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EVALUATION OF ENZYME ACTIVITIES BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY: HMGCoA REDUCTASE AND CHOLESTEROL 7α-HYDROXYLASE

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SUMMARY

Methods are described for the evaluation of HMGCoA reductase and cholesterol 7α-hydroxylase activities. The methods are based on the measurement by selected-ion monitoring of mevalonate, formed from HMGCoA and of 7α-hydroxycholesterol formed from cholesterol, respectively. The methods are as sensitive as those based on the use of radioisotopes but less time-consuming because quantitation is carried out on total lipid extracts. In the case of cholesterol 7α-hydroxylase, the procedure does not require the use of exogenous cholesterol as the substrate, so problems related to its equilibration with endogenous microsomal cholesterol are avoided.

INTRODUCTION

Cholesterol metabolism is regulated mainly through the modulation of the activity of two enzymes: 3-hydroxy-3-methyl-glutarylCoA (HMGCoA) reductase, which catalyses the formation of mevalonate (MVL) from HMGCoA[1], and cholesterol 7α-hydroxylase, found to be the rate-determining step in the synthesis of bile acids[2]. Both enzymes are located in the endoplasmic reticulum and their activities show diurnal variation, possibly regulated through phosphorylation–dephosphorylation of the enzyme proteins[1,3,4].

In view of the critical role played by these enzymes in cholesterol metabolism, many efforts have been made to develop rapid and sensitive methods for the measurement of their activities *in vitro*.

Results reported here show the advantages of using the selected-ion monitoring

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(SIM) technique for the evaluation of HMGCoA reductase and cholesterol 7α-hydroxylase activities.

EXPERIMENTAL

Determination of HMGCoA reductase activity

Liver microsomes were obtained as described previously[5] in the presence of 50 m*M* sodium fluoride to inhibit protein dephosphorylation, and were suspended in 300 m*M* potassium chloride, containing 50 m*M* phosphate, 1 m*M* EDTA, and 5 m*M* dithiotreitol (1 mg protein/ml). The suspension, usually 0.1–0.5 ml, was incubated for 30 min at 37°C in the presence of 20 m*M* EDTA, 25 m*M* glucose-6-phosphate, 2 U/ml of glucose-6-phosphate dehydrogenase, 2.7 m*M* $NADP^+$, and 0.15 m*M* HMGCoA at pH 7.2; the final volume was 0.2–1 ml. When the total form of the enzyme was under investigation, the incubation was preceded by treatment of the microsomal proteins with a phosphorylase phosphatase preparation, as described previously[5]. Incubation was stopped with 0.05–0.25 ml of 5 *M* sodium hydroxide. In blank samples, microsomal proteins were inactivated with 5 *M* sodium hydroxide before the incubation. A calibration curve was also prepared in each experiment by addition of known amounts of mevalonate to blank samples.

Deuterated MVL (1.0 μg) was added to each sample as the internal standard. The compound was prepared by synthesis as described previously[6] and was a mixture of tetradeuterated and pentadeuterated molecules. The relative amount of the two labelled forms was found to be 80:20 from the intensities of the ions at *m/e* 191 and 192 ($M^+ - 15$) in the mass spectrum of the trimethylsilyl (TMS) derivative of the compound.

After addition of the internal standard the suspensions were acidified with hydrochloric acid and shaken at 37°C for 1 h. Samples were then extracted with diethyl ether. The organic phase was discarded, and the aqueous phase was saturated with sodium sulphate and extracted three times with diethyl ether. The residue obtained by evaporation of the solvent of the organic phase was refluxed with 0.02 ml of Sylon for 10 min at 60°C to obtain the TMS ethers. Aliquots of the solution were analysed by SIM. Analyses were carried out using a Varian MAT 112 S gas chromatograph–mass spectrometer under the following conditions: electron energy, 70 eV; emission current, 1.5 mA; electron multiplier, 2.5 kV; molecular separator and ion source temperature, 280°C. Ions focused were at *m/e* 187 and 150 for the TMS derivatives of MVL and of its deuterated analogue, respectively. An OV-17 3% silanized column (3 m × 2 mm I.D.) kept at 190°C was used; both the injector and the detector were at 290°C, and the helium flow-rate was 15 ml/min.

