## STABLE WATER-SOLUBLE VITAMINS AND COENZYMES. VII. KINETICS AND

MECHANISM OF THE HYDROLYSIS OF THE CALCIUM SALT OF HOMOPANTOTHENIC

ACID

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UDC 615.27:577.164.141].033

The calcium salt of homopantothenic acid (pantogam, I) is an agent for metabolic therapy.

The purpose of this work was to investigate the process of hydrolysis of I in aqueous solutions for the development of a stable injection form of I.

#### EXPERIMENTAL

Pharmacopoeia quality substances were used in the work: I (VFS 42-834-79) and  $\gamma$ -aminobutyric acid (aminalon, II; FS 42-1019-75). Analytical reagents, as well as buffer aqueous and aqueous alcohol solutions, were prepared according to [3]. An acid solution of formaldehyde was prepared by mixing 0.1 ml of a 40% solution of formalin, 40 ml of 1 N hydrochloric acid, and 10 ml glacial acetic acid. The ionic strength was varied by varying the concentration of the compoents of the buffer solution (sodium acetate and hydrochloric acid) by dilution at a constant ratio.

The hydrolysis of I was studied in the temperature range  $60-90^{\circ}C$  (±0.1 °C). The course of the reaction was monitored according to the change in the concentration of I, which was determined spectrophotometrically according to the reaction product with the sodium salt of 1,2-naphthoquinone-4-sulfonic acid (III) on a Specord UV VIS instrument using one centimeter cuvettes; accuracy of the method 2%.

# Methods of Kinetic Investigations

A 0.1-0.5-g weighed sample of I was dissolved in 100 ml of aqueous or aqueous alcohol buffer solution. The solution obtained, with an initial concentration of I  $1.98 \cdot 10^{-3}$ -9.91.10<sup>-3</sup> M, was placed in a thermostatically controlled vessel, equipped with a magnetic mixer and reflux condenser, or poured into ampuls, which were then exposed in a constanttemperature chamber. Samples were collected periodically from the reaction mixture, including the zero sample (t = 0), for a total of seven samples. A 5-ml portion (exact volume) was collected with a pipette from each sample, cooled to 20°C. The sample was transferred to a 50-ml volumetric flask; depending on the pH the medium was neutralized according to 0.02% phenolphthalein with 0.1 N solutions of sodium hydroxide or hydrochloric acid and brought up to the mark with water (series of solutions A, concentration of I 0.19.10  $0.99 \cdot 10^{-3}$  M). A 2-10-ml portion was collected from each solution of series A, placed in a 25-ml flask, 1 ml of a 1% solution of borax and 1 ml of a 0.5% solution of III were added, and the mixture exposed for 10 min on a boiling water bath. After cooling to room temperature, 1 ml of an acid solution of formaldehyde and 1 ml of 0.1 N sodium thiosulfate were added. The contents of the flask were mixed and the volume of the solution brought up to the mark with water. The solution obtained (series B) was exposed at room temperature for 20 min, and the optical density was measured at  $\lambda = 465$  nm. The control solution, prepared in the same way as solution B but without the solution A, was placed in a reference cuvette. The optical density to be measured, Dt, corresponds to the content of II in the reaction mixture at the moment of time t, while the difference of the optical densities  $D_t - D_o$  is the optical density of a solution of series B, obtained from the zero sample, corresponds to the change in the content of II in the course of the reaction, beginning with t = 0.

A parallel determination of the content of II was made at the time of the end of the reaction, i.e., at the stage of the maximum degree of conversion of the initial I to II.

All-Union Vitamin Scientific-Research Institute, Moscow. Translated from Khimikofarmatsevticheskii Zhurnal, Vol. 18, No. 7, pp. 865-870, July, 1984. Original article submitted May 30, 1983.

