INVESTIGATIONS IN THE FIELD OF ACYL GROUP CARRIERS. XVI. HYDROLYTIC STABILITY AND ENZYMATIC DEPHOSPHORYLATION OF D-PANTOTHENIC ACID 4'-PHOSPHATE

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Samples of D-pantothenic acid 4'-phosphate (I) have been investigated by IR spectroscopy in dimethyl sulfoxide and deuterated water and in the form of a thin film without a solvent. The vibrations of the carbonyl and amide groupings in (I) as compared with pantothenic acid (II) are found at higher frequencies in all media, which indicate the greater strength of these bonds. It is assumed that the resistance of (I) to hydrolysis as compared with (II) is due to the inclusion of the phosphate in a macrocycle by the formation of hydrogen bonds between the phosphate, hydroxy, amide, and carboxy groups of the molecule. The complete dephosphorylation of (I) has been achieved in concentrations of 0.0075 and 0.005 mM with the aid of a dialyzed preparation of orthophosphate monoester phosphohydrolase (E.C. 3.1.3.1).

The quantitative determination of D-pantothenic acid (I) and of other precursors of the biosynthesis of the coenzyme form of the vitamin (CoA) is based mainly on microbiological analysis [1], some defects of which have already been discussed repeatedly [2, 3]. In recent years, gas-chromatographic methods for determining (I) have been proposed which are based on the chromatography of products of the hydrolytic cleavage of the vitamin — β-alanine or pantolactone [4, 5]. The latter variant of gas-chromatographic analysis has acquired some acceptance in the practice of vitaminological investigation [6], including the determination of the pantothenic acid balance in man [3]. A further development of this procedure is a method of determining CoA by its hydrolytic cleavage to pantolactone [7]. These investigations have not taken into account the fact that in biological tissues and liquids considerable amounts of 4'-phosphopantothenic acid (II) are present [3, 8], the formation of pantolactone from which is extremely problematic. Nevertheless, 4'-phosphopantothenic acid is not only the end-product of the metabolism of pantothenate-containing coenzymes in biological materials and an intermediate in the biosynthesis of CoA but is also an active metabolite with specific biochemical functions [9].

Continuing our investigations on the synthesis of pantothenic acid derivatives [10] and the study of their biological activity [9], the necessity has arisen for developing a method for the quantitative determination of compound (II). However, on the hydrolysis of (II) with 18% hydrochloric acid we detected only 30% of pantolactone, while the similar hydrolysis of (I) led to the complete cleavage of the amide bond [11]. Compound (II) is still more resistant to alkaline treatment. These facts are in harmony with observations reported previously on the stability of the phosphate and amide groupings of the phosphate (II) to hydrolytic cleavage [12].

The increased resistance of the amide bond to hydrolytic cleavage in the phosphate (II) as compared with (I) is connected with the introduction of the phosphate grouping and its interaction with other functional groups of the molecule, which obviously leads to a conformational change of the whole compound.

The comparative results of the IR spectra of both the acids and their esters together with a consideration of Dreiding models of these compounds permit a possible reason for the

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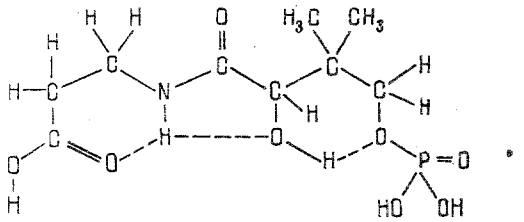
TABLE 1. IR Frequencies of the Carbonyl and Amide Vibrations, cm^{-1}

Substance	DMSO			D_2O			Without a solvent		
	$\nu_{C=O}$ (COOH)	$\nu_{C=O}$ (CONH)	δ_{NH} (CONH)	$\nu_{C=O}$ (COOH)	$\nu_{C=O}$ (CONH)	ν_{COO^-}	$\nu_{C=O}$ (COOH)	$\nu_{C=O}$ (CONH)	δ_{NH} (CONH)
D-Pantolactone	1783 (607)*			1765 (554)					
I	1718 (362)	1665 (410)	1518 (228)	1710	1636 (442)	1578	1726	1648	1538
II	1736 (592)	1668 (418)	1524 (235)	1722 (400)	1643 (400)		1736	1655	1515
Ethanol (I)	1731	1664	1519						
Ethanol (II)	1730	1665	1518						

*Molar coefficient of absorption, ε.