From the SIM analysis, the ratio of the intensities of the peaks in the traces of ions at *m/e* 187 ($M^+ - 15$) and 150 ($M^+ - 15 - CH_2CO$) at the retention time of TMS-MVL was obtained. The amount of mevalonate formed during incubation of the microsomes was estimated from this ratio on the basis of the calibration curve obtained by plotting the ratio of intensities of the above-mentioned ions in blank samples with added MVL, *versus* the added amount of MVL.

Determination of cholesterol 7α-hydroxylase activity

Washed liver microsomes (0.15–0.20 mg protein), prepared as described pre-

viously[7], were incubated in 0.1 *M* phosphate buffer containing 2 m*M* $NADP^+$, 20 m*M* glucose-6-phosphate, 9 m*M* mercaptoethanol, 4 m*M* magnesium chloride, 30 m*M* nicotinamide, 3 U/ml glucose-6-phosphate dehydrogenase; the final volume was 0.2 ml and the pH was 7.4. After 30 min at 37°C in a Dubnoff incubator the reaction was stopped by the addition of 20 volumes of chloroform–methanol (2:1, v/v) and 1.0 μg of 5α-cholestane as the reference compound. Zero time controls were run in all the experiments using microsomes previously inactivated by the addition of the chloroform–methanol mixture. The 7α-hydroxycholesterol content in the lipid extracts obtained from incubated samples and from zero time controls was measured by SIM. For this purpose, the extracts obtained from incubations were silylated with N,O-bis-trimethylsilyltrifluoroacetamide (BSTFA)–pyridine (4:1, v/v) at 60°C for 1 h. The reaction mixture was then directly analysed by SIM using a Varian MAT 112 S mass spectrometer, focused on ions at *m/e* 372 (M^+), 443 ($M^+ - 15$) and 456 ($M^+ - 90$) for the detection of cholestane and of the TMS ethers of cholesterol and 7α-hydroxycholesterol, respectively. Conditions for the SIM analysis were as described above for the determination of HMGCoA reductase, as far as the mass spectrometry conditions are concerned. Gas chromatographic conditions were as follows: a glass silanized column (1 m × 2 mm I.D.) packed with 3% SP-2250 was used; the column temperature was 280°C, the helium flow-rate 20 ml/min, and the injector and detector temperatures were 300°C.

Reference curves were obtained from the analysis of standard samples containing 1.0 μg of cholestane and variable amounts of either 7α-hydroxycholesterol or cholesterol. Peak intensities of the monitored ions were measured from the selected ion current trace at the retention time of each tested compound and ratios of the peak heights for *m/e* 456/372 and 443/372 were plotted against the known values of nanomoles of 7α-hydroxycholesterol per milligram of protein or nanomoles of cho-

