# Improved methods for the study of hepatic HMG CoA reductase: one-step isolation of mevalonolactone and rapid preparation of endoplasmic reticulum

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**Summary** Two new methods are described for the study of hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase. (*1*) Endoplasmic reticulum was rapidly prepared by diluting a 10,000 *g* supernatant with buffer containing 8 mM calcium chloride. The yield of protein and the specific activity of HMG CoA reductase in the pellet subsequently obtained by low speed centrifugation were nearly identical to those in the microsomal pellet prepared by ultracentrifugation. This technique may be particularly useful in studies of the rapid, in vitro modulation of the enzyme. (*2*) Mevalonolactone was extracted into benzene from the HMG CoA reductase assay mixture with an efficiency of 58%. There was less than 1% extraction of HMG CoA, acetoacetate, or β-hydroxybutyrate. The extracted mevalonolactone was at least 98% pure as judged by thin-layer chromatography with four different solvent systems. These improved methods should significantly aid studies of the physiological importance of HMG CoA reductase.

**Supplementary key words** cholesterol biosynthesis · calcium precipitation of microsomes · benzene extraction

3-Hydroxy-3-methylglutaryl CoA reductase (mevalonate:NADP oxidoreductase [acylating CoA], EC 1.1.1.34) (HMG CoA reductase) is a microsomal enzyme which catalyzes the NADPH-dependent reduction of HMG CoA to mevalonate. As the rate-limiting enzyme of cholesterol biosynthesis, HMG CoA reductase is strictly regulated (1). In liver, the level of HMG CoA reductase is controlled by the nutritional and hormonal state of the animal (1). In addition, the catalytic activity of the enzyme is subject to more rapid control; enzyme activity is quickly inhibited during cholesterol feeding (2) and is modulated, at least in vitro, by MgATP (3–5) and cyclic AMP (3, 6). Regulation of HMG CoA reductase is defective in lymphocytes from mice with lymphocytic leukemia (7), in hepatic tumor tissue (8, 9), and in fibroblasts from patients with familial hypercholesterolemia (10).

In yeast, HMG CoA reductase is soluble and has been assayed spectrophotometrically by following the

Abbreviations: HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TLC, thin-layer chromatography; ER, endoplasmic reticulum; TCA, trichloroacetic acid.

HMG CoA-dependent oxidation of NADPH (11). In liver, however, this approach is not feasible because the endoplasmic reticulum contains many other NADPH-utilizing enzymes. Rather, assay methods have been based on the conversion of radioactive HMG CoA to mevalonate. Many techniques have been devised for the isolation of mevalonate or its derivative mevalonolactone: gas–liquid chromatography (12), celite (13) and anion exchange (14) column chromatography, and thin-layer chromatography (TLC) (15–17). The most widely used assay for HMG CoA reductase is a lengthy procedure which requires repeated extraction of the incubation mixture with ether, separation of mevalonolactone on TLC (16), and the use of double-labels (17) to correct for incomplete recovery. Shapiro and co-workers (5) recently described a method that bypassed ether extraction by direct application of the deproteinized assay mixture to TLC sheets. An alternative approach, based on the spectrophotometric measurement of coenzyme A, has also been reported (18).

The study of hepatic HMG CoA reductase is also hindered by a competing enzyme, HMG CoA lyase, which is released from mitochondria even during careful homogenization (19, 20). Thus, endoplasmic reticulum must be isolated by high-speed centrifugation before HMG CoA reductase can be assayed. Unfortunately, the length of this procedure may restrict studies of the rapid modulation of the enzyme.

In this paper we describe two new methods for the study of HMG CoA reductase: the one-step isolation of mevalonolactone by extraction into benzene, and the rapid precipitation of endoplasmic reticulum with $Ca^{2+}$.

