Improved assay of 3-hydroxy-3-methylglutaryl coenzyme A reductase

STANLEY GOLDFARB and HENRY C. PITOT

McArdle Laboratory for Cancer Research, University of Wisconsin Medical Center, Madison, Wisconsin 53706

SUMMARY Two improvements are described for the assay of HMG CoA reductase. These are a simple synthesis of the substrate precursor HMG-3-^{14}C anhydride and a double-label (^{14}C and ^{3}H) method for determining the amount of mevalonate-3-^{14}C that is formed from the substrate.

SUPPLEMENTARY KEY WORDS 3-hydroxy-3-methylglutaric anhydride · double-label assay · thin-layer chromatography · cholesterol biosynthesis

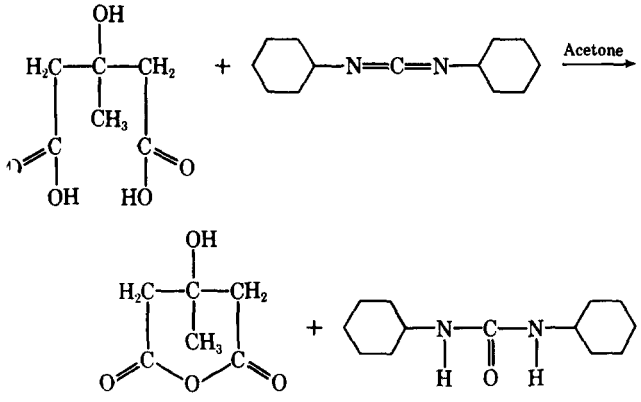
HMG CoA reductase (mevalonate:NADP oxidoreductase [acylating CoA], EC 1.1.1.34) is believed to be an important rate-controlling enzyme for hepatic cholesterol biosynthesis in animals (1, 2). The assay of the enzyme in tissues is complex, requiring the incubation of washed microsomes with the substrate, HMG-3-^{14}C CoA in the presence of NADPH or an NADPH generating system, and the subsequent extraction, chromatographic separation, and quantitation of the product, mevalonate-3-^{14}C. The recent development of a reliable thin-layer chromatographic method for the isolation of mevalonate (3) has considerably simplified the procedure, which formerly required column (4) or gas–liquid (5) chromatographic separations. However, difficulty in synthesizing HMG-3-^{14}C anhydride, which is used for the preparation of HMG-3-^{14}C CoA, and the tedious repeated extraction of mevalonate with ethyl ether prior to thin-layer chromatography remained obstacles to the routine performance of large numbers of these tests. We have resolved both of these problems by developing a simple synthesis for HMG-3-^{14}C anhydride and by devising a double isotopic label method (^{3}H and ^{14}C) that obviates the need for quantitative extraction of mevalonate.

Materials. HMG-3-^{14}C acid and DL-mevalonic-5-^{3}H acid (dibenzylethylenediamine salt) were obtained from

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; G-6-P, glucose-6-phosphate; EDTA, ethylenediaminetetraacetate.

New England Nuclear Corp., Boston, Mass. HMG acid (mp 110°C) was purchased from K & K Laboratories, Inc., Plainview, N.Y., and was dried over BaO at 1–2 mm of Hg for 24 hr prior to use. *N,N'*-Dicyclohexylcarbodiimide was from Schwarz BioResearch, Inc., Orangeburg, N.Y. Coenzyme A (free acid, chromatopure) was from P-L Biochemicals Inc., Milwaukee, Wis. NADP, DL-mevalonic acid lactone, G-6-P disodium salt, and G-6-P dehydrogenase type XI were from Sigma Chemical Co., St. Louis, Mo. Triethanolamine HCl, A grade, was from Calbiochem, Los Angeles, Calif. 2-Mercaptoethanol and deoxycholic acid, sodium salt, were from Matheson, Coleman and Bell, Cincinnati, Ohio. Benzene and acetone were redistilled before use and the benzene was stored over CaH_2 to ensure its dryness. Chromogram silica gel sheets (without fluorescent indicator) were from Eastman Kodak Co., Rochester, N.Y. Scintisol Complete was from Isolab, Inc., Elkhart, Ind. The infrared absorption spectra were determined on a Beckman IR-10 infrared spectrophotometer.