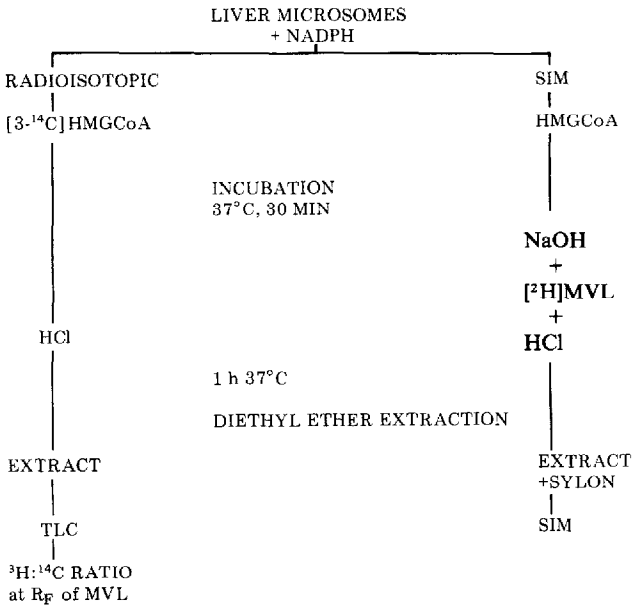

Fig. 1. Radioisotopic and SIM procedures for the evaluation of HMGCoA reductase activity.

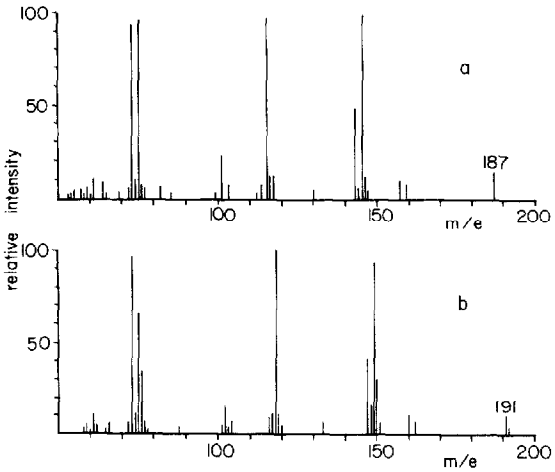

Fig. 2. Mass spectra of the TMS derivatives of (a) MVL and (b) deuterated MVL.

lesterol per milligram of protein. In the analysis of lipid extracts obtained after incubation of microsomes or from the zero time controls, the above peak height ratios were also measured and the nanomoles of sterol per milligram of protein were obtained from the calibration curve. The activity of the enzyme was calculated as the difference between the nanomoles of 7α-hydroxycholesterol per milligram of protein found after incubation and those in the corresponding zero time control.

RESULTS AND DISCUSSION

As shown in Fig. 1, the more commonly used assay of HMGCoA reductase requires the incubation of washed microsomes with [3-^{14}C]HMGCoA in the presence of NADPH and subsequent extraction, chromatographic separation and quantitation of the radioactive MVL formed during the incubation[8]. The addition of tritiated

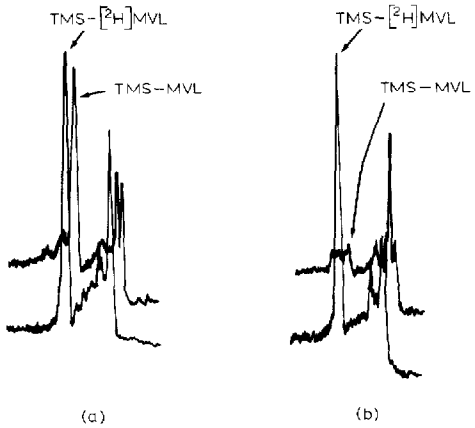

Fig. 3. Typical analysis by SIM of the extracts obtained (a) after 30 min incubation at 37°C of rat liver microsomes with HMGCoA and (b) under the same conditions using microsomes inactivated with sodium hydroxide.

