notes on methodology

Determination of hepatic 3-hydroxy-3-methylglutaryl CoA reductase activity in man

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Summary Procedures were developed for the determination of the activity of the microsomal enzyme 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase, EC 1.1.1.34) in human liver. The enzyme assay could be carried out with as little as 20 mg of fresh liver tissue, thus making the method applicable to specimens obtained by percutaneous liver biopsy. Experiments were carried out to determine optimal assay conditions and to establish the identity and radiopurity of the reaction product formed from 3-^{14}C-labeled 3-hydroxy-3-methylglutaryl CoA. The specific activity of the enzyme was measured in a number of patients with different disorders of lipid metabolism.

The rate-determining step in the biosynthesis of cholesterol from two-carbon precursors is the formation of mevalonic acid from HMG CoA (3-hydroxy-3-methylglutaryl CoA). This reaction is catalyzed by the enzyme HMG CoA reductase (mevalonate:NADP oxidoreductase [acylating CoA], EC 1.1.1.34) (1). In order to study the activity of hepatic microsomal HMG CoA reductase in patients with disorders of lipid metabolism and to assess the effects of treatment, procedures were developed to assay the activity of the enzyme in the relatively small amounts of liver tissue obtained by percutaneous liver biopsy. This report describes the preparation of the microsomal fraction from 20–65 mg of liver, characterizes those properties of the enzyme activity that must be known to establish an optimal assay system, and establishes the identity and radioactive purity of the reaction product, mevalonolactone.

Methods

Preparation of substrate. [3-^{14}C]HMG CoA was prepared by reacting [3-^{14}C]HMG anhydride with reduced CoA (2). The labeled anhydride was synthesized from [3-^{14}C]HMG by the method of Goldfarb and Pitot (3), with some modifications. A solution of 10 mg of [3-^{14}C]HMG (New England Nuclear Corp., Boston, Mass.) and 44 mg of unlabeled HMG (0.3 mmole), mp 108°C, in 1.5 ml of acetone was added to a solution of 103 mg of dicyclohexylcarbodiimide (0.5 mmole) in 2.0 ml of acetone. The reaction mixture was stirred at 30°C for 10 min. A precipitate of dicyclohexylurea formed, which was filtered by suction and discarded. The filtrate was evaporated at room temperature in vacuo. The solid residue was washed with 5 ml of cold benzene (previously dried over anhydrous calcium sulfate) to remove unreacted dicyclohexylcarbodiimide, and the crude HMG anhydride was filtered and purified by crystallization from benzene. Yield of HMG anhydride, mp 101.5°C (2), was 36 mg (74% based on HMG); specific activity, 2440 dpm/nmole.

Clinical procedure. Liver tissue was obtained from 14 individuals who were hospitalized at the Manhattan Veterans Administration Hospital. Liver biopsies were removed at abdominal surgery in eight patients: cholecystectomy was performed for cholesterol cholelithiasis in four subjects, and subtotal gastrectomy with vagotomy was performed for chronic peptic ulcer disease in four subjects. Percutaneous liver biopsy was performed in six other patients. All biopsies were obtained between 9 and 11 a.m.; the subjects had been fasted since the previous evening. Informed consent was obtained from each subject. All 14 patients listed in Table 3 were in good condition and showed no evidence of other serious illness. Liver function and blood coagulation tests were normal. The patients were fed the regular hospital diet for at least 1 wk prior to surgery and no subject exceeded ideal weight by more than 5%. No drugs were administered to these subjects except for chloral hydrate, which was given as a bedtime sedative. The surgical biopsies were performed by Dr. Richard Kessler, Assistant Chief of Surgery at the Manhattan Veterans Administration Hospital. In no instance was significant bleeding noted.

Percutaneous liver biopsies were performed by the Menghini technique utilizing a 16-gauge Menghini needle. The right intercostal approach with the patient in the supine position was employed. The weight of liver tissue removed was 20–65 mg, and no complications were encountered. Liver histology was normal in all patients.

