Metabolic variables of cholesterol during squalene feeding in humans: comparison with cholestyramine treatment[1]

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Abstract Squalene, a key intermediate of cholesterol synthesis, is present especially in olive oil. Regulation of cholesterol metabolism by dietary squalene in man is unknown, even though olive oil users in Mediterranean areas have low serum cholesterol levels. We have investigated absorption and serum levels of squalene and cholesterol and cholesterol synthesis with the sterol balance technique and serum levels of cholesterol precursors in humans during squalene feeding (900 mg/d for 7–30 days). The results were compared with those during cholestyramine treatment. Fecal analysis suggested that about 60% of dietary squalene was absorbed. Serum squalene levels were increased 17 times, but serum triglyceride and cholesterol contents were unchanged. The squalene feeding significantly ($P<0.05$) increased serum levels of free (1.7–2.3 times) and esterified (1.9–2.4 times) methyl sterol contents, while elevations of free and esterified Δ^8-cholestenol and lathosterol levels were inconsistent. Cholestyramine treatment modestly augmented free methyl sterol levels (1.3–1.7 times), less consistently than those of esterified ones, while, in contrast to the squalene feeding, serum contents of free and esterified Δ^8-cholestenol and lathosterol were dramatically increased (3.3–8 times). Neither of the treatments significantly affected serum plant sterol and cholestanol levels. The squalene feeding had no consistent effect on absorption efficiency of cholesterol, but significantly increased (paired t-test, $P<0.05$) the fecal excretions of cholesterol and its nonpolar derivatives coprostanol, epicoprostanol, and coprostanone (655 ± 83 SE to 856 ± 146 mg/d) and bile acids (212 ± 24 to 255 ± 24 mg/d), indicating an increase of cholesterol synthesis by about 50%. We suggest that a substantial amount of dietary squalene is absorbed and converted to cholesterol in humans, but this squalene-induced increase in synthesis is not associated with consistent increases of serum cholesterol levels. The clearly increased serum contents of esterified methyl sterols may reflect stimulated tissue acyl CoA: cholesterol acyltransferase (ACAT, EC 2.3.1.26) activity during squalene feeding as these sterols are not esterified in serum. —**Strandberg, T. E., R. S. Tilvis, and T. A. Miettinen.** Metabolic variables of cholesterol during squalene feeding in humans: comparison with cholestyramine treatment. *J. Lipid Res.* 1990. **31:** 1637–1643.

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Low prevalence of coronary heart disease (CHD) in Mediterranean countries has been connected with high intake of olive oil in this region (1). Besides monounsaturated fatty acids, olive oil also contains substantial amounts of squalene, the intake of which can reach up to 200 mg/day (2). Squalene is a key intermediate of cholesterol synthesis and when absorbed may be converted to cholesterol. In fact, earlier studies have shown that dietary squalene is converted to cholesterol in experimental animals (3–5) and that it also inhibited the activity of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase (EC.1.1.1.34) (6). Preliminary findings also indicate that in humans dietary squalene can enhance cholesterol synthesis and modify receptor activity for LDL apoB metabolism (7). Thus, individuals on a squalene-rich diet may actually resemble those on a cholesterol-rich diet, which makes the connection of rich olive oil consumption with the low CHD prevalence interesting (1). On the other hand, it is possible that endogenous cholesterol formed from squalene differs from exogenous cholesterol in biliary secretion and influence on LDL receptor activity (7).

Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; campesterol, cholest-5-en-24-methyl-3β-ol; cholestanol, 5α-cholestan-3β-ol; cholesterol, cholest-5-en-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; dihydrolanosterol, 4,4,14α-trimethyl-5α-cholest-8-en-3β-ol; $\Delta^{8,24}$-dimethylsterol, 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol; Δ^8-dimethylsterol, 4,4-dimethyl-5α-cholest-8-en-3β-ol; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; lanosterol, 4,4,14α- trimethyl-5α-cholest-8,24-dien-3β-ol; Δ^8-cholestenol, 5α-cholest-8-en-3β-ol; HMG-CoA, hydroxymethylglutaryl-coenzyme A; lathosterol, 5α-cholest-7-en-3β-ol; LCAT, lecithin:cholesterol acyltransferase; Δ^8-methostenol, 4α-methyl-5α-cholest-8-en-3β-ol; methostenol, 4α- methyl-5α-cholest-7-en-3β-ol; sitosterol, cholest-5-en-24-ethyl-3β-ol; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein.