MVL prior to the extraction step obviates the need for quantitative extraction from the aqueous medium. This method, although more sensitive than assays based on the measurement of either coenzyme A formed[9] or NADPH oxidized[10] during the enzyme reaction, is time-consuming and presents the inconvenience that HMGCoA used as the substrate usually contains impurities giving rise to high blank values. This is a drawback particularly when the enzyme activity to be measured is low. We have therefore set up a method by which mevalonate enzymatically derived from HMGCoA is determined using the SIM technique. Synthetic deuterated MVL is used as the internal standard and quantitation is carried out after conversion of the enzymatically formed MVL into the lactone, followed by treatment with a silylating agent to obtain the TMS derivatives. Ions monitored for the detection of the derivatives of MVL and of its deuterated analogue are at *m/e* 187 ($M^+ - 15$) and 150, respectively[6]. A characteristic ion in the mass spectrum of TMS-MVL is found at *m/e* 145 (Fig. 2) and it probably originates from the opening of the lactone ring and cleavage of the bond between carbon atoms 2 and 3. The ion derived from this fragmentation is found in the mass spectrum of the deuterated analogue at *m/e* 149 (Fig. 2) and may therefore have the structure $(C(C^2H_3OTMS)CH_2C^2HHO) - 15^+$. The signal at *m/e* 150 may be due in part to the natural isotopic abundance and in part to the ion $(C(C^2H_3OTMS)CH_2C^2H_2O) - 15^+$ originating from the pentadeuterated molecules. The two ions at *m/e* 187 and 150 were chosen for the detection of MVL and of the internal standard because they are not detectable in the extracts of blank samples. Moreover, the two ions do not interfere. This appears from the mass spectra shown in Fig. 2 and from the reference curve obtained from the SIM analysis of mixtures of authentic MVL with fixed amounts of deuterated MVL. In fact, plotting the ratio of the intensities of ions at *m/e* 187 and 150 *versus* the ratio of the amount of MVL and deuterated MVL a line is obtained with an intercept on the *y*-axis that does not differ from zero. The high correlation coefficient of the line ($r > 0.999$) also shows the linearity of the assay in a wide range of mevalonate concentrations (0.05–10 nmol/ml). SIM analyses of the extracts from incubation of rat liver microsomes and from a blank sample obtained by inactivation of enzyme proteins before the incubation are shown in Fig. 3.

As far as the sensitivity is concerned, it can be calculated that by the double-isotope procedure described by Goldfarb and Pitot[8], when 0.1 mg of microsomes with 5 pmol min^{-1} (mg protein)$^{-1}$ enzyme activity is incubated in the presence of 6 nmol of HMGCoA for 30 min a maximum of 0.25% of the substrate radioactivity is associated with mevalonate. Under these conditions, careful chromatographic purification of the compound from impurities with similar R_F is required. Owing to the presence in liver microsomal preparations of either endogenous mevalonate or of any other compound that by fragmentation gives an ion at *m/e* 187, blank values of the SIM measurement were found to correspond[6] to 3.6 ± 0.07 pmol min^{-1} (mg protein)$^{-1}$. The signal intensity in the trace of the ion at *m/e* 187 at the retention time of TMS-MVL is therefore more than twice that in blank samples when the enzyme assay is carried out under the conditions described above. This renders the SIM method particularly useful when liver specimens have very low enzyme activity and when low amounts of microsomal proteins are available as in the case of those obtained from human liver biopsies.