After the biopsy the tissue was placed immediately into a 5-ml beaker containing ice-cold homogenizing solution, 1 ml for the percutaneous biopsies and 3–4 ml for the surgical specimens. The time needed to transport the tissue from patient to laboratory was less than 20 min.

Preparation of liver microsomes. The homogenizing medium contained: sucrose, 0.3 M; nicotinamide, 0.075 M; EDTA, 0.002 M; and mercaptoethanol, 0.02 M. All of the manipulations described in this section were per-

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl or 3-hydroxy-3-methylglutaric acid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

formed either in a cold room (4°C) or with cooling in an ice bath. The tissue was dried by placing it on sterile gauze for about 5 sec, and weighed. The tissue was then homogenized in a 15-ml centrifuge tube (Corning no. 8441) with a loose-fitting pestle (radial clearance 0.5 mm), using the homogenizing medium described above. The procedure was standardized by employing a motor-driven pestle at 1500 rpm and homogenizing for 10–15 sec with four up and down excursions of the pestle. Sufficient homogenizing medium was added to obtain a 5–10% homogenate (w/v). With weights of tissue greater than 100 mg, the liver tissue was extruded through a tissue press (Harvard Apparatus Co., Millis, Mass.) or chopped with a sterile blade before homogenization.

The homogenate was then centrifuged at 11,000 *g* for 10 min to sediment nuclei, cell debris, and the mitochondrial fraction. The supernatant solution was transferred into a 4-ml polycarbonate tube (no. 2804, International Equipment Co., Needham Heights, Mass.) and centrifuged at 100,000 *g* for 60 min. The sedimented microsomes were washed with 1 ml of homogenizing medium and centrifuged again at 100,000 *g* for 60 min. The supernatant solution was decanted and the sedimented microsomal pellet was homogenized in the same centrifuge tube (radial clearance of pestle, 0.5 mm) with a volume of homogenizing medium to give a solution of known concentration (5–10%). Protein concentrations were determined by the method of Lowry et al. (4). With histologically normal liver tissue, the amount of microsomal protein obtained was 23.8 ± 4 (SD) mg/g wet weight of liver. Identical yields of microsomal protein were obtained from the livers of the patients with duodenal ulcer and those with cholelithiasis.

Assay of HMG CoA reductase activity. The procedure was similar to that previously described for the rat liver enzyme (5). In order to establish optimal conditions, the assay system was modified with respect to the NADPH-generating system, composition of incubation medium, reaction time, substrate concentration, and protein concentration. The final composition of the assay system was decided upon on the basis of experiments designed to establish optimal assay conditions. Some of these studies (those dealing with the effects of pH, substrate and protein concentrations, and the time course of the reaction) are described in Results.

The standard assay system contained, in a volume of 0.85 ml: phosphate buffer, pH 7.2, 100 mM; $MgCl_2$, 3 mM; NADP, 3 mM; glucose-6-phosphate, 10 mM; glucose-6-phosphate dehydrogenase, EC 1.1.1.49, 3 enzyme units; mercaptoethanol, 20 mM; R,S-[3-^{14}C]HMG CoA, 0.4 mM; microsomal protein, 0.1–0.4 mg. The final pH of the complete assay system was 7.4 after all of the components of the system had been added. The buffering capacity of the phosphate buffer was adequate to keep the pH at 7.4 during the incubation period.

TABLE 1. Identification of biosynthetic mevalonolactone obtained from incubation of human hepatic microsomes with [3-^{14}C]HMG CoA[a]

	Specific Radioactivity
	dpm/μmole
[3-^{14}C]Mevalonolactone from preparative TLC	317
[3-^{14}C]Mevalonolactone from Celite partition column	277
After addition of carrier	19
[3-^{14}C]Mevalonodiaminodiphenylmethane	
1st crystallization	18
2nd crystallization	18

[a] The biosynthetic [3-^{14}C]mevalonolactone obtained from a large-scale incubation experiment of microsomes with [3-^{14}C]HMG CoA was separated from the incubation mixture by ether extraction and then applied to a preparative TLC plate. Subsequent purification steps are described in Results.

