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Measurement of cholesterol synthesis in man by isotope kinetics of squalene

(mevalonate/sterol balance/unsteady state)

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ABSTRACT **A method for measuring the rate of total daily cholesterol synthesis in man has been developed through isotope kinetic studies of squalene biosynthesis after intravenous administration of [^{14}C]mevalonic acid. Plasma squalene becomes rapidly labeled, reaching maximal specific activity approximately 100 min after mevalonate administration and then decays exponentially to reach undetectable levels in 12 hr. The rate of daily squalene synthesis equals the percent dose of mevalonate converted to cholesterol divided by the area under the specific activity curve of squalene; the fraction of the dose of mevalonate converted to cholesterol is calculated by the simultaneous injection of [^{3}H]- and [^{14}C]cholesterol in plasma.**

The premise that squalene and cholesterol synthetic rates are equivalent was tested. In seven patients it was found that the mean daily cholesterol synthesis rates estimated simultaneously by sterol balance and by squalene kinetic methods agreed within 8%. In addition, fractional conversions of mevalonic acid to cholesterol were highly correlated with cholesterol synthesis rates. Maximum estimates of the pool sizes and half-lives of metabolically "active" squalene also were obtained.

This measurement of daily cholesterol synthesis by squalene kinetics minimizes patient inconvenience, is suitable for outpatient studies, and yields results in 4 weeks or less. Because of the rapidity of the rate of squalene synthesis, the results obtained reflect cholesterol synthesis over a period of less than 10 hr and are therefore uniquely applicable to unsteady state situations.

The measurement of cholesterol synthesis rates in intact animals and man is currently carried out in two ways: by the sterol balance methods developed in this laboratory (1) and by studies of cholesterol kinetics using compartmental analysis (2) or input-output analysis (3) after administration of radioactive cholesterol. Both methods demand the existence of the metabolic steady state, in which there is neither net gain nor loss of total body cholesterol. The present report describes a new approach to the measurement of cholesterol synthesis rates, in which squalene synthesis is measured and equated to that of cholesterol.

MATERIALS AND METHODS

Materials. DL-[2-^{14}C]Mevalonolactone (17.5 mCi/mmol) was purchased from Amersham/Searle Corp., Arlington Heights, Ill., and DL-[5-^{14}C]mevalonolactone (12.2 mCi/mmol) from Schwarz/Mann, Orangeburg, N.Y. [*1,2*-^{3}H]cholesterol (40 mCi/mmol) was purchased from New England Nuclear, Boston, Mass.

Preparation of Radioisotopic Materials for Intravenous Infusion. The purity of the two radioactive mevalonolactones was routinely checked by thin-layer chromatography (benzene:acetone = 1:1) (4) (>98% pure). These lactones, stored in benzene solution, were dried under N_2 and were converted to the free acids by mixing with 8 ml of sterile physiological Na-bicarbonate by ultrasonication (5 min) and incubation at room temperature for 2 hr; an aliquot was taken for determination of radioactivity. The mevalonic acid (MVA) was sterilized by Millipore filtration (Swinnex-25 filter units, 0.45 μm) into a sterile injection vial. To assure complete transfer of labeled MVA, incubation tubes and syringes were washed twice with 2 ml of Na-bicarbonate solution through the filter units into the sterile vial. [*1,2*-^{3}H]Cholesterol was purified by thin-layer chromatography on Silica Gel G (benzene:ethyl acetate = 4:1).

Procedures

(*1*) **Administration of Isotopic Materials.** Thirty microcuries of [*1,2*-^{3}H]cholesterol dissolved in 1.5 ml of ethanol was mixed into 150 ml of physiologic saline for intravenous infusion. At the same time [^{14}C]MVA (250 μCi) was administered intravenously as a single dose through a Y connection on the infusion set. After completion of the labeled infusion mixture, a fresh batch of saline (50 ml) was infused through the tubing as a rinse. Then, in order to estimate the amount of measured isotopic material *not* administered to the patient, the residue on all glassware and apparatus contacted by [^{14}C]MVA and [^{3}H]cholesterol was extracted with methanol; this was deducted from that added to the infusion, to get the doses actually given.

