Isolation and purification of 3-hydroxy-3-methylglutaryl-coenzyme A by ion-exchange chromatography

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Summary For precise determination of the catalytic activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), the HMG-CoA employed as substrate must be free of HMG, CoA, and other inhibitors of HMG-CoA reductase activity. The standard purification of HMG-CoA by paper chromatography gives poor resolution of HMG-CoA from CoA and may be accompanied by some decomposition of HMG-CoA. We describe a simplified procedure for synthesis and for isolation from the reaction mixture of homogeneous, high specific activity [3-^{14}C]HMG-CoA free of HMG, CoA, or nonpolar contaminants. Isolation of HMG-CoA utilizes ion-exchange chromatography in a gradient of ammonium formate, which is subsequently removed by lyophilization. The methods are proposed for use in the preparation or isolation of HMG-CoA. —**Williamson, I. P., and V. W. Rodwell.** Isolation and purification of 3-hydroxy-3-methylglutaryl-coenzyme A by ion-exchange chromatography. *J. Lipid Res.* 1981. **22:** 184–187.

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While the activity of solubilized, partially purified HMG-CoA reductase (EC 1.1.1.34) may be assayed spectrophotometrically (1), the assays of choice for

Abbreviations: HMG, 3-hydroxy-3-methylglutaric acid; TLC, thin-layer chromatography; DEAE, diethylaminoethyl; DCC, dicyclohexylcarbodiimide.

microsomal preparations are, with few exceptions, variations of the thin-layer radio-chromatographic technique of Shapiro et al. (2, 3). Direct estimation of product mevalonolactone without prior TLC has been reported (4, 5), but has yet to find wide acceptance. For all radioisotopic assays that measure the product (mevalonate), the nonpolarity of mevalonolactone relative to HMG or HMG-CoA is exploited to effect resolution by TLC (2, 3) or to partition mevalonolactone into a toluene-based scintillation fluor (4, 5). Sensitivity in either assay depends both on the specific radioactivity of the HMG-CoA used as substrate and on its purity (5). The presence in HMG-CoA of nonpolar contaminants of chromatographic/partition behavior similar to mevalonolactone will greatly increase zero time blanks, thus reducing sensitivity. Such impurities may form in HMG-CoA solutions which are repeatedly frozen and thawed (6).

HMG-CoA generally is purified by paper chromatography in *n*-butanol–acetic acid–water (5, 7). While this removes nonpolar contaminants, resolution of HMG-CoA from CoA is poor, and application of sample to the paper may be accompanied by some decomposition of HMG-CoA.[1] Ion-exchange chromatography on amininated supports (DEAE-cellulose or DEAE-Sephadex) has been employed to purify acetyl-CoA and malonyl-CoA (8). A disadvantage of the LiCl elution gradient most commonly employed is that subsequent removal of LiCl by solvent extraction or gel filtration is tedious and reduces yields. Using DEAE-cellulose eluted with ammonium formate, Smith (9) resolved methylmalonyl-CoA from CoA and other compounds. Subsequent lyophilization yielded salt-free methylmalonyl-CoA. We have applied this approach to chromatography of HMG-CoA.

EXPERIMENTAL

Chemicals

[3-^{14}C]HMG, *R,S*-[3-^{14}C]HMG-CoA, and D,L-[2-^{3}H]mevalonic acid lactone were from Amersham Corp., Arlington Heights, IL. Coenzyme A (grade III-L, lithium salt), 5,5′-dithiobis(2-nitrobenzoic acid), formic acid, ammonium formate, and N,N′-dicyclohexylcarbodiimide were from Sigma Chemical Co., St. Louis, MO. Ready-Solv scintillation fluor was from Beckman Instruments, Inc., Palo Alto, CA. DEAE-cellulose (Cellex-D, capacity 0.67 meq/g) was from Biorad Labs, Richmond, CA. Prior to use, the Cellex-D was treated to remove fines, precycled as recommended by the manufacturers, and equilibrated with 0.1 M ammonium formate, pH 4.4. Ammonium formate buffers were prepared by dissolving appropriate quantities of ammonium formate in 90% of the desired final volume of water, adjusting to pH 4.4 with formic acid, and diluting to the desired final volume. The 5- and 10-ml reaction vessels used in the synthesis of HMG-CoA were standard-taper ®Bantamware (Kontes, Vineland, NJ).

Determination of radioactivity

Samples, 5–20 μl, from column eluates were counted in 4.0 ml of Ready-Solv scintillation fluor in a Beckman Model LS 100 scintillation spectrometer. Efficiency of ^{14}C counting was 60%.