Determination of liver cholesterol. Since the amount of liver tissue was limited, the concentration of liver cholesterol was determined in an aliquot (0.1 ml) of the whole homogenate used for the preparation of the microsomal fraction. This aliquot, containing 2–10 mg of liver, was hydrolyzed for 3 hr by refluxing with 5 ml of a solution of 25% KOH in 95% ethanol (w/v). The reaction mixture was cooled to room temperature, diluted with an equal volume of water, and extracted twice with 20 ml of *n*-hexane. The hexane layers were combined, washed with 10–15 ml of water, and dried over anhydrous Na_2SO_4. The hexane was filtered and evaporated under a stream of nitrogen at 40°C. The residue left after evaporation of the hexane was dissolved in $CHCl_3$, and cholestane was added as internal standard. The concentration of cholesterol in this solution was determined by GLC, using a Barber-Colman Selecta 5000 gas chromatograph with hydrogen flame detectors. The samples were injected onto a 180 cm × 4 mm glass column packed with 3% QF-1 on 80–100 mesh Gas-Chrom Q (Applied Science Laboratories, Inc.). The column temperature was 245°C.

Results

Identification of biosynthetic mevalonolactone. The identity and radioactive purity of the labeled mevalonolactone formed from [3-^{14}C]HMG CoA by human liver microsomes were established as follows. The assay system was scaled up 10-fold, and the labeled mevalonolactone formed plus unlabeled carrier was extracted with ether. After evaporation of the ether, the residue was applied as a band to a preparative TLC plate (silica gel G, layer thickness, 2 mm) and developed with acetone–benzene 1:1 (v/v) (5). The mevalonolactone band was removed from the plate and eluted with acetone. The specific radioactivity of the mevalonolactone was determined by scintillation counting and GLC (5). This mevalonolactone of known specific activity (Table 1) was subjected to column partition chromatography, using 0.1 N HCl as the stationary phase and $CHCl_3$ as the mobile phase (6). Within the

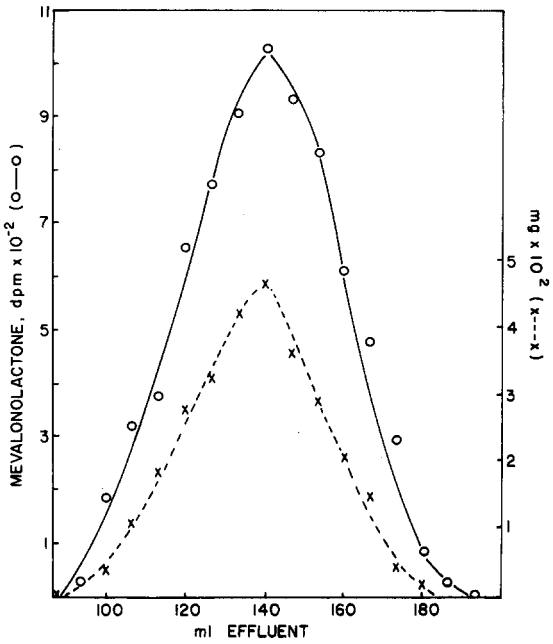

Fig. 1. Column partition chromatography of biosynthetic [3-^{14}C]mevalonolactone obtained by large-scale incubation. Column fractions were assayed for mevalonolactone by GLC and for ^{14}C by scintillation counting.

precision of measurement (estimated to be ±7%), the specific radioactivity of the mevalonolactone remained constant throughout the mevalonolactone band (Fig. 1). The column fractions containing mevalonolactone were combined, and an additional amount of unlabeled carrier was added. Reaction with diphenylmethylamine resulted in formation of the crystalline derivative [3-^{14}C]mevalonoaminodiphenylmethane (7). The specific radioactivity of the derivative remained constant during two recrystallizations from benzene–*n*-hexane mixtures (Table 1).

