis of compounds (I), (II), (V), and (VI) at various pH values. About 15 μmole of substance was dissolved in 1 ml of distilled water, and 0.1-ml portions of the solution were transferred to eight test tubes. To each tube was added 0.1 ml of the appropriate buffer solution (pH 0.5–7.3) and the mixture was incubated at 37° C for 1 hr. Then the

samples were cooled with ice and transferred completely to chromatograms. The hydrolysates of compounds (I) and (II) were chromatographed in system 1 and the amount of nucleotide and nucleoside formed in each tube were determined together with the amount of unchanged nucleotidyl (5' → N) phenylalanine. The hydrolysates of compounds (V) and (VI) were chromatographed in system 2 and in each sample the amounts of uridine 3'(2'), 5'-diphosphate and uridine 3'(2')-phosphate, together with the amount of unchanged compound (V) or (VI) were determined.

Compound	R_f in systems				U_{rel}	
	1	2	3	4	pH 4.5	pH 8.0
Uridine 5'-phosphate	0.09	0.30	0.20	0.12	0.94	0.96
Uridine 3'(2')-phosphate	0.12	0.34	0.42	0.43	1	1
Uridine 3'(2'), 5'-diphosphate	0.03	0.23	0.13	0.10	1.56	1.40
Uridine	0.57	0.72	0.90	0.54	0.25	0.30
Adenosine 5'-phosphate	0.08	—	0.20	—	0.71	0.84
Adenosine	0.64	—	—	—	0.10	0.13
Uridylyl (5' → N) phenylalanine (I)	0.28	—	0.36	0.40	1.0	0.92
Adenylyl (5' → N) phenylalanine (II)	0.32	—	—	—	—	—
Methyl ester of uridylyl (5' →N) phenylalanine (III)	0.54	—	0.73	0.66	0.85	0.63
Methyl ester of adenylyl (5' →N) phenylalanine (IV)	0.56	—	—	—	—	—
3'(2')-phosphoryluridylyl (5'→ N) phenylalanine (V)	0.12	0.45	—	—	1.50	1.26
Methyl ester of 3'-phosphoryluridylyl (5' → N) phenylalanine (VI)	0.15	0.54	0.67	—	1.42	1.20
Methyl ester of 2', 3'-cyclophosphoryluridylyl (5' → N) phenylalanine (VII)	0.46	0.69	0.80	—	1.24	0.94

Taking the sum of all the UV-absorbing compounds in the sample as 100%, the degree of hydrolysis of the phosphoramide bond was calculated from the amount of nucleotide [for (I) and (II)] or uridine 3'(2'), 5'-diphosphate [for (V) and (VI)], and the degree of hydrolysis of the phosphate bond from the amount of nucleoside [for (I) and (II)] or uridine 3'(2')-phosphate [for (V) and (VI)].

Hydrolysis of adenylyl (5' → N) phenylalanine (II) and its methyl ester (IV). The hydrolysis of compounds (II) and (IV) was carried out similarly in hydrochloric acid of various concentrations at 40° C for 15 min. The hydrolysates were chromatographed in system 1 (see Fig. 2).

Summary

The hydrolysis of nucleotidyl (5' → N) phenylalanines with free carboxy groups has been studied. It has been found that in an acid medium the cleavage of the phosphate bond takes place simultaneously with that of the phosphoramide bond. The introduction of a second phosphate group into the 3'(2')-position of the nucleotide increases the cleavage of the phosphate bond. The hypothesis has been put forward that the cleavage of the phosphate bond in nucleotidyl (5'→N) amino acids in an acid medium is determined by the state of the carboxy group.

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