Measurement of squalene in human tissues and plasma: validation and application

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Abstract A method is described for accurate and reproducible measurement of squalene in plasma, feces, urine, bile, and tissue that depends on isolation by alumina column chromatography after mild saponification and on measurement by gas-liquid chromatography. Recoveries from all tissues exceeded 80% and from plasma 96%; losses were accurately corrected by appropriate additions of squalane as an overall recovery standard.

Squalene measurements in more than 20 foodstuffs showed a 5000-fold variation, the richest source being olive oil (7 mg/g); however, the mean intake/person•day in the United States appears to be in the range of 30 mg. Squalene concentrations in more than 25 human tissues also varied widely; the highest levels were in skin (about 475 μg/g dry weight) and adipose tissue (about 275 μg/g), while only moderate amounts were found in sites of active cholesterol synthesis (liver, 75 μg/g; small intestine, 42 μg/g). There was a direct relationship between plasma levels of squalene and triglycerides but not with cholesterol. Plasma squalene levels rose strikingly with increased dietary squalene and varied directly but not consistently with cholesterol synthesis rates. The large amounts excreted in skin surface lipids are presumed to reflect de novo synthesis in the skin, rather than transfer from plasma; only trivial amounts were excreted in feces and urine.

Supplementary key words gas–liquid chromatography • internal standards • stability during storage • diet squalene • hyperglyceridemia

In 1964 Goodman (1) demonstrated the presence of small amounts of metabolically active squalene in human and rat plasma, with most of the labeled squalene transported in the very low density (d <1.019) fraction after intravenous administration of [2-^{14}C]mevalonate. He proposed that the small plasma pool of squalene (about 35 μg/dl) was in rapid equilibrium with a much larger squalene pool in the liver, estimated by Langdon and Bloch (2) to be about 25 μg/g wet weight. Goodman's paper summarized the literature on the natural occurrence of squalene in various oils, as well as its important place in the biosynthesis of cholesterol.

Since 1964 a number of interesting reports have appeared in regard to squalene metabolism, notably the isolation of sterol-carrier proteins from liver that are believed to transport squalene as well as a wide range of sterols within the liver cell (3–5), the finding of increased plasma levels in the febrile phase of human Hong Kong influenza (6), the identification of squalene in human arterial atheromata (7), and the finding by Miettinen that levels of cholesterol precursors in plasma (C_{28-29} sterols and squalene) rise with increasing rates of whole body synthesis of cholesterol (8, 9). A recent report from this laboratory (10) has documented the very sizable secretion of squalene by the human skin along with other waxes, alcohols, and sterols: in five patients the daily secretion of squalene from the total skin surface ranged from 125–475 mg/day.

The present study was prompted largely by our efforts to devise a method for the measurement in man of total body synthesis of cholesterol in the *unsteady state*, a pressing need in certain studies of the mechanisms of action of hypocholesterolemic drugs and diets. On theoretical grounds it seemed reasonable to predict that the measurement of squalene synthesis rates would satisfy this need, in view of the rapidity of squalene synthesis and the small pool size of metabolically active squalene: the basic assumption was that squalene synthesis would equal cholesterol synthesis. Experiments along these lines are now in progress (11), but as support for the basic underlying premises it was clear from the onset that we needed considerably more detailed information about squalene metabolism in man than was available in the literature.

The present report describes the validation of a simplified method for measuring squalene concentrations in various body fluids and tissues. We also present (*a*) the results of applying this method to the measurement of squalene in a number of common foodstuffs; (*b*) plasma levels in relation to squalene intake and to plasma cholesterol and triglyceride concentrations; (*c*) concentrations in a wide variety of human tissues; (*d*) relationships between plasma squalene levels and rates of cholesterol synthesis; and (*e*) routes of excretion of squalene.

MATERIALS

Standards

Squalene and squalane were purchased from Eastman Kodak Company, Rochester, N. Y. Squalene ($C_{30}H_{50}$, mol

Abbreviations: GLC, gas–liquid chromatography

wt 410.70; 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) was found to be 97.5% pure by proton nuclear magnetic resonance, gas–liquid chromatography (GLC) and mass spectrometry; the major impurity was pristane, with trace amounts of squalane. Squalane ($C_{30}H_{62}$, mol wt 422.80; 2,6,10,15,19,23-hexamethyltetracosane) also was more than 97% pure by the above criteria. As an internal GLC standard, we used 5α-cholestane (Steraloids, Inc., Pawling, N.Y.). Over the last 15 years' use this standard has proven to be 97–99% pure; the impurities in this standard, which do not appear on GLC, have not been identified.