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As there are few previous data on the effects of squalene feeding in humans (2, 7, 8), we designed the present experiments to explore short-term effects of dietary squalene on cholesterol metabolism in humans. The effects of squalene were compared with those of cholestyramine, which is known to increase the activity of HMG-CoA reductase and cholesterol production by a different mechanism, i.e., by bile acid malabsorption-induced depletion of hepatic cholesterol. Thus, we could detect possible differences between excess exogenous (dietary) squalene causing suppression of HMG-CoA reductase activity but stimulation of overall synthesis of cholesterol, and excess endogenous squalene formed through the stimulation of HMG-CoA reductase activity.

SUBJECTS AND METHODS

Subjects

The subjects of the squalene group (n = 9, five male/four female) were long-term hospital patients in stable condition suffering mainly from cerebrovascular disorders. The cholestyramine group (n = 6, three male/three female) consisted of hospital patients with cardiovascular disorders and different degrees of hypercholesterolemia. None of the patients showed clinical signs of malabsorption, renal, thyroid, or liver diseases. The groups were comparable as to relative body weight, fasting blood glucose, and serum triacylglycerol levels. Informed consent was obtained from the subjects and the study protocol was approved by the Ethical Committee of the hospital.

Diets and treatments

Prior to the studies the subjects of both groups were on a standard hospital diet for several weeks; the mean intakes of cholesterol and squalene were 300 and 7 mg/day, respectively.

Squalene was administered in capsules (300 mg) three times daily with meals. The minimum feeding period before serum sterol analyses was 1 week; in six subjects the squalene feeding was extended to 30 days. Eight grams of cholestyramine resin (Questran, Lääkefarmos, Finland) was given four times daily with meals. In two subjects serial analyses of serum sterols were performed during treatment with squalene, squalene + resin, and resin alone.

Studies

Cholesterol absorption, fecal steroid measurements, and quantitation of cholesterol, noncholesterol sterols, and squalene in serum were performed before and during treatment. In three subjects in the squalene group, lipoprotein analyses were also performed by sequential ultracentrifugation using an increasing salt gradient (9). VLDL was separated by ultracentrifugation for 18 h at d 1.006 g/ml. LDL was separated by spinning the infranatant for 24 h at d 1.0637 g/ml. LDL was removed and the bottom portion was centrifuged for 48–60 h at d 1.2108 g/ml to separate HDL. At 30 days of squalene feeding, fractional cholesterol absorption (10) and fecal sterol output in six subjects were measured from stools collected for 3 days during a 7-day administration, three times a day, of a capsule containing 200 mg of Cr_2O_3, 0.11 μCi of [4-^{14}C]cholesterol, and 0.14 μCi of [22,23-^{3}H]sitosterol. The two labeled sterols were obtained from Radiochemical Centre, Amersham, UK.

Determinations

Serum triacylglycerol levels were measured by standard laboratory methods (11). Measurements of squalene and sterols in serum and fecal samples were performed as recently described in detail (12, 13). Briefly, squalene, free and esterified cholesterol and its precursor sterols, as well as other noncholesterol sterols, cholestanol, and plant sterols (campesterol and sitosterol) were quantitated by thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) methods. In order to eliminate the effect of varying lipoprotein levels, the squalene and noncholesterol sterol values were expressed in relation to cholesterol.

Fecal steroids were quantitated by GLC on an SE-30 capillary column (14) and the cholesterol balance technique was used to assess total cholesterol synthesis (fecal elimination of cholesterol as neutral sterols[2] and bile acids minus dietary cholesterol). We also calculated intestinal total cholesterol flux (15) (fecal neutral sterol/1 – fractional cholesterol absorption) and endogenous cholesterol flux (total flux minus dietary cholesterol) which indirectly reflects biliary cholesterol secretion. Assuming that the absorption of dietary and biliary cholesterol is similar, the total, dietary, and biliary cholesterol fluxes multiplied by fractional cholesterol absorption reflect total, dietary, and biliary cholesterol absorption, respectively.

Statistics

The results were analyzed using the t-test (paired t-test when appropriate). P values < 0.05 were considered statistically significant. Mean ± SE values are given in the text.

