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INVESTIGATIONS ON ACYL GROUP CARRIERS.

XIII. A NEW METHOD OF SYNTHESIS OF D-(+)-S-SULFOPANTETHEINE AND ITS CONVERSION INTO VITAMIN B_3 COENZYME FORMS

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Recently, new evidence has been obtained on the presence of new derivatives of D-pantothenic acid, other than coenzyme A (CoA) and its bioprecursors, in biological systems [1]. Of particular interest are the S-sulfo derivatives of D-pantetheine, 4'-phosphopantetheine, and 3-dephospho-CoA, which are the growth factors for *Bifidobacterium bifidum* (bifidobacteria) constantly present in human intestine and playing an important role in the gastrointestinal functioning, especially in young children [2]. D-pantethine [3] also shows a pronounced ability to stimulate *in vitro* growth of bifidobacteria, but its clinical effect was high, apparently, due to the fact that it does not reach lower portions of the colon. At the same time, it was found that S-sulfo derivatives of pantethine significantly increase the bifidobacteria content of the infant intestine showing, thus, better promise as drugs for the therapy of gastrointestinal diseases than D-pantethine [4]. In this connection we continued our studies on the synthesis of new D-pantethine derivatives [5] and developed a new approach to the synthesis of D-(+)-S-sulfopantetheine [1], as well as the first synthesis of its L isomer. Considering that compound I is a derivative of D-pantothenic acid, we studied the possibility of converting it into the coenzyme forms of D-pantothenic acid and its ability to take part in the *in vivo* acetylation reactions of the aromatic amines.

In the early seventies, Japanese workers [4] carried out the synthesis of compound I by sulfonation of D-pantethine with sulfites or bisulfites of the alkali metals in the presence of catalytic amounts of copper, iron, or cobalt ions. However, the use of D-pantethine, obtained from the derivatives of D-pantothenic acid as an oil, as the starting material requires multistage chromatographic purification [6] which is an essential drawback of the method. Thus, we developed a new method of preparing compound I, starting with calcium D-pantothenate, an industrial by product, and S-sulfocysteamine (II). Compound II was prepared in a high yield by a modification of a known method [7] through opening the ethyleneimine ring with sodium thiosulfate at pH 4.0-4.5:

$$\overline{CH_2CH_2NH} + Na_2S_2O_3 \xrightarrow{HCl} H_2NCH_2CH_2SSO_3H + NaCl.$$

As a basis for the synthesis of I we used the method of mixed anhydrides, developed for the preparation of D-pantethine [6] and its S-acyl derivatives [5] and consisting in the condensation of the mixed anhydride of D-pantothenic and carboxylic acids (III) with cysteamine and its derivatives in an organic solvent. Because S-sulfocysteine and its salts are insoluble in organic solvents, a new variation of the reaction of mixed anhydride III and compound II was carried out in aqueous media leading to the formation of I in a sufficiently high yield. Analogously, L-(−)-S-sulfopantetheine was prepared from L-pantothenic acid.

We also developed another method of preparing compound I consisting in the opening of the aziridine ring of N-pantothenoylethylenimide (IV), which was obtained from mixed anhydride III and ethylenimine [5], using sodium thiosulfate. Since an alkali is formed in this reaction, neutralization of the solution with a mineral acid is indispensable for its success. The best pH is 4.0-5.0, which practically completely prevents the decomposition of the desired product I under the action of alkali. This method is sufficiently simple as it

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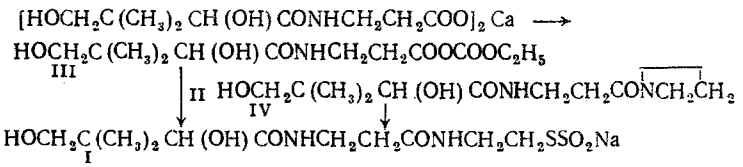
is carried out without the isolation of any intermediates.



The aqueous solution of I is passed through a column packed with an ion-exchange resin in the required salt form for purification and conversion into the salts of alkali or alkaline earth metals. The calcium salt of I, obtained in such a manner, was chromatographically homogeneous and its structure was confirmed by infrared and NMR data. The infrared spectrum of compound I was very similar to the spectrum of D-pantethine [6]. The absorption in the region 1200, 1040, and 640 cm^{-1} is characteristic for the S—SO_3 stretching vibrations [8] and do not appear in the spectrum of D-pantethine. The prepared calcium salt of I was identical with a sample of the same compound given to us by Prof. Z. Tamura of Tokyo University, to whom goes the authors' sincere appreciation.