Stereospecifically tritiated squalene was prepared for us by Dr. George Popják, University of California at Los Angeles, by incubation of [5-^{3}H]mevalonate (New England Nuclear, Boston, Mass.) in a rat liver homogenate, as previously described by him (12). It had a specific activity of 214 μCi/μmole, and was radiochemically pure by thin-layer and gas–liquid radiochromatography.

Sources of biological samples

Plasma was obtained from several sources: freshly pooled plasmas from 10 supposedly healthy blood donors, generously furnished by the New York Blood Center (Dr. Carlos Ehrich), and individual, fresh specimens from patients under study in the metabolic ward of this Hospital. Feces samples were obtained from patients engaged in metabolic studies. Bile was obtained from the gall bladder at surgery through the cooperation of Dr. William Nickel, New York Hospital. Tissue specimens also were collected at surgery by Dr. Nickel, but the majority of these specimens were furnished by the New York City Medical Examiner's Office from apparently undiseased victims of sudden accidental death (these specimens were collected within 24 hr of death). Food samples were obtained by purchase in the open market.

ANALYTICAL PROCEDURE

After mild saponification, the nonsaponifiable materials were extracted with petrol ether and subjected to alumina column chromatography for clean separation of hydrocarbons from sterols. Squalene was measured by GLC of the hydrocarbon fraction. The lowest practical detection limit was found to be about 10 ng/ml plasma. Specific details are given in the following paragraphs.

Plasma or bile (2–4 ml), feces (1 g of a pipettable homogenate), or tissue (100–200 mg wet weight) were mixed in a 50 ml glass stoppered centrifuge tube with 10 ml of methanol–KOH. For plasma, bile and fecal homogenates the final concentration of KOH was 10% (w/v), of methanol 70%. For tissue analyses, 3 ml of distilled water were added and the final concentration of KOH was 20%, of methanol 70%. A known amount of squalane, the overall recovery standard (alternatively, [^{3}H]squalene), was added at that stage, prior to saponification at 70°C for 2 hr. The nonsaponifiable fraction was recovered by three extractions with 20 ml of petrol ether (bp 30–60° C) (*n*-hexane for bile), which were pooled and concentrated on a rotary evaporator before being transferred quantitatively with petrol ether to an 8 × 1 cm alumina column.

For preparation of these columns 5 g of alumina oxide (neutral Alumina, AG 7, 200-mesh, Bio-Rad Laboratory, Richmond, Calif.) was heated overnight at 105° C, then reduced in activity by addition of 50 μl distilled water. Squalene (and squalane) were eluted with 50 ml of petrol ether. To recover cholesterol and other sterols it was necessary to elute subsequently with chloroform (100 ml); in tests with [^{3}H]-squalene and [^{14}C]cholesterol, there was no cross contamination of the hydrocarbon and sterol fractions.

After addition of a known amount of 5α-cholestane (about 2 μg for plasma, 20–200 μg for bile and tissues) to the hydrocarbon fraction as a GLC recovery standard, the fraction was concentrated to near dryness by rotary evaporation and quantitatively transferred to a 15 ml stoppered vial; final drying of the specimen for application to a GLC column was carried out in a nitrogen stream. Six-foot columns (4 mm ID) of 1% Dexsil 300 on 100–200 mesh Supelcoport (Supelco, Inc., Bellefonte, Pa.) held at 235° C (flash heater 265° C, detector 255° C) had a resolving power of 2800 plates for squalene; nitrogen was used as carrier gas with an inlet pressure of 30 psi. Under these conditions the retention times relative to squalane (=1.00) were: squalene 1.55, 5α-cholestane 2.07, and cholesterol 4.92. A Packard gas chromatograph with hydrogen flame ionization detector, Model 7600, was used, coupled to a digital integrator for quantitation (Hewlett Packard Co., Palo Alto, Calif., Model 3370B).

Radioactivity measurements with external quench corrections were made on separate aliquots of the hydrocarbon fraction in a Packard Tri-Carb liquid scintillation spectrometer, Model 3390, with an absolute activity analyzer (Packard Model 544).