## Materials and methods

*Animals.* Male Sprague-Dawley rats (Flow Laboratories, Dublin, Virginia) were exposed to light from 4 P.M. to 4 A.M. and to dark from 4 A.M. to 4 P.M. for at least 7 days before use and had continuous access to water and Purina Lab Chow (Ralston Purina Co., St. Louis, Mo.).

*Chemicals.* Dithiothreitol, glutathione, *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES), digitonin, nicotinamide, and the sodium salts of NADP and glucose-6-phosphate were obtained from Calbiochem, La Jolla, Calif. Glucose-6-phosphate dehydrogenase was from Boehringer Mannheim, New York. Mevalonolactone was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. HMG CoA was from P-L Biochemicals, Milwaukee, Wisc. Tris (hydroxymethyl) amino methane (Trizma Base) and EGTA were from Sigma Chemical Co., St. Louis, Mo. LSC Complete and Instabray were from Yorktown Research, New Hyde Park, N. Y. Chromagram TLC Sheets (Silica gel G) were from Eastman Kodak Co., Rochester, N. Y. Rhodamine B, sodium sulfite, EDTA, and all solvents (reagent grade) were purchased from J. T. Baker Chemical Co., Phillipsburg, N. J.

Sodium [1-$^{14}$C]acetate, [3-$^{14}$C]HMG CoA, [3-$^{14}$C]-HMG acid, DL-[2-$^{14}$C]mevalonic acid (dibenzylethylene diamine salt), [4-$^{14}$C]cholesterol, potassium DL-[3-$^{14}$C]-$\beta$-hydroxybutyrate, and [1-$^{14}$C]palmitic acid were purchased from New England Nuclear, Boston, Mass. Biosynthetic [$^{14}$C]acetoacetate was a gift from Dr. John Amatruda. The free acid of [$^{14}$C] mevalonate was prepared from an aqueous solution of the dibenzylethylenediamine salt by the addition of excess sodium bicarbonate followed by extraction of the liberated amine into ether. After any remaining ether was evaporated under nitrogen, the aqueous solution of mevalonate was neutralized.

*Preparation of endoplasmic reticulum.* A rat weighing 150–300 g was killed by cervical dislocation between 9 and 10 A.M. The liver was immediately removed and chilled in cold buffer (0–4°C) containing 225 mM sucrose and 25 mM Tris · HCl, pH 7.8. After clotted blood and connective tissue were trimmed off, the liver was weighed, minced, and homogenized with two strokes of a loose-fitting, motor-driven Teflon pestle in the above buffer (2.5 ml buffer/g of liver) with 5 mM glutathione. The homogenate was centrifuged in an International refrigerated centrifuge (Model B-20) at 10,000 *g* for 20 min at 4°C. The supernatant beneath the floating fat layer was used for preparation of endoplasmic reticulum (ER) both by a modification of the calcium precipitation technique of Kamath and coworkers (21–23) and by centrifugation at 105,000 *g* for 60 min at 4°C in a model L2-50 ultracentrifuge (Beckman Instruments, Palo Alto, Cal.).

To precipitate ER with $Ca^{2+}$, 1 ml of the 10,000 *g* supernatant was mixed with 10 ml of cold (4°C) 12.5 mM sucrose containing 8 mM $CaCl_2$, pH 7.5. After 3 min, ER was sedimented by centrifugation at 100 *g* for 10 min at 4°C. The supernatant was removed by gentle suction through a Pasteur pipette. Microsomal pellets prepared by either method were resuspended in buffer (24) containing 100 mM sucrose, 50 mM KCl, 40 mM $KH_2PO_4$, 30 mM EDTA, and 20 mM dithiothreitol, pH 7.2 (resuspension buffer).