[2]In this report, neutral sterols (NS) refer to fecal cholesterol and its nonpolar derivatives coprostanol (5β-cholestan-3β-ol), epicoprostanol (5β-cholestan-3α-ol), and coprostanone (5β-cholestan-3-one) (excluding bile acids and cholestanol).

RESULTS

Serum cholesterol and lipoproteins

Both free and esterified serum cholesterol contents were significantly reduced by cholestyramine treatment, whereas squalene feeding significantly increased free (paired *t*-test, $P<0.05$) but not esterified or total cholesterol concentrations in serum at 7 days (**Table 1**). However, the difference was not significant at 30 days of feeding and serial analyses of serum cholesterol with and without squalene suggested that squalene feeding had no consistent effect on serum cholesterol levels. Serum cholesterol measured in different lipoproteins of a few subjects showed no consistent changes (**Table 2**). Feeding squalene for 30 days did not significantly affect serum triacylglycerol levels which were 118.7 ± 29.2 mg/dl (mean + SE,

TABLE 1. Effects of squalene feeding (900 mg/day) and cholestyramine treatment (32 g/day) on serum levels of squalene, cholesterol, and noncholesterol sterols (mean ± SE)

Lipid	Squalene (n = 9)			Cholestyramine (n = 6)	
	Basal	7 Days (n = 9)	30 Days (n = 6)	Basal	7 Days
	mg/dl				
Cholesterol					
Free	53 ± 6	56 ± 6[a]	52 ± 9	63 ± 10	48 ± 9[a]
Ester	127 ± 14	132 ± 15	122 ± 21	147 ± 21	113 ± 20[a]
Total	180 ± 20	188 ± 20	174 ± 29	210 ± 32	161 ± 28[a]
	μg/100 mg cholesterol				
Squalene	13 ± 3	210 ± 156[a]	165 ± 65[a]	31 ± 18	37 ± 14
Lanosterol					
Free	24 ± 3	51 ± 5[a]	64 ± 6[a]	22 ± 3	37 ± 5[a]
Ester	4 ± 1	7 ± 2	10 ± 3[a]	2 ± 0.3	2 ± 1
$\Delta^{8,24}$-DMS[b]					
Free	24 ± 4	56 ± 8[a]	76 ± 16[a]	21 ± 3	34 ± 3[a]
Ester	1 ± 0.1	2 ± 0.3[a]	3 ± 1[a]	2 ± 1	2 ± 0.3
Δ^{8}-DMS					
Free	19 ± 4	37 ± 6[a]	50 ± 14	13 ± 2	20 ± 2[a]
Ester	1 ± 0.2	3 ± 0.4[a]	4 ± 1[a]	1 ± 0.07	1 ± 0.2
Δ^{8}-Methostenol					
Free	24 ± 5	40 ± 6[a]	53 ± 16	16 ± 3	26 ± 2[a]
Ester	6 ± 1	13 ± 2[a]	12 ± 2[a]	6 ± 1	9 ± 1[a]
Methostenol					
Free	26 ± 4	54 ± 6[a]	67 ± 16[a]	21 ± 2	27 ± 1[a]
Ester	9 ± 1	19 ± 3[a]	24 ± 6[a]	8 ± 1	9 ± 1
Δ^{8}-Cholestenol					
Free	17 ± 5	20 ± 4	23 ± 7	6 ± 1	45 ± 9[a]
Ester	14 ± 4	19 ± 3	19 ± 6	8 ± 1	33 ± 4[a]
Lathosterol					
Free	173 ± 25	229 ± 31	232 ± 43	140 ± 14	647 ± 101[a]
Ester	69 ± 9	93 ± 9	90 ± 13	59 ± 4	193 ± 26[a]
Desmosterol					
Free	36 ± 4	55 ± 5[a]	58 ± 5[a]	30 ± 4	68 ± 14[a]
Ester	41 ± 5	67 ± 9[a]	65 ± 13[a]	43 ± 4	76 ± 10[a]
Campesterol					
Free	137 ± 13	118 ± 14[a]	122 ± 18	147 ± 18	156 ± 25
Ester	114 ± 12	113 ± 12	107 ± 16	126 ± 21	132 ± 28
Sitosterol					
Free	119 ± 17	115 ± 17	112 ± 19	131 ± 28	171 ± 44
Ester	97 ± 12	95 ± 14	88 ± 14	99 ± 21	130 ± 36
Cholestanol					
Free	89 ± 17	158 ± 78	100 ± 39	123 ± 17	72 ± 15
Ester	109 ± 12	112 ± 16	111 ± 22	130 ± 5	159 ± 32

Squalene and sterols were quantitated by TLC and GLC.
[a] $P < 0.05$ versus basal values (paired *t*-test).
[b] Dimethylsterol.

