SUMMARY

It has been established that gossypulin from cotton plant seeds undergoes complex conformational changes in the pH interval from 2 to 13. Sodium phytate stabilizes the protein molecule at pH 2 and 3.

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SEPARATION OF PANTOTHENIC ACID DERIVATIVES AS PRECURSORS FOR THE BIOSYNTHESIS OF THE ACETYLATION COENZYME BY CHROMATOGRAPHY ON DEAE-DELLULOSE

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The possibility of the chromatographic separation of pantothenic acid derivatives — 4'-phosphopantothenate, pantethine, 4'-phosphopantetheine, dephospho-CoA and CoA — on a column of DEAE-cellulose (DE-11 or Servacel DEAE 23 SH) has been studied, with their detection by radiometric, enzymatic, or spectrophotometric methods. The affinity of pantothenic acid and that of pantotheine for the ion-exchange resins used are identical. The phosphorylated derivatives of these compounds, which are eluted at higher values of a concentration gradient of lithium chloride, also have identical chromatographic characteristics. The separation of dephospho-CoA and CoA is not achieved on the chromatographic system investigated, but the elution of the fractions to free them from the retained nucleotide-containing precursors of the coenzyme can be used effectively for analytical purposes.

The chemical or enzymatic synthesis of the acetylation coenzyme — pantetheine adenine nucleotide diphosphate (CoA) — is performed in several stages by the successive transformation of its vitaminic precursor — pantothenic acid (I) [1-3]. In the biosynthesis of CoA, intermediate metabolites are 4'-phospho-(I) (II), 4'-phosphopantothenylcysteine, 4'-phosphopantetheine (III) (PPN), and dephospho-CoA (IV). However, among other PAA compounds isolated from biological material must be mentioned pantethine (V), diphosphopantethine (the disulfide derivatives of pantetheine and (III), respectively), and the sulfo derivatives of these two compounds [4]. At least seven derivatives of the vitamin, including the free form of (I) and CoA have been identified in the hepatocytes of higher animals [5], but their accurate differential determination has proved impossible in spite of the simultaneous use of six analytical methods.

Our preceeding communication in this journal [6] related to the quantitative gas-chromatographic determination of pantolactone, which arises on the acid hydrolysis of (I) [7] and compounds containing it [8]. This method is extremely convenient for pharmaceutical chemical purposes [9], although the microbiodetermination of (I) still remains the method of choice [10]. These methods permit analytical problems to be solved with adequate accuracy both in the process of the chemical transformation of compound (I) and also in biochemical investigations.

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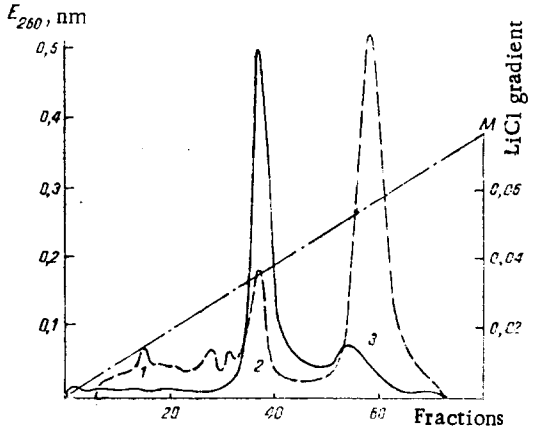

Fig. 1. Spectrophotometric analysis of the fractions of boiled aqueous solutions of Fluka CoA (dashed line; amount of CoA in the sample 1.4 mg, cellulose DE-11; 1, 2, 3 — chromatographable components of the system; respectively (I), (III), and (CoA) and P.-L. Biochemicals dephospho-CoA (full line; amount 0.8 mg, Servacel DEAE 23 SH cellulose).

The problem remains the preliminary separation of the metabolites of (I), since their physicochemical properties are similar. We have investigated the possibility of separating compounds (I-V) by the use of ion-exchange chromatography on a column of DEAE-cellulose, which was first proposed for the isolation of the reaction mixture of (III) [11] and has already been used for studying the biotransformation of (I) in animal livers [12].

As follows from the experimental results illustrated in Fig. 1, chromatography on DEAE-cellulose permits the separation of compounds containing (I) to be effected fairly successfully. In a CoA preparation subjected to labeled hydrolysis, 59% (starting from 100% of a Fluka native preparation of CoA) of the coenzyme (eluted in fractions 50-70) and of the products of its hydrolysis (eluted in fractions 12-18 and 25-40) were detected. On the whole, the chromatogram was similar to that in the separation of liver extracts, in which free (I), (III), and CoA were identified in the fractions mentioned. It remains unclear whether (II) arises during the hydrolysis of the coenzyme or is this compound present among the precursors of CoA in the native tissue. A similar observation can be made in relation to the question of identifying three other intermediates in the biosynthesis of CoA — 4'-phosphopantothenylcysteine, (V), and (IV).

