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Separation of (3-^{14}C) 3-Hydroxy-3-methylglutaric acid from (3-^{14}C) 3-Hydroxy-3-methylglutaryl CoA using Sephadex G-15 column chromatography.[1]

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RINGKASAN

Satu teknik baru menggunakan kromatografi turus Sephadex G-15 untuk mengasingkan asid (3-^{14}C) 3-Hidroksi-3-metilglutarik daripada (3-^{14}C) 3-Hidroksi-3-metilglutaril KoA telah dimajukan. Teknik ini adalah lebih mudah dan cepat, dan amatlah berguna di dalam pengajian dimana (3-^{14}C) 3-Hidroksi-3-metilglutaril KoA diperlukan tanpa cemaran oleh asid (3-^{14}C)3-Hidroksi-3-metilglutarik.

SUMMARY

A new method has been developed for the separation of (3-^{14}C) 3-Hydroxy-3-methylglutaric acid from a (3-^{14}C) 3-Hydroxy-3-methylglutaryl CoA preparation using Sephadex G-15 column chromatography. This method is simple, rapid and is useful in studies where the preparation of (3-^{14}C) 3-Hydroxy-3-methylglutaryl CoA is required to be free from (3-^{14}C) 3-Hydroxy-3-methylglutaric acid contamination.

INTRODUCTION

HMG CoA reductase (EC. 1.1.1.34) is the key enzyme in the regulation of cholesterol biosynthesis (Rodwell *et al.*, 1976). The enzyme is commonly assayed by measuring the ^{14}C-mevalonic acid produced when ^{14}C-HMG CoA is used as the substrate in the enzyme incubation mixture. ^{14}C-HMG CoA can be prepared by reacting ^{14}C-HMG anhydride with CoA according to the method of Goldfarb and Pitot (1971). Such a preparation consists of unreacted CoA and ^{14}C-HMG acid in addition to ^{14}C-HMG CoA. It can be used directly in the radioassay for the reductase or else purified to remove ^{14}C-HMG acid either by paper chromatography (Fogelman *et al.*, 1975), or by extraction with ether (Baqir and Booth, 1977).

As an addition to these methods, we have now developed a much simpler and rapid technique for the removal of ^{14}C-HMG acid from a ^{14}C-HMG CoA preparation by column chromatography on Sephadex G-15.

MATERIALS AND METHODS

Chemicals: Coenzyme A (the Li salt), HMG acid and N,N-dicyclohexyl-carbodiimide were purchased from Sigma, Chemical Co., St. Louis, Missouri, and (3-^{14}C)-HMG acid was from New England Nuclear, Boston, Mass., U.S.A. Sephadex G-15 was obtained from Pharmacia, Uppsala, Sweden.

Preparation of ^{14}C-HMG anhydride: ^{14}C-HMG anhydride was prepared according to the method of Goldfarb and Pitot (1971), and the specific activity of all preparations was 200 μCi/mmole, with a yield of 60%.

Preparation of ^{14}C-HMG CoA: Coenzyme A (31 mg) and ^{14}C-HMG anhydride (5 mg) were reacted under the conditions described by Louw *et al.* (1969) except that the addition of saturated $KHCO_3$ solution was omitted, since this normally resulted in a pH of above 8.0. Instead, the pH of the reaction was continuously monitored with a pH meter, and kept to a value of 7.6 by careful addition of small amounts of 5N KOH solution as needed. The anhydride was added in several small additions, adjusting the pH as necessary following each addition. The reaction was completed within a few minutes, after which the pH

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Abbreviations used: HMG CoA = 3-hydroxy-3-methylglutary coenzyme A; HMG acid = 3-hydroxy-3-methylglutaric acid; CoA = reduced coenzyme A.

of the solution remained at 7.6 and the nitroprusside test (Louw *et al.*, 1969) for free-SH groups was negative. The pH of the solution, containing both ^{14}C-HMG CoA and ^{14}C-HMG acid in a final volume of about 3.0 ml, was adjusted to 5.5 with 5 N HCl. This solution usually contained 75% ^{14}C-HMG CoA, as judged by paper chromatography of an aliquot, prepared as described for Fig. 2.

Purification of ^{14}C-HMG CoA on a Sephadex G-15 column

The Sephadex G-15 column (2 × 32 cm) was prepared according to the manufacturer's specifications and was equilibrated with 10 mM potassium phosphate buffer (pH 6.5). In order to check that the column bed was homogenous, a mixture (1.0 ml) of Blue Dextran (2mg/ml) and potassium ferricyanide (2mg/ml) were loaded and eluted with the above buffer. Both these compounds separated with well-defined boundaries.