*HMG CoA reductase assay.* Microsomal suspensions, 0.1–0.8 mg of protein in 100 $\mu$l, were added to 50 $\mu$l of resuspension buffer in 25-ml screwcap tubes. Assays were started by the addition of 50 $\mu$l of a cofactor–substrate mixture to give the following con-

centrations: 2 mM NADP, 30 mM glucose-6-phosphate, 1.75 IU/ml of glucose-6-phosphate dehydrogenase, and 0.2 mM DL-[3-$^{14}$C]HMG CoA (0.4 $\mu$Ci/$\mu$mole). Incubations were carried out at 37°C in a shaking water bath (120 oscillations/min) and stopped after 20–60 min by the addition of 100 $\mu$l of 12 N HCl. After addition of 20 $\mu$moles of carrier mevalonolactone, samples were incubated for 30 min at 37°C to allow complete lactonization of mevalonate. A saturating amount of sodium sulfite[1] (0.5–0.6 g) was added to each tube, raising the pH of the reaction mixture to 6.5. These conditions enhance the retention of HMG CoA in the aqueous phase.

Mevalonolactone was isolated from the incubation mixture by two successive extractions with 7.5 ml of benzene. A 10-ml aliquot of the pooled benzene extracts was transferred to a scintillation vial and dried. After the addition of 50 $\mu$l of water and 10 ml of Instabray, samples were counted in a Beckman liquid scintillation spectrometer (LS-133) with a counting efficiency for $^{14}$C of 58%. Data were corrected for quenching with the external standard-channels ratios. Mevalonate recovery was assessed in duplicate for each experiment by adding [$^{14}$C]mevalonate (100,000 dpm) to tubes containing 100 $\mu$l of 12N HCl and all assay components. Samples were then carried through the above procedure beginning with the lactonization step. The average variability between duplicate tubes for extraction of [$^{14}$C]mevalonolactone into benzene was ±3%. The blank value for this reductase assay (HCl added prior to assay) was 100 dpm in the total benzene extract. A similar blank value was attained when NADP was omitted from the assay. These background dpm were subtracted before calculation of reductase activity.

To compare this new method for isolating mevalonolactone with the technique of Shapiro, Imblum, and Rodwell (16), mevalonolactone from incubation mixtures was extracted twice into 10 ml of ether; after the ether was evaporated, the residue was transferred in 200 $\mu$l of acetone to an activated TLC sheet divided into four 5 cm-wide channels. The sheets were developed in acetone–benzene 1:1 for 75 min and dried. Mevalonolactone was visualized with rhodamine B (25), cut out, and counted in 50 $\mu$l of $H_2O$ and 10 ml of Instabray. Data were corrected for the slight quenching effect of rhodamine B. Blank values (HCl added prior to assay) for this method were similar to those for benzene extraction. With this procedure [$^{14}$C]mevalonate was extracted with an efficiency of 61 ± 5% (mean ± standard error of the mean for seven determinations).

*Protein determination.* Protein concentrations were determined by a modification of the biuret method (26). To remove dithiothreitol, which interferes with protein determination by this method, microsomal samples were precipitated with 5% trichloroacetic acid (TCA). After centrifugation, the pellet was resuspended in water and reprecipitated with TCA. Before mixing with the biuret reagent the protein pellet was dissolved by the addition of 1 ml of $H_2O$, 0.3 ml of 10% NaOH, and 0.2 ml of 5% deoxycholate.

*HMG CoA lyase assay.* HMG CoA lyase was assayed by the conversion of nonvolatile [3-$^{14}$C]HMG CoA to volatile [3-$^{14}$C]acetoacetate (20). The reaction was started by adding 80 nmoles of [3-$^{14}$C]HMG CoA to an incubation mixture containing 100 $\mu$l of a 10,000 *g* supernatant or microsomal suspension and 250 $\mu$l of resuspension buffer, and was conducted with shaking at 37°C. At timed intervals, 40-$\mu$l aliquots of the reaction mixture were transferred to glass scintillation vials containing 0.3 ml of 6 N HCl. The acidified samples were dried at 95°C in a forced-draft oven. After the addition of 1 ml of $H_2O$ and 10 ml of Instabray, samples were counted to determine the amount of unreacted [$^{14}$C]HMG CoA. This method was capable of detecting as little as 1 milliunit of HMG CoA lyase activity (20).