The ability of D-(+)-S-sulfopantetheine to be used by the animal organism and be converted into CoA was studied in our experiments on white rats weighing 50-60 g, by administering to them a synthetic meal devoid of vitamin B_3 for five weeks [9]. After pronounced avitaminosis and slow weight gain developed, 30-40% of the experimental animals were administered a single subcutaneous dose of 3.3 mg/kg (equimolar with respect to pantothenate and equivalent to the dose used in clinical practice) of calcium-D-pantothenate, D-pantethine, and compound I. After 6 h the animals were decapitated, and the tissues were dipped in liquid nitrogen and used for determining the activity of the coenzyme forms of pantothenic acid by using the reaction of acetylation of p-aminobenzoic acid (PABA) [10, 11]. The acetylation of PABA was investigated in mature mongrel rats after intraperitoneal administration of PABA in a dose of 1 mg [12].

Investigation of the level of the coenzyme forms of pantothenic acid (total content of CoA, dephospho-CoA, and 4'-phosphopantetheine) showed that, with a deficiency of vitamin B_3, there is a definite lowering of the content of these forms in the liver, heart, kidneys, brain, and skeletal muscles of the experimental animals (Table 1). Six hours after a single administration of the pantothenate, normal activity of the coenzyme forms in the animal tissue was observed. After the introduction of pantethine, this indicator was significantly increased, while the activity in the liver, heart, kidneys, and skeletal musculature exceeded the level characteristic for the control animals.

A single injection of compound I to the experimental animals stimulated more than four times the activity of the coenzyme forms of pantothenic acid in the liver; the increase of the CoA level in the skeletal muscle tissue is significant, while, less significantly, the activity increased in the cerebellum and was unchanged in the myocardium. At the same time, there was a significant decrease in the activity of the coenzyme forms in the renal tissue as compared with the initial level.

These data show a high capacity of compound I to penetrate through the hepatocyte membrane and the skeletal muscle cells, while in the myocardium and in the cerebellum, at the time of the experiment, there was no increase in CoA activity. The decrease in the level of the active coenzyme forms of pantothenate in the kidneys is obviously due to an accumulation of the excretion products of metabolism of I or, possibly, of I itself and its 4'-phosphate, which were identified earlier in the urine of the animals that had received an injection of ^{14}C-pantethine [14].

We have also studied the effect of I on the acetylation of PABA in normal animals *in vivo* with a parenteral administration of the preparation in doses equimolar to the pantothenate (3.3 and 30 mg/kg). The latter did not change the acetylation reaction of PABA and the percentage of the PABA aceto form in the aromatic amines excreted with urine, while the injection of I, especially in large doses, decreased the excretion of the acetylated PABA in the daily urine. Thus, the introduction of all excretable forms of PABA with urine decreased as with the introduction of pantothenate at a dose of 3.3 mg/kg (Table 2), which led to a relatively slight decrease of the aceto form in the excreted PABA.

TABLE 1. Level of CoA and Its Bioprecursors in the Tissues of White Rats Lacking Vitamin B_3 (M ± m)

Group	Tissue				
	liver	heart	kidney	brain	muscles
	μg acetylated PABA/g of tissue				
Control	560±0	310±18	210±11	115±8	45±8
Avitaminosis	230±28*	190±14*	160±20*	65±9*	15±7*
Avitaminosis + calcium pantothenate	620±55.6	260±32	200±0	60±7*	35±10.7
Avitaminosis + pantethine	640±48	400±26*	320±24*	120±10	100±0*
Avitaminosis + I	1140±88*	180±34*	100±0*	90±9*	90±9*

*Here and in Table 2, $P < 0.05$ (calculated by comparison with the control).

TABLE 2. Effect of Parenteral Administration of Calcium D-pantothenate and D-(+)-S-sulfopantetheine on the Acetylation of PABA, Administered Intraperitoneally in a Dose of 1 mg into White Rat (M ± m)

Preparation	Dose, mg/kg	PABA content in daily urine			
		total	free	acetylated	% acetyl form
		μg			
Control	—	583±32	333±32	250±40	42.6±4.8
Calcium pantothenate	3,3	408±16*	242±24*	167±24	40,3±5,7
» »	30	500±61	283±16	217±40	39,7±3,9
I	5,15	433±16*	292±16	142±16*	32,5±2,8
I	49,1	400±48*	275±32	125±24*	31,4±5,1

The decrease in the acetylation intensity of PABA on the administration of I is additional confirmation of the conversion of that compound into the active vitamin form, capable of regulating the reactions of biosynthesis of CoA. In a given case, obviously, there is a decrease in the biosynthesis of the active forms of pantothenate capable of participating in acetylation reactions [15], analogously to the action of larger doses of pantothenate or pantethine [16].

Thus, the ability of I to penetrate the biomembranes and act as a bioprecursor of the coenzyme forms of pantothenate in individual strains of microorganisms [17] was shown in experiments with animals for the first time.

The acute toxicity of compound I (LD_{50}) on intraperitoneal administration to white mice was 2.01 g/kg, which is lower than the corresponding indicator in commercial preparations of calcium D-pantothenate (1.44 g/kg [18]). The ability of I to be converted into the active coenzyme forms of pantothenic acid in the liver of animals lacking the vitamin and the relatively low toxicity of the preparation, has raised the question of its possible parenteral administration in cases of primary or secondary vitamin B_3 deficiency.