Synthesis of HMG-3-^{14}C Anhydride. The synthesis of HMG-3-^{14}C anhydride used in this laboratory is based on the classical nucleophilic attack on a carbodiimide by an anionic carboxyl group of a dicarboxylic acid, with subsequent ring anhydride formation (6, 7). When dissolved in acetone, HMG acid reacts with *N,N'*-dicyclohexylcarbodiimide within several minutes to form the soluble HMG anhydride and insoluble dicyclohexylurea as indicated in the following equation:



Method. 1 mmole (162 mg) of HMG-3-^{14}C acid (SA 2.02 × 10^5 dpm/mole) was dissolved in 2 cc of acetone in a glass-stoppered test tube. 1 mmole (206 mg) of *N,N'*-dicyclohexylcarbodiimide dissolved in 2 cc of acetone was then added, and the mixture was incubated with mechanical agitation at 37°C for 2 hr. It was then cooled on ice, and the precipitated dicyclohexylurea (90% of expected yield) was filtered off. After evaporating the acetone from the supernatant solution, the residue was redissolved in hot benzene and crystallization was allowed to proceed slowly. The needlelike crystals of HMG-3-^{14}C anhydride that formed (70% of expected yield) had a mp of 102–102.5°C (lit. 102–103°C) and showed the expected infrared absorption spectrum (1.0 mg/150 mg KBr) of this compound, with a hydroxyl band at 3500 cm^{-1} and two anhydride bands, one at 1805 cm^{-1} and one at 1750 cm^{-1}.

Double-label Assay of HMG CoA Reductase. HMG-3-^{14}C was synthesized from 35 μmoles of HMG-3-^{14}C anhydride and 33 μmoles of coenzyme A by adding the reagents to ice-cold water at pH 7.5 and subsequently adjusting the pH to 5.5 (8). The HMG-3-^{14}C CoA was then assayed by paper chromatography using Whatman filter paper No. 3 MM and the solvent system *n*-butanol–acetic acid–water 5:2:3 (9). Two radioactive peaks were identified. The substance in the larger peak, containing 80% of the total eluted radioactivity, had an R_F of 0.35, fluoresced under ultraviolet light, and was presumed to be HMG-3-^{14}C CoA (9). A second, nonfluorescent, radioactive peak with an R_F of 0.75 was thought to be HMG-3-^{14}C acid, and a third, very faintly fluorescent, nonradioactive spot with an R_F of 0.17 was considered to be unreacted coenzyme A (9). Over 95% of the total radioactivity could be accounted for after elution of the radioactive spots, and it was calculated that 85% of the original coenzyme A was converted to HMG-3-^{14}C CoA. The substrate, without purification, was added to an incubation mixture that included an NADPH generating system, Na_2EDTA, and 2-mercaptoethanol (10) at pH 7.3 (11). Protein determinations were performed on the washed microsomes at two protein levels to ensure that the enzymic activity was linear with microsomal concentration (12). The mevalonate-3-^{14}C that formed during incubation was then determined by the double-label method described below.

Method. Washed microsomes were prepared by homogenizing liver with a loose-fitting Potter-Elvehjem homogenizer in 4 vol of pH 7.3 buffer containing 0.05 M triethanolamine HCl, 0.25 M sucrose, 0.01 M Na_2EDTA, and 0.01 M 2-mercaptoethanol, centrifuging for 15 min at 12,000 *g*, carefully decanting the upper one-half of the supernatant solution to avoid contamination with mitochondria, and finally centrifuging. This postmitochondrial fraction was centrifuged at 80,000 *g* for 1 hr in a Spinco 30 or 30.2 rotor. The supernatant liquid was discarded and the microsomes were resuspended in the same buffer and centrifuged for another hour at 80,000 *g*. The microsomal pellets were resuspended in a solution containing 0.1 M triethanolamine HCl, 0.02 M Na_2EDTA, and 0.01 M 2-mercaptoethanol at pH 7.3 and assayed for protein by a modification of the biuret reaction (13) employing 0.5% sodium deoxycholate to reduce turbidity due to lipid as well as a turbidity blank to correct for turbidity due to glycogen.

