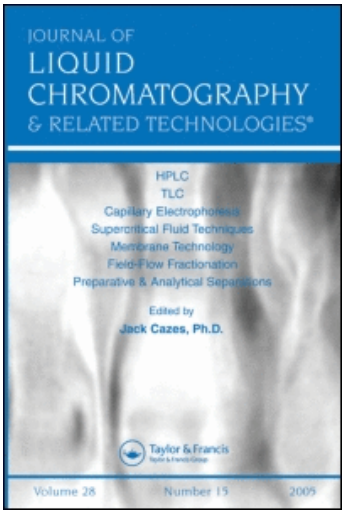

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DETERMINATION OF PROPIONYL-CoA DEHYDROGENASE ACTIVITY IN *SALMONELLA TYPHIMURIUM* LT-2 CELLS BY HPLC

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ABSTRACT

A reverse-phase high-performance liquid chromatographic system was developed for the detection of propionyl-CoA dehidrogenase activity in the catabolic pathway of propionate from bacterial extracts, in the presence of some interferent activities.

The method is also useful for the separation and identification of free CoASH and some of its derivatives (acetyl-CoA, propionyl-CoA and acrylyl-CoA).

INTRODUCTION

Propionate es a suitable source of carbon and energy for the growth of Salmonella typhimurium

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LT2 (1). However, the pathway used by this bacteria for the utilization of this substrate is not known.

In a number of bacterial systems, different pathways of propionate metabolism have been reported involving acrylyl-CoA as an intermediate compound. Acrylyl-CoA is a product of the dehydrogenation of propionyl-CoA, the reaction being catalyzed by propionyl-CoA dehydrogenase. The presence of this enzyme has been demonstrated in different microorganisms and its activity is usually dependent on flavin nucleotides (2, 3, 4, 5, 6, 7), except in Moraxella where it is dependent on adenine ones (8).

The determination of this enzymatic activity by conventional methods, such as electron acceptors or systems of electron acceptors (2-6 dichlorophenol indophenol-phenazine methosulphate), in Salmonella was not practicable, owing to presence in the cell-free extracts of an acyl-CoA hydrolase limiting the supply of propionyl-CoA to the oxidative enzyme.

The present paper describes a reverse-phase system of HPLC to detect propionyl-CoA dehydrogenase activity, by determination of substrate and product concentrations.

MATERIALS AND METHODS

Bacteria and culture methods

The organism used was Salmonella typhimurium LT2 obtained from the Department of Biochemistry, University of Leicester (England).

The cells were grown aerobically at 37°C in M-63 medium (9) supplemented with the appropiate carbon source (propionate 20 mM, acetate 30 mM, glucose

10 mM). The growth was followed in a Spectronic 20 (Bausch & Lomb) at 680 nm. In these conditions, 1.0 absorbance corresponds to 0.68 mg dry weight cell/ml or 1.3 x 10^9 cells.

Preparation of cell-free extracts

Bacteria from 100 ml of medium were harvested in the late logarithmic phase of growth, suspended in 4 ml of 50 mM tris (hydroxymethyl) aminomethane hydrochloride buffer, pH 7.0, washed twice in the same buffer, and desrupted by exposure to ultrasonic oscillations in a Sonifier B-12 cell disruptor (Branson Sonic Power Company, Danbury, Connecticut) (operating at a peak-to-peak amplitude of 8 to 9 μm) at 0°C for 30s x 2.

Protein estimation

Soluble protein was measured either colorimetrically by the Biuret method (10), with crystalline bovine serum albumin as standard, or spectrophotometrically (11).

Preparation of the samples of enzymatic assay for analysis by HPLC.

The incubation mixture corresponding to propionyl-CoA dehydrogenase (5 ml) at 30°C contained: 50 m of tris-HCl or potassium phosphate buffers, pH 7.0, 0.80 mol of propionyl-CoA and 200 μl of bacterial extracts (approx. 0.8 mg of protein).

Aliquots of 0.5 ml were removed periodically. The proteins were precipitated in 6% perchloric acid. After centrifuging once in an Eppendorf 5414 centrifuge for 5 minutes, the pH of the supernatant was

adjusted to ca. 6.0 with KOH; and again to remove the precipitated potassium perchlorate, the supernatant was filtered, degassed and injected into the liquid chromatograph.