TABLE 1. Coeffi-				
cient of Extinction				
(ε1)	$(\varepsilon_1)$ of the Product			
of th	e Rea	action of		
II wi	th Il	I		
	1	1 -		
M Fa		εıat		
O <sup>4</sup> teni	ity	· .		
	pti	max		
<u>U di li</u>	6.0	465 nm		
0.791	0.235	2971		
	0,238	3009		
1 100	0,240	3034		
1,180	0.375	3162		
	0.375	3162		
1,582	0,495	3129		
	0,500	3160		
j	0,510	3224		
		ε <sub>av</sub> 3112		

TABLE 2. Maximum Degree of Conversion of I to II

H <sup>d</sup> ntration the on mix- 10 <sup>3</sup> , M		Condition of reaching of max- imum degree of conversion		sample ction re after lent with		num e of con- n, %
	Conce of I in reactive	time, h	temper- ature, C	of reaction treatment of the second s	$\epsilon_{2}/\epsilon_{1}^{\dagger}$	Maxir degree versio
0,8 0,9 1,1 12,5	1,65 1,65 1,65 1,65	$1 \\ 1,5 \\ 2 \\ 1,5 \\ 1,5$	100 100 100 100	5871 5934 5745 5682	1,89 1,91 1,85 1,83	94,3 95,3 92,3 91,3

\*The value of  $\varepsilon_2$  is unchanged for 2 h after completion of the reaction.  $^{\dagger} \boldsymbol{\epsilon}_{1}$  is the coefficient of extinction of II,  $\epsilon_1 = 3112$ .

To obtain the value of the optical density  $D_{\infty}$ , corresponding to this value, 5 ml of the reaction mixture was placed in a 50-ml volumetric flask, 20 ml of water and 2.5 ml of 2 N hydrochloric acid were added, the mixture boiled for 1.5 h on a water bath, and after cooling, neutralized according to phenolphthalein with 2 N sodium hydroxide and brought up to the mark with water (solution C). Then the content of II was determined in solution C (see series A), and D was established.

The value of the effective rate constant  $k_{eff}$  was calculated according to a first-order kinetic equation.

$$k_{\text{eff}} = \frac{12,303}{t} |\mathbf{g}| \frac{1}{1-X};$$
$$X = \frac{D_t - D_0}{D_\infty - D_0}.$$

### RESULTS AND DISCUSSION

Compound I is the closest analog of the potassium salt of D-pantothenic acid (IV), the kinetics and mechanism of the hydrolysis of which were outlined in [3]. There is only one study on the hydrolysis of I; in addition to a colorimetric determination and the physicochemical properties of homopantothenic acid (I) it cites the values of the rate constant of the reaction at one temperature,  $90 \pm 0.1^{\circ}C$  [4]. From the data cited it follows that the pH range 5.0-6.0 corresponds to the greatest stability of I.



Fig. 1. Semilogarithmic plots of the kinetic curves of hydrolysis of I in aqueous solution. a) At 90°C and pH 1.06 (1), 1.24 (2), 1.28 (3), 1.31 (4), 1.60 (5); b) at 60°C and pH 7.33 (1) 6.08 (2), 5.95 (3), 5.57 (4); c) at 90°C and pH 10.30 (1), 9.75 (2), 9.44 (3).



Fig. 2. Dependence of the effective rate constant of hydrolysis of I (log  $k_{eff}$ ) in aqueous buffer solutions on the pH values at 60°C.

Fig. 3. Dependence of the effective rate constant of hydrolysis of I (log  $k_{eff}$ ) on the temperature (T) at pH 1.03 (1) and 10.10 (2).

Fig. 4. Dependence of the effective rate constant of acid hydrolysis of I (log  $k_{\mbox{eff}})$  on the dielectric constant (e) at 60°C and pH 4.3-4.5.

Just as in the case of IV, the hydrolysis of I in acid, neutral, and alkaline media proceeds with cleavage of the C-N amide bond, as a result of which II or its salt is formed; they are determined in the course the kinetic experiment by a spectrophotometric method according to the colored product with III. For a determination of the mole fraction of decomposed I, the amount of II formed is related to the amount of I, analyzed by the same method, according to VFS 42-834-79, with the exception of the fact that the spectrophotometric determination is performed in water.