Calculations

The amount of squalene in the sample is calculated in terms of the relative areas of the squalene and squalane peaks in the final GLC analysis, where squalane was added at the outset as an overall recovery standard:

$$\text{Squalene in sample } (\mu g) = \frac{\text{squalene area}}{\text{squalane area}} \times \mu g \text{ squalane added}$$

The recovery of squalane through the several purification steps is calculated in terms of the relative areas of squalane and of 5α-cholestane added as a second recovery standard immediately prior to GLC analysis:

$$\%\text{ recovery of squalane} = \left[\left(\frac{\text{squalane area}}{\text{5α-cholestane area}} \times \mu g \text{ 5α-cholestane added} \right) \div \mu g \text{ squalane added} \times 100 \right]$$

Precautions

The high rate of secretion of squalene by the human skin

TABLE 1. Linearity of GLC response to squalene using 5α-cholestane and squalane as internal standards[a]

Compound of Interest	Internal Standard	Recovery	
μg added	*μg added*	*μg ± SD*	%
Squalane recovery with 5α-cholestane as standard			
1.93	3.99	1.89±0.02	97.9
3.85	3.99	3.86±0.01	100.2
5.78	3.99	5.70±0.02	98.6
7.70	3.99	7.94±0.02	103.1
11.57	3.99	11.82±0.01	102.1
19.25	3.99	19.31±0.00	100.2
38.50	3.99	38.89±0.01	101.0
Squalene recovery with 5α-cholestane as standard			
1.91	3.99	1.87±0.01	97.9
3.82	3.99	3.80±0.00	99.5
5.73	3.99	5.74±0.01	100.2
7.64	3.99	7.86±0.02	102.8
11.46	3.99	11.46±0.01	99.0
19.10	3.99	19.17±0.00	100.3
38.20	3.99	38.42±0.01	100.5
Squalene recovery with squalane as standard			
1.91	3.85	1.87±0.01	97.9
3.82	3.85	3.85±0.03	100.8
5.73	3.85	5.58±0.02	99.4
7.64	3.85	7.58±0.02	99.2
11.46	3.85	11.57±0.01	100.9
19.10	3.85	19.17±0.03	100.3
38.20	3.85	38.28±0.02	100.2

[a] All analyses were performed in triplicate.

leads to gross contamination of laboratory glassware unless exceptional care is taken to render every item squalene-free. Only after some experience is gained with the method is it feasible not to use polyvinyl gloves prerinsed with petrol ether.

After routine cleaning of glassware, including that in which biologic samples are to be collected, we sonicate (Crest Ultrasonics Corp., New York, N. Y.) for 3 min in detergent; then, after rinsing away the detergent in distilled water, we sonicate once again in distilled water before air-drying. Immediately prior to use of the glassware, it is rinsed in chloroform–methanol 2:1 and then air-dried.

Oxidation of squalene during the analytical procedure has not proven to be troublesome, since all evaporations are carried out at room temperature and finally under N_2. Repeat analyses of samples of plasma and feces after storage periods at 4° C up to 6 months have shown no significant changes, suggesting that squalene in biologic materials is resistant to oxidation under these conditions.

VALIDATION OF ANALYTICAL PROCEDURE

The usefulness of the procedure depends on the demonstration that the GLC responses of squalene, squalane, and 5α-cholestane are linear in proportion to weight over a wide range. Evidence to support this conclusion is shown in **Table 1**, where recoveries of the three materials are related to each other over 20-fold concentration ranges. Each mixture of two compounds was analyzed in triplicate. It can be seen that coefficients of variation never exceeded 1% and that the percentage recoveries in the three sets of comparisons were very close to 100. Further evidence on reproducibility is given in **Table 2,** in which replicate analyses were carried out on biologic materials to which [^{3}H]squalene as well as squalane were added as recovery standards. The coefficients of variation for plasma never exceeded 2%. It is seen that the recoveries of radioactive squalene and of squalane were essentially the same. This suggests that squalene was not selectively lost or oxidized during the analysis; that the tritium label was not exchangeable during the procedure; and thus that squalane is an ideal recovery standard. The recoveries of internal standards were only about 88% in analyses of feces and 82% for bile, as contrasted to 96% or more in plasma; nevertheless these were high enough, in our estima-

TABLE 2. Reproducibility of squalene analysis in plasma, bile and feces (mean values ± SD)

Material Analyzed	n	Recovery of Radioactive Internal Standard[a]	Recovery of Squalane Relative to 5α-cholestane[a]	Squalene Concentration (Corrected for Recoveries)
		%	%	*μg/dl*
Plasma				
fresh pool[b]	16	97.8±2.44	96.1±2.09	26.2± 1.58
stored pool[c]	4		97.1±1.18	26.4± 1.81
Bile[d]	4		81.8±5.50	103.9±10.9
Feces[e]	4	88.2±2.35	87.5±2.30	4.75± 0.05 (μg/g wet weight)

[a] [^{3}H] squalene and/or squalane were added as overall recovery standards; 5α-cholestane as GLC internal standard.