With the aim of a more accurate analysis of the chromatographic properties of the derivatives of (I), we have effected the synthesis of (I), (II), and (V) labeled with radionuclides and an unlabeled preparation of (III) and have chromatographed them on a column of DEAE-cellulose in comparison with CoA preparations, including [^{3}H]-CoA. Figure 2 illustrates generalized results of chromatographic investigations, from which it is clear that ion-exchange chromatography on DEAE-cellulose permits only a limited separation of the PAA metabolites. Compounds (I) and (V), and also their phosphorylated derivatives, chromatograph with practically identical characteristics. Thus, it becomes obvious that the radiochromatogram obtained of extracts of tissue liver in the form of three peaks of metabolites of (I) essentially reflects the presence of at least five compounds (peak I — (I) and (V); peak II — (II) and (III); and peak III — CoA). At the same time, the method used permits an effective separation of (I) and (V) from their phosphorylated derivatives, which is important for monitoring the chemical or enzymatic transformation of compound (I) [14].

The question of the chromatographic properties of (IV), which is detected, like CoA, by the direct spectrophotometry of the eluates at 260 nm must be considered. As a sample we used a P.-L. Biochemicals high-purity preparation of (IV). In view of the poor stability of the compound, however, the possibility of partial hydrolysis must be assumed. This was confirmed by the results of chromatography (Fig. 1), from which it follows that (IV) chromatographs in the fractions corresponding to the 0.03 and 0.05 M concentrations of the gradient. The exact relationship of the chromatographic properties of (IV) and those of (III) and CoA remains problematical, but it is not a matter of doubt that this precursor of the synthesis of CoA also chromatographs in fractions of the other metabolites of (I).

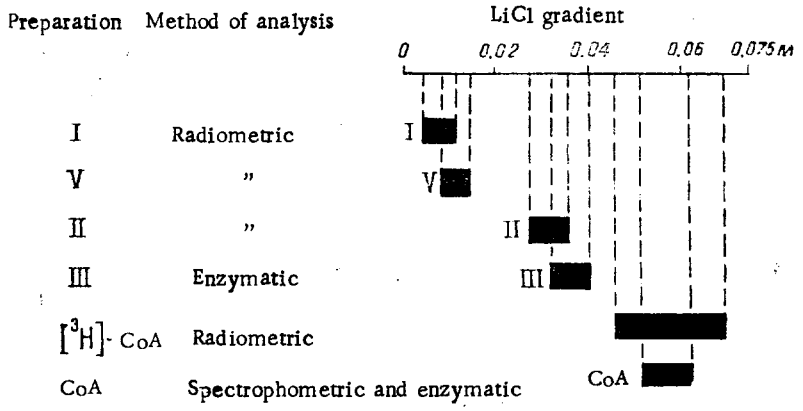

Fig. 2. Comparative evaluation of the binding on an anion-exchange resin of separations of pantothenate in a linear gradient of lithium chloride and their possible analysis by a radiometric method (0.1 ml of fraction in 10 ml of dioxane scintillator) and by an enzymatic method using a system of acetylating enzymes from pigeon liver — the acetylation apoenzyme [4].

EXPERIMENTAL

Chromatography on DEAE-cellulose. The investigation was carried out with sodium D-[1-^{14}C]pantothenate (^{14}C-SPA) and [^{3}H(G)]-CoA with specific activities of 55.2 mCi/mmole and 1.3 Ci/mmole, respectively (FRG), CoASH (from Switzerland), CoA (lithium salt) and dephospho-CoA (from USA), and PPN (from Japan). We synthesized the calcium [^{14}C]pantothenate (^{14}C-CPA), 2.4 mCi/mmole, the [^{14}C]PPA, 0.3 mCi/mmole, and the D-pantethine 4,4"-diphosphate (PTP).