observed differences to be found. The hydrolysis of (I) is facilitated by the intramolecular nucleophilic attack of the γ-hydroxy group on the carbon of the amide bond [13]. In the circumstances, the position of the neighboring hydroxyl (α-OH) in the cis position to the C = O bond of the amide grouping and, apparently, the spatial propinquity of the carboxy group [14] promote the hydrolysis of pantothenic acid. In (II) the nucleophilic attack of the γ-hydroxyl cannot be performed without the preliminary hydrolysis of the phosphate; the α-OH has a tendency to form an intramolecular hydrogen bond with the phosphate, and in so doing to take up the trans position with respect to the amide carbonyl. The increased resistance of the phosphate bond to hydrolysis under acid and alkaline conditions (in an alkaline medium the hydrolysis of the amide bond of the phosphate is insignificant [15]) is probably connected with the inclusion of the phosphate in a macrocycle by the formation of hydrogen bonds between the phosphate, hydroxyl, amide, and carboxy groups. In a paper on the conformational calculation of the phosphopantothenic moiety of CoA [16] it was shown that the most stable conformations are those in which a macrocycle is formed. By analogy with [16] it is possible to write for (II):



The IR spectra of both the acids and their esters were measured in a thin film and in the solvents dimethyl sulfoxide (DMSO) and deuterated water (D_2O) at concentrations of 0.1-0.2 M. The choice of such solvents, complicating the interpretation of the spectra because of their coordination with different centers of the molecules and having pronounced absorption in the IR spectra is due to the extremely low solubility of the acids in ordinary sol-

TABLE 2. Hydrolysis of Phosphopantothenate by Alkaline Phosphatase in Different Times of Incubation (% on the amount added)

Derivatives	Concentration, mM	Alkaline phosphatase, 0.1 mg/ml		Alkaline phosphatase, 1 mg/ml	
		3 h	24 h	3 h	24h
II	1.0	20.6	38.5	40.0	43.5
	0.033	41.3	44.8	65.9	45.7
Calcium salt of (II)	1.0	30.8	37.8	38.8	44.8
	0.033	39.5	39.5	51.8	60.6
Sodium salt of (II)	1.0	35.6	40.5	42.0	51.3
	0.033	51.0	48.3	66.8	74.7

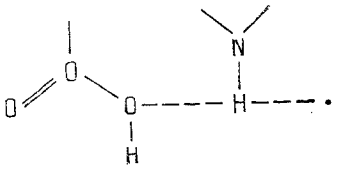
vents of low polarity.

As compared with (I), the vibrations of the carbonyl and amide groups in (II) are found at higher frequencies in all media (Table 1), which, in general, indicates a greater strength of these bonds. In the spectra of (II), broad bands of the P = O bond are observed at 1200 cm^{-1} (in a film) and at 1260 cm^{-1} (in DMSO) and of the C-O-P grouping at 1000 cm^{-1} (in a film). The replacement of the terminal hydroxyl by phosphate is also clearly shown in the region of the stretching vibrations of the OH, NH, COOH, and P-OH groups (a decrease in the intensity of the band with its separation into two at 3260-3280 and 3420 cm^{-1}, while in the spectrum of (I) in DMSO one strong absorption band is observed with ν_{max} 3360 cm^{-1} and an increase in the absorption background in the 2000-3000 cm^{-1} region).

Taking into account the contamination with the pantolactone that is normally present in both the acids in very small amounts, we determined intensity of the carbonyl and amide bands. In the case of solutions in D_2O, for (I) we give the intensity only for $\nu_{C=O}$ of the amide bond, since this acid is largely ionized in water. Together with $\nu_{C=O}$ of the carboxy group at 1710 cm^{-1}, a strong band of the COO^- ion is observed at 1578 cm^{-1}; under these conditions, compound (II) is not ionized. In D_2O, in both acids complete deuterium exchange takes place at the NH bond (see Table 1).

The higher values of the frequencies of the NH deformation vibration (more accurately, $\delta_{NH} + \nu_{C-N}$) at 1520-1540 cm^{-1} in the spectra of (II) compared with (I) show a stronger hydrogen bond in the first case ($\Delta\nu$ 6-7 cm^{-1} in DMSO and in a film). According to Perahia and Cebe [16], NH can participate in an intramolecular hydrogen bond simultaneously with carbonyl and hydroxy groups.