Assay of HMG-CoA

HMG-CoA was assayed by absorption at 260 nm, by determination of alkali-releasable thiol (10–12), and by conversion to mevalonolactone by rat liver HMG-CoA reductase (7).

RESULTS AND DISCUSSION

Ion-exchange chromatography of HMG-CoA, HMG, CoA, and mevalonate

HMG-CoA is readily resolved from HMG, CoA, and mevalonic acid lactone by chromatography on DEAE-cellulose eluted with a gradient of ammonium formate. (**Fig. 1**).

Synthesis of HMG-anhydride and of HMG-CoA

The method employed closely parallels that of Goldfarb and Pitot (13) but omits recrystallization of HMG-anhydride, adds reactants in the reverse order for conversion of HMG-anhydride to HMG-CoA, and is carried out by techniques that minimize transfer of materials. The procedure given is for synthesis of [3-^{14}C]HMG-CoA of specific activity 14.3 dpm/pmol suitable for assay of HMG-CoA reductase activity.

Prepare, just prior to use, 3–4 ml of a solution of *N,N′*-dicyclohexylcarbodiimide (DCC) in distilled acetone, 3.2 mg per ml. Transfer the [3-^{14}C]HMG (250 μCi, 4.8 μmol, sp act 52.1 μCi/μmol) to a 5-ml round-bottom flask (ground glass neck) with successive portions of about 1.5 ml of redistilled acetone.[2]

[1] Amersham Corp., in describing the paper chromatographic purification of HMG-CoA, performs chromatography without prior drying of the applied HMG-CoA solution.

[2] If the radioisotope was initially dry, proceed. If the radioisotope was dissolved in a hydroxylic solvent, this must be removed by flash evaporation prior to adding DCC. The dry [3-^{14}C]HMG is then redissolved in 1.5 ml of acetone.

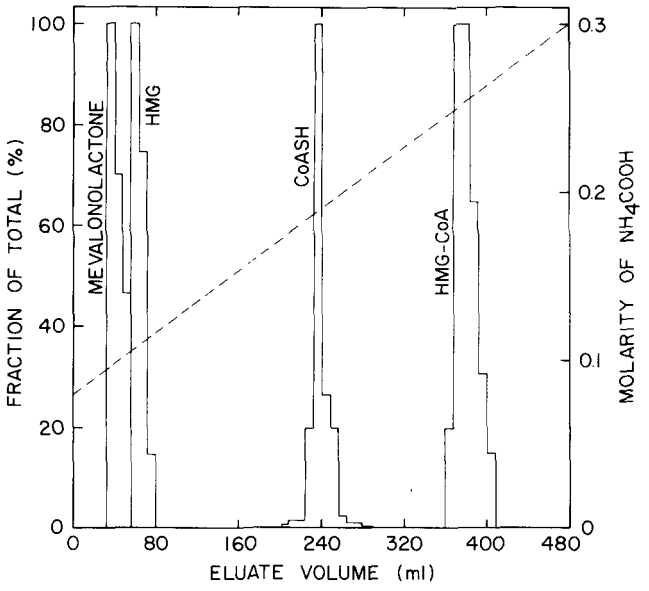

Fig. 1. Chromatography of standards of HMG-CoA, HMG, CoA, and mevalonic acid lactone. The column measured 1.5 × 26 cm (46 ml bed volume). A mixture of [5-^{3}H]mevalonolactone, [3-^{14}C]HMG-CoA, [^{3}^{14}C]HMG and CoASH were applied in 2.5 ml of water to a column previously equilibrated with 0.1 M NH_4COOH, pH 4.4. Gradient elution was begun immediately after addition of the sample. The mixing chamber and reservoir contained 500 ml each of 0.1 M and of 0.3 M NH_4COOH, pH 4.4, respectively. CoA was measured at 260 nm. Other compounds were detected by scintillation counting. Data for ^{3}H (mevalonolactone) are corrected for spill of ^{14}C from HMG into the ^{3}H channel. Data for each compound are expressed relative to the fraction present in the peak tube for each component (100%).