A 1.5 ml aliquot of the crude ^{14}C-HMG CoA preparation was loaded on to the column and eluted with the same buffer. After collecting buffer equalling the void volume of the column (35 ml), fractions of 1.5 ml each were collected. The flow rate was 140 ml/hr at room temperature. A 10 μl aliquot of all the fractions was assayed for radioactivity in 5 ml scintillation fluor (μ5g) PPO, 0.1 g POPOP/1 dioxan) using a Nuclear Chicago Isocap/300 counter. From examination of the pattern of elution of radioactivity, the fractions containing ^{14}C-HMG CoA (A in Fig. 1) were pooled and freeze dried by vacuum sublimation using the apparatus shown in Plate 1. The residue obtained was redissolved in distilled water (pH 5.5) to give, for our purposes, a final concentration of 5 mM of ^{14}C-HMG CoA and then stored frozen. When analysed by paper

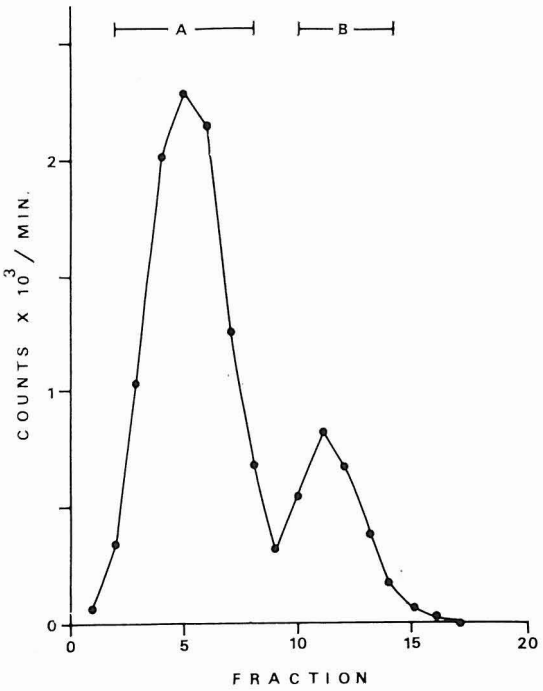

Fig. 1. The elution profile of a mixture of ^{14}C-HMG CoA and ^{14}C-HMG acid from a Sephadex G-15 column (bed dimensions, 2 × 32 cm), using 10 mM potassium phosphate buffer (pH 6.5). The void volume was 35 ml and the flow rate 75 ml/hr at room temperature.

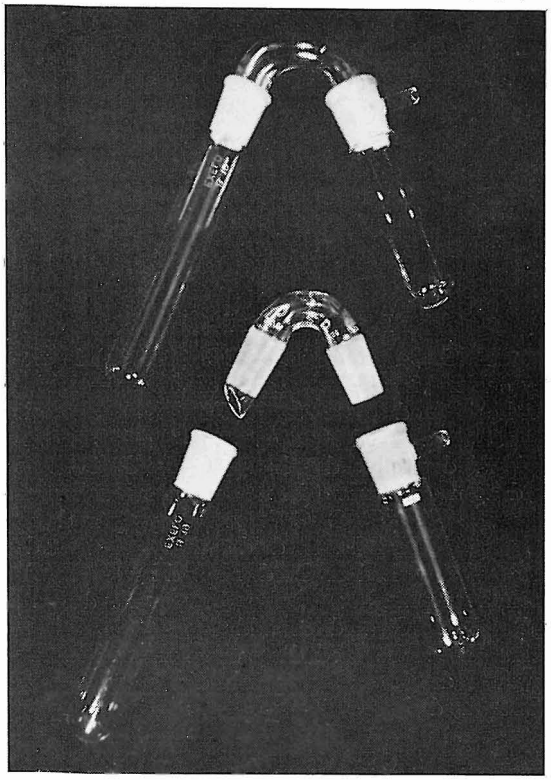

Plate 1. Apparatus for Freeze-drying Fraction "A"

The Fraction "A" was placed in the Rittenberg tube with the side arm and the apparatus assembled as shown. After snap freezing the aqueous content in liquid nitrogen, the whole apparatus was evacuated with a mechanical pump and the vacuum sealed. The empty limb of the apparatus was then immersed in the liquid nitrogen. The transfer of pure water from the tube at room temperature to the tube immersed in the liquid nitrogen occurs by the process of sublimation and refreezing, thus leaving behind the residue of ^{14}C-HMG CoA.

chromatography, as described for Fig. 2, no deterioration of this preparation was observed even after six months storage.

The purification step took about $2\frac{1}{2}$ hr from the time the sample of ^{14}C-HMG CoA preparation was loaded on to the column. After use the column was stored in the above buffer containing sodium azide (0.02%, w/v) as an antimicrobial agent.