TABLE 2. Cholesterol, squalene, and precursor sterol concentrations in different lipoproteins during squalene feeding (mean ± SE, n = 3)

Lipid	VLDL		LDL		HDL	
	Basal	7 Days	Basal	7 Days	Basal	7 Days
			mg/dl			
Total cholesterol	10 ± 3	10 ± 3	199 ± 11	201 ± 3	34 ± 3	30 ± 3
			μg/100 mg cholesterol			
Squalene	141 ± 50	1316 ± 813[a]	25 ± 7	89 ± 25[a]	39 ± 31	105 ± 59[a]
Methyl sterols[b]						
Free	119 ± 41	192 ± 18[a]	153 ± 106	206 ± 81[a]	171 ± 79	309 ± 30[a]
Ester	32 ± 20	38 ± 6	25 ± 9	34 ± 12[a]	24 ± 12	60 ± 6[a]
Lathosterol						
Free	135 ± 53	218 ± 43[a]	137 ± 41	218 ± 55[a]	141 ± 36	208 ± 47[a]
Ester	47 ± 9	85 ± 8[a]	44 ± 10	62 ± 10[a]	51 ± 8	72 ± 9[a]

[a] $P < 0.05$ versus basal values (paired t-test).
[b] Sum of five methyl sterols.

n = 6) before and 124.0 ± 23.0 mg/dl during squalene feeding.

Serum squalene and cholesterol precursor sterols (Tables 1 and 2)

Squalene feeding increased serum squalene concentrations almost 17-fold while cholestyramine had no effect (Table 1). Serum contents of five measured methyl sterols (free and esterified) rose consistently during squalene feeding (1.7- to 2.4-fold) while the respective increases were less during cholestyramine treatment (1- to 1.7-fold). The opposite was true for Δ^8-cholestenol and lathosterol which clearly increased more during cholestyramine treatment (3.3- to 8-fold) than squalene feeding (1.2- to 1.4-fold). Serum desmosterol levels increased significantly during both treatments. Extension of squalene feeding from 1 to 4 weeks gave virtually similar results.

The different effects of the two treatments on the precursor sterol levels are illustrated in two subjects who were first on squalene alone, then on squalene + resin, and finally on resin alone (**Fig. 1**). It can be noted that both the trimethyl sterol, lanosterol, and the monomethyl sterol, methostenol, behaved quite similarly.

Analysis of the precursors in different lipoproteins without and with squalene feeding revealed that the bulk of squalene and precursor sterols was found in LDL but in terms of μg/100 mg lipoprotein cholesterol, the highest concentration of squalene was in VLDL (Table 2). During squalene feeding, the squalene content increased more in VLDL (9.3-fold) than in LDL or HDL (3.6- and 2.6-fold, respectively). The precursor sterol contents increased quite similarly in different lipoproteins during the squalene feeding.