Obtention of intracellular acyl-CoA intermediates for analysis by HPCL

The method was essentially that of Bagnara and Finch, as modified slightly by Long (12). Cells (approx. 70 mg dry weight) were harvested and suspended in 1.5 ml of 1.2 M $HClO_4$ at 0°C; the suspension was shaken in a mixer and stood in ice for 15 min before centrifuging at 30000 x g for 10 min at 4°C. The supernatant was then neutralized with 1.08 M KOH containing 0.24 M $KHCO_3$. The $KClO_4$ precipitate was removed by centrifuging at 30000 x g at 4°C and the supernatant was stored in ice prior to analysis. Just before the injection this solution was filtered and degassed.

Liquid chromatography conditions

Analyses were performed with a liquid chromatograph equipped with a constant flow pump rate (Constametric IIG, Laboratory Data Control, Riviera Beach, FL, U.S.A.) and a Rheodyne injector (Laboratory Data Control) with a 20 µl sample loop.

Sample detection was effected at 254 nm with a variable wavelength absorbance detector Model 441 (Waters Associates, Milford, MA, U.S.A.). The response was monitored with a Linear recorder with a scale expansor.

All results were obtained by using a 1 x 10 cm column of reverse phase, µBONDAPAK C18, with 10

m of particle size (Waters Associates), and precolumn (2 x 10 mm) of the same resine.

Eluents were 0.220 M KH_2PO_4 buffers, pH 4.0, containing 20% of methanol and 0.05% (v/v) of thiodiglycol. Solvents were mixed, filtered through a 0.45 m Millipore filter, type HA, and degassed by vacuum filtration just before use.

The elution was isocratic and the flow rate was 1 ml/min, requiring, under these conditions, a pressure in the system of 340-350 p.s.i.

All tests were performed at room temperature.

The void volume, Vo, was determined from retention volumes found in several samples not retained at the given eluting conditions, and was about 3.3 ml.

Chemicals

Acetyl-CoA and propionyl-CoA were prepared according to Simon and Shemin's method (13), while the Wieland and Köppe's method (14, 15) was used for acryl-CoA synthesis.

The purity of the synthetized acyls was tested by the following methods: nitroprussiate (16), 5-5' dithiobis-2-nitrobenzoic acid (DTNB) and HPLC, using standard conditions of analysis. In all cases purity was higher than 95 per cent.

CoASH, crystalline bovine serum albumin and thiodiglycol were obtained form Sigma (St. Louis, MO, U.S.A.) Tris (hydroxymethyl) aminomethane hydrochloride, acetic and propionic anhydrides, acrylic acid and the sources of carbon form Merck (Darmstadt, F.R.G.). All other reagents were of the highest purity commercially available.

RESULTS

The separation of CoASH, acetyl-CoA, propionyl-CoA and acrylyl-CoA, under isocratic conditions, is shown in Fig. 1. The solvent stock was 220 mM potassium phosphate buffer pH 4.0, ca. 20% methanol and 0.05% (v/v) thiodiglycol, with a flow rate of 1 ml/min; thiodiglycol was added to the solvent in order to avoid oxidation of CoA during analysis (17).

Elution under these conditions showed the following peaks and retention times: acrylyl-CoA 4 and 6.5 minutes, CoASH 5.5 and 12 minutes, acetyl-CoA 9 minutes and propionyl-CoA 20 minutes.

The retention times of CoASH and acyls of CoA are dependents on the methanol concentrations at constant pH and ionic strength. Changes in the amount of methanol in the mobile phase from 22% to 12% gave caused large increases in the retention times, which nevertheless permitted peak identification and separation, although propionyl-CoA eluted very late (55-60 minutes) with a flattened peak. Methanol at 20% afforded the most favourable conditions for separation and the correct speed for the process.

The 20 μl injections, effected separately, of each one of the standard compounds at different concentrations permitted the plotting of standard curves, linear relationship being obtained between the injected amount and the peak height.

Reproductibility was good as the variations of the peak height in replicate runs were less than 2%.