[b] This single pool consisted of plasma from freshly drawn bloods of ten donors; generously furnished by the N.Y. Blood Center.

[c] Same as (*b*) but analysis after storage at 4° C for 6 months.

[d] Gallbladder bile samples obtained during exploratory laparotomy in four patients without hepatic-biliary disease.

[e] Stool collections under steady state conditions in four patients fed squalene-free diets (13).

TABLE 3. Squalene content in various foods

(μg/g wet weight, mean of duplicate analyses)

	Food Fat		
Olive oil[a]	1989–6855	Safflower oil	37.2
Cod liver oil	520.3	Cottonseed oil	27.8
Corn oil	278.9	Lard	22.5
	Meats		
Sweet breads	27.8	Kidney	18.7
Beef	20.3	Liver	18.5
	Poultry		
Chicken		Duck	
Skin	29.6	Skin	14.0
Dark meat	16.4	Dark meat	20.5
White meat	31.5	White meat	36.4
Turkey		Egg yolk	47.0
Skin	22.3		
Dark meat	29.5		
White meat	13.7		
	Fish		
Halibut	96.9	Tuna (canned in polyunsaturated vegetable oil)	30.0
Flounder	50.0		
	Dairy Products		
Butter (Hotel Bar)	61.3	Margarine (Kraft's Parkay)	16.9
Cheese		Milk	
Parmesan	95.5	Whole homogenized	2.4
Munster	34.0	Skim	1.5
	Fruit, Vegetable, Nuts		
Avocado	1.9	Almond	13.2
Artichoke (heart or leaf)	1.7	Peanut	22.3
Cashew	9.5	Pistachio	15.1
		Walnut	0.9
	Typical USA diet[b] 24–38 mg/2000 calories		

[a] Samples of three brands were analyzed in duplicate.

[b] Homogenate of foodstuffs designated by a USDA survey (1965) to be representative of mean intake/person•year in the USA population (Table 6-1 in Ref. 14).

tion, to give confidence in any final results corrected by reference to the recoveries of appropriate internal standards.

The squalene contents of pooled plasmas, fresh and after 6 months' storage at 4° C, were almost identical. This finding suggests that *retrospective analyses of squalene are reliable, so long as uncontaminated glassware is used throughout and samples are stored at 4°C.*

APPLICATION OF THE METHOD

In the following section we will describe a few applications of the method in assessing certain parameters of squalene metabolism in man. The results to be presented are intended to be illustrative rather than comprehensive.

Squalene levels in foods

The primary source of commercial squalene is shark liver oil, in which concentrations of 50–80% are reached. (To our knowledge, shark liver is not eaten by man, even in the Orient.)

TABLE 4. Plasma squalene concentration in patients fed diets containing various amounts of squalene

	Squalene-Free Diet[a]	High-Squalene Diet[b,c]
	μg squalene/dl plasma ± SD	
Patient, A. G. 53 yr male hyperglyceridemia	64 ± 1.6 (n = 3)	4555 ± 12.3[b] (n = 3)
Patient, R. B. 59 yr female hypercholesterolemia	24 ± 1.3 (n = 3)	129 ± 30.4[b]; (n = 4) 896 ± 19.8[c] (n = 6)
Patient, E. H. 29 yr female hypercholesterolemia	36 ± 16.8 (n = 6)	79 ± 20.7[c] (n = 5)

[a] All diets were eucaloric in that body weight was maintained with less than 2% variation during steady state periods of 2–6 weeks' duration. The composition was liquid formula with squalene-free cottonseed oil comprising 45% of total daily calories.

[b] This formula diet contained olive oil as 45% of total calories. Squalene is endogenous in olive oil and the daily squalene intake was 692 and 375 mg per day in patients A.G. and R.B., respectively.

[c] These plasma squalene concentrations were reached after feeding the cottonseed oil formula with added squalene. Daily squalene intakes were 350 and 369 mg, respectively, in patients R.B. and E.H.

Table 3 lists the concentrations of squalene in various foodstuffs that are prominent in the diets of the USA population. Of the food fats, olive oil is uniquely rich in squalene, but cod liver oil and corn oil contain significant quantities also. Poultry, red meats, and organ meats have about the same content, whereas halibut and flounder are somewhat richer in squalene.

A more useful benchmark is our finding that the average squalene intake of the USA population approximates 24 mg per 2000 calories per person per day. This figure is based on analyses of a homogenate of the foods most commonly eaten in this country, prepared with regard to the items and quantities of each that were identified in a survey published in 1965 by the U.S. Department of Agriculture (summarized recently in Table 6-1 of ref. 14).[1]

Salad oils constituted 4.7% of total edible fat in this tabulation; in the homogenate we prepared, corn oil was selected as the salad oil. Had olive oil been taken instead of corn oil, the squalene content of this homogenate could have reached 38 mg/2000 calories.