EXPERIMENTAL

The infrared spectra were recorded on a UR-10 (Carl Zeiss, German Democratic Republic) spectrophotometer using Vaseline oil. NMR spectra were taken on a Hitachi R-20A (60 MHz) instrument in D_2O using 2,2-dimethyl-2-silapentanesulfonate as the internal standard. The specific rotation was determined on an A-1-EPL instrument.

S-sulfocysteamine (II). Hydrochloric acid (2N, 25 ml, pH 4.5) is added dropwise to a solution of ethylenimine (2.15 g) in methanol (40 ml) and sodium thiosulfate (15 g) in water (40 ml), and the reaction mixture is refluxed for 7 h. After concentration to a small volume, the precipitate is recrystallized from water to yield II, 6.54 g, 83.3%, mp 182-185°C (with decomposition). Reported [7] mp 183-185°C (with decomposition). Infrared spectrum: 1020

(SO_3H), 1190 (S=O), 1230 (SO_3H), 1480 (NH_3^+), and 1610 (NH_3^+) cm^{-1}.

D-(+)-S-sulfopantetheine (I). A. To a solution of III, obtained by a known method [6] from calcium D-pantothenate (12.5 g) and ethyl chlorocarbonate (5.13 g), is added dropwise a solution of II (10 g) in 12% aqueous sodium hydroxide (25 ml). The reaction mixture is stirred at 0°C for 1 h and then at 20°C for 8 h, concentrated to dryness, and the residue treated with methanol (50 ml). The insoluble part is removed by filtration and the filtrate concentrated. The residue is dissolved in water (50 ml) and extracted with ethyl acetate (3 × 30 ml). The aqueous layer is passed through a column packed with KU-2 (Na^+-form) resin and the resin is washed with water to neutral reaction. After concentration, the residue is dissolved in ethanol (50 ml) and precipitated with a ninefold volume of anhydrous ether. Filtration yields the sodium salt of I, 12.8 g 64.1%, $[\alpha]_D^{20}$ + 12.8° (c 2, water). NMR spectrum (D_2O):0.85 (singlet, $C(CH_3)_2$), 3.41 (CH_2OH), 3.85 (CHOH), 2.25 (triplet, CH_2CO), 2.59 (CH_2S), and 3.24 ($NHCH_2$) ppm. Calculated for $C_{11}H_{21}N_2O_7S_2$: C 34.73; H, 5.56; N, 7.37%. Found: C, 35.05; H, 5.27; N, 7.49%. B. To the mixed anhydride obtained from calcium D-pantothenate (10 g) in a procedure analogous to the one described above is added, at −5°C, ethylenimine (2 g) and triethylamine (5 g) in ethyl acetate (70 ml). The reaction mixture is stirred at 0°C for 1 h and, then, at 20°C for 1 h, the precipitate is collected and washed with ethyl acetate (10 ml). The filtrate is concentrated to dryness and the residue dissolved in ethanol (40 ml). Sodium thiosulfate (12 g) in water (40 ml) is then added to the ethanolic solution and adjusted to pH 4.0-4.5 with 2 N hydrochloric acid. After 1 h standing, the precipitate is collected and the filtrate concentrated under reduced pressure. The residue is dissolved in methanol (50 ml), filtered with the addition of active charcoal, and the filtrate extracted with ethyl acetate (3 × 20 ml). The aqueous layer is passed through a column packed with KU-2 × 8 (Ca^{++} form) resin, washed with water, and the aqueous washings concentrated. The residue is dissolved in methanol (50 ml), the insoluble portion removed, and the filtrate precipitated with a ninefold excess of anhydrous ether. The precipitate is filtered to yield the calcium salt of I, 8.8 g, 55.6%, $[\alpha]_D^{20}$ + 13.1° (c 2, water). Reported [4]: $[\alpha]_D^{20}$ + 13.9° (c 2, water). Calculated for $C_{22}H_{42}CaN_4O_{14}S_4$: C, 35.00; H, 5.61; N, 7.42%. Found: C, 35.08; H, 5.85; N, 7.84%. The calcium salt of I, obtained as described in B and purified on a column of KU-2 (Ca^{++} form), is dissolved in methanol and precipitated with a fivefold excess of acetone. Yield, 8.72 g, 50.8%. Calculated for $C_{22}H_{42}CaN_4O_{14}S_4 \cdot CH_3COCH_3$: C, 36.94; H, 5.95; N, 6.89%. Found: C, 37.22; H, 6.27; N, 6.54%.

Calcium Salt of L-S-sulfopantetheine. This is prepared from L-pantothenic acid in a procedure analogous to that described in A. Yield, 66.5%; $[\alpha]_D^{20}$ −12.8° (c 2, water).

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