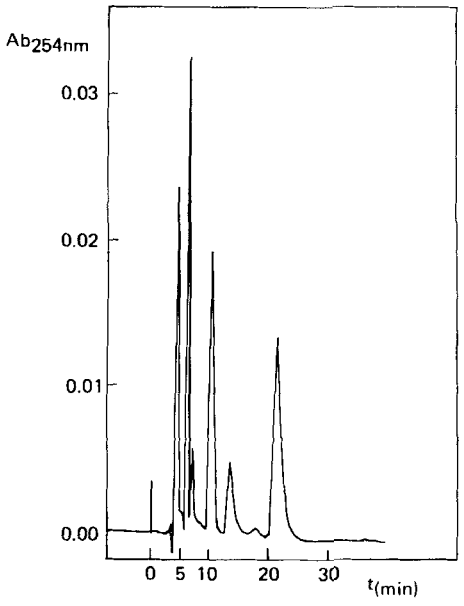

Figure 1.- HPLC elution pattern of standars of acrylyl-CoA (0.272 nmol, t_R = 4.0 min nmol t_R = 6.5 min), CoASH (1.80 nmol, t_R= 5.5 min and t_R =12.0 min), acetyl-CoA (2.72 nmol, t_R= 9.0 min) and propionyl-CoA (2.72 nmol, t_R= 20 min). The solvent stock was 220 mM potassium phosphate buffer, pH 4.0, 20% methanol and 0.05 % thiodiglicol; 20 µl were injected into the chromatograph. Detection was at 254 nm. Full seale deflection represents 0.05 absorbance units.

Determination of intracellular activated compounds

The above conditions were applied to the determination of free CoASH and intracellular activated compounds in Salmonella typhimurium cells grown in differents substrats.

The solutions obtained with the method described in Materials and Methods were filtered, and ali

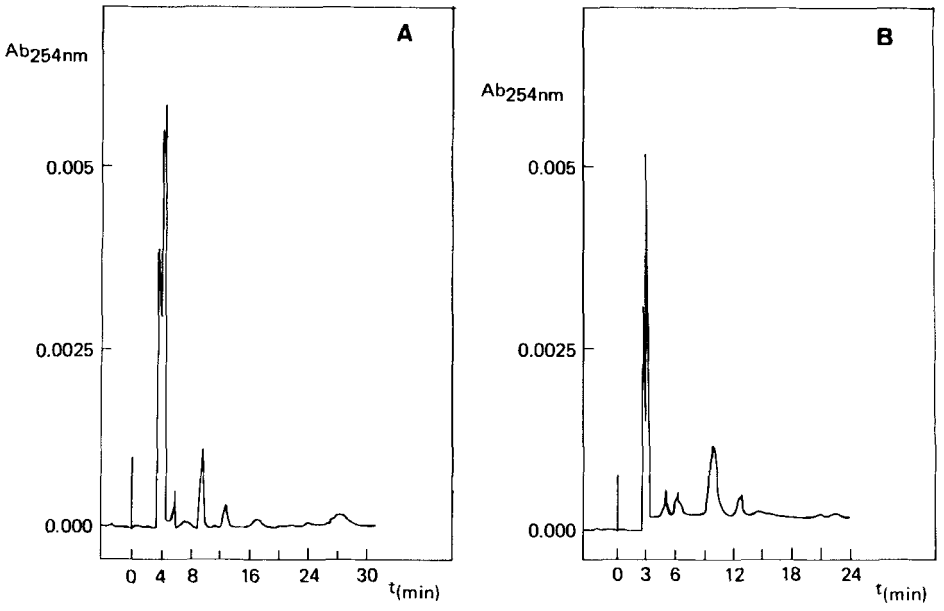

Figure 2.- HPLC elution of endogenous acyls of CoA and CoASH from S. typhimurium LT2 cells grown on: (A) glucose, (B) acetate, (C) lactate and (D) propionate. Chromatographie conditions were similar to those described for Figure 1.

quots of 20 µl injected in the cromatograph. Figs. 2a, 2b, 2c and 2d show the chromatograms corresponding to glucose, acetate, lactate and propionate-growth cells, respectivelly.

Free CoASH and acetyl-CoA were detected in all cases. The chromatogram for the propionate-grown cells shows, additionally, peaks corresponding to propionyl-CoA, results which is verify its role as intermediares in the propionate degradation.

