SYNTHESIS OF [^{3}H]-LABELED CoA AND 4′-PHOSPHOPANTOTHENATE BY THE ENZYME SYSTEM OF BREVIBACTERIA

V. I. Rezyapkin, V. I. Zubko, V. M. Kopelevich, M. I. Tsibul'skaya, L. A. Kudryacheva, T. I. Khomich, and A. G. Moiseenok

UDC 577.15:579.66

Using the method of synthesizing the disulfide form of CoA in a system with dried cells of Brevibacterium ammoniagenes developed previously, we have obtained labeled CoA with a yield of 15% calculated on the initial of [^{3}H]-pantothenate introduced into the reaction medium. The purity of the preparation obtained, after purification using ion-exchange chromatography was 95% in terms of radioactivity. A possible pathway for the production of labeled phosphopantothenate in a system with a cell-free extract of Br. ammoniagenes is considered. According to preliminary results, the phosphorylation of the pantothenate amounted to 30-40%. This system is unsuitable for obtaining labelled phosphopantetheine.

CoA and its precursors take part in the functioning and regulation of fundamental metabolic pathways [1, 2]. Because of this, these compounds are widely used in the practice of biochemical studies, many of which require labelled preparations. However, the domestic industry does not produce CoA and its main precursors, although substrates for their synthesis (cysteine, ATP) are available in radionuclide-labeled form. Foreign firms mass-produce from labeled compounds of this series only panthothenate (PAA), while the synthesis of labeled [^{3}H]-CoA is carried out to special order by the firm NEN (Germany).

CoA and its precursors can be obtained both chemically [3] and by biochemical methods [4-8]. The latter are clearly preferable in obtaining a CoA preparation for medicinal purposes and also for the biochemical process of introducing a radionuclide into CoA.

In the Vitaminy NPO [Scientific Production Combine], a method has been developed for obtaining CoA disulfide with the aid of dried cells of *Br. ammoniagenes*. In the present work we have used this method for obtaining labeled CoA. In addition, we have performed investigations on the possibility of synthesizing labeled precursors of CoA — phosphopantothenate (PPA) and phosphopantetheine (PPN) with the aid of cell-free extracts of *Br. ammoniagenes*.

Preparation of Labeled CoA. As the source of the label for obtaining labeled CoA we used [^{3}H]-PAA. The incubation medium contained, in addition to the PAA, the other substrates for the enzymatic synthesis of the coenzyme (ATP and cysteine). In this experiment, as the polyenzyme preparation containing all the necessary enzymes for the biosynthesis of CoA we used dried cells of *Br. ammoniagenes*. After incubation at 32°C for 18 hours, and heat treatment (100°C) followed by centrifugation, the supernatant liquid was analyzed by high-performance liquid chromatography as described in the Experimental part. The analysis showed (Fig. 1) that the inclusion of the label in the CoA amounted to about 20%. The [^{3}H]-CoA obtained was isolated by ion-exchange chromatography on DEAE-Toyopearl 650 M. As the result of purification we obtained a preparation of [^{3}H]-CoA with a yield of 15% on the initial radioactive material added to the reaction medium in the form of [^{3}H]-PAA. The purity of the preparation deduced from its radioactivity was 95%, and that found by photospectrometric analysis was 80% (Fig. 2). Thus, the preparation of [^{3}H]-CoA disulfide obtained was suitable for use in biochemical experiments. From this preparation it is possible to obtain labeled dephospho-CoA after treatment with DTT and phosphoprotein phosphatase, which is capable of dephosphorylating CoA [12].

Preparation of Labeled Precursors of CoA. As is known, *Br. ammoniagenes* cells possess the highest panthothenate kinase activity of all the microorganisms investigated [9]. It has also been shown that panthothenate kinase is capable of

Institute of Biochemistry, Academy of Sciences of the Belarus Republic, Grodno. Vitaminy Scientific—Production Association, Moscow. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 428-431, May-June, 1993. Original article submitted October 23, 1991.