Incubations were carried out at microsomal levels of 0.25–1.0 mg of protein in an incubation mixture containing 0.06 μmole of DL-HMG-3-^{14}C CoA (SA 202,000 dpm/μmole), 1 μmole of NADP, 3 μmoles of Na_2G-6-P, and 1 IU of G-6-P dehydrogenase and made up to 1 cc volume with 0.1 M triethanolamine HCl, 0.02 M Na_2-EDTA, and 0.01 M 2-mercaptoethanol, pH 7.3. After 0.5 hr incubation at 37°C the reaction was stopped by the addition of 0.1 cc of 10 N NaOH.

Exactly equal amounts of DL-mevalonic-5-^{3}H acid (usually 50,000 dpm) were next added to each alkalinized incubation mixture. Carrier DL-mevalonic acid lactone, 20 μmoles, was added and the mixture was acidified with 0.2 cc of concentrated HCl in order to lactonize the mevalonate; the mixture was then left overnight at room temperature after the tubes were stoppered. Excess Na_2SO_4 was added and two extractions with 20 vol of ethyl ether were carried out. The ether was then evaporated under nitrogen and the residue was taken up in about 200 μl of acetone, spotted on chromogram silica gel sheets, and developed with acetone–benzene 1:1 (3). The product of the reaction was identified as mevalonate-3-^{14}C by its cochromatography with synthetic mevalonate-5-^{3}H on thin-layer plates. The silica gel containing the mevalonolactone, in the R_F region between 0.6 and 1.0, was scraped directly into scintillation vials containing 10 cc of Scintisol Complete and ^{3}H and ^{14}C were determined simultaneously in Packard Tri-Carb spectrometers, models 3375 or 3380. Quenching was corrected for by the external standard method. The amount of mevalonate-3-^{14}C formed was determined by the equation:

$$\mu\text{moles of mevalonate-3-}^{14}\text{C formed} = \frac{\text{mevalonate-5-}^{3}\text{H added (dpm)}}{\text{mevalonate-5-}^{3}\text{H recovered (dpm)}} \times \frac{\text{mevalonate-3-}^{14}\text{C recovered (dpm)}}{\text{sp. act. HMG-3-}^{14}\text{C CoA (dpm/}\mu\text{mole)}}$$

With two extractions of 20 vol of ethyl ether, recovery of mevalonate was regularly between 50 and 70%, a level quite adequate for the determination of mevalonate-3-^{14}C. Care was taken that the dpm of mevalonate-5-^{3}H added was at least 15 times the dpm of mevalonate-3-^{14}C formed to ensure statistical validity of the double-label method. In addition, the amount of radioactive impurity in the mevalonate-5-^{3}H was chromatographically determined (usually 3–5%), since this did not migrate with the mevalonolactone.

Discussion. In the original description of the synthesis of HMG CoA (8), HMG anhydride was first formed by refluxing HMG acid and acetic anhydride with boiling benzene for 1 hr. After evaporating the mixture to a residue, the anhydride was recrystallized from hot benzene. In agreement with Louw, Bekersky, and Mosbach (9), we have found that this anhydride synthesis is unreliable. It was of interest to learn from their report that acetic acid was needed in the reflux mixture in order for the HMG anhydride synthesis to proceed. Based on this observation these workers devised a modification of the original synthesis which incorporated acetic acid in the reaction mixture. However, in our hands this modification has also proved unreliable, since we were not able to obtain needlelike crystals, but only slowly forming prismatic crystals. These probably represented 3-acetoxy-3-methylglutaric anhydride, although they were not further identified. In addition to being an entirely dependable method, the synthesis using the carbodiimide is much faster and simpler than the original acetic anhydride method or its modification.

The use of a double-label assay of mevalonate-^{14}C circumvents the need for quantitative isolation of mevalonate, which requires 10 extractions with ethyl ether (14). A previously described alternative to the complete isolation of mevalonate-^{14}C (10) required the addition of a known amount of mevalonate, two extractions with ether, separation by thin-layer chromatography, resuspension of adsorbed mevalonate in acetone, and the taking of one aliquot for scintillation counting and another aliquot for colorimetric determination of mevalonate (15). From these data the extraction efficiency of mevalonate and total formed mevalonate-^{14}C could be determined. We have found the double-label method to be a much easier and faster way to determine extraction efficiency. It may be used in practically all laboratories equipped for standard scintillation counting.

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