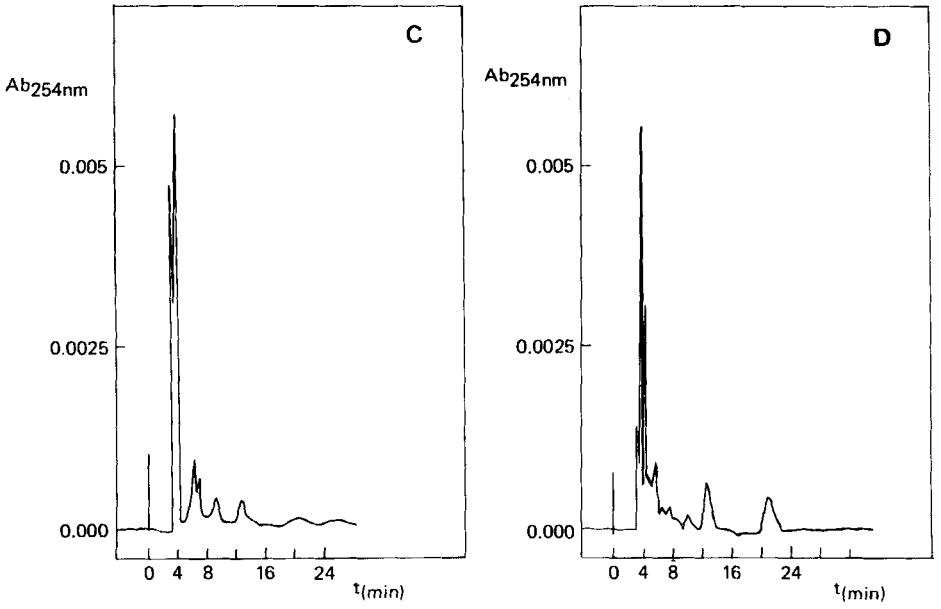

Figure 2 C & D

All chromatograms show the elution of some substances with the elution-front, presumably adenine nucleotides.

Propionyl-CoA dehydrogenase activity

Determination of propionyl-CoA dehydrogenase activity was effected by analysing the formation of the product and the consequent disappearance of substrate at different reaction times.

Using the method described in Material and Methods and the standard chromatographic conditions, aliquots of 0.5 ml of reaction solution were removed at 0, 10 and 30 minutes, deproteinized, filtered, de-

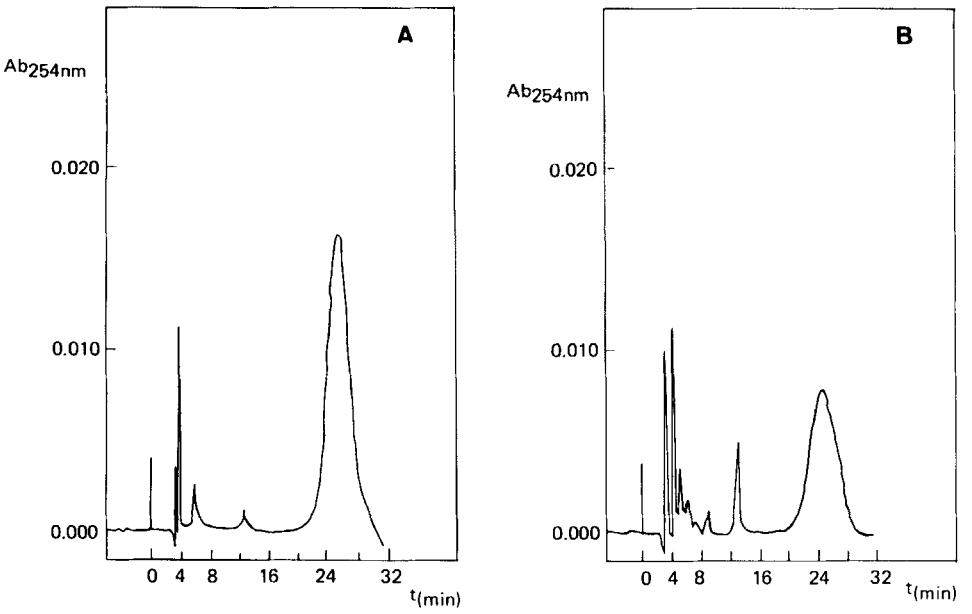

Figure 3.- HPLC elution of acrylyl-CoA, propionyl-CoA and CoASH from reaction mixture to assay the propionyl-CoA dehydrogenase activity.