*Assessment of cholesterol formation during the HMG CoA reductase assay.* An aliquot of the benzene extract from an assay mixture and 0.8 mg of carrier cholesterol in ethanol were dried together in a 12-ml centrifuge tube. The residue was dissolved in 4 ml of acetone–ethanol 1:1 and mixed with 2 ml of 0.5% digitonin in 50% ethanol (27, 28). The samples were allowed to stand for at least 3 hr at room temperature. The cholesterol digitonides were sedimented by centrifugation and washed, first with 3 ml of diethyl ether–acetone 1:1 and then with 3 ml of diethyl ether. The precipitate was dried, transferred to a scintillation vial with 2 ml of chloroform–methanol 2:1, and dried again. One ml of methanol and 10 ml of LSC scintillator were added to each vial prior to counting.

*Assessment of palmitate formation during the HMG CoA reductase assay.* An aliquot of the benzene extract from an assay mixture was dried, and the residue was dissolved in acetone and applied to a TLC sheet. Unlabeled palmitate and mevalonolactone were spotted as standards. The sheet was developed in ether–benzene–ethanol–acetic acid 40:50:2:0.2 until the solvent front had migrated at least 10 cm. The sheet was dried and sprayed with rhodamine B.

[1] Edwards, P., personal communication.

TABLE 1. Comparison of ultracentrifugal and calcium-precipitation methods for the isolation of endoplasmic reticulum

| Yield | $Ca^{2+}$ precipitation | Ultracentrifugation |
|---|---|---|
| Protein[a] | 22.6 ± 0.9% | 24.0 ± 0.6% |
| HMG CoA Reductase (nmoles/min/mg) | 0.49 ± 0.1 | 0.48 ± 0.2 |

[a] Results are expressed as the percent of protein in the 10,000 *g* supernatant which sedimented as ER.

The methods for isolation of endoplasmic reticulum and assay of HMG CoA reductase are described in Materials and Methods. Data given are means ± SEM for parallel determinations in duplicate on seven different liver preparations.

Channels were cut into 1 cm strips which were then placed in scintillation vials and counted in 10 ml of Instabray. Palmitate ($R_f$ 0.8–0.9) and mevalonolactone ($R_f$ 0.45–0.55) were well separated in this solvent system.

## Results and discussion

**Table 1** compares the properties of endoplasmic reticulum (ER) prepared by calcium-precipitation and by ultracentrifugation. Both the yield of protein and the specific activity of HMG CoA reductase were nearly identical for the two methods. Neither preparation contained detectable levels of HMG CoA lyase, an enzyme easily released from mitochondria during homogenization (20) and capable of competing with HMG CoA reductase for substrate. Microsomes prepared by either method showed less than 1% of the HMG CoA lyase activity present in the 10,000 *g* supernatant. However, since HMG CoA lyase requires $Mg^{2+}$ for activity (29), the EDTA present in the resuspension buffer might have prevented detection of all of the lyase present.