## Stoichiometry and Order of the Reaction

Tables 1 and 2 present the values of the coefficients of extinction of the products of the reaction of III with a known sample of II ( $\varepsilon_1$ ) and a sample of the reaction mixture taken at the end of the reaction ( $\varepsilon_2$ ). From the ratio  $\varepsilon_2/\varepsilon_1 \simeq 2$  it follows that in the hydrolysis of 1 mole of I, 2 moles of II (or a salt of II) are formed. The maximum degree of conversion

TABLE 3. Dependence of the Effective Rate Constants of the Hydrolysis of I on the Initial Concentration

Temperature, °C	pН	Initial concen- tration of I• 10 <sup>3</sup> , M	<sup>k</sup> . 10⁴, h − <b>1</b> eff
60	4,44,5	1,98 5,94 9,91	2,61 2,52 2,43

TABLE 4. Dependence of the Effective Rate Constant of Hydrolysis of I on the pH value in Weakly Acid Medium at 60°C

pН	<sup>k</sup> eff <sup>. 103</sup> , h <sup>-1</sup>
4,31	0,148
5,12	0,060
5,19	0,067
5,45	0,084
5,95	0,090
6,08	0,188

TABLE 5. Dependence of the Effective Rate Constant of the Hydrolysis of I on the Dielectric Constant

Medium	Alcohol concen- tration,% by weight	ε (25 °C)	<sup>k</sup> eff <sup>-104,</sup> ti <sup>-1</sup>
Ethanol- water The same » » Water	50 35 20 0	47,5 57,8 67,1 78,3	1,34 0,90 0,82 0,77

TABLE 6. Comparison of  ${\rm k}_{\rm eff}$  of the Rates of Acid and Alkaline Hydrolysis of IV and I at 90°C

Compound IV		Compound I		
pН	<sup>k</sup> eff <sup>·10<sup>3</sup></sup> ·min <sup>-1</sup>	pН	<sup>k</sup> eff <sup>·103</sup> min <sup>-1</sup>	
1,55 1,77 2,17 2,87 3,07 9,37 9,55 9,76 9,82	5,84 3,78 2,14 1,46 1,33 2,09 2,53 3,39 3,53	$\begin{array}{c} 1,20\\ 1,26\\ 1,61\\ 2,35\\ 2,63\\ 9,44\\ 9,50\\ 9,75\\ 10,30\\ \end{array}$	$7,50 \\ 3,09 \\ 0,89 \\ 0,35 \\ 0,22 \\ 1,79 \\ 1,84 \\ 2,49 \\ 4,39$	

of I to II during acid and alkaline hydrolysis is 91-95%. In both cases of hydrolysis the reaction is practically irreversible. The hydrolysis of I in acid, neutral, and alkaline media proceeds as a first-order reaction with respect to the initial compound, which is demon-

strated by the linearity of the semilogarithmic plots of the kinetic curves for various pH values (Fig. 1). Under set experimental conditions (constant pH, ionic strength), the values of the effective rate constants do not depend on the initial concentration of I (Table 3).

Dependence of the Effective Rate Constant of the Hydrolysis of I on the pH of the Medium. The influence of pH on the rate of hydrolytic decomposition of I was studied in the range of pH values 2.0-10.2. Figure 2 presents the dependence of the effective rate constant of hydrolysis of I ( $k_{eff}$ ) on the hydrogen ion concentration in logarithmic coordinates. As can be seen from Fig. 2, increasing the pH to 5.0 leads to a sharp drop in the value of  $k_{eff}$ , which reaches minimum values in the range of pH 5.0-5.5 and then increases sharply when the pH is increased to 10.2. The minimum values of  $k_{eff}$  at 60°C are cited in Table 4. The general form of the function is characteristic of specific acid-base catalysis [1]. The range of pH corresponding to optimum stability of I is somewhat shifted in the more acid direction in comparison with IV.