(A) Chromatogram corresponding to the reaction mixture at time O. (B) Idem at 10 mins. (C) Idem at 30 mins. Chromatographic conditions were similar to those described for Figure 1.

gassed and injected (20 μl). Figs. 3a, 3b and 3c respectively show the chromatograms corresponding to those times.

In these profiles it is remarkable that when the reaction occurs the concentration of propionyl-CoA is reduced, while that of acrylyl-CoA is increased at the same time as free CoASH appears.

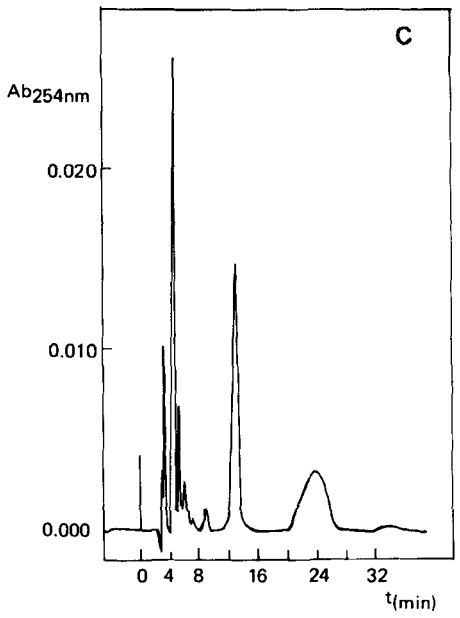

Figure 3C

By comparing the peaks shown in the chromatograms with the standard curves for the quantification of acyls of CoA, it was possible to follow the evolution of corresponding concentrations of different compounds; therefore propionyl-CoA goes from 0.14 mM to 0.082 mM and 0.035 mM; acrylyl-CoA from 0.0 mM to 0.006 mM and 0.016 mM; and finally free CoASH from 0.005 mM to 0.022 mM and 0.6 mM.

These results suggest the presence of propionyl-CoA dehydrogenase activity in cell-free extracts; likewise, the existence of an enzyme with hydrolasic activity that disrupts the acyl-CoA in free CoASH and the corresponding compound is also indicated.

DISCUSION

Propionyl-CoA dehydrogenase activity has been detected for the first time in Salmonella typhimurium cells grown in propionate as a carbon source through HPLC. The existence of that activity has been described in the propionate anabolic pathway of Clostridium propionicum (8, 19) and of Megasphaera elsdenii (20, 21), but no assay to determine the enzyme activity by these workers could be applied to S. typhimurium owing to the presence of a hydrolase enzyme in the bacterial extracts. An acyl-CoA hydrolase with a similar action has been reported in Moraxella lwoffi (8), and in the β-oxidation of fatty acids in animal tissues (22, 23, 24). Presumably, the hydrolase action in S. typhimurium is an artifact created by the extraction procedure resembling the behaviour of different synthetases of activated compounds (e.g. ATP synthetase) that could effect "in vitro" the opposite of the "in vivo" function.

The attempt to separate the dehydrogenase and hydrolase activities by ammonium sulphate fractioning and gel filtration chromatography was unsuccessful. However, using HPLC enabled the evaluation of both activities acrylyl-CoA being determined as the reaction product with the concomitant presence of free CoASH.

It has been reported (25) that CoASH can be separated from acetyl-CoA in a reversed-phase system with ion-pair chromatography, using tetrabutylammonium ion as counter ion, but the system was not evaluated for measurements of CoASH in biological extracts. Ingebretsen and Farstad (26) have described a method to

determine HS-CoA in biological samples and in presence of dephospho-CoA and acetyl-CoA, and Halvorsen and Skrede (27) to determine HS-CoA and its anabolic precursors.

Taking into account these methods we have developed an HPLC method to determine propionyl-CoA dehydrogenase activity, the values obtained being of the same order as those described for Escherichia coli E-26 (5, 6) and Megasphaera elsdenii (21) by radioactivity and thin layer chromatography. Futhermore, the method described in this work is quicker and more accurate than other methods.

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