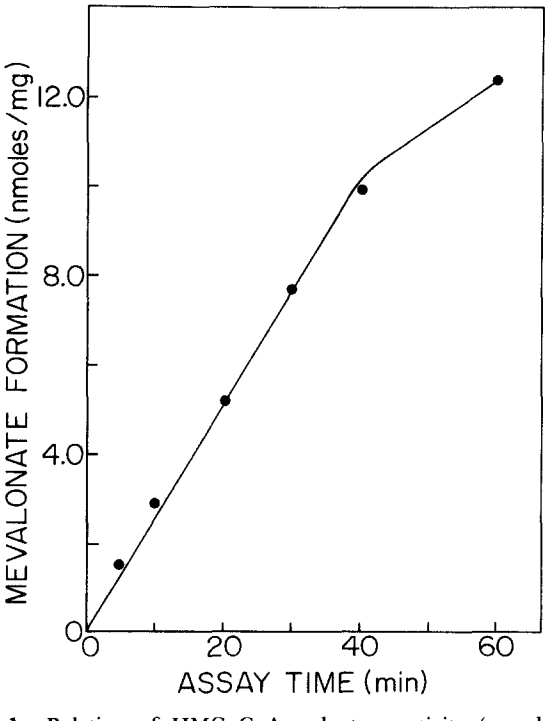


**Fig. 1.** Relation of HMG CoA reductase activity (mevalonate formation) to incubation time. Each tube contained 0.44 mg of microsomal protein. The assay procedure is described in Materials and Methods. Each point represents the mean of duplicate determinations on three different microsomal preparations.

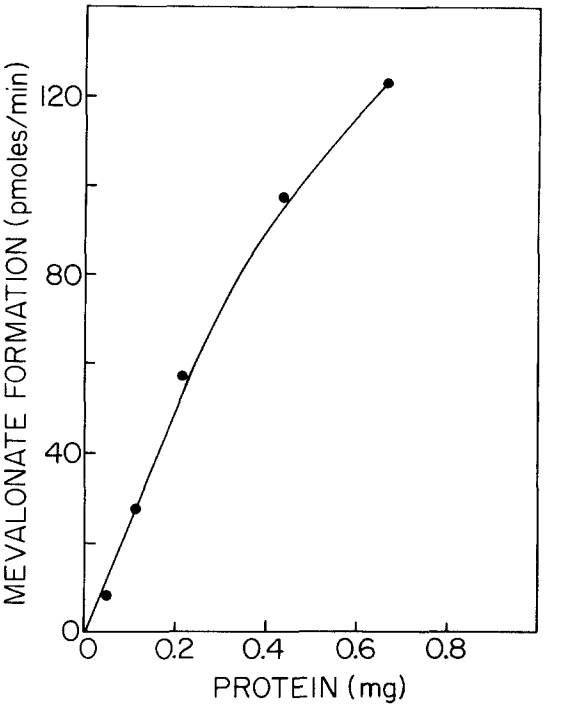


**Fig. 2.** Relation of HMG CoA reductase activity (mevalonate formation) to the amount of microsomal protein present during the assay. The assay procedure is described in Materials and Methods. The incubation time was 45 min. Each point represents the mean of duplicate determinations on three different microsomal preparations.

No precipitate formed when liver cytosol (supernatant from centrifugation at 105,000 *g* for 60 min) was diluted 10-fold with 8 mM $CaCl_2$ in 12.5 mM sucrose. Nor did the homogenization buffer form a precipitate when mixed with $Ca^{2+}$. These results further support the specificity of $Ca^{2+}$-precipitation for endoplasmic reticulum. No precipitate formed when 10,000 *g* supernatant was mixed with 12.5 mM sucrose. Thus, $Ca^{2+}$ is absolutely required for the precipitation of ER.

The precipitation of ER with $Ca^{2+}$ required a 10-fold dilution of the 10,000 *g* supernatant with 12.5 mM sucrose. Thus, ER could be prepared from undiluted supernatant by ultracentrifugation in the presence of 8 mM $Ca^{2+}$. The specific activity of HMG CoA reductase in ER prepared in this way was similar

to that in ER prepared by ultracentrifugation in the absence of $Ca^{2+}$.

The success of the precipitation of ER with $Ca^{2+}$ depended on the buffer in which the liver was homogenized. For example, no precipitation occurred when the buffer consisted of 100 mM Tris · HCl, 30 mM nicotinamide, 10 mM $KP_i$, and 1 mM $MgCl_2$. However, $Ca^{2+}$-precipitation was successful with any of the following buffers: 250 mM sucrose; 225 mM sucrose, 25 mM Tris · HCl; 210 mM D-mannitol, 70 mM sucrose, 2 mM HEPES.

