AN INVESTIGATION OF THE STABILITY OF THE PHOSPHATE ESTER BOND IN D-PANTOTHENIC ACID 4'-PHOSPHATE

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The hydrolysis of the phosphate ester bond in D-pantothenic acid 4'-phosphate in strongly acid (9 and 6 N HCl), weakly acid, weakly alkaline, and strongly alkaline (5 N KOH) aqueous media has been studied. In strongly acid and strongly alkaline media the phosphate ester bond of (I) breaks down completely in 3 and 2.5 h, respectively. The rate of hydrolysis at pH values between 1.3 and 11 remains constantly low but almost doubles in the presence of lanthanum salts at pH 8-9 and in the presence of lithium salts at pH 4 and a molar ratio of (I):Me^+ = 1:1.

The vitaminic properties of pantothenic acid are determined by its structural role in the biosynthesis of pantetheine adenine nucleotide diphosphate (the acetylation coenzyme CoA) and of phosphopantetheine proteins with the participation of which various oxidative and biosynthetic reactions of the metabolism are performed. The multistage nature of the system for the biosynthesis of CoA from the vitamin finds its reflection in the broad spectrum of natural compounds of pantothenate present in cell material, which includes free pantothenic acid, 4'-phosphopantothenic acid, 4'-pantothenoylcysteine, 4'-phosphopantetheine, pantethine, dephospho-CoA, acyl-CoA, CoA-S-S-CoA, and others [1].

In vitaminological investigations, just as in analytical chemistry of pantothenate-containing compounds, in addition to microbiological methods for the analysis of pantothenic acid and vitamin-containing preparations, at the present time the highly specific method of gas—liquid chromatography based on the detection of pantolactone — the end-product of the hydrolysis of the vitamin — is finding wide use [2-4]. In spite of certain investigations of the hydrolysis of CoA [5], the formation of pantolactone from phosphate derivatives of pantothenate in acid or alkaline medium is extremely problematical, which is connected with the stability of this molecule because of the formation of a macrocycle [6]. Consequently, the quantitative determination of the lactone cannot be achieved without the previous cleavage of the phosphate ester bond. There is practically no information in the literature on the degree of stability of phosphate bonds under the conditions of hydrolysis of phosphopantothenates, with the exception of limited investigations in their identification [7].

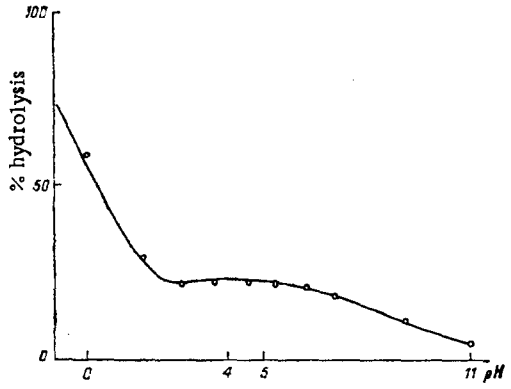

Fig. 1. Hydrolysis of the phosphate ester bond in D-pantothenic acid 4'-phosphate at various pH values.

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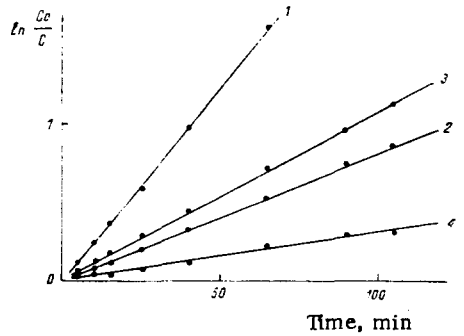
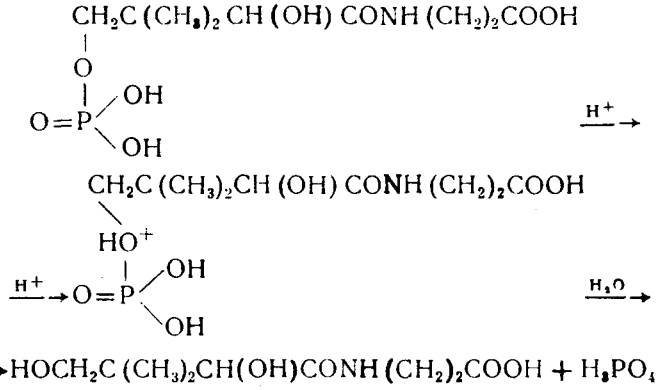


Fig. 2. Kinetics of the hydrolysis of D-pantothenic acid 4'-phosphate. Hydrolysis conditions: 1) 9 N HCl, 90°C; 2) 9 N HCl, 70°C; 3) 6 N HCl, 90°C; 4) 6 N HCL, 70°C.