Add 3.8 mg (23.2 μmol) of [^{12}C]HMG.[3] Add 2.0 ml (6.4 mg, 31 μmol) of DCC solution, stopper the flask, and incubate the contents, with gentle shaking, at 37°C for 2 hr. Place the flask on ice until its contents reach 0°C (20 min) to precipitate dicyclohexylurea. Using vacuum filtration and a small (1.5-cm diameter) Buchner funnel, filter the flask contents into a 10-ml round-bottom flask and wash the precipitated dicyclohexylurea with small portions of ice-cold acetone. Transfer the filtrate and washings to a rotary flash evaporator and evaporate to dryness (warm water bath). Add 44 mg (54 μmol) of lithium CoA dissolved in 3.0 ml of ice-cold water that contains 5–6 drops of saturated K_2CO_3 solution (a volatile buffer) and sufficient 1 N KOH (a few μl) to achieve pH 8.[3] The pH may be monitored using thin (0.5 mm) slivers of paper. While the HMG-anhydride is freely water-soluble, dicyclohexylurea is not. Undissolved dicyclohexylurea in the reaction flask can be ignored. Place the flask on ice, monitoring the pH occasionally, and adding a few μl of 1 N KOH as needed to maintain the pH at 8. After 5 min, add sufficient 1 N HCl to lower the pH to 4–5. HMG-CoA is stable at this pH. Further workup may be postponed, and the crude HMG-CoA solution may be stored at −20°C. Alternatively, the entire reaction mixture may be applied directly to a DEAE-cellulose column for purification.

[3] More or less carrier HMG may be added to produce [3-^{14}C]-HMG-CoA of a desired final specific activity. This will require proportional changes in the quantities of DCC and CoA used. We employ a twofold excess of CoA over HMG-anhydride. Yields are significantly lower if a 1 to 1.5-fold molar excess is used. If a four-fold excess of CoA is used, yields should approach 100%.

Ion-exchange chromatography

Apply the solution of crude [3-^{14}C]HMG-CoA to a 1.5 × 23 cm column of DEAE-cellulose equilibrated with 0.1 M ammonium formate, pH 4.4, wash it in with this buffer, and elute with an 0.1–0.3 M gradient of ammonium formate, pH 4.4. The mixing chamber and reservoir contain 250 ml each of 0.1 and 0.3 M ammonium formate, respectively. Flow rate 1.1 ml/min; 4.5 ml fractions. HMG-CoA is obtained free of HMG or CoA (**Fig. 2**). Combine all fractions (#70–90) that contain HMG-CoA and remove the water and ammonium formate by lyophilization. Depending on the efficiency of the lyophilizer used, it may be necessary to dissolve the lyophilized material in 25–30 ml of water and repeat the lyophilization one or more times to remove all trace of ammonium formate. Ammonium formate may be detected using Nessler's reagent. Dissolve the salt-free [3-^{14}C]HMG-CoA in 4–5 ml of water, adjust the pH to 6, and store at

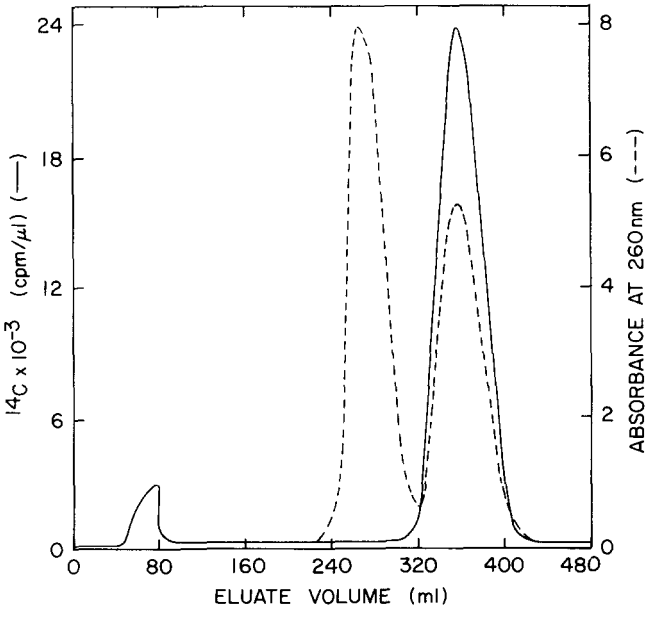

Fig. 2. Resolution of HMG-CoA from a synthesis reaction mixture. Conditions are given in the accompanying text. Symbols are: (– – –), absorbance at 260 nm, (——) ^{14}C in 5-μl portions of each fraction.

−20°C. Yield 70% (based on HMG), specific activity 14.3 dpm/pmol.

For laboratories that perform only the occasional assay of HMG-CoA reductase activity, purchase of commercial [3-^{14}C]HMG-CoA is indicated. However, the 1980 price of [3-^{14}C]HMG-CoA ($10.40/$\mu$Ci) is an order of magnitude above that for [3-^{14}C]HMG ($1.06/$\mu$Ci) (14). For laboratories that assay reductase on a routine basis, preparation of pure [3-^{14}C]HMG-CoA from [3-^{14}C]HMG thus presents economic advantages. Obstacles to this approach, which include the manipulation and crystallization of small quantities of radioactive materials and the drawbacks of paper chromatography, are obviated by the above procedures. In addition, the chromatographic method described should be applicable to the quantitative analysis of the concentration of HMG-CoA in biological fluids.

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