Clearly, the daily squalene intake in this country must vary widely, depending on individual food preferences. However, a 2000-calorie diet rich in fish and salads dressed with olive oil could furnish as much as 200 mg squalene, suggesting this as a tentative maximum intake for man, except under highly artificial conditions (see below).

Plasma levels and their determinants

Table 2 shows that human plasma pooled from 10 healthy

[1] An analysis of this diet homogenate, in terms of major and minor nutrients, minerals, vitamins, etc., has been completed recently in this laboratory; a manuscript is in preparation.

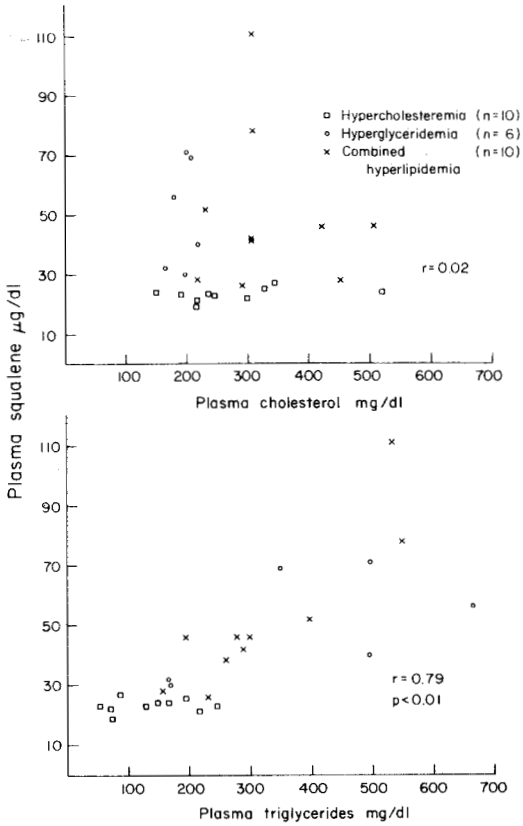

Fig. 1. Mean levels of plasma squalene, cholesterol and triglycerides in 26 patients held at constant body weight on low squalene or squalene-free diets. 138 analyses were made: in four patients single determinations were carried out, but in 18 instances one point represents the mean of more than 5 analyses made in a single patient on a variety of dietary and drug regimens. There was no correlation between plasma squalene and cholesterol concentrations. However, squalene and triglyceride concentrations were linearly correlated with an r value = 0.79, $P < 0.01$.

blood donors eating ad libitum (presumably their diets contained about 25 mg squalene per 2000 calories, Table 3) contained 26 µg squalene/dl plasma. Individual analyses of plasmas from 26 healthy fasting normolipidemic donors on ad libitum diets revealed 33.7 ± 11.0 µg squalene/dl plasma (mean ± SD), with a range of 18.0 to 72.4 µg/dl. **Table 4** shows that three patients fed much larger squalene intakes had substantially higher plasma squalene levels. In addition there was considerable person-to-person variation, the causes of which remain to be explored. However, it is noteworthy that Patient 1 had marked hyperglyceridemia at the time of this experiment, his plasma glycerides averaging 1000 mg/dl, total cholesterol 200 mg/dl; by contrast, Patients 2 and 3 had triglyceride levels of 85 and 60 mg/dl, respectively. Whether the high squalene levels achieved by Patient 1 simply reflect a solvation effect in the triglyceride core of his elevated very low-density lipoprotein fraction, or some other peculiarity of absorption and/or disposal of dietary squalene, remains to be explained.

Analyses of 138 plasma samples taken from 26 patients at various stages in medical management of various forms of hyperlipidemia showed (**Fig. 1**) that squalene levels were distinctly lower in hypercholesteremia (<35 µg/dl) than in hyperglyceridemia or mixed hyperlipidemia. There was a strong correlation between plasma triglyceride and squalene concentrations, though none with plasma cholesterol levels.

Squalene levels in human tissues

A wide variety of human tissues has been examined for their squalene and cholesterol concentrations. **Table 5** presents the data; they are too few to warrant statistical analysis, but serve to give some tentative impressions of the relative amounts in different tissues. Since we could see no differences between analyses of tissues obtained at autopsy or at surgery, these data have been combined. Unfortunately, it was not practical to measure coincident plasma levels.