The present work had as its aim a study of the hydrolysis in strongly acid, weakly alkaline, weakly acid, and strongly alkaline aqueous media of the phosphate bond in D-pantothenic acid 4'-phosphate (I), which is a regulatory metabolite of the biosynthesis of pantothenate-containing coenzymes in biological materials and an active metabolite with specific biochemical functions [8, 9].

In strongly acid solutions inorganic phosphate is split out, the rate of hydrolysis rising with an increase in the acidity of the medium (Fig. 1). The results obtained in this way on the catalysis by hydrogen ions of the process under investigation agree well with ideas on the attack of protons in an acid medium on the nucleophilic oxygen atom, which facilitates the splitting out of the phosphoric acid.



The not very fast cleavage of the phosphate ester bond is due to the influence of the α-hydroxy group which apparently decreases the nucleophilicity of the oxygen atom.

The hydrolysis of the phosphate bond in the presence of 9 and 6 N hydrochloric acid was studied in detail. Under these conditions a first-order reaction was observed, as follows from Fig. 2, plotted by the method of least squares. The rate constants at 90 and 70°C in 9 and 6 N HCl were, respectively, $k_1 = 0.0256$ min^{-1}; $k_2 = 0.0081$ min^{-1}; $k_3 = 0.0107$ min^{-1}, and $k_4 = 0.0032$ min^{-1}. It was found from the Arrhenius equation that the apparent activation energy of hydrolysis, E, was 59.5 kJ/mole. In the process of hydrolysis, the amide bond was also cleaved, as a result of which 4'-phosphopantoic acid (II) was formed and therefore the measured hydrolysis constants were the sums of the rates of the decomposition of (I) and (II).

The hydrolysis of the phosphate bonds of (I) in a weakly acid medium at pH 1.5-6.5 took place slowly. Furthermore, there was no increase in the rate of decomposition at pH 4, as is characteristic for the majority of monoesters of phosphoric acid, when they are present in the form of monoanions. The increase in the rate of hydrolysis under these conditions is usually ascribed to an attack of the hydrogen atom of the monoanion or the solvent, which facilitates the splitting out of the alkoxy group of the phosphate ester [10]. The anomalous behavior of (I) is explained by the interaction of spatially close groups: by the inclusion of the phosphate in a macrocycle through the formation of hydrogen bonds between the phosphate hydroxyl and the amide and carboxy groups [6].

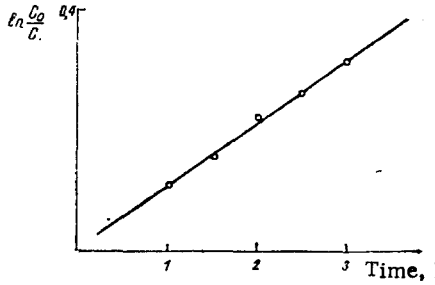


Fig. 3. Kinetics of the hydrolysis of D-pantothenic acid 4'-phosphate at pH 4.

The kinetics of the hydrolysis of the ester bond of (I) was studied at pH 4. The linear dependence of ln (C_0/C) on the time (Fig. 3) is an indication of a first-order reaction with a rate constant $k = 0.0017$ min^{-1}.

Between pH values of 7 and 1, the hydrolysis of (I) took place very slowly. A further increase in the basicity of the medium caused an acceleration of the breakdown of the phosphate ester bond. Thus, when the reaction was performed in 5 N KOH hydrolysis was complete after 2.5 h.