By including both EDTA (or EGTA) and dithiothreitol (or glutathione) in the buffer used to resuspend $Ca^{2+}$-precipitated ER, the observed HMG CoA reductase activity was enhanced by at least 3-fold over that seen when neither was included. When the resuspended ER was frozen at −40°C with dry ice/ethanol and stored for 1 month, there was no loss of HMG CoA reductase activity.

Endoplasmic reticulum could be prepared in 20 min by $Ca^{2+}$-precipitation, whereas 2 hr were required for the ultracentrifugal method. Furthermore, the pellet of $Ca^{2+}$-precipitated ER was only loosely packed and, therefore, much easier to resuspend than the gel-like pellet obtained by ultracentrifugation.

HMG CoA reductase activity in $Ca^{2+}$-precipitated ER (0.44 mg per assay) was linear for at least 40 min (**Fig. 1**). Enzyme activity in a 45-min assay was linear with the amount of protein up to 0.3 mg (**Fig. 2**). ER prepared by this technique was used in all subsequent experiments.

TABLE 2. Efficiency of extraction of various compounds into benzene

| Compound | Extraction (%) | (n) |
|---|---|---|
| Mevalonolactone | 58 ± 2 | (15) |
| HMG CoA | 0.37 ± 0.2 | (5) |
| HMG Acid[a] | 2.1 ± 0.4 | (5) |
| Acetoacetate | 0.94 ± 0.3 | (4) |
| β-Hydroxybutyrate | 0.39 ± 0.06 | (5) |
| Acetate | 0.016 ± 0.002 | (5) |
| Cholesterol | 75 ± 3.3 | (5) |
| Palmitate | 73 ± 3.0 | (5) |

[a] Most of the extracted $^{14}C$-labeled HMG acid co-chromatographed [silica gel G TLC sheets developed with acetone–benzene (1:1)] with a contaminant present in the commercial preparation of $^{14}C$-labeled HMG. After purification on the same TLC system, only 0.4% of the $^{14}C$-labeled HMG acid was extracted into benzene.

Mevalonate or one of the above compounds ($^{14}C$-labeled) was added to a tube containing 100 μl of 12 N HCl and all components of the HMG CoA reductase assay except cofactors. After samples were incubated for 30 min at 37°C to simulate the lactonization step, saturating amounts of sodium sulfite (0.5–0.6 g) were added. Samples were then extracted twice with 7.5 ml of benzene, and an aliquot of the pooled benzene extract was transferred to a scintillation vial, dried, and dissolved in scintillation fluid for counting. The number of determinations for each compound is indicated by n. Data given are means ± SEM. When samples were extracted twice with ether under the above conditions, 83% of the mevalonolactone and 1.3% of the HMG CoA were extracted.

TABLE 3. Radiochemical purity of benzene-extracted mevalonolactone

| Solvent System | Radiochemical Purity (%)[a] Biosynthetic | (n) | Commercial | (n) |
|---|---|---|---|---|
| Acetone–benzene 1:1 | 98 ± 0.1 | (6) | 99 ± 0.2 | (6) |
| *n*-Butanol | 98 ± 0.6 | (4) | 98 ± 0.5 | (3) |
| *n*-Butanol–propionic acid–water 50:25:10 | 98 ± 0.4 | (2) | 100 ± 0.01 | (3) |
| Ether–benzene–ethanol–acetic acid 40:50:2:0.2 | 98 ± 0.4 | (6) | 99 ± 0.2 | (4) |

[a] Radiochemical purity is defined as the percentage of total counts in a TLC channel which migrated with standard mevalonolactone.