(*2*) **Bleedings.** An in-dwelling intravenous cannula (Medicut, Aloe Medical, St. Louis, Mo.) in the antecubital vein opposite to that used for the infusion permitted blood sampling into EDTA vacutainer tubes over the next 10-hr period. Blood was drawn (10 ml) at accurately timed intervals: 5, 15, 30, 60, 75, 90, 100, 110, and 120 min, then hourly until 9 hr. The needle was kept patent between bleedings by slow infusion of normal saline. Patients sat up in bed throughout this procedure, ate meals, and had fluids ad lib. They returned to the clinic for bleedings of 10 ml (nonfasting) at weeks 3, 4, and 5.

Special precautions, described elsewhere (5), were taken to insure that all glassware used in collecting and storing plasmas was squalene-free.

(*3*) **Specific Activity (SA) Determinations.** *Squalene.* Plasma squalene measurements were made as described elsewhere (5), using 3 ml of plasma for each determination.

Abbreviations: MVA, mevalonic acid; SA, specific radioactivity; I_T, input (or synthesis) rate.

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Radioactivity measurements were made on separate aliquots of the same extract subjected to gas-liquid chromatography by methods described (6). Since the radioactivity levels are low, even at the peak of the squalene curve (100–600 dpm above background), it is important to carry out quench corrections and counting efficiencies by addition of an internal standard to each sample, discarding counts that are less than three times background (usually 7 hr after MVA dosage).

Cholesterol. Plasma cholesterol SAs ([^{14}C] and [^{3}H]) were measured by gas-liquid chromatography in petrol ether extracts after mild saponification. In samples containing radioactive squalene, cholesterol was eluted from the alumina columns with chloroform after squalene had first been eluted with petrol ether. [^{3}H]- and [^{14}C]cholesterol radioactivities were plotted against time as follows:

$$\%\ \text{dose/g}\ [^{3}\text{H}]\text{cholesterol} = \frac{\text{SA}\ [^{3}\text{H}]\text{cholesterol (dpm/g)}}{\text{Total dpm}\ [^{3}\text{H}]\text{cholesterol administered}} \quad [1]$$

$$\%\ \text{dose/g}\ [^{14}\text{C}]\text{cholesterol} = \frac{\text{SA}\ [^{14}\text{C}]\text{cholesterol (dpm/g)}}{\text{Total dpm}\ R\text{-}[^{14}\text{C}]\text{MVA administered}} \quad [2]$$

When *RS*-[5-^{14}C]MVA was used, *R*-[^{14}C]MVA = 50% of dpm in the racemate. When *RS*-[2-^{14}C]MVA was used, *R*-[^{14}C]MVA = 41.6% of dpm in the racemate, since in the conversion of this compound to cholesterol one of every six radioactive C_2 atoms is lost ($\frac{1}{2} \times \frac{5}{6} = 41.6\%$).

(4) Calculation of Synthesis Rates and Pool Sizes of Squalene. On a squalene-free diet:

$$\text{Squalene synthesis (mg/day)} = \frac{\text{Dose of squalene (dpm)}}{\text{Area under SA curve (dpm/mg·day)}} \quad [3]$$

where the area = $\int_0^\infty$ SA plasma squalene dt; the dose of squalene (dpm) = dose of *R*-[^{14}C]MVA (dpm) × fractional conversion of MVA to cholesterol; and the fractional conversion of MVA to cholesterol = the ratio of the % dose per g of [^{14}C]cholesterol to the % dose per g of [^{3}H]cholesterol in plasmas sampled 3 weeks or more after the initial infusion. To simplify the above, Eq. [3] can be rewritten as follows:

$$\text{Squalene synthesis (mg/day)} = \left[\frac{\text{dpm}\ [^{14}\text{C}]\text{cholesterol}}{\text{dpm}\ [^{3}\text{H}]\text{cholesterol}}\ \text{(at 3 weeks, etc.)} \times \text{dose of}\ [^{3}\text{H}]\text{cholesterol at time zero}\right] \div \text{area under SA curve of squalene (dpm/mg·day)} \quad [4]$$

To convert squalene synthesis to cholesterol synthesis, a correction must be made for the difference in their molecular weights, i.e., multiplication of the squalene synthesis rate (mg/day) by 0.942 (= 386/410).

