Effect of Olive Oil and Squalene on Cholesterol Mobilization in the Rat.* (24571)

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Squalene is considered a precursor in biosynthesis of cholesterol(1,2). Olive oil is unique in that it is the only commonly used vegetable fat which contains squalene in appreciable amounts (31% to 64% of the nonsaponifiable fraction, or about 0.5% of the oil)(3,4,5). It also contains a higher percentage of oleate and a lower percentage of linoleate than do corn, soy and cottonseed oils. Coconut oil, in contrast, contains little oleate or linoleate, but it contains a high proportion of glycerides of saturated acids with shorter chain length than palmitic acid. Young female rats fed 10% olive oil with 1% cholesterol had higher serum cholesterols than did matched groups of rats fed other oils cited above, with the same synthetic diets. High serum cholesterol figures were observed only in cholesterol-fed females and not in cholesterol-fed males. It seemed possible, therefore, that when cholesterol synthesis was inhibited by cholesterol feeding, dietary squalene might be serving either as a stimulant of, or a precursor for, synthesis of some ovarian hormone.

Materials and methods. Intact rats. Squalene,† at approximately maximum level furnished by olive oil, was added to diets containing 10% cottonseed and 10% coconut oils, respectively.‡ Diets containing 10% of new and specially prepared olive oil were also fed, with and without cholesterol. Weanling rats in groups of 10 males and 10 females were given the diets, (Table I) for 7 weeks. They were then sacrificed, and liver and serum lipids were determined. Methods were those reported previously(7).

Results. Data for serum and liver lipids and cholesterol are given in Table I. Serum cholesterols were again much higher ($P<0.01$) in olive oil-cholesterol-fed females than in companion groups fed either cottonseed or coconut oils. Serum levels of cholesterol in females fed squalene and cholesterol with cottonseed oil showed some increase over the group fed cholesterol only. When squalene was given with coconut oil and cholesterol, increases approached significance ($P<0.05$). Serum values were raised only in cholesterol-fed females.

Both liver lipid and liver cholesterol values (mg/liver) were significantly higher ($P<0.01$) in squalene- and cholesterol-fed male as well as female rats given coconut oil than in their companion groups with cholesterol but without squalene. Rats of both sexes given olive oil with cholesterol likewise had significantly higher liver lipids and cholesterol than did those given cottonseed or coconut oil with otherwise identical diets. The higher values for liver lipids and cholesterol observed when squalene was given to cholesterol-fed males suggested the possibility that squalene might in some way be accelerating retention of liver glyceride and cholesterol. Whether this was a direct effect or one mediated by a hormone was not clear.

Castrates. To test whether squalene affected the level of serum and liver cholesterol in the absence of hormones secreted by the gonads, male and female castrates were studied. Rats of each sex were placed, at

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† Eastman—Technical grade.

‡ The diets contained: 10% albumin; 5% vit-free casein; 10% fat; .05% squalene (when fed); 1% vit. B mix; 1% fat-soluble vit. mix (in cottonseed oil); 1% cholesterol (when fed); and sucrose to 100%. The vitamins furnished/kg of diet were: 15,000 USP units A; 1,000 USP units D; 5 mg menadione; 400 mg mixed tocopherol; 4 mg thiamine; 4 mg riboflavin; 2 mg pyridoxine; 10 mg calcium pantothenate; 2 mg folacin; 1.5 mg biotin; 10 mg p-aminobenzoic acid; 10 mg niacin; 100 mg ascorbic acid; 500 mg inositol; and 900 mg choline. This diet differed from the 10% fat diets described in(3) in that it furnished a slightly higher percentage of choline.

TABLE I. Lipids and Cholesterol: Intact Rats. Means and stand. errors. (10 rats/group.)

Fat in diet*	Sex	Gain body wt, g	Liver					Serum
			Wt, g	Total cholesterol		Free cholesterol, % moist wt	Lipids		Total cholesterol, mg %
				% moist wt	mg/liver		% moist wt	mg/liver	
Ol	♂	223	9.6	.39 ±.03†	38 ± 4.3	.23	5.2 ± .19	500 ± 49	88 ± 6
	♀	155	5.9	.30 ±.01	18 ± 1.1	.25	5.1 ± .18	300 ± 17	72 ± 5
Ol + C	♂	240	11.9	3.97 ±.29	474 ±54.0	.33	15.1 ±1.7	1814 ±262	96 ± 8
	♀	162	6.7	4.23 ±.49	290 ±41.0	.36	13.9 ±1.4	946 ±116	272 ±47
CsO + C	♂	209	9.6	2.80 ±.30	270 ±34.0	.31	12.5 ± .7	1203 ± 90	77 ± 3
	♀	157	6.5	2.05 ±.33	134 ±25.0	.33	9.7 ± .9	624 ± 72	114 ±15
CsO + C + Sq	♀	167	6.9	3.10 ±.30	214 ±21.0	.35	12.8 ±1.0	890 ± 76	147 ±19
CnO	♂	222	8.5	.30 ±.01	25 ± 1.4	.23	4.7 ± .3	398 ± 29	71 ± 3
	♀	155	5.4	.25 ±.01	14 ± 3.7	.24	5.0 ±3.7	260 ± 22	77 ± 6
CnO + Sq	♂	223	10.2	.37 ±.04	36 ± 4.8	.22	5.6 ± .3	546 ± 48	76 ± 5
	♀	138	6.0	.31 ±.04	19 ± 2.4	.27	6.0 ± .8	368 ± 56	76 ± 5
CnO + C	♂	232	9.5	1.47 ±.02	135 ± 2.0	.29	8.0 ± .7	735 ± 65	96 ± 8
	♀	157	6.1	.79 ±.01	48 ± 5.0	.32	6.1 ± .6	377 ± 49	117 ±11
CnO + C + Sq	♂	224	11.5	2.53 ±.35	303 ±52.0	.30	16.9 ±2.8	2092 ±452	82 ± 8
	♀	156	6.5	1.93 ±.21	125 ±13.0	.33	8.8 ± .6	569 ± 38	153 ±13

* Abbreviations: Ol = olive oil; C = cholesterol; CsO = cottonseed oil; Sq = squalene; CnO = coconut oil.