Properties of the microsomal assay system. The relationship between reaction rate and pH of the incubation medium is illustrated in Fig. 2. The optimal pH was approximately 7.5. In the standard assay system containing

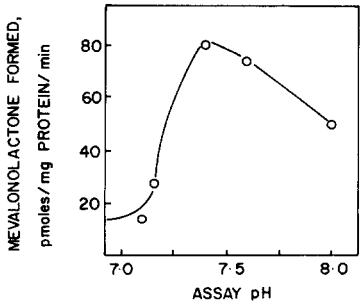

Fig. 2. Microsomal HMG CoA reductase activity of human liver as a function of assay pH. Standard assay conditions except for pH of buffer. The *final* pH of the incubation mixture was used in plotting the curve because the pH of the complete assay system differed from that of the buffer by 0.1–0.2 pH units. However, the pH of the assay system did not change during the incubation period.

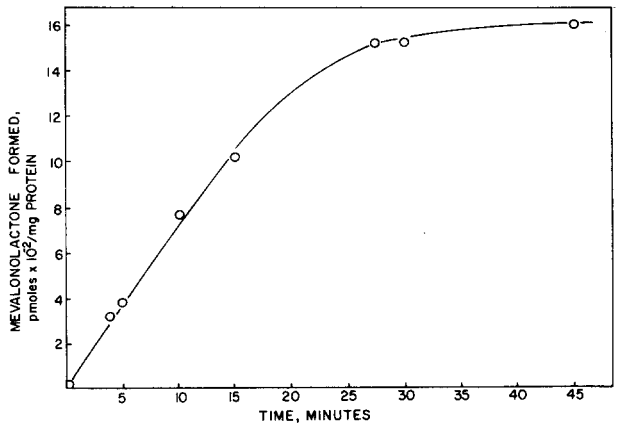

Fig. 3. Time course of enzymatic reduction of S-HMG CoA. Standard assay conditions except for incubation time.

0.1–0.4 mg of protein per tube, the rate of formation of mevalonolactone was linear with time during a 20-min period (Fig. 3). The effect of substrate concentration on reaction rate is shown in Fig. 4. The enzyme required a substrate concentration of 160 μmoles of S-[3-^{14}C]HMG CoA/l for maximal velocity. Therefore, in the standard assay system a 200 μM concentration of S-HMG CoA was used to ensure that the enzyme was saturated with substrate. The relationship between reaction rate and enzyme concentration is illustrated in Fig. 5. Proportionality was observed when the protein concentration ranged from 0 to 0.45 mg/tube in the standard assay system.

Effect of tissue weight and of microsomal protein concentration on HMG CoA reductase activity. In order to show that observed enzyme activities were independent of the weight of tissue available for the assay, liver tissue from two subjects was divided into several sections of different weight prior to homogenization, and HMG CoA reductase activity of the sections was then determined. The results are summarized in Table 2. In each patient, HMG CoA reductase activity was very similar within the precision of measurement (estimated to be ±5%) in all specimens, even though the weight of tissue used varied by

TABLE 2. Relationship between weight of liver tissue and HMG CoA reductase activity

Experiment No.	Weight of Liver Tissue	Approximate Concentration of Tissue in Whole Homogenate (w/v)	Weight of Microsomal Protein in Assay System	HMG CoA Reductase Activity[a]
	mg	*%*	*mg*	*pmoles*
1	1130	10	0.40	46
	35	6	0.24	51
	40	7	0.26	52
2	65	8	0.42	72
	25	4	0.21	77

[a] Mevalonolactone formed per milligram protein per minute.