The data show a very wide variation in squalene concentration and in squalene/cholesterol ratios. Of all tissues, skin had the highest squalene level (on a dry weight basis), which is not surprising in view of the very sizable secretion of squalene by the sebaceous glands of the skin that can amount to 125–475 mg/day/person (10). Of the other organs and tissues, adipose tissue was the second richest in squalene, standing in a class by itself; since cholesterol synthesis in human adipocytes is exceedingly low (15), this finding suggests sequestration of squalene due to solvation. The third group, all with concentrations above 100 µg/g dry weight, consisted of lymph node, arterial intima, myocardium, skeletal muscle, and kidney. The contrast between arterial intima and media is striking for both squalene and cholesterol. Among the remaining tissues that contained less than 100 µg/g, it is interesting to find liver and small intestine, the same organs in which cholesterol synthesis is considered to proceed most vigorously.

In the 27 tissues, the squalene/cholesterol ratio (µg/mg) ranged from essentially zero (erythrocytes) to nearly 100 (adipose tissues). In 19 of 27 tissues this ratio was 6.5 (liver) or less. In an intermediate group of three tissues the ratios varied from 14 to 19 (lymph node, pancreas, myocardium), while three other tissues had strikingly higher ratios: skeletal muscle 42, skin 65, adipose tissue 90–97. The high ratio in adipose tissue lends to the idea that squalene may be sequestered with triglyceride by solvation. On the other hand, the tissues characterized by highest cholesterol synthesis capacities did not have high squalene/cholesterol ratios (liver 6.5, small intestine 3.9), except for skin.

Plasma levels and cholesterol synthesis rates

Miettinen (8, 9) showed that in man increased cholesterol synthesis rates were reflected by increased levels of C_{28-29} methyl sterols; in rats, plasma squalene levels also were increased. We attempted to extend his findings by analyzing the plasma levels of squalene in outpatients involved in a double-blind trial of cholestyramine vs. placebo (conducted by Dr. Richard Hoffman, Staten Island Hospital, Staten Island, New York, whose cooperation is gratefully acknowledged). We expected to find higher squalene levels during

TABLE 5. Squalene and cholesterol concentrations in 27 human tissues

	n	Squalene Concentration				Cholesterol Concentration				Squalene (μg)/ Cholesterol (mg)
		μg/g wet weight		μg/g dry weight[a]		mg/g wet weight		mg/g dry weight[a]		
Bulk tissues		*mean*	*range*	*mean*	*range*	*mean*	*range*	*mean*	*range*	*mean*
Adipose tissue										
Subcutaneous fat	3	309.9	164.0–473.3	389.9	266.2–555.1	1.6	1.5–1.7	1.8	1.5–2.0	96.7
Abdominal fat	4	159.0	91.0–280.5	178.5	106.7–309.3	1.3	1.3	1.5	1.5	89.8
Skin	4	148.4	65.3–328.8	478.1	208.0–1067.0	1.1	1.0–1.3	3.5	3.1–4.0	64.5
Arterial wall										
Intima	5	40.0	29.9–53.3	134.5	58.0–199.8	14.5	2.6–26.6	46.2	11.6–87.0	4.6
Media	8	3.2	0.9–7.6	12.0	3.7–28.9	3.7	1.5–8.8	14.4	5.6–38.5	1.0
Skeletal muscle	4	25.0	15.8–31.2	114.4	68.5–138.5	0.6	0.5–0.8	2.8	2.3–3.4	42.0
Connective tissue										
Dura mater	6	11.1	2.8–17.0	81.1	10.1–194.8	1.6	1.2–2,1	7.8	5.6–13.6	4.7
Biceps tendon	4	7.2	1.7–15.3	21.0	4.5–46.2	2.6	0.4–4.7	7.5	1.1–13.9	3.4
Falx cerebri	8	6.3	1.2–13.7	24.1	5.9–62.3	9.8[b]	2.1–17.2	38.8	8.1–58.6	0.7
Erythrocytes	2	0.2	0.1–0.4			1.2				
Endrocrine organs										
Adrenal	4	22.1	16.1–31.8	64.9	48.7–95.8	84.3	33.3–114.5	234.8	100.0–350.7	0.4
Testis	2	7.1	6.2–8.2	43.0	38.7–47.3	3.2	2.9–3.5	19.7	18.5–20.8	2.2
Thyroid	4	4.7	3.4–7.6	20.8	14.4–31.1	1.8	1.2–2.3	7.8	6.0–10.7	2.7
Ovary	2	1.4	0.8–1.9	8.1	4.5–11.6	2.3	1.5–3.0	13.5	8.1–18.8	0.6
Other organs										
Lymph nodes	3	52.2	36.8–78.1	140.3	103.4–150.7	4.2	2.4–5.9	15.0	4.0–23.7	16.0
Pancreas	4	29.9	6.8–68.4	97.9	21.0–190.6	2.4	2.2–2.8	9.2	6.2–10.7	14.3
Myocardium	5	24.4	20.6–29.4	117.4	105.3–136.7	1.3	1.2–1.4	6.2	5.8–6.8	19.1
Liver	5	21.8	17.4–27.9	75.1	56.3–92.3	3.6	2.8–5.6	12.4	9.9–18.8	6.5
Large intestine	2	19.5	19.0–19.9	71.1	58.1–84.1	1.9	1.9	7.7	5.7–9.7	10.4
Kidney	5	18.4	12.9–23.1	100.8	70.8–121.7	3.1	2.6–3.9	17.3	15.1–19.7	5.8
Gall bladder	3	9.1	4.6–15.2	32.7	25.5–43.0	2.9	1.8–3.6	10.8	9.7–12.6	3.0
Small intestine	2	8.2	8.0–8.3	42.2	41.1–43.4	2.1	2.0–2.2	11.0	10.6–11.4	3.9
Cerebellum	3	7.3	4.7–12.0	43.7	28.3–72.6	12.9	8.0–18.3	69.0	50.8–80.9	0.4
Prostate	1	6.8		31.0		2.3		12.8		2.6
Cerebrum	3	5.6	3.1–8.0	22.7	14.5–30.9	19.3	18.3–20.0	85.8	75.1–96.4	0.2
Lung	3	5.3	2.5–9.3	30.2	14.8–53.0	3.4	3.2–3.7	19.4	17.4–21.3	2.7
Uterus	3	1.9	1.7–2.0	10.4	8.2–11.6	2.2	1.5–2.9	12.1	7.4–16.8	0.9
Spleen	3	1.7	1.2–2.2	7.3	4.9–10.9	3.9	3.8–4.1	18.8	18.0–19.8	0.4