Problems in the *in vitro* assay of microsomal cholesterol 7α-hydroxylase are

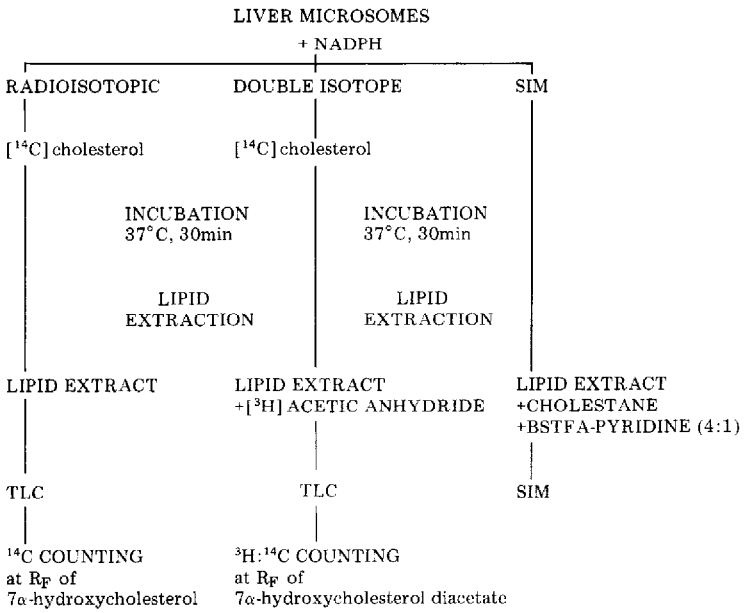

Fig. 4. Radioisotopic and SIM procedures for the evaluation of cholesterol 7α-hydroxylase activity.

more complicated because the enzyme utilizes microsomal cholesterol as the substrate[2]. The activity of this enzyme is generally estimated from the incorporation of the label of radioactive cholesterol into 7α-hydroxycholesterol in incubations of liver microsomes in the presence of NADPH (Fig. 4). Exogenous cholesterol is added to the microsomal suspension either as an acetone solution or as a suspension obtained with detergents[2]. Isolation of 7α-hydroxycholesterol from the total lipid extract is then achieved by column chromatography, which eliminates most of the unchanged cholesterol, followed by thin-layer chromatography (TLC). It was demonstrated[11,12], however, that accessibility of exogenous cholesterol to the enzyme depends on its physical state in the incubation mixture. Equilibration of the exogenous with the endogenous substrate is not complete and is lower when acetone solutions instead of suspensions with detergents are used to add cholesterol to the medium[11,12]. Moreover, we have recently shown[13] that when cholesterol 7α-hydroxylase is not saturated by the endogenous substrate as it occurs in liver microsomes of male rats, equilibration increases with the exogenous cholesterol concentration. It appears, therefore, that correct estimation of this enzyme activity in microsomes can be achieved only by measuring the absolute amount of 7α-hydroxycholesterol formed from the endogenous substrate. For this purpose a double-isotope procedure was described by Mitropoulos and Balasubramaniam[12] implying incubation of [^{14}C]cholesterol with microsomes in the presence of NADPH, extraction, and isolation of 7α-hydroxycholesterol by TLC. The compound is then treated with [^{3}H]acetic anhydride (Fig. 4) and the diacetate obtained is further purified by TLC. The absolute amount of 7α-hydroxycholesterol in the samples is computed from the ^{3}H:^{14}C ratio of the diacetate. The method was improved by Shefer *et al.*[14], who showed that purification of 7α-hydroxycholesterol before its conversion into the diacetate is not necessary. The

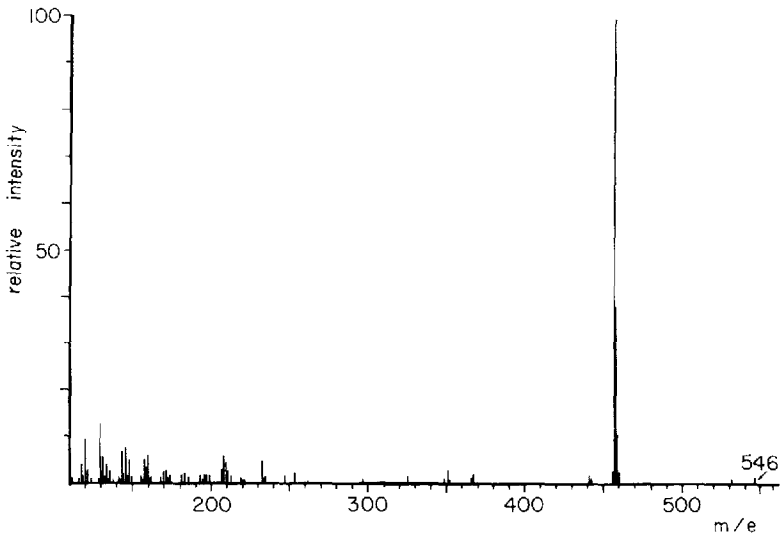

Fig. 5. Mass spectrum of the TMS derivative of 7α-hydroxycholesterol.