Influence of Temperature. The dependence of the effective rate contant of hydrolysis of I in acid and alkaline media on the temperature in the temperature range 60-90°C is described by an Arrhenius equation (Fig. 3). The average value of the effective activation energy is  $20.2 \pm 0.2$  kcal/mole at pH 1.0 and  $22.4 \pm 0.6$  kcal/mole at pH 10.1. The average value of the effective activation energy of hydrolysis of I in acid medium is somewhat higher than the corresponding value for IV, which at pH 1.2 is  $18.9 \pm 0.4$  kcal/mole, which is evidence of greater reactivity of IV.

Influence of the Ionic Strength and Dielectric Constant of the Medium. The dependence of the effective rate constant of acid hydrolysis of I on the ionic strength ( $\mu$ ) was investigated under conditions of a constant pH value (3.5-3.6) and concentration of I (9.91·10<sup>-3</sup> M). At values of  $\mu$  0.26, 0.41, and 1.0, the effective rate constants of hydrolysis of I ( $k_{eff}$ ·10<sup>4</sup> h<sup>-1</sup>) are 5.65, 6.07, and 8.60, respectively, i.e., when the ionic strength is increased more than fourfold, the rate constant increases by a factor of 1.5, and a negligible salt effect is observed.

The influence of the dielectric constant ( $\varepsilon$ ) on the effective rate of acid hydrolysis of I was studied in ethanol-water solvent mixtures at pH 4.3-4.5 ( $\mu = 0.01$ ; 60°C. The dielectric constants were calculated as described in [3]. Table 5 presents the values of  $k_{eff}$  found experimentally for various compositions of the medium (the change in  $\varepsilon$  with the temperature is rather small and was not considered in the calculations). The results obtained are evidence that the effective rate constant of the hydrolysis of I is almost independent of the polar properties of the medium. The dependence constructed in a plot of log  $k_{eff}$  versus  $1/\varepsilon$  (the parameters of the Kirkwood equation for ion-dipole interaction) is linear with a small slope (Fig. 4).

The observed dependences on the electrostatic and polar properties of the medium (increase in  $k_{eff}$  with increasing ionic strength and decreasing dielectric constant of the medium) are characteristic of the interaction of an ion with a dipole molecule. However, on account of the greater dispersal of the charge in the transition state, these effects for I, just as for IV, are negligible.

<u>Mechanism of the Reaction</u>. By analogy with the hydrolysis of IV, the hydrolytic decomposition of I should be classified as a reaction of specific acid-base catalysis (the catalysts are  $H_3O$  + and  $OH^-$ , respectively), the rate of which is described by the expression:

$$-\frac{d[AH + A^{-}]}{dt} = k_{H_3O^+}[H_3O^+][AH] + k_1[AH] + k_{OH^-}[OH^{-}][A^{-}] + k_2[A^{-}], \qquad (1)$$

where  $[AH + A^{-}]$  is the total concentration of dissociated and nondissociated homopantothenic acid (V); [AH] and  $[A^{-}]$  are the concentrations of undissociated V and its anion, respectively;  $k_{H_{30}} + k_{OH}^{-}$  are the rate constants of the limiting step of acid and alkaline hydrolysis, respectively;  $k_1$  and  $k_2$  are the rate constants of noncatalytic conversions of undissociated and dissociated forms, respectively. Since hydrolysis proceeds slowly in the absence of acids and bases (see Table 4, Fig. 2), the terms  $k_1[AH]$  and  $k_2[A^{-}]$  can be neglected; then Eq. (1) takes the form:

$$-\frac{d[AH + A^{-}]}{dt} = k_{H_3O^+}[H_3O^+][AH] + k_{OH^-}[OH^{-}][A^{-}].$$
(2)



Fig. 5. Dependence of the effective rate constant (log  $k_{eff}$ ) of acid hydrolysis of I on the acidity of the medium (a) and alkaline hydrolysis of I on the concentration of hydroxyl ions (b) at 90°C.