Commercial [$^{14}C$]mevalonate and [$^{14}C$]mevalonate synthesized during the HMG CoA reductase assay were lactonized and extracted into benzene as described in Materials and Methods. An aliquot of each benzene extract was dried, and the residue was dissolved in acetone and spotted on a TLC sheet divided into 4 channels. After the spots were dried in an air stream, the sheets were developed in one of the above solvent systems until the solvent front had migrated at least 10 cm from the origin. Sheets were sprayed with rhodamine B to visualize mevalonolactone. Each channel was cut into 1 cm strips which were placed in separate scintillation vials and counted in 10 ml of Instabray. The number of samples examined with each solvent system is indicated by n. Data given are means ± SEM.

The method used here for precipitation of endoplasmic reticulum (ER) with $Ca^{2+}$ is a modification of the procedure described by Kamath and co-workers (21–23) for preparation of rat liver ER. Those workers demonstrated that $Ca^{2+}$-precipitated ER was comparable to ER prepared by ultracentrifugation with respect to yield of protein, glucose-6-phosphatase, $Na^+$–$K^+$–$Mg^{2+}$ activated ATPase, 5′-nucleotidase, aminopyrine demethylase, aniline hydroxylase, cytochrome P-450, cytochrome $b_5$, phospholipids, cholesterol, and RNA. Electron micrographs revealed both smooth ER and rough ER with intact ribosomes (23). $Ca^{2+}$-precipitation has been employed to prepare ER from brain (30), adrenal, ovary, and testis of rat (31), housefly abdomen (32), and southern army worm midgut (32), as well as from rat and mouse liver (21–23, 32). This paper has described the first application of this technique to the study of HMG CoA reductase.

This $Ca^{2+}$-precipitation method facilitates in vitro studies of the rapid modulation of HMG CoA reductase. For example, we previously reported that preincubation of a 10,000 *g* supernatant from rat liver for 20 min at 37°C activated by 2 to 5-fold the subsequent rate of cholesterol synthesis from acetate, while cholesterol synthesis from mevalonate was not activated (33). In further studies, no stimulation of HMG CoA reductase could be demonstrated in ER prepared by ultracentrifugation from preincubated

10,000 *g* supernatant. However, when ER was prepared from preincubated supernatant by $Ca^{2+}$ precipitation, 2 to 4-fold activation of HMG CoA reductase was observed (6).

The remainder of this paper describes an improved technique for the isolation of mevalonolactone. **Table 2** shows the extraction into benzene of mevalonolactone, HMG CoA, and several other compounds that might be formed from HMG CoA during the HMG CoA reductase assay. The efficiency of mevalonolactone extraction (58%) was similar to that reported for ether extraction followed by TLC (16). Less than 0.5% of the substrate $^{14}$C-labeled HMG CoA was extracted. Similarly, there was very little extraction of HMG acid, acetoacetate, β-hydroxybutyrate, or acetate. Although both cholesterol and palmitate were efficiently extracted into benzene, neither was synthesized from $^{14}$C-labeled HMG CoA under the conditions of the HMG CoA reductase assay.

**Table 3** compares the radiochemical purity of benzene-extracted mevalonolactone from two sources: commercial [$^{14}$C]mevalonate and [$^{14}$C]-mevalonate synthesized during the HMG CoA reductase assay. In each of the four solvent systems, [$^{14}$C]mevalonolactone from both sources was at least 98% pure. This result suggests that no benzene-extractable compounds other than mevalonate were synthesized from $^{14}$C-labeled HMG CoA during the assay. Since further purification of mevalonolactone on TLC was unnecessary, the time required to isolate mevalonolactone for counting was decreased by at least two-thirds. This benzene extraction method and the ether extraction–TLC procedure of Shapiro and co-workers (16) gave nearly identical values for HMG CoA reductase activity: 0.098 and 0.102 nmoles/min/mg, respectively, for one particular microsomal preparation.

The two new methods described here for measuring HMG CoA reductase activity greatly reduce the time required to prepare endoplasmic reticulum and to quantitate [$^{14}$C]mevalonolactone. These improvements should significantly aid the continued study of the physiological and pathological regulation of HMG CoA reductase.

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