During the first 100 min after MVA injection, the SA curve of squalene increases to reach a peak and then declines exponentially thereafter (Fig. 1). The area under the squalene curve and the maximum half-life of the decay exponential are calculated by input-output analysis (7). The pool size of metabolically "active" squalene (8) is calculated:

$$\text{"Active" pool}\ (M_{Sq}), \frac{t\tfrac{1}{2}_{max} \times I_T}{0.693} \quad [5]$$

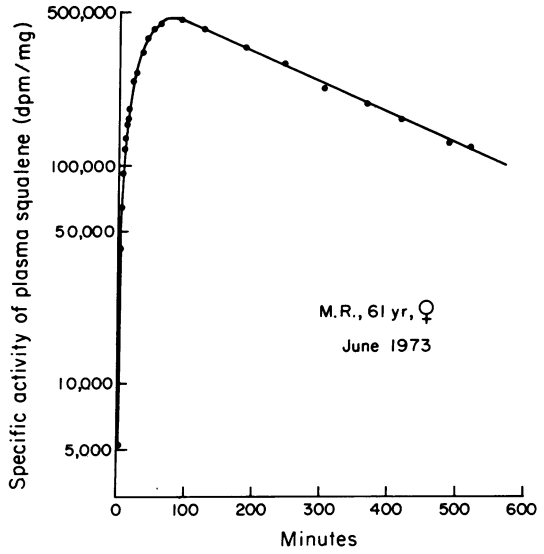

FIG. 1. SA-time curve of plasma squalene after intravenous administration of 250 μCi of DL-[2-^{14}C]MVA to Patient 3.

where the "active" squalene pool (M_{Sq}) is defined as that portion of the total body squalene pool involved directly in cholesterol synthesis, and I_T is the input (or synthesis) rate of squalene. As in the case of the $t\frac{1}{2}$, M_{Sq} also is a maximum estimate. To calculate these parameters by extrapolation of the logarithmically linear decay curve to time zero is theoretically unsound, we believe, since the squalene curve is obtained by formation from a precursor rather than by administration of squalene itself.

Ancillary procedures

Diets were essentially squalene-free. This was assured by use of orally administered liquid formula feedings (9), calories being adjusted to maintain body weights constant (vitamins and minerals were given as supplements), and by solid foods diets that on analysis were shown to be exceedingly low in squalene.

Stools for sterol balance measurements were collected and analyzed as described elsewhere (1), using the internal standards chromic oxide (10) and β-sitosterol (11).

RESULTS

Table 1 presents the relevant clinical information in seven patients studied to date, as well as kinetic data obtained by the labeled squalene technique. Since the major purpose of this report is to present the evidence validating the squalene kinetic method for computing cholesterol synthesis rates, the sterol balance data in six of the seven experiments are shown as benchmark determinations of cholesterol synthesis.

Three other patients also have been studied by this technique. Their data have not been included because of technical and metabolic faults. The eight studies reported in detail seem homogeneous to us by the following criteria: absence of changes in body weight and plasma levels of cholesterol during the test period, complete stool collections (judged by recoveries of the inert marker, chromic oxide), strict adherence to a low-squalene diet, and a smooth clinical course.

Squalene kinetics

A typical SA-time curve for plasma squalene after intravenous administration of [^{14}C]MVA is shown in Fig. 1. Plasma squalene and cholesterol became ^{14}C-labeled within 1.5 min.