RESULTS

The elution profile from the Sephadex G-15 column of the mixture of ^{14}C-HMG CoA and ^{14}C-HMG acid is shown in Fig. 1, using a flow rate of 75 ml/hr at room temperature. Increasing the flow to 140 ml/hr did not alter the elution pattern, so this higher flow was routinely adopted to save time. The radiochemical nature of the fractions A and B (from Fig. 1) was shown to be

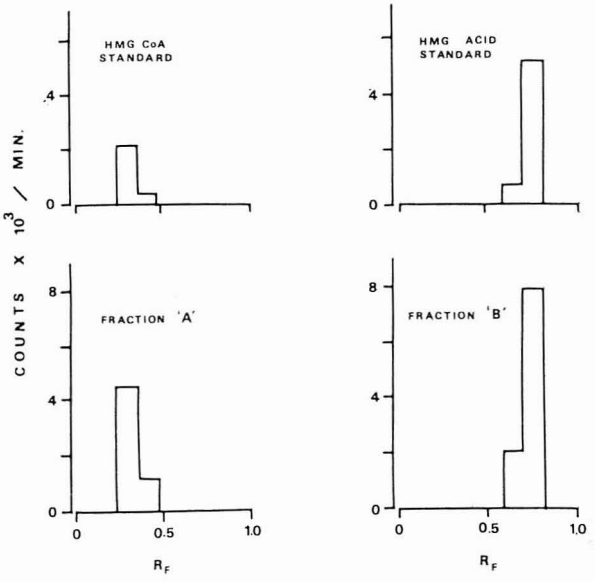

Fig. 2. Paper chromatography of an aliquot of the pooled fractions A and B respectively, using Whatman 3 MM paper and butanol : acetic acid : water (5 :2 :3) solvent. The chromatography was carried out in an ascending direction at room temperature. After drying, segments of 1.5 cm each were cut along the chromatogram strip and assayed for radioactivity in 5.0 ml dioxan scintillation fluor (5 g PPO, 0.1 g POPOP/ 1). The standard ^{14}C-HMG CoA was prepared and purified as described by Fogelmann et al. *(1975).*

^{14}C-HMG CoA and ^{14}C-HMG acid respectively (Fig. 2).

As can be seen from Fig. 2, the purified ^{14}C-HMG CoA was free from ^{14}C-HMG acid. The recovery of ^{14}C-HMG CoA was typically greater than 95%. A small loss occurred when the fractions near the leading edge of the ^{14}C-HMG acid peak (see Fig. 1) were discarded to avoid contamination. The ^{14}C-HMG acid was recovered after the fractions containing it were pooled, concentrated by freeze drying and finally extracted with ether (Baqir and Booth, 1977).

DISCUSSION

The purification method described herein is based on the large difference in the molecular weights of HMG CoA (MW 912) and HMG (MW 162). In the case of CoA (MW 768) however, the difference is relatively much less and, therefore, it is likely to be present in the purified ^{14}C-HMG CoA preparation. The exact amount was not determined but one can assumed that this would be less than 25% of the original amount used since the yield of ^{14}C-HMG CoA was generally 75%. The unreacted CoA cannot be separated from HMG CoA by paper chromatography (Suzuki *et al.*, 1975) or ether extraction (Baqir and Booth, 1977).

Free CoA is known to inhibit the activity of the solubilized and purified HMG CoA reductase from rat liver (Kawachi and Rudney, 1970) and yeast (Kirtley and Rudney, 1967). The amount of free CoA used by these workers, however, was 5 to 30 fold greater than that expected to be present in an equivalent concentration of ^{14}C-HMG CoA purified as described in this work. They also reported that the extent of inhibition by free CoA was dependent on whether pre-incubation of the reaction mixture was carried out or otherwise.

There has so far been no report on the inhibitory effect of free CoA on microsomal HMG CoA reductase activity. In fact Hulcher and Oleson (1973) were able to use the formation of free CoA during the HMG CoA reductase reaction as the basis for measuring enzyme activity in pigeon liver microsomes. Their data do not show any evidence of end product inhibition by free CoA formed during the reaction.

Nevertheless, when it is essential that free CoA is absent in the ^{14}C-HMG CoA preparation, the addition of excess HMG anhydride would ensure its complete conversion to HMG CoA in the reaction mixture (Baqir and Booth, 1977; Suzuki *et al.*, 1975). The unreacted HMG acid can then be removed from HMG CoA by the

technique described herein. This method is simple and rapid and is useful for preparing ^{14}C-HMG CoA free from HMG acid for use in studies of enzymes which metabolise HMG CoA, in particular the reductase (EC 1.1.1.34), the deacylase (EC 3.1.2.5) and the lyase (EC 4.1.3.4).

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