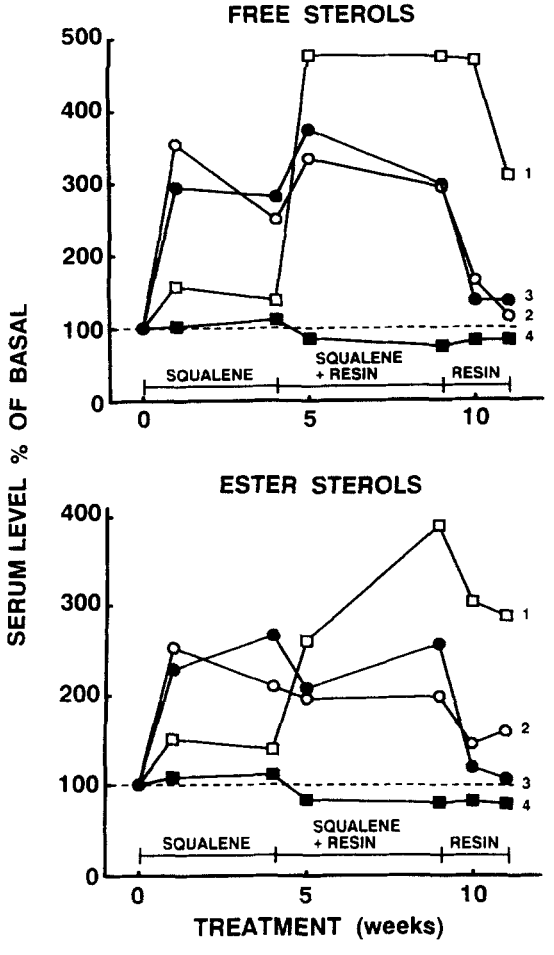

Fig. 1. Serial alterations of serum cholesterol and precursor sterols during squalene and cholestyramine treatment. Each point is mean of two subjects. Basal value is indicated by the dotted line; 1, lathosterol; 2, methostenol; 3, lanosterol; 4, cholesterol.

TABLE 3. Effects of 7-day squalene feeding on fecal lipid excretions, cholesterol absorption, and fluxes in six subjects (mean ± SE)

	Basal	During Squalene Feeding
Dietary intake (mg/d)		
Cholesterol	300	300
Squalene	7	907[a]
Cholesterol absorption (%)	38 ± 4	39 ± 3
Fecal excretion (mg/d)		
Squalene	2 ± 0.2	359 ± 71[a]
Lanosterol	5 ± 0.03	9 ± 2
Neutral sterols (NS)[b]	655 ± 83	856 ± 146[a]
Bile acids (BA)	212 ± 24	255 ± 24[a]
NS + BA	867 ± 102	1110 ± 160[a]
Plant sterols	257 ± 33	249 ± 23
Cholestanol	12 ± 2	13 ± 2
Cholesterol synthesis (mg/d)	563 ± 126	844 ± 191[a]
Cholesterol flux (mg/d)		
Total intestinal	1091 ± 102	1603 ± 320
Biliary	791 ± 102	1303 ± 320
Cholesterol absorption (mg/d)		
Total	398 ± 24	626 ± 139
Dietary	114 ± 12	116 ± 8
Biliary	284 ± 20	511 ± 135

Cholesterol absorption was measured by continuous double isotope technique; fecal lipid excretions were quantitated by gas-liquid chromatography on a capillary column. Cholesterol synthesis is the mean of NS and BA minus dietary intake of cholesterol; total intestinal cholesterol flux is NS divided by 1 minus fractional cholesterol absorption; and biliary cholesterol flux is total flux minus dietary cholesterol. Fractional absorption multiplied by total, dietary, and biliary fluxes represent their individual absorption rates.

[a] $P < 0.05$ versus basal values (paired t-test).

[b] Includes cholesterol and its nonpolar derivatives coprostanol, epicoprostanol, and coprostanone.

Serum plant sterols and cholestanol

Squalene feeding signficantly decreased free campesterol levels (Table 1), but overall the reduction in the esterified, free, or total plant sterol levels was insignificant. The combined sitosterol + campesterol levels were significantly decreased ($P<0.05$, paired t-test), however. Cholestanol contents were not significantly affected.

Fecal lipids and cholesterol absorption (Table 3)

Squalene feeding increased fecal squalene excretion from 2 to 359 mg/day, suggesting that about 60% of the 907 mg/day of dietary squalene was absorbed.

Efficiency of cholesterol absorption from the intestine was not changed, but both fecal bile acid and neutral sterol outputs were augmented significantly by 20% and 31%, respectively. The calculations showed that cholesterol synthesis was increased by ca. 50% from 563 to 844 mg/day during the squalene treatment. Although total and biliary cholesterol fluxes and absolute cholesterol absorption (mg/day) were also increased in some of the subjects during the squalene feeding, the differences were not statistically significant. Cholestanol and plant sterol excretions were virtually unaltered.

DISCUSSION

Provided that dietary squalene is not metabolized during the intestinal passage, the present fecal analyses suggest that about 60% of intestinal squalene is absorbed in humans. However, the fecal data (Table 3) show that the loss of squalene during the intestinal passage (907 mg – 358 mg = 549 mg) is clearly more than the increase of cholesterol synthesis (844 mg – 563 mg = 281 mg). This would mean that some daily squalene had disappeared (549 mg – 281 mg = 268 mg) possibly due to intestinal squalene degradation and/or to retention of the squalene-derived cholesterol somewhere in the body.