The hydrolysis of (I) is catalyzed by metal ions. At pH 4, lithium salts proved to be active catalysts. In a weakly alkaline medium at pH 8-9 the most active were lanthanum salts at a molar ration of (I) to Me^+ of 1:1. Under these conditions the rate of the reaction almost doubled. Ions of the metals magnesium, calcium, berylium, and aluminum had no effect on the rate of hydrolysis.

It has been established that the phosphate ester bond in (I) is unstable in the strongly acid and strongly alkaline media. The ions of certain metals have a substantial influence in accelerating hydrolysis.

EXPERIMENTAL

An accurately weighed sample of (I) (about 80 mg) was dissolved in acid or alkali or a buffer solution in a 25-ml measuring flask and the solution was made up to the mark and mixed. Portions (0.2 ml) of the resulting solution were placed in a glass tube with a volume of 2 ml which were then sealed and kept in a thermostat at 70-80°C. Tubes were removed after predetermined intervals of time, hydrolysis was stopped by rapid freezing in a mixture of acetone and solid carbon dioxide, and the tubes were opened, the contents were thawed out and were transferred quantitatively to test tubes, and inorganic phosphorus was determined [11]. The completeness of hydrolysis was checked by incinerating samples with the subsequent determination of inorganic phosphorus.

Hydrolysis in the presence of metallic salts was carried out at 90°C for 3 h. In each experiment there was a control with no metal present.

SUMMARY

The hydrolysis of the phosphate ester bond in D-pantothenic acid 4'-phosphate in strongly acid (9 N and 6 N HCl), weakly acid, weakly alkaline, and strongly alkaline (5 N KOH) in aqueous media has been studied. In strongly acid and strongly alkaline media, the phosphate ester bond of (I) broke down completely in 3 and 2.5 h, respectively. The rate of hydrolysis in the pH range of 1.3-11 remained constant and low but almost doubled in the presence of lanthanum salts at pH 8-9 and of lithium salts at pH 4 at a molar ratio of (I) to Me^+ of 1:1.

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CHOICE OF A METHOD OF PREPARING SAMPLES FOR RECORDING THE RADIOACTIVITY OF COMPLEXES OF ^{14}C-MACROTETROLIDE ANTIBIOTICS WITH ALKALI-METAL PICRATES

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A comparison of the method of preparing samples for recording the radioactivity of complexes of ^{14}C-macrotetrolide antibiotics with the picrates of univalent cations using a correction for color quenching and the method with the washing out of the water-soluble (including colored) components has shown the possibility of obtaining adequate reproducible results by both methods.

Macrotetrolide antibiotics produced by various strains of streptomycetes are ionophoric compounds that, in nonaqueous media, form strong complexes with the ions of univalent cations, especially ammonium, and this has been made the basis of the quantitative determination of these compounds by an extraction-spectrophotometric method in the presence of an excess of alkali-metal picrates [1, 2]. In this connection, it is important to study the distribution of the macrotetrolide antibiotics themselves between the organic and aqueous phases. The most suitable method for estimating these compounds in the different phases is the radioindicator method. However, the measurement of the radioactivity of complex multicomponent systems requires the conditions for obtaining a reproducible and comparable result to be clarified beforehand. With this aim, in samples containing ^{14}C-labeled macrotetrolides, picric acid, and alkali-metal picrates, we first determined the true activity of the samples of ^{14}C-macrotetrolides in ZhS-106 scintillation fluid using the method of internal standards to introduce corrections for quenching (Table 1, variant 1). Samples of ^{14}C-macrotetrolides with the addition of picric acid and ammonium and sodium picrates were estimated by the same program (Table 1, variants 2, 3, and 4).

TABLE 1. Activities of Preparations of ^{14}C-Macrotetrolides in the Presence of Picric Acid and of Ammonium and Sodium Picrates

Experimental conditions	Activity of the sample		Counting efficiency	Error, %
	disint./min	error,%		
1. ^{14}C-Macrotetrolides	17391±460	2,64	0.9263±0.0010	0,11
2. ^{14}C-Macrotetrolides + picric acid	3125±149	4,79	0,7645±0,0332	4,34
3. ^{14}C-Macrotetrolides + ammonium picrate	2817±67	2,97	0,7137±0,0161	2,26
4. ^{14}C-Macrotetrolides + sodium picrate	2010±81	4,03	0.8694±0,0191	2,20

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