THE ENZYMATIC PREPARATION OF L(+)-3-HYDROXY(3-^{14}C)BUTYRATE

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SUMMARY

L(+)-3-hydroxy(3-^{14}C)butyrate was prepared by treating the D,L-racemic mixture with the stereospecific D-(-)-3-hydroxybutyrate dehydrogenase enzyme. The unfavorable equilibrium position of the dehydrogenase was overcome by the addition of acetaldehyde and alcohol dehydrogenase. The D-isomer was converted by this treatment to acetoacetate. The unreacted L(+)-3-hydroxy(3-^{14}C)butyrate was then isolated by preparative silica gel chromatography in an overall yield of 65%.

Key words: L(+) and D(-)-3-Hydroxy(3-^{14}C)butyrate

INTRODUCTION

The uptake and metabolism of ketone bodies has been studied with 3-^{14}C-labelled DL-3-hydroxybutyrate, D-3-hydroxybutyrate and acetoacetate. Recently, the L-isomer of 3-hydroxybutyrate has been shown to label the sterol and fatty acid components of brain, spinal cord and skin from 18-day old rats better than the D-isomer[1]. In order to continue our studies of the metabolism of each isomer, an efficient high-yield procedure for the preparation of L(+)-3-hydroxy(3-^{14}C)-butyrate had to be developed.

The preparation of the L(+)-3-hydroxy(3-^{14}C)butyrate from a racemic mixture has been previously described in which the D-isomer was converted to acetoacetate

0362-4803/80/0317-0365$01.00

Received April 17, 1979
Revised May 8, 1979

by the sterospecific D-3-hydroxybutyrate dehydrogenase enzyme.[1] However, this procedure removes only 80-85% of the D-isomer from the racemic mixture and optically pure L(+)-3-hydroxy-(3-^{14}C)butyrate could only be obtained after repeated treatment with the dehydrogenase enzyme followed by thin layer chromatography.

This report details the modification of the above procedure which overcomes the unfavorable equilibrium constant for the conversion of 3-hydroxybutyrate to acetoacetate[2] by the addition of acetaldehyde and alcohol dehydrogenase to the reaction mixture. This coupled enzyme reaction converts over 98% of the D-isomer in the racemic mixture to acetoacetate and enables the L-isomer to be recovered after silica gel thin layer chromatography in an overall yield of 65%. For the reasons mentioned above the following procedure was developed.

EXPERIMENTAL

DL-3-hydroxy(3-^{14}C)butyrate (500 μCi, 19 μCi/μmole, Amersham-Searle) was dissolved in 2.5 ml water and placed in a 10 ml reaction vial. To the reaction vial was added 1.0 ml of 0.25 M Na_2HPO_4/KH_2PO_4 phosphate buffer (pH 7.8), 0.05 ml of alcohol dehydrogenase enzyme solution (EC 1.1.1.1., 233 units/mg protein, 2.0 mg/ml, Sigma Chem. Co., Cat. No. A3263), 0.25 ml of freshly prepared 200 mM acetaldehyde, 0.50 ml of 5 mM disodium nicotinamide adenine dinucleotide (NAD^+) and 0.60 ml of water. The mixture was heated to 37°, and the reaction initiated by the addition of 0.10 ml of 3-hydroxybutyrate dehydrogenase enzyme suspension in 3.2 M $(NH_4)_2SO_4$ (EC 1.1.1.30., 6.2 units/mg protein, 5 mg/ml, Sigma Chem. Co., Cat. No. H6126). After 1 hour, the reaction mixture was acidified by the addition of 0.10 ml of 6 N $HClO_4$ and heated at 60° for 45 min. The acetoacetate initially formed was quantitatively decarboxylated to acetone and CO_2 under these conditions.

After cooling to room temperature, the reaction mixture was neutralized with 0.30 ml of 2 N KOH and the acetaldehyde and radioactive acetone removed by lyophilization overnite in a hood vented to the outside. The L(+)-3-hydroxy(3-^{14}C) butyrate was selectively extracted by first dissolving the freeze-dried residue in 1.0 ml of water, followed by 9.0 ml of absolute ethyl alcohol. The precipitate

was removed by centrifugation at 400 g for 10 min. The precipitate was washed 3 times with 2 ml aliquots of 90% alcohol. The washings were added to the supernatant fraction and the volume reduced to 0.5 ml by lyophilization. Any precipitate formed was removed by centrifugation. The supernatant was then applied to two preparative silica gel thin layer glass plates (20 x 20 cm, 500 μ, Analtech). The plates were developed in ethyl ether/acetic acid/water (70:10:3) and the L(+)-3-hydroxy(3-^{14}C)butyrate band located by scanning the chromatograms with a Geiger counter. The L(+)-3-hydroxy(3-^{14}C)butyrate band (R_f, 0.84) was scraped from the plates and eluted from the silica gel with 80% ethanol. The eluants were combined, the pH adjusted to 7.0, and lyophilized to dryness.

The purity of the L(+)-3-hydroxy(3-^{14}C)butyrate was determined by treating a small portion of the freeze-dried preparation with a freshly prepared D-3-hydroxybutyrate dehydrogenase/alcohol dehydrogenase mixture. After 1 hour, the reaction mixture was chromatographed on analytical silica gel plates (100 μ, Eastman) in ethyl ether, acetic acid/water (70:10:3). Acetoacetate and 3-hydroxybutyrate are separated in this system with Rf's of 0.56 and 0.79 respectively. It was found that the L(+)-isomer was 98% pure and that the overall yield starting with the racemic D,L-mixture was 65%.

In order to establish the limits of the reaction, a series of experiments were conducted in which

(a) The 3-hydroxybutyrate concentration was varied from 2.5 to 30 mM at constant concentrations of NAD^+ (2 mM) and acetaldehyde (32 mM),

(b) The NAD^+ concentration was varied from 0.25 to 10 mM at constant concentrations of 3-hydroxybutyrate (5 mM) and acetaldehyde (32 mM), and

(c) The acetaldehyde concentration was varied from 20 to 350 mM at constant concentrations of 3-hydroxybutyrate (5 mM) and NAD^+ (2 mM).

The remaining reaction conditions were as described in the text. The reactions were stopped at the end of 60 and 120 min. with perchloric acid, and after reneutralization with KOH, an aliquot from each was assayed enzymatically for D-3-hydroxybutyrate [2] that remained in the reaction mixture.

The results of the series of experiments established that acetaldehyde concentrations up to 350 mM did not interfere with the enzymatic conversion of D-3-hydroxybutyrate to acetoacetate. Furthermore, over 95% of the conversion to acetoacetate occurred within 1 hour and the optimum concentration range for NAD^+ was 0.5 to 2 mM and for 3-hydroxybutyrate, less than 5 mM.

REFERENCES

1. Webber R.J. and Edmond J. - J. Biol. Chem. 252: 5222 (1977)
2. Krebs H.A., Mellanby J. and Williamson D.H. - Biochem. J. 82: 96 (1962)