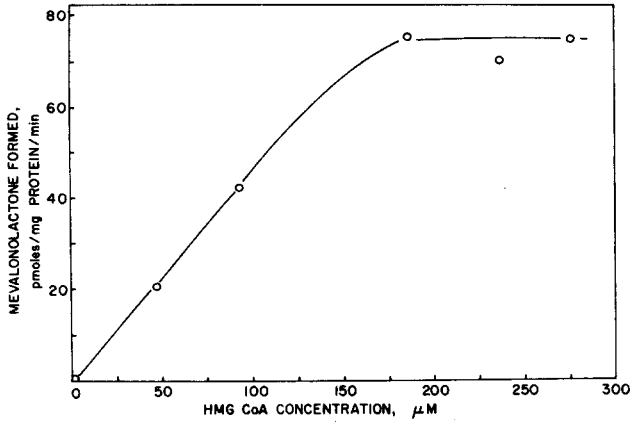

Fig. 4. Effect of substrate concentration (S) HMG CoA) on microsomal HMG CoA reductase activity. Standard assay conditions except for substrate concentration.

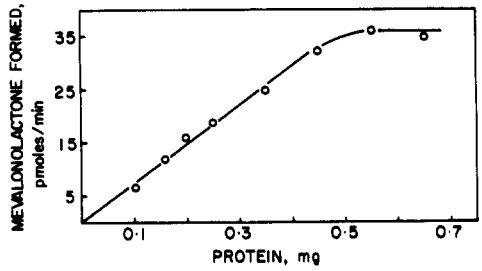

Fig. 5. Effect of increasing amounts of microsomal protein on the rate of reduction of S-HMG CoA. Standard assay conditions except for protein concentration.

a factor of about 30 in patient 1 and by a factor of about 2.5 in patient 2.

Clinical application of the HMG CoA reductase assay. The levels of hepatic HMG CoA reductase activity from eight subjects with cholesterol cholelithiasis and four control subjects with chronic peptic ulcer disease are reported in Table 3. In the gallstone subjects the specific enzyme activities ranged from 70.0 to 90.0 (pmoles of mevalonolactone formed/mg protein/min) with a mean of 80.7. This activity was significantly higher than the mean value of 60.5 observed in the control subjects. The hepatic cholesterol concentration ranged from 4.9 to 11.0 mg/g wet weight in the gallstone subjects with a mean of 7.0. This value is significantly higher than the average of 4.1 mg/g observed in the controls.

Table 3 further lists HMG CoA reductase activities obtained in two subjects with different abnormalities of lipid metabolism. The highest activity of HMG CoA reductase was observed in a patient with cerebrotendinous xanthomatosis, an inherited disease characterized by tendon and tissue xanthomatous deposits and unusually low plasma cholesterol concentrations (8). Hepatic HMG CoA reductase activity was below the average of the control group in a patient with type II hyperlipoproteinemia with xanthomatosis.

Discussion

The results of this study indicate that human hepatic HMG CoA reductase activity can be evaluated in small specimens of liver tissue obtained by percutaneous biopsy. In several species, particularly the rat, there exists considerable evidence that HMG CoA reductase is rate-limiting for cholesterol biosynthesis. Since the pathway of cholesterol biosynthesis in man is very similar to that of the rat,