[a] Dried at 60° C for 3 days or until no further weight was lost.
[b] mg/ml packed cells; from Farquhar, J. W. 1962. Human Erythrocyte Phospholipids. *Biochem. Biophys. Acta.* **60**: 80–89.

cholestyramine treatment, since this drug is known to cause a marked increase in cholesterol synthesis (16), and indeed 18 patients given the drug had a mean plasma squalene level of 84 μg/dl (SD ± 110), while 9 other patients on placebo medication had a mean level of 56 ± 28 μg/dl. It is obvious that the range of variation in both groups was very large, due perhaps to the uncontrolled (and presumably different) levels of squalene intake among these outpatients.

However, under conditions where dietary intakes of squalene are negligible (as can be achieved with formula feeding regimens), it is possible that plasma squalene levels may vary directly with cholesterol synthesis rates. Some support for this has been shown in Fig. 1: since synthesis rates have been shown to be higher in some patients with hyperglyceridemia than in those with hypercholesteremia (17–19), it is relevant to find that there was a direct correlation between squalene and triglyceride levels in these patients fed squalene-free formulas. Low squalene concentrations were seen consistently in hypercholesteremic patients and in successfully treated hyperglyceridemic patients.

Hypocaloric diets and fasting are thought to cause a reduction in cholesterol synthesis (20). Six patients studied during weight maintenance and then during weight reduction on hypocaloric diets (squalene-free) showed a fall in mean plasma squalene levels from 32.9 ± 5.8 μg/dl (n = 11) to 24.1 ± 8.5 μg/dl (n = 12) ($P < 0.005$). These changes were independent of plasma triglyceride changes: significant increases in glyceride levels occurred in two patients, decreases in two, and insignificant changes in two.

Excretion of squalene

Reference has been made to previous work in this laboratory that defined the large excretion of squalene by the human skin (10). In a subsequent related report (21) it was shown that a major proportion of the cholesterol secreted by the skin is made there de novo, in contrast to being transferred through the skin from plasma. Thus, it seems likely that the skin secretion of squalene also reflects de novo synthesis rather than transfer from the plasma.