The pronounced shift in the direction of higher frequencies ($\Delta\nu$ 10-18 cm^{-1}) with a change in intensity on passing from (I) to (II) is observed for $\nu_{C=O}$ of the carbonyl group more remote from the phosphate ring of the molecule. This fact obviously reflects a difference in the strengths of the acids and in the participation of the acid group in intra- and intermolecular associates. It is interesting to note that the frequency of the vibrations of the carbonyl group of compound (II) has the same value in DMSO and in a film without a solvent. It is known that DMSO forms strong hydrogen bonds with the hydroxy group of carboxylic acids ($-\Delta H \sim 11$ kcal/mole [17, 18]), and in a film such a hydrogen acceptor may be the phosphate, as is confirmed by a fall in $\nu_{P=O}$ of the substance measured in the solid state as compared with in DMSO. The self-association of pantothenic acid 4'-phosphate may form dimers or polymers. This effect of the formation of a hydrogen bond through the OH of the carbonyl group is accompanied by a rise in the frequency of its carbonyl [19]. The high sensitivity of $\nu_{C=O}$ in (II) to the solvent (on passing from DMSO to water, $\Delta\nu$ is 14 cm^{-1}, while in (I), it is 8 cm^{-1}) may indicate that in water an intramolecular ring is also formed through the OH of the carboxy group, i.e.,



The carbonyl group of the amide bond participates in the internal stabilization of the molecule to a smaller degree ($\Delta\nu$ if 3-7 cm^{-1} in comparison with (I)) and strongly responds to the influence of the solvent ($\Delta\nu$ 12-29 cm^{-1}), while in (I) this influence predominates only slightly.

It is interesting to note that in the ethyl esters of both acids measured in DMSO the frequencies of the carbonyl and amide bonds are the same (± 1 cm^{-1}) being 1730, 1665, and 1518 cm^{-1} for $\nu_{C=O}$ of the ester and amide groups and for δ_{NH}, respectively (see Table 1).

Thus, the absence of a nucleophilic attack of the hydroxyl in the gamma-position of the carbon of the amide bond and the change in the conformational state of the molecule with the formation of a macrocycle through hydrogen bonds between the phosphate, hydroxy, NH, and COOH groups are apparently the main causes of the greater resistance of pantothenic acid 4'-phosphate to hydrolysis in comparison with pantothenic acid itself.

In view of the high stability of pantothenic acid 4'-phosphate under the conditions of chemical hydrolysis, the first step in the development of a method for its quantitative analysis may be enzymatic dephosphorylation of this compound to pantothenic acid. Since nonspecific enzymatic dephosphorylation systems (in particular, acid and alkaline phosphatases, 5'-nucleotidase, nucleotide pyrophosphatase) take part in the catabolism of CoA and of 4'-phosphopantetheine to (I) and (II) in biological systems [3, 9], fundamental interest is presented by the investigation of the hydrolysis of (II) by orthophosphate monoester phosphohydrolase (E.C. 3.1.3.1).

Substance (II) and its sodium and calcium salts are hydrolyzed by alkaline phosphatase with the liberation of organic phosphorus. The presence of magnesium ions is necessary for the manifestation of the activity of the enzyme with respect to the substrate, the optimum concentration of Mg^{2+} being 1-2 mM. The optimum pH of the hydrolysis of (II) (2 mM) by alkaline phosphatase in the presence of magnesium ions (1 mM) in 0.05 M aminotris(hydroxymethyl)methane buffer is 8.7, and in 0.05 mM diethanolamine buffer it is 9.0. When the concentration of (II) is lowered to 0.015 mM in aminotris(hydroxymethyl)methane buffer, the optimum pH falls to 7.5-8.0, which corresponds to figures given in the literature [20].

The values of K_M determined by the method of double reciprocals amount to 1.1 mM for the sodium salt and 0.8 mM for the calcium salt of (II) in an aminotris(hydroxymethyl)methane buffer, pH 9.0, concentration of Mg^{2+} 1 mM 37°C (K_M for p-nitrophenyl phosphate, the generally adopted substrate of the enzyme, is 0.3 mM).

The incubation of solutions of (II) or its sodium or calcium salts with the alkaline phosphatase taken in a concentration of 0.1 mg/ml under the optimum conditions led in 3 h to the hydrolysis of 20-40% of the substances (Table 2). Increasing the time of incubation to 24 h had little effect on this process. A tenfold increase in the concentration of the enzyme raised the amount of substances hydrolyzed by 5-25%. A lowering of the concentration of of (II) to 0.03 mM led to the more complete hydrolysis of this compound, but not to more than 75% in 24 h. Only lowering the concentration of the sodium salt of (II) to 0.0075 and 0.005 mM permitted complete dephosphorylation to be achieved in the presence of alkaline phosphatase on incubation for 2.5 h.