The products  $k_{H_30}$ +  $[H_30^+]$  and  $k_{OH}$ - $[OH^-]$  are the effective rate constants of acid and alkaline hydrolysis of I, which proceeds as a first-order reaction at a constant pH of the medium. The value of  $k_{H_30}$ + was found from the experimental dependence of  $1/k_{eff}$  on  $1/h_o$  (where  $h_o \simeq [H_30^+]$  at an activity coefficient assumed equal to one) in the range of pH 1.26-2.63, where V is in an undissociated form (pK of V is 4.5 at 25°C [4]). The straight line obtained (Fig. 5a) intercepts a segment on the y axis equal to  $1^{k}H_30^+$  with slope  $1/(k_{H^30}^+\cdot K_B)$ , where  $K_B$  is the equilibrium constant of formation of the transition state during hydrolysis. We find the values  $k_{H_30}^+ = (2.1 \pm 0.3) \cdot 10^{-3} \text{ min}^{-1}$ ,  $K_B = 49 \pm 5 \text{ liter/mole by}$  the method of least squares.

By analogy with an acid hydrolysate of substituted amides, it is assumed that acid hydrolysis of I, like that of IV, proceeds according to a bimolecular acid hydrolysis mechanism  $A_{AC^2}$  [2, 3]. The limiting step of the process is the conversion of the active inter-

mediate form  $H_2O^+ - C - NH_2R'$ , where  $R = -CH(OH)C(CH_3)_2CH_2OH$ ,  $R' = -CH_2CH_2CH_2COOH$ , to the

reaction products, accompanied by cleavage of the acyl carbon-nitrogen bond. In an  $A_{AC2}$  mechanism a dependence of the reactivity on the steric effects of substituents close to the reaction site is observed [2]. The inhibiting effect of the substituent increases with its size. The molecules of homopantothenic (V) and pantothenic (VI) acids have the general formula R=C(0)NHR' and differ in substituents R' at the nitrogent R' = --CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>COOH for V and R' = --CH<sub>2</sub>CH<sub>2</sub>COOH for VI (in both compounds R = --CH(OH)C(CH<sub>3</sub>)<sub>2</sub>CH<sub>2</sub>OH). In I the substituent R' is one CH<sub>2</sub> group larger. Precisely this may explain the fact that the hydrolysis of I in acid medium at the same pH values occurs several times more slowly than the acid hydrolysis of IV (Table 6).

By analogy with the alkaline hydrolysis of amides, for the acid hydrolysis of I we should assume a  $B_{AC2}$  mechanism [2, 3]. The reaction is considered as reversible addition of an OH<sup>-</sup> ion to the molecule of I at the carbonyl group. Decomposition of the active intermediate form determines the rate of alkaline hydrolysis. The mechanism of biomolecular basic hydrolysis  $B_{AC2}$  is characterized by a negligible decrease in the rate in a homologous series of substituents close to the reaction site.

A comparison of the values of the effective rate constants under conditions of alkaline hydrolysis (at close pH values) confirms this pattern (see Table 6). The mechanism of the alkaline hydrolysis of I confirms a linear dependence of the effective rate constant on the basicity of the medium in the range of pH 9.4-10.3 (where I is entirely dissociated) in a plot of  $1/k_{eff}$  versus  $1/[OH^-]$ . The straight line obtained (Fig. 5b) intercepts a segment on y axis equal to  $1/k_{OH}$ . The value of  $k_{OH}$ -, found according to the method of least squares, is  $(5.4 \pm 0.7) \cdot 10^{-3} \text{ min}^{-1}$ ; the equilibrium constant of the transition state is equal to  $(1.7 \pm 0.1) 10^{+1}$  liters/mole.

Since the numerical value of the rate constant of acid hydrolysis is somewhat lower than of alkaline hydrolysis, while the equilibrium constants of formation of the intermediate form in both cases of hydrolysis are rather large, the interval of pH values corresponding to the greatest stability of I is shifted in the acid direction (5.0-5.5). For IV the rate constants of alkaline and acid hydrolysis differ less:  $4.3 \cdot 10^{-3}$  and  $3.3 \cdot 10^{-3}$  min<sup>-1</sup>; the pH range corresponding to the greatest stability of IV is closer to neutral (5.7-6.2).

Thus, as a result of our kinetic investigation we studied the influence of various factors on the stability of I and determined the conditions ensuring its greatest stability in solutions.

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