method is still time-consuming however, and more importantly, it implies the addition to microsomal preparations of a cholesterol suspension prepared with detergents, which were shown by Sanghvi *et al.*[15] to inhibit the activity of cholesterol 7α-hydroxylase in the *in vitro* assay. Procedures based on SIM allow one to overcome this problem. In fact Bjorkhem and Danielsson[11] were able to measure by this technique the 7α-hydroxycholesterol enzymatically formed from microsomal cholesterol using a trideuterated internal standard. We therefore developed a rapid and simple procedure for the determination of cholesterol 7α-hydroxylase[16]. For this purpose SIM analysis is carried out on the total lipid extract obtained from the incubation of microsomal suspensions in the presence of NADPH without any added substrate (Fig. 4). After 1 h at 60°C in BSTFA–pyridine (4:1) the extract is directly analysed. In addition to the ions monitored for the detection of cholestane used as the internal standard (*m/e* 372, M^+) and TMS-7α-hydroxycholesterol (*m/e* 456, $M^+ - 90$) (Fig. 5), the ion at *m/e* 443 ($M^+ - 15$) is also focused in order to determine the amount of cholesterol in the extract. A typical analysis of a lipid extract is shown in Fig. 6. The enzyme activity is computed from the ratio of the intensities of ions at *m/e* 456, at the retention time of TMS-7α-hydroxycholesterol, and at *m/e* 372 at the retention time of cholestane in extracts obtained from incubated samples minus the ratio in blanks prepared by inactivation of enzyme proteins before the incubation. Fig. 7 shows the calibration curve prepared by SIM analyses of blank samples to which increasing amounts of 7α-hydroxycholesterol were added. The intercept on the *y*-axis of the line obtained by regression analysis represents the amount of 7α-hydroxycholesterol in the microsomes used for the preparation of the calibration curve. Microsomal cholesterol levels are computed from the ratio of intensities of ions at *m/e* 443/372. This ratio was also found to increase linearly with the cholesterol concentration in the incubation medium[16]. Therefore cholesterol 7α-hydroxylase activity and microsomal cholesterol levels can be evaluated simultaneously by this method.

The sensitivity of the method depends on the basal level of 7α-hydroxycholes-

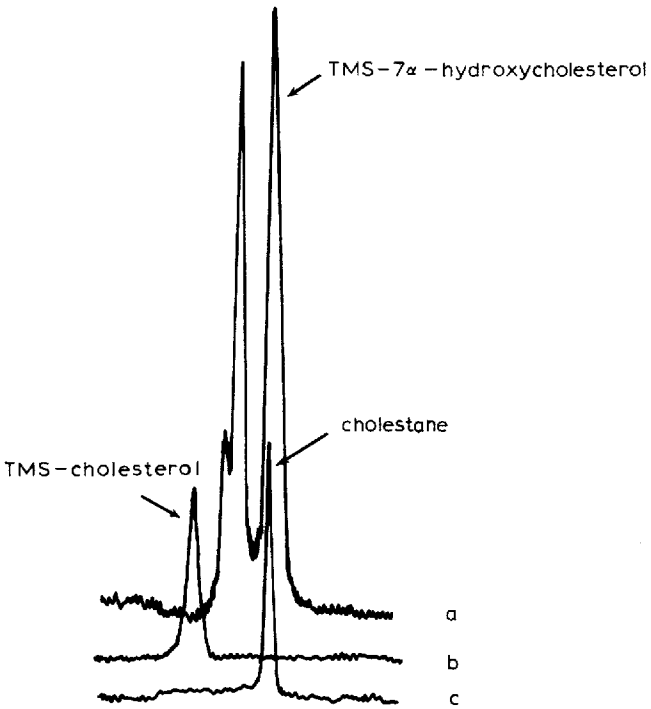

Fig. 6. Typical analysis by SIM of lipid extracts obtained after incubation of rat liver microsomes with NADPH. The ion at *m/e* 456 (a) was monitored at a sensitivity three times greater than that of ions at *m/e* 443 (b) and 372 (c).