0009-3130/93/2903-0365$12.50

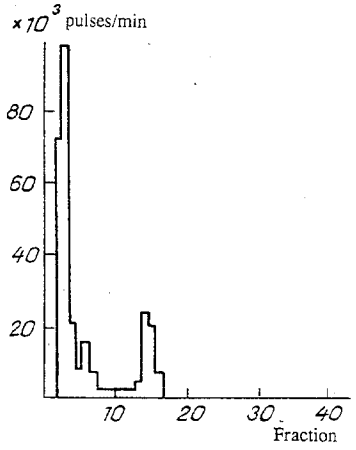

Fig. 1. HPLC of the products of the enzymatic synthesis of [^{3}H]-CoA.

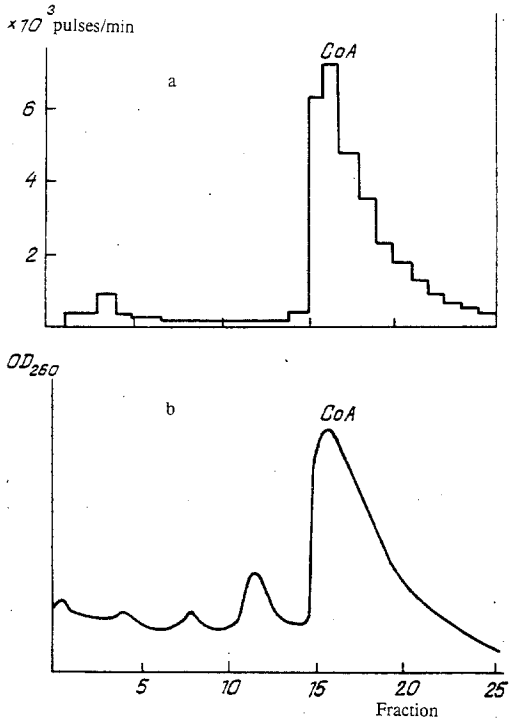

Fig. 2. Analysis by radioactivity (*a*) and by optical density (*b*) of a preparation of [^{3}H]-CoA purified by HPLC.

phosphorylating both PAA and pantetheine (PN) at practically equal rates. This capacity can be used in the biotechnological production of [^{14}C]-PPA and [^{3}H]-PPN.

The incubation medium for obtaining the labeled PPA and labeled PPN (from [^{3}H]-pantetheine) was similar to the medium used previously for determining pantothenate kinase activity [10]. Into a sample (1 ml) was introduced the cell-free extract containing 2 mg of protein, and [^{14}C]-PAA or [^{3}H]-PN with a total radioactivity of $2 \cdot 10^5$ pulses/min in 1 ml. The samples were incubated for 0.5-6 h with an interval of 30 min at 37°C. After the reaction had stopped, the amount of labeled PPA was determined by a method described previously [10]. The samples containing [^{3}H]-PZ as substrate, after separation on DEAE-Sephadex A-25 [10], were analyzed by the HPLC method [11].

The experiment showed that the phosphorylation of [^{14}C]-PAA amounted to 30-40% in relation to the substrate added to the medium. It was possible to separate the [^{14}C]-PPA from the labeled substrate by ion-exchange chromatography, and this was made the basis of the determination of the activity of the pantothenate kinase.

When the cell-free extracts were incubated in the presence of [^{3}H]-PN with subsequent analysis of the reaction products by the HPLC method, [^{3}H]-PPN was detected in insignificant amounts. In view of the circumstance that the biological material

used contained a complete set of enzymes for the biosynthesis of CoA [8], the low yield of [^{3}H]-PPN can be explained by the fact that it is the substrate for the following stage in the biosynthesis of CoA. However, the possibility is not excluded of the partial hydrolysis of the PN in a pantothenase reaction. To obtain PPN it is possible to use a purified preparation of panthothenate kinase and also other methodological stratagems taking into account the kinetic characteristics of the enzymes involved in the biosynthesis of CoA.