Table 1. Clinical information, squalene kinetics, and rates of daily synthesis of cholesterol in eight patients by two methods

Patient: Age (yr), sex, body weight (kg)	Clinical diagnoses*	Diet†	Plasma squalene		Fractional conversion of MVA to cholesterol§	Squalene kinetics				Cholesterol synthesis rates (mg/day)	
			Concentration (μg/dl)	Content‡ (mg)		Dose of squalene (dpm × 10^{-6})	Area under squalene curve (dpm × 10^{-3}/mg-day)	$t_{½}$ max (hr)	"Active" pool¶ (M_{Sq}) (mg)	Sterol balance method	Squalene kinetic method
1a. BE: 47, F, 92	MH, CHD, Ob, X	40% cottonseed oil FF	20.6	0.85	0.654	128.82	68.82	2.73	307	1517	1762
1b. BE: 47, F, 92	MH, CHD, Ob, X	40% cottonseed oil FF	30.9	1.28	0.621	154.54	89.43	5.19	539	1617	1626
2. AJ: 73, M, 61	NL, CHD, COPD	Solid foods	25.1	0.69	0.403	98.88	114.44	4.08	212	670	813
3. MR: 61, F, 83	HC, Ob	Solid foods	25.1	0.94	0.419	128.04	122.76	3.57	224	—	982
4. RK: 58, F, 44	HC; cholestyramine (16 g/day)	40% lard FF	13.9	0.28	0.988	194.84	91.90	2.46	314	2300	1995
5. VC: 44, F, 41	NL, AIP	40% cottonseed oil FF	19.0	0.35	0.313	266.50	337.34	2.71	129	780	743
6. RS: 57, M, 64	HC, CHD, X	Solid foods	47.1	1.35	0.469	126.03	86.09	3.30	290	996	1378
7. AA: 66, F, 69	MH, CHD, Ob, X	Solid foods	18.1	0.56	0.385	96.53	92.11	3.30	208	691	987
Mean values ± SD			25.0 ± 10.3	0.79 ± 0.39	0.532 ± 0.218			3.42 ± 0.89	278 ± 122	1224 ± 611	1329 ± 491

* Abbreviations: NL, normolipidemia; MH, mixed hypercholesterol- and hyperglyceridemia; HC, hypercholesterolemia; CHD, coronary heart disease; Ob, obesity; X, xanthomatosis; COPD, chronic obstructive pulmonary disease; AIP, acute intermittent porphyria.

† Diet: squalene-free formula feeding (FF), or weighed solid foods (low in squalene) with 3-day rotating menus. All patients were maintained at constant body weight before and during these tests, and drugs were not administered (except in Patient 4). All FFs contained 40% of total calories as fat.

‡ Total content (mg) = plasma volume (dl) × plasma concentration (μg/dl), where plasma volume (dl) = body weight (kg) × 0.45.

§ Fractional conversion of administered dose of *R*-[^{14}C]MVA to [^{14}C]cholesterol.

¶ "Active" pool of squalene = a maximum estimate of that pool directly involved in cholesterol biosynthesis.

The peak of the squalene curve was reached in 100–120 min in this and six other patients; thereafter, the decline in SA was logarithmically linear as long as radioactivity in squalene was precisely measurable (about 7 hr).

The assembled squalene kinetic data are shown in Table 1. The total mass of metabolically "active" squalene, M_{Sq}, varied from 129 to 539 mg. Since the total plasma pool of squalene is only about 1/200 of M_{Sq} or less, it is evident that radioactive squalene synthesized from mevalonate moved rapidly from sites of synthesis into the plasma. The decay exponentials, $t_{½max}$, varied from 2.46 to 5.19 hr.

Fractional conversion of MVA to cholesterol

The two plasma cholesterol SA-time curves obtained in a typical experiment are shown in Fig. 2. The upper figure illustrates the shapes of the curves from 0 to 24 hr, the lower from 0 to 80 days. (Note the different ordinates for the two sets of curves.)

The upper part of Fig. 2 shows the familiar disappearance and reappearance of labeled cholesterol given intravenously as a finely particulate emulsion: this is considered to be due to temporary sequestration of cholesterol in the reticuloendothelial system (12). In contrast, newly biosynthesized [^{14}C]cholesterol appeared rapidly, reached a peak much earlier than the intravenously administered cholesterol, and then declined. The two curves did not become parallel until about 2 weeks, after which they remained parallel for as long as they were reliably measurable (up to 1 year in other experiments). Although the forces contributing to the earlier disequilibrium are not completely understood, for present purposes it is sufficient to note that an equilibrium eventually is established, since an accurate calculation of fractional conversion of MVA to cholesterol depends on attainment of this equilibrium. In our experience to date, the two curves have always become parallel in 2 weeks or less; thus, the conversion calculation can be made at any time thereafter.