As the ion-exchange materials we used cellulose DE-11 with a capacity of 1 meq/g (United Kingdom) and Servacel DEAE 23 SH, 0.75-0.90 meq/g (Hungary). Solutions were boiled in a water bath for 10 min with the aim of creating conditions analogous to those used in the extraction of similar compounds from animal tissues. To 8 ml of the aqueous solution were added 1 ml of 0.1 M Tris-HCl buffer, pH 7.3 [17] and 1 ml of dithiothreitol (DTT) to give a concentration of 0.01 M, and, after having been shaken at +4° for 30 min, the mixture was deposited on a column (1 × 12 cm) with the ion-exchange material. The substances were eluted with 100 ml of water and with a linear gradient of 0.075 M LiCl in 0.003 M HCl (400 ml). The rate of elution was 1 ml/min and the fraction volume was 5 ml. An aliquot after the separation of the radiolabeled compounds was transferred into dioxane scintillator and the radioactivity was measured in a Mark 2 scintillation counter (USA). PPN, PTP, and dephospho-CoA were determined by the acetylation reaction [4], and CoA and dephospho-CoA also by direct spectrophotometry at 254 nm, using a LKB Uvicord-III instrument (Sweden).

[1-^{14}C]Pantolactone. A solution of 3.6 g of ammonium chloride, 4.4 g of potassium cyanide and K^{14}CN in 15 ml of water was added to a solution of 6 g of α,α-dimethyl-β-hydroxypropionaldehyde in a mixture of 100 ml of water and 40 ml of ethanol cooled to 8°C at such a rate that the temperature of the reaction mixture did not rise above 10°C. Then it was stirred at 8-10°C for 1 h and at room temperature for 1 h. After this, 15.6 ml of 30% HCl was added and the reaction mixture was heated at 80-85°C for 3 h, neutralized with Na_2CO_3 to pH 4.5, and evaporated to dryness. The residue was dissolved in water and the solution was extracted with chloroform (3 × 15 ml). The chloroform extracts were dried over calcined sodium sulfate and filtered. The filtrate was evaporated to dryness and the residue was distilled in vacuum at 112-114°C (2-5 mm Hg). This gave 3.2 g of [1-^{14}C]pantolactone (35.3%).

[^{14}C]Pantothenic acid was synthesized as described in [15].

D-Pantothenic acid 4'-phosphate was obtained by the phosphorylation of pantothenic acid with cyanoethyl phosphate in pyridine with dicyclohexylcarbodiimide [16].

[1-^{14}C]Pantethine. A suspension of 0.4 g of β-alethine dihydrochloride in 6 ml of ethanol was treated with 3.9 ml of 4.7% sodium methanolate, the mixture was stirred for 30 min, the precipitate was filtered off, and the solvent was evaporated off to dryness. The residue

was treated with 0.31 g of [1-^{14}C]pantolactone and a drop of sodium methanolate solution. The mixture was fused at 65°C for 3 h, at 100°C for 2 h, and at 120°C for 20 min. Then it was dissolved in methanol, the solution was filtered, and the filtrate was precipitated with ether and left to stand at 0°C for 5 h. The ether was decanted off, the residue was dissolved in methanol, and the solvent was distilled off in vacuum. This gave 0.35 g (68%) of product.

Pantethine 4',4"-Diphosphate. A mixture of 1.11 g of pantethine and 1.35 g of 8-quinolyl phosphate was dried by evaporation with three 15-ml portions of pyridine. The residue was dissolved in 20 ml of pyridine, and 810 mg of anhydrous copper chloride was added, the mixture was stirred at 80°C for 5 h, and then 4 ml of water was added and it was stirred at 20°C for 12 h. The reaction mixture was evaporated to dryness and the residue was washed with acetone and chloroform. The residue was dissolved in 20 ml of water, the solution was filtered, the filtrate was passed through a column of Dowex 50WX8 resin (H^+ form), which was then washed with water, and the aqueous eluated (∿400 ml) were concentrated to 15 ml, treated with an aqueous solution of potassium hydroxide to pH 7.5, and filtered, and the filtrate was evaporated to dryness in vacuum. The residue was dissolved in 5 ml of water, the solution was filtered from an insoluble fraction, the filtrate was precipitated with 100 ml of ethanol, and the precipitate was separated off and dried. The yield of the compound was 0.73 g (46.2%).

The analytical results for all the compounds corresponded to the calculated figures.

SUMMARY

1. Pantothenic acid derivatives — pantothenate, 4'-phosphopantothenate, pantethine, 4'-phosphopantetheine, dephospho-CoA, and CoA — chromatograph on a column of DEAE-cellulose in the form of three groups of fractions corresponding to concentrations of lithium chloride gradient of 0.01, 0.03, and 0.05 M. The first group includes pantothenate and pantethine, the second includes 4'-phosphopantothenate and 4'-phosphopantetheine, and the third group includes CoA and, possibly, dephospho-CoA.

2. Ion-exchange chromatography permits the effective separation of phosphorylated and nonphosphorylated pantothenate and pantethine, but the isolation of the nucleotide-containing metabolites remains problematical.

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