EXPERIMENTAL

In this work we used DEAE-Sephadex A-15 from Pharmacia (Sweden). [^{14}C]-PAA with a specific radioactivity of 57 mCi/mmole from NEN (Germany), [^{3}H]-PAA with a specific radioactivity of 2×10^7 pulses/min·mg (synthesized in the laboratory) with a degree of purity of 85%, ATP from the firm Reanal (Hungary), and DEAE-Toyopearl 650 M from the firm Toyo Soda Mfg. Co. Ltd. (Japan), and also other compounds of domestic production.

Instruments. Mark 2 liquid scintillation counter from the firm Chicago Nuclear (USA), UZD N-2G ultrasonic disintegrator of domestic production, and a Liquochrom-2010 high-pressure chromatograph (Hungary).

Cell-free extracts of *Br. ammoniagenes* were obtained in the following way. Bacterial cells were grown at 28°C for 24-28 h. The culture medium was similar to that used previously by Japanese authors [7]. The yield of biomass was about 5 g/liter of medium. The cells were washed with potassium phosphate buffer, pH 6.5, followed by centrifugation at 5000 g for 15 min. The washed cells were suspended in 0.01 M potassium phosphate buffer, pH 6.5, and were disrupted in the UZD N-2G ultrasonic disintegrator at 22 kHz for 5 min. The supernatant obtained after the centrifugation of the disrupted cells (10,000g for 15 min) was used as the cell-free extract.

The medium for obtaining the labeled [^{3}H]-CoA contained [4]: [^{3}H]-PAA, 5 μmole/ml; L-cysteine 10 μmole/ml; ATP, 15 μmole/ml; $MgSO_4$, 10 μmole/ml; dried *Br. ammoniagenes* cells, 750 mg/ml; and 0.1 M potassium phosphate buffer, pH 8.0, in a total volume of 5 ml.

After incubation at 32°C for 18 h with constant stirring, the reaction was stopped by boiling for 5 min. The precipitate was removed by centrifugation at 5000g. The supernatant liquid was used for the isolation of purified labeled CoA.

Purification of the CoA. The supernatant liquid containing the labeled CoA was deposited on a column (1.8 × 12)* containing DEAE-Toyopearl 650M (HCO_3 form). After absorption, the column was washed with water and with 0.17 N NH_4HCO_3 until there was no longer any radioactivity in the eluate. The CoA was eluted with 0.27 N NH_4HCO_3 at the rate of 30 ml/h. The fractions (with a volume of 5 ml) with the highest counts were combined and diluted with H_2O to decrease the concentration of the salts. An aliquot of the material obtained was subjected to rechromatography on a similar column with DEAE-Toyopearl 650M but with elution by 0.4 N NH_4HCO_3. Samples containing the labeled coenzyme were combined and evaporated and were suspended in ethanol followed by reevaporation several times. In the final stage, the contents of the flask were evaporated to dryness and the residue obtained was dissolved in 1 ml of distilled water.

Analysis of the [^{3}H]-CoA Obtained. The [^{3}H]-CoA disulfide obtained was converted into the reduced form by preliminary treatment with dithiothreitol (DTT) for 1 h at room temperature (the concentration of DTT in the sample was 20 mM). The preparation was analyzed for its [^{3}H]-CoA content on a Liquochrom-2010 chromatograph (Hungary) using a column (1.4 × 250 mm) and a precolumn (4.6 × 50 mm) containing μ-Bondapak C (10 μM). The mobile phase used was 20 mM KH_2PO_4, pH 5.0—methanol (91.5:8.5). Rate of elution 1 ml/min, fraction volume 1 ml. Detection was based on radioactivity and absorption at a wavelength of 260 nm.

Protein was determined by Lowry's method [13].

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