TABLE 3. HMG CoA reductase activity and cholesterol concentration of human liver

Patient	Sex	Age	Diagnosis	Wet Weight of Liver Tissue	HMG CoA Reductase Activity	Liver Cholesterol Concn
				mg	*pmoles/mg/min*	*mg/g*
K.S.	F	26	Cholelithiasis	40[a]	77	9.4
J.T.	M	68	Cholelithiasis	120	86	6.0
I.K.	M	66	Cholelithiasis	500	70	—[b]
K.M.	F	56	Cholelithiasis	200	79	11.0
R.P.	M	54	Cholelithiasis	50[a]	89	5.6
A.S.	M	26	Cholelithiasis	700	77	—[b]
T.S.	F	52	Cholelithiasis	50[a]	90	5.3
J.G.	M	21	Cholelithiasis	65[a]	78	4.9
					80.7[c] ± 2.4[d]	7.0[c] ± 1.0[d]
J.R.	M	69	Duodenal ulcer	330	64	5.5
J.R.	M	44	Duodenal ulcer	540	65	3.5
R.E.	M	42	Duodenal ulcer	380	50	3.5
F.B.	M	57	Duodenal ulcer	120	63	3.8
					60.5 ± 3.5[d]	4.1 ± 0.48[d]
J.C.	M	33	CTX[e]	50[a]	264	2.2
P.C.	M	42	Type II hyperbeta-lipoproteinemia	40[a]	47	6.5

[a] Percutaneous needle biopsy.
[b] Not determined.
[c] Differs significantly from control group ($P < 0.01$).
[d] Standard error of the mean.
[e] Cerebrotendinous xanthomatosis.

it is probably valid to conclude that hepatic HMG CoA reductase is rate-limiting in man. Obviously, this conclusion must be verified by additional experimentation.

The optimal conditions for the assay of microsomal HMG CoA reductase of human liver differ somewhat from those described previously for the rat liver reductase (5). The former required larger concentrations of substrate (per milligram of microsomal protein) to saturate the enzyme, and proportionality between reaction rate and microsomal protein concentration was observed over a narrower range of protein concentration than in rat liver. The feasibility of the assay procedure may be ascribed to the stability of HMG CoA reductase in the relatively dilute (4–10%) homogenates prepared from percutaneous biopsies. A similar stability had been observed previously in the case of microsomal HMG CoA reductase of rat intestinal mucosa (5).

The enzyme activities shown in Table 2 are apparently not a function of sample size, as would be the case if the micro procedure led to a rapid denaturation of the enzyme. It seems likely that in a diseased liver, enzyme activities in an overtly damaged section of tissue will differ from those found in apparently normal areas. In the present studies only those activities found in *histologically* normal tissues have been measured. It will be important to obtain definite information on the distribution of HMG CoA reductase activity throughout the nondiseased liver. In previous studies in the rat, enzyme activity was distributed homogeneously throughout the liver.[1] If this finding can be shown to be applicable to human liver, it would greatly enhance the value of a single determination of enzyme activity. An additional factor may have to be considered in interpreting the results of human HMG CoA reductase assays: In the rat it has been conclusively demonstrated that the activities of hepatic and intestinal HMG CoA reductases possess a diurnal rhythm. These rhythmic changes in enzyme activity seem to be related to the feeding pattern of the animals, but the detailed mechanism underlying these changes is not understood. In the present study we attempted to minimize the potential effects of a diurnal variation of the human enzyme (which has not been demonstrated as yet) by obtaining liver tissue from all subjects at approximately the same time (10 a.m.) and after an overnight fast. Adherence to a uniform experimental design appears to be necessary in studies of human HMG CoA reductase until data on the diurnal rhythm of this enzyme become available.

[1] Shefer, S., and E. H. Mosbach. Unpublished observations.

We would like to point out that the present paper is merely a feasibility study showing that the enzyme assay is capable of detecting low as well as high enzyme activities. Considerable work with larger groups of subjects carefully matched by age and sex will be required to ascertain the significance of the enzyme activities reported here. It is hoped that the technique described in this paper coupled with the assay of cholesterol 7α-hydroxylase activity will permit a rapid evaluation of relative rates of cholesterol biosynthesis and catabolism, since the results can be obtained within 8–24 hr after the tissue sample arrives in the laboratory. Such information should be valuable in determining the nature of the metabolic defect in certain disorders of lipid metabolism, and it might be helpful in the selection of appropriate therapy and in evaluating the effects of treatment. The long-term usefulness of such enzyme assays can be determined only after it has been demonstrated that changes in enzyme activity correlate reasonably well with observed changes in the metabolism of sterols and bile acids.

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