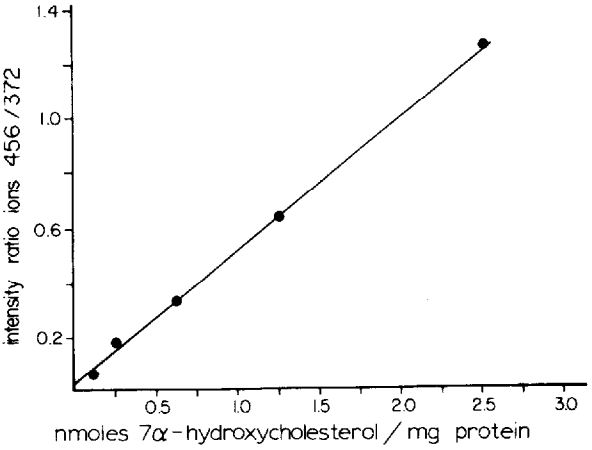

Fig. 7. Calibration curve for the measurement of cholesterol 7α-hydroxylase in rat liver microsomes. From the intercept on the *y*-axis of the regression line, 7α-hydroxycholesterol in the microsomes used for the preparation of the curve was 50 ± 5 pmol (mg protein)$^{-1}$.

TABLE I

7α-HYDROXYCHOLESTEROL LEVELS AND CHOLESTEROL 7α-HYDROXYLASE ACTIVITY IN LIVER MICROSOMES

Results are mean ± S.E. of the number of livers shown in brackets.

Animal species	*7α-Hydroxycholesterol [pmol (mg protein)$^{-1}$]*	*Cholesterol 7α-hydroxylase [pmol min^{-1} (mg protein)$^{-1}$]*
Male rat	70 ± 10	8.3 ± 2.5
Female rat (5)	57 ± 9	7.4 ± 2.0
Female hamster (6)	67 ± 6	2.9 ± 0.4*
Female guinea pig (5)	72 ± 9	18 ± 1.8
Human biopsy women (4)	133 ± 59	n.d.**

* Microsomes from twelve hamsters were assayed; the reported values are the mean ± S.E. of animals shown in brackets in which the activity was over the detection limits. Some of the data shown were reported previously[13,17].

** Not detectable.

terol in liver microsomal preparations. In microsomal suspensions prepared from male rat liver this level was found to be 70 ± 10 pmol (mg protein)$^{-1}$ (refs. 13 and 17) and no significantly different values were found in livers from female rats, hamsters and guinea pigs (Table I). Variability in the levels found in human liver biopsies was too high to allow comparison with the other species tested. If the activity of cholesterol 7α-hydroxylase is 2 pmol min^{-1} (mg protein)$^{-1}$ in a given preparation of microsomes, the amount of 7α-hydroxycholesterol formed during 30 min incubation is 60 pmol (mg protein)$^{-1}$, of the same order of magnitude of the basal levels of 7α-hydroxycholesterol. Because the reproducibility of the assay is high enough, activities even lower than 1 pmol min^{-1} (mg protein)$^{-1}$ can be evaluated. This value is indeed much lower than that found in the liver of control fasted rats but still too high to measure the activity in the fasted hamster and in human liver biopsies (Table I).

The methods described for the determination of HMGCoA reductase and cholesterol 7α-hydroxylase activities are examples of the usefulness of the SIM technique in the evaluation of enzyme activities. With a sensitivity comparable with that obtained using radioisotopes, SIM presents higher specificity and permits one to solve particular problems, such as the equilibration of exogenous with endogenous substrates[18,19], which are familiar to scientists involved in research on lipid biochemistry.

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