Fractional conversions of MVA to cholesterol ranged from 0.40 to 0.65 in the six patients maintained at constant body weight and not taking drugs (all except Patient 4, see below). That fraction of MVA not converted to cholesterol presumably moves into other pathways: ubiquinone, prenoic acids, fatty acids, ketones, and CO_2 (13). The conversion fractions in Table 1 correlated closely ($P < 0.01$) with cholesterol synthesis rates ($r = 0.92$ versus squalene kinetics method, $r = 0.97$ versus sterol balance method).

The exceptional patient (no. 4) had been taking 16 g of cholestyramine per day for 18 days, a drug known to increase greatly the daily synthesis of cholesterol (14); a high conversion rate (in this case, 0.988) would be expected with increased synthesis.

Squalene and cholesterol synthesis rates

Table 1 compares the rates of daily synthesis of cholesterol obtained by the present squalene method with those obtained by sterol balance methods in seven experiments in six patients. In four experiments the two sets of data agreed within 14%, and in the other three experiments, within 17–30%. In an overall comparison the cholesterol synthesis rate

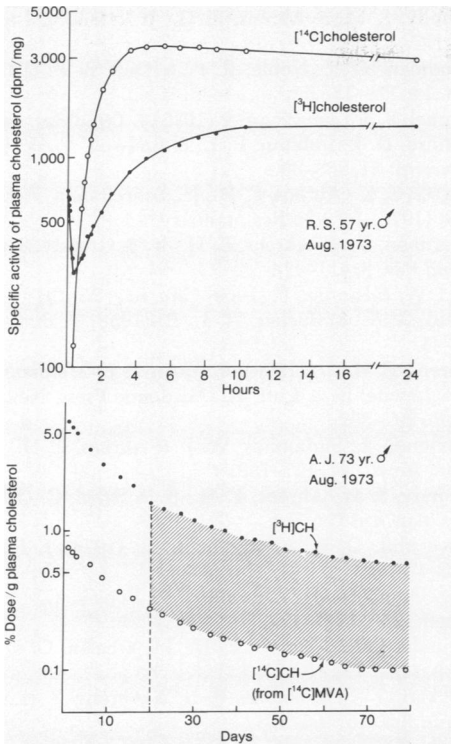

FIG. 2. (Upper) SA-time curves of [^{3}H]cholesterol (30 μCi administered intravenously at time zero) and [^{14}C]cholesterol (biosynthesized from 250 μCi of DL-[2-^{14}C]MVA given intravenously at time zero) for first 24 hr in Patient 6. (Lower) As above, but in Patient 2 and for 80 days. (Note the different ordinate expressions.)

was 8% higher by the squalene method; the means were not significantly different.

DISCUSSION

The present method of describing the kinetics of plasma squalene is based on two premises: that the diet is low in squalene (so that total daily squalene input is wholly ascribable to synthesis) and that metabolically "active" squalene in tissues equilibrates rapidly with plasma squalene. Our experimental results appear to substantiate the latter assumption, but definitive proof of the equilibrium between plasma squalene and "active" squalene in tissues awaits the performance of experiments in animals. Since previous studies in other laboratories have furnished ample evidence that squalene is an obligatory precursor of cholesterol, it seems reasonable at this point to presume that the rate of squalene synthesis in man can be equated with that of cholesterol synthesis.

Comparisons of Squalene and Cholesterol Synthesis Rates. The correspondences between the data obtained by the squalene method and by the sterol balance method were reasonably good, although the data obtained by squalene kinetics were higher than the balance data, on average.

The squalene input data would be falsely high if labeled squalene were lost through the skin. However, repeated measurements of skin surface squalene have failed to show any significant excretion of radioactive squalene (<0.1% of the dose of squalene in three studies). Falsely high results also would be found if squalene were lost in urine or feces. However, repeated assays of squalene radioactivity in feces and urine have been negative (<0.1% of dose of squalene in two studies). An untested possibility is that a small amount of labeled squalene may be sequestered in one or another tissue and is released so slowly that we fail to detect its conversion to cholesterol. In Patient 1, isolated adipocytes obtained by needle aspiration were assayed at the peak of the labeled plasma squalene curve and also 2 hr later; although the mass of squalene was consistently large, no radioactivity above background was detected.

