CHROM. 5530

Thin-layer chromatographic separation of ^{14}C-labelled branched-chain α-keto acids and hydroxamic acids derived from the corresponding enzymatically formed acyl-CoA compounds

Conversion of volatile carboxylic acids to the corresponding non-volatile hydroxamic acids is a convenient method for achieving a quantitative yield after chromatographic separation[1]. Furthermore, conversion to the hydroxamic acids is useful for the determination of acyl phosphates, esters and thioesters originating from enzymatic reactions[2], especially acyl-coenzyme A compounds.

In this paper, we describe a method for the measurement of acyl-CoA formed enzymatically from α-ketoisocaproic acid, α-keto-β-methylvaleric acid and α-ketoisovaleric acid by the branched-chain keto-acid oxidase complex[3]. Owing to its very low activity in mammals, this multi-enzyme system can be tested only by using ^{14}C-labelled α-keto acids as substrates[4]. A hereditary enzyme deficiency in this metabolic step in man causes the maple syrup urine disease[5].

Material and methods

^{14}C-Labelled branched-chain α-keto acids were obtained by oxidative deamination of the corresponding L-amino acids (purchased from the Radiochemical Centre, Amersham, Great Britain) by a modification of the method described by MEISTER[6]. Hydroxamic acids were prepared from the respective ethyl esters[7], isovaleric acid ethyl ester, α-methylbutyric acid ethyl ester and isobutyric acid ethyl ester. Ready-made silica gel thin layers (0.25 mm) on plastic foils and other chemicals were obtained from Merck, Darmstadt, G.F.R.

The enzymatic test will be described in detail elsewhere[4]. From the acyl-CoA formed in this way, the hydroxamic acid was made by addition of a neutralized hydroxylamine solution. 10 μl of each incubation assay was applied to the silica gel plate and chromatographed for 90 min in a solvent mixture consisting of chloroform, isopropyl alcohol, and methanol (11:3:1) without prior saturation of the chamber. Radioactivity distribution on the chromatogram was detected with a thin-layer chromatographic scanner II (Berthold, Wildbad, G.F.R.). The individual spots were subsequently cut out of the foil and the radioactivity was determined quantitatively in a liquid scintillation counter (Fa. Packard). Reproducible results were obtained with a modified liquid scintillator solution consisting of toluene and ethanol (75:25).

Results and discussion

The method yields similar results for the determinations of isovaleric hydroxamate (R_F = 0.80), α-methylbutyric hydroxamate (R_F = 0.80) and isobutyric hydroxamate (R_F = 0.77). An excess of unlabelled hydroxamic acid in the sample prior to chromatography was generally required for adequate separation, probably because of the adsorption of small amounts of hydroxamic acid on the silica gel during chromatography. This cause has been demonstrated by adding different concentrations of unlabelled hydroxamic acid to the sample of the same incubation assay (Fig. 1). The samples that contained a low concentration of the hydroxamic acid showed large tailing and incomplete separation of the hydroxamic acid and α-keto acid. Following

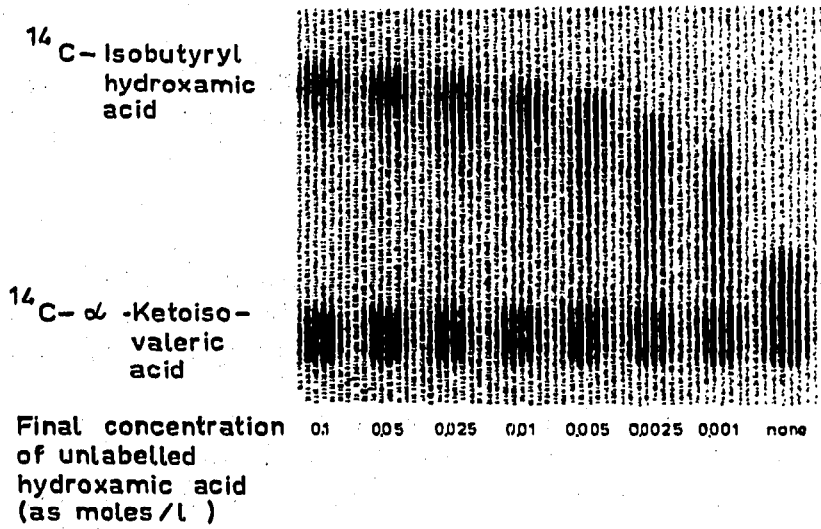

Fig. 1. Distribution of radioactivity on the chromatogram (thin-layer chromatographicscanner II, Labor. Prof. Berthold, Wildbad, G.F.R.). Influence of decreasing amounts of unlabelled isobutyrylhydroxamic acid added prior to chromatography on the separation of ^{14}C-hydroxamic acid and ^{14}C-α-ketoisovaleric acid.

addition of about a thousand-fold excess of unlabelled hydroxamic acid (final concentration about 0.1 *M*), the labelled spot appeared distinct and well separated from a spot, containing both α-keto acid and hydroxyiminocarboxylic acid, which is derived from the α-keto acid by treatment with hydroxylamine. It should be mentioned that detection of the excess of hydroxamic acid is possible using a spray of 5% solution of $FeCl_3$ in 0.1 *N* HCl. This is an additional advantage which makes radioactive monitoring unnecessary.

Institute of Human Genetics,
University of Hamburg,
Hamburg (G.F.R.)

H. W. RÜDIGER
M. NIKISCH
H. W. GOEDDE

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Received June 14th, 1971