EXPERIMENTAL

IR spectra were measured on a Perkin-Elmer model 180 spectrophotometer. The spectra of compound (I) and (II) were obtained under the same conditions without a solvent, in DMSO, or Na_2O at concentrations of 0.1-0.2 M in fluorite cells with layer thicknesses of 0.08 and 0.12 mm. The spectra of pantolactone, with allowance for the presence of which as an impurity the molar absorption coefficients ε were determined, were measured in DMSO and D_2O, and the spectra of the esters of both acids in DMSO.

The absorption frequencies were determined with an accuracy of 1 cm^{-1}.

The chromatography of the compounds was performed in an ascending flow on Filtrak FN-3 chromatographic paper (GDR) in the following solvent systems: 1) butan-1-ol—acetic acid—water (5:2:3); 2) isopropanol—25% ammonia—water (7:1:2).

D-Pantothenic Acid 4'-Phosphate (II). A solution of 0.62 g of ethyl pantothenate and 0.8 g of 2-cyanoethyl phosphate in pyridine was evaporated to dryness. The residue was dissolved in 10 ml of anhydrous pyridine, and then 0.56 g of N,N'-dicyclohexylcarbodiimide was added and the mixture was stirred at room temperature for 24 h. Then, 5 ml of water was added and, after the mixture had been stirred for 0.5 h, the N,N'-dicyclohexylurea that had precipitated was filtered off. The filtrate was evaporated to dryness, the residue was dissolved in water, barium hydroxide solution was added to pH 7.5, the mixture was filtered, the filtrate was evaporated to dryness, the residue was dissolved in 10 ml of 10 N ammonia, the solution was left for 6 h, and the solvent was evaporated off in vacuum. The residue was dissolved in 10 ml of water and the aqueous solution was passed through a column of the resin Amberlite IR-120 (H^+ form), the column was washed with water, the aqueous eluates were evaporated to dryness, and the residue was dried in vacuum over P_2O_5. The yield of compound (II) was 0.23 g (30%), R_f 0.45 (system 1), 0.34 (system 2).

Ethyl D-Pantothenate. Hydrogen chloride was passed through a solution of 0.4 g of D-pantothenic acid in 30 ml of ethanol until there was no further increase in weight. The reaction mixture was evaporated to dryness and the residue was dissolved in chloroform. The

chloroform solution was washed with 5% hydrochloric acid and with water, was dried with Na_2SO_4, and was evaporated to dryness. This gave 0.15 g (33%) of ethyl D-pantothenate with R_f 0.85 (system 1), 0.91 (system 2).

The ethyl ester of D-pantothenic acid 4'-phosphate was obtained in a similar manner to ethyl pantothenate. Yield 29%, R_f 0.88 (system 1), 0.93 (system 2).

Enzymatic Dephosphorylation. Alkaline phosphatase from chick intestine (Reanal) was dialyzed before use against aminotris(hydroxymethyl)methane buffer, pH 8.0, to eliminate inorganic phosphorus. The hydrolysis of compound (II) with alkaline phosphatase was determined from the liberation of inorganic phosphate. To 1 ml of a buffered solution of compound (II) was added 0.1 ml of a solution of alkaline phosphatase and incubation was carried out at 37°C. The reaction was stopped by the addition of 1 ml of 5% trichloroacetic acid. The inorganic phosphorus was determined by the method proposed by Chem et al. [21], and in the case of the hydrolysis of low concentrations of compound (II) (0.005-0.015 mmole) by the method of Gribanov and Bazanov [22]. The completeness of hydrolysis was checked by the combustion of samples with the subsequent determination of inorganic phosphorus.

CONCLUSION

1. The resistance of D-pantothenic acid 4'-phosphate to hydrolysis, as compared with pantothenic acid itself, is due mainly to the change in the conformational state of the molecule with the formation of a macrocycle formed by hydrogen bonds in the phosphate, hydroxy, amide, and carboxy groups.

2. The complete hydrolytic cleavage of low concentrations of the sodium salt of D-pantothenic acid 4'-phosphate by a preparation of orthophosphate monoester phosphohydrolase (E.C. 3.1.3.1) has been achieved.

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