On the other hand, there is good reason to know that the balance method consistently underestimates cholesterol synthesis by 10–15%: urinary steroids derived from cholesterol normally total as much as 50 mg/day, and losses of plasma cholesterol through the skin also may occur (in six patients this loss averaged 35 ± 8 mg/day, ref. 15). There also is a systematic loss of fecal neutral steroid sulfates: in 10 patients this averaged 26 mg/day but never exceeded 66 mg/day (6). All of these outputs are neglected in the customary application of the sterol balance method.

The mean levels of cholesterol synthesis measured in six studies by balance and by squalene kinetics agreed within 8%, although the largest individual difference (Patient 7) was 30%. When comparisons were made of large numbers of cholesterol turnover rates obtained by sterol balance methods (1) and by kinetic studies of cholesterol itself (3) (44 such comparisons have been completed in this laboratory), the means agreed within 20%; however, in individual patients the discrepancies amounted to as much as 50%. Therefore, and in view of the several known sources of error in all these methods, we feel that the comparisons shown in Table 1 warrant the tentative conclusion that MVA-squalene kinetics can give a satisfactory estimate of daily cholesterol synthesis rates. Obviously, any statistical evaluation of the new and older methods awaits the acquisition of many more experimental comparisons.

Repetition of Test. Investigators may wish to obtain a second, third, etc., set of synthesis data on the same patient, either for the sake of testing reproducibility or to gauge the effect on synthesis of a change in regimen (new diet, drug, etc.). The present procedure permits this repetition as early as 6 weeks after the first test, provided that care is taken to make appropriate deductions of residual radioactivity in cholesterol from the results obtained during the second test. Table 1 lists results obtained in two successive tests in Patient 1 that were only 4 weeks apart.

Present Uncertainties. Although the data presented have encouraged us in our search for simpler and more precise measures of cholesterol synthesis rates, a number of important variables remain to be explored. (*a*) Effect of dietary squalene: though the mean daily intake of squalene in the U.S.A. has been estimated to be less than 50 mg/day (5), certain individuals may ingest up to 200 mg/day, especially those eating diets rich in olive oil. (*b*) Results in different forms of hyperlipidemia: a strong correlation has been shown (5) between plasma triglyceride and plasma squalene concentrations, and it remains to be determined whether cholesterol synthesis rates obtained by the present procedure are valid in patients with very high triglyceride levels. (*c*) Diurnal variations in cholesterol synthesis rates: since it is well established that in rats there are marked diurnal variations in cholesterol biosynthesis (16), this possibility must also be explored in man.

Advantages and Future Applications. While the advantages of the present method have been enumerated in the final paragraph of the Abstract, the opportunity to measure

cholesterol synthesis in the unsteady metabolic state seems to us its chief attraction. By contrast, currently accepted techniques (sterol balance and cholesterol kinetic studies) demand the steady state, which in turn imposes burdens on patients and on the laboratory that are avoided in the application of the squalene kinetic method.

We anticipate that the squalene kinetic method will help us to resolve a number of dilemmas for which no other solution is presently available—dilemmas created by our inability to distinguish cholesterol synthesis from cholesterol flux. For example, during neomycin administration there is a large increase in neutral steroid excretion in feces, yet it has not been possible to distinguish what part of this is due to increased cholesterol synthesis and what part to efflux of cholesterol from tissues (17). Similar problems still exist in regard to the mode of action of clofibrate (18), β-sitosterol (19), and other drugs, and to the effects of increased caloric intake or of isocaloric exchange of carbohydrate for fat calories (20). In animal studies it may now be possible to dissect out the effects of body growth on cholesterol synthesis, since growth is characteristically an unsteady metabolic state (21). We expect that the ability to carry out repeated measurements of cholesterol synthesis at relatively short intervals by the present method will allow us to clarify some of these uncertainties.

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