Since squalene feeding reduced HMG-CoA reductase activity in rat liver (6), it is obvious that the baseline cholesterol synthesis (563 mg/day) from acetate and mevalonate was inhibited by dietary squalene. On the other hand, animal experiments have indicated that squalene feeding increases tissue squalene and cholesterol contents (3–6), reflecting stimulated cholesterol production from squalene. Labeled squalene administered to rats is converted to methyl sterols and rapidly to cholesterol mainly in the liver, with only trace amounts in the fat tissue (16). In addition, the markedly increased esterified methyl sterol contents in the present study and in earlier animals studies may indicate enhanced ACAT activity as a consequence of increased tissue cholesterol content (5, 6).

In any event, fecal steroid output suggests that a substantial amount of dietary squalene was absorbed and at least some of it was converted to cholesterol and bile acids in human subjects, indicating enhanced cholesterol synthesis. However, the increase of cholesterol production was not reflected in serum cholesterol levels.

Differences of precursor sterol profiles during the squalene and cholestyramine treatments were quite similar to those seen earlier in experimental animals (6). The increase of methyl sterols during the squalene feeding was more prominent in the rat probably due to the relatively high squalene dose. The difference between squalene and cholestyramine may be related to HMG-CoA reductase activity, which is low during squalene feeding and activated by cholestyramine. We have earlier hypothesized that the accentuated increase of methyl sterols as compared to Δ^8-cholestenol and lathosterol by squalene feeding is due to an inhibition of hepatic methyl sterol oxidase during the low HMG-CoA reductase ac-

tivity (6). Cholestyramine-induced enhancement of cholesterol synthesis activates HMG-CoA reductase activity and methyl sterol oxidase so that formation of Δ^8-cholestenol and lathosterol is more effective, and the increase of methyl sterols is less marked.

It has been noted earlier in the rat that squalene feeding and especially cholestyramine treatment decrease both hepatic and serum phytosterol levels (17). The mechanism may be either decreased absorption or preferential secretion of plant sterols into bile. In the present human experiment only squalene feeding tended to lower serum plant sterol contents. Since biliary cholesterol secretion was frequently increased, enhanced biliary sterol output, known to regulate serum plant sterol levels in normal subjects (18), was apparently the major regulatory factor.

An increased load of newly synthesized cholesterol from dietary squalene may have some effects on subsequent cholesterol metabolism different from those after the feeding of exogenous cholesterol. Biliary cholesterol excretion is actually markedly enhanced by stimulated cholesterol synthesis as compared to enhanced dietary cholesterol (19). In addition, squalene feeding appears to reduce hepatic LDL-receptor activity in man (7) only when the serum precursor levels are increased high enough; a moderate increase of newly synthesized cholesterol may cause preferential secretion of cholesterol and bile acids to bile and feces. Thus, due to effective sterol elimination, the sterol balance of hepatocytes may have changed only modestly. This and inconsistently enhanced cholesterol absorption may then be reflected in modest changes in LDL kinetics and serum cholesterol level. Squalene feeding has only a low risk of atherosclerosis in experimental animals (4). Cholesterol feeding in humans usually has little effect on bile acid synthesis and can reduce LDL removal due to apparent reduction of hepatic LDL apoB receptors (7).

Experiments in rats showed that squalene feeding significantly increased hepatic ACAT activity and esterified cholesterol in liver even in short-term experiments (6). In the present study we did not measure hepatic lipids or enzyme activities directly. However, the increase of serum esterified methyl sterols suggests activated hepatic ACAT in our squalene-fed subjects; methyl sterols are not esterified by LCAT in serum (20), but are effectively esterified by ACAT in the liver (21). Whether enhanced hepatic ACAT activity might lead to clinically significant cholesterol accumulation during long-term squalene consumption is presently unknown.

Cholesterol-lowering effects of monounsaturated fat (22) may counterbalance harmful side effects of squalene in olive oil. In fact, squalene-rich olive oil effectively reduced serum cholesterol levels in humans despite markedly increased squalene and precursor sterol levels (8). There are recent data reporting increased hepatic cholesterol concentrations in rats fed olive oil as compared with rats fed coconut fat (23). The difference in the hepatic cholesterol accumulation could partly be explained by squalene content in olive oil. New epidemiological data suggest increased serum cholesterol levels in Cretan men who still have high olive oil consumption (24). Together these findings may favor the use of monounsaturated oils without squalene content in order to maximize the favorable hypolipidemic properties of these oils.

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