The data in Table 2 showed that squalene levels in bile were much higher than in plasma, and random samples of feces of patients on squalene-free diets also contained squalene. In order to estimate total excretion of squalene in feces, and as a background for future studies of squalene absorption, we measured the daily excretion rates of fecal squalene in three patients at constant body weight under controlled conditions on squalene-free, normal-cholesterol diets (two stool collections each). On weighed low-squalene solid foods, S.H. excreted 1.46 and 1.70 mg squalene per day; A.J. 2.95 and 2.58 mg/day; B.E., who was maintained solely on a squalene-free diet, excreted 0.66 and 0.44 mg/day. Whether squalene is degraded in its passage through the intestinal tract of man cannot yet be stated with certainty, but in vitro incubations of fresh human feces with labeled and unlabeled squalene/squalane mixtures have shown no changes in the ratio of squalene to its indicator.

We have analyzed pooled urine samples from four patients for squalene radioactivity at appropriate intervals after the intravenous administration of 250 μCi [2-^{14}C]mevalonate. Although plasma specific activity usually rose over 300,000 dpm/mg, there were no detectable counts found in any of the urinary nonsaponifiable fractions.

DISCUSSION

The method described for measurement of squalene mass (and specific activity, where appropriate labeling has occurred) appears to be accurate and reproducible, provided certain precautions are taken to avoid contamination by squalene secreted from skin. We believe it represents a significant improvement in precision over previous nonspecific methods based on isolation and iodimetry (22); it is somewhat simpler than the methods used by Brooks (7) and Goodman (1); the isolation procedure resembles that of Eidinoff et al. (23). Recoveries of squalene from biologic samples exceeded 82% in all cases, and with the use of internal standards the appropriate corrections could be made easily and accurately.

A number of applications of the method have been described, but much more remains to be undertaken if we are to gain a better understanding of the metabolism in man of this key intermediate in biosynthesis of cholesterol. The data in Table 5 demonstrate the widespread occurrence of squalene in almost all body tissues; a minimum estimate of total body squalene (based on the concentrations found in discrete organs and bulk tissues but exclusive of nervous and vascular systems) could reach 2.6 g. Yet, our studies of squalene kinetics (11) indicate that the size of the pool of metabolically active squalene in man (i.e., that which is rapidly converted to cholesterol) is only about 300 mg. This finding that the equilibration between the active and inactive pools of squalene must be extremely sluggish simply confirms the earlier studies of squalene compartments in rat liver by Popják (24) and by Loud and Bucher (25) which indicated that the active squalene pool was very small in comparison with the inactive pool. Why this disequilibrium exists remains to be explained. The transfer of squalene across certain membranes also must be very slow: at the peak of the radioactive squalene curve following intravenous administration of [^{14}C]mevalonate we found no radioactivity and very small mass in the squalene fraction of washed erythrocytes, and in aspirated adipocytes no radioactivity despite a very significant mass of squalene.

We are currently investigating the possible usefulness of measuring cholesterol synthesis rates in the unsteady metabolic state by determining the kinetics of squalene turnover in the plasma (11). The data in the present report force us to investigate, in the future, the reasons for the high content of squalene in gallbladder bile, the rate of absorption of dietary squalene and the degree of its degradation during transit through the intestinal canal, the mode of transport of squalene in plasma lipoproteins and in bile, and the effects of absorbed squalene on rates of synthesis of cholesterol. If absorbed squalene reaches the active sites of cyclization of squalene to lanosterol, thus increasing total daily cholesterol synthesis, as suggested by Channon's early work (26), it will be of interest to determine whether this endogenously produced increment of biosynthesized cholesterol exerts an effect on the negative feedback control of cholesterol synthesis, so far demonstrated in man only for exogenous cholesterol (27, 28).

ADDENDUM

Two additional references to squalene contents of foods have been noted since this paper was completed. Fresh tuna meat was reported to contain 0.014 mg squalene per g wet weight, mackerel and bonito 0.011, mullet 0.002 (R. W. Lewis. 1971. Squalene distribution in fish with normal and pathologically fatty livers. *Int. J. Biochem.* **2**: 609–614); avocado pear (skinned) 0.044 mg/g wet weight; eggplant 0.0024; carrots, apple, squash, banana, peas, mushroom 0.00012 or less (R. W. Lewis. 1972. Squalene content of plant tissues. *Phytochemistry.* **11**: 417–419).

There is no consensus whether human atheromata contain an elevated content of squalene. Brooks et al. (7) reported 1000 μg/g wet weight in aortic plaques, whereas a recent report by Lewis (R. W. Lewis. 1975. Squalene content of atheromatous plaques. *Atherosclerosis.* **22**: 637–640) noted no values higher than 1.3 μg/g in plaques, compared to 0.46 in normal aorta. In our study (Table 5) we report 40 μg squalene per g wet weight of normal aortic intima, a 100-fold higher concentration than that recorded by Lewis, but only one-twenty fifth that reported by Brooks et al. (7).

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