† Stand. errors: $\frac{\sqrt{\frac{\Sigma d^2}{n-1}}}{\sqrt{n}}$.

weaning, on the 10% coconut oil diets (a) without, and (b) with added cholesterol. Subgroups, on each diet, were fed squalene. After approximately 3 weeks, the animals were gonadectomized. They were continued on respective diets for 4 more weeks, so that age and total time on diet were comparable with those of intact rats.

The data in Table II show that addition of squalene to a cholesterol-rich diet had little effect on serum or liver cholesterol of castrates of either sex. The data for intact and for castrate rats are not strictly comparable because of consistently higher liver lipid and cholesterol values of cholesterol-fed castrates. Nevertheless, it is significant that intact animals fed squalene with cholesterol had liver cholesterol values twice as high as those of intact animals fed cholesterol only, while, in castrates, there were no significant differences between liver cholesterols of squalene-cholesterol-fed groups and those fed cholesterol only. Serum values for castrates were not significantly affected by addition of squalene to cholesterol-rich diets. Female castrates did, however, have higher mean serum cholesterols than male castrates. Variations within groups were considerable.

Discussion. The structure of squalene offers many possibilities for formation of cis and trans isomers. It is possible that the commercial squalene which was fed may have had the lesser effect on serum cholesterol because of presence of unnatural isomers. The squalene of olive oil might be different and possibly more active.

On the other hand, data for olive oil-fed rats can hardly be interpreted without consideration of the high oleate content of this oil (75%-85%). Cottonseed oil is richer in linoleate (about 50%), but coconut oil owes its low melting point to its content of saturated acids of short to medium chain length. Dietary oleate and linoleate both seem to facilitate deposition of liver cholesterol in the rat, and large percentages of oleic acid have been found in both liver cholesterol esters and glycerides. The observed effects of olive oil might have been a summation of a specific action of squalene, plus a mass effect of a large supply

TABLE II. Lipids and Cholesterol: Castrates. 10% coconut oil diet. Means and stand. errors.

Diet supplement	No. & sex	Wt gain, g	Liver wt, g	Liver: Total cholesterol % moist wt	Liver: Total cholesterol mg/liver	Liver: Total lipids % moist wt	Liver: Total lipids mg/liver	Liver: Free cholesterol, % moist wt	Serum cholesterol, mg %
None	7 ♂	206	8.2	.27 ±.01*	22 ± 1.2	4.3 ±.3	355 ± 28	.23 ±.01	79 ± 7
	8 ♀	176	6.7	.27 ±.01	18 ± 1.0	5.7 ±.4	378 ± 24	.23 ±.01	80 ± 4
Squalene only	14 ♂	201	7.5	.29 ±.01	22 ± .9	4.9 ±.1	369 ± 12	.24 ±.01	68 ± 3
	12 ♀	182	6.8	.30 ±.01	20 ± .8	5.1 ±.2	345 ± 13	.24 ±.01	86 ± 5
Cholesterol only	8 ♂	199	8.9	2.50 ±.30	226 ±38.0	10.1 ±.6	904 ± 79	.31 ±.02	102 ±15
	8 ♀	180	7.6	2.00 ±.20	154 ±17.0	9.2 ±.4	700 ± 41	.30 ±.01	129 ±19
Cholesterol + squalene	11 ♂	203	9.1	2.23 ±.23	200 ±20.0	9.1 ±.6	811 ± 47	.30 ±.01	94 ± 9
	13 ♀	170	7.2	1.71 ±.10	122 ±11.0	8.4 ±.5	599 ± 39	.26 ±.02	134 ±16

* Stand. errors: $\frac{\sqrt{\frac{\Sigma d^2}{n-1}}}{\sqrt{n}}$.

of oleate on absorption and esterification of cholesterol and accumulation of glyceride.

Coconut oil furnishes very little oleic acid. Moreover, a considerable proportion of its fatty acids of short chain length can be expected to be absorbed *via* the portal circulation, rather than to accompany cholesterol through the lymphatics. That feeding squalene increases liver cholesterol in the coconut oil-fed animals is, therefore, the more perplexing, as is its effect on serum cholesterol of females only. The data for coconut oil-cholesterol-fed females seem reasonable only if we postulate that, where cholesterol synthesis is inhibited by cholesterol feeding, squalene is a more direct intermediate than cholesterol for synthesis of an ovarian hormone. This hormone, like estradiol(6), might raise the level of circulating cholesterol. It would also be necessary to assume that squalene either directly influenced liver lipid deposition, or that another hormone-assisted mechanism was active in producing high liver lipid and cholesterol values in male rats fed squalene with coconut oil and cholesterol. The possibility cannot, of course, be ruled out that some very active impurity may have been present both in the olive oil and in the squalene fed.

The large standard errors of serum cholesterol values for olive oil- and squalene-fed females were due to the fact that three-fourths of the animals had higher than the mean values given, while the rest were within normal range for the diet without squalene. The above might be expected if the effect of squalene on serum cholesterol were felt only during certain phases of the estrous cycle.

Summary. Much higher values for serum cholesterol were observed in 2 series of female rats fed 1% cholesterol with diets containing 10% olive oil than in the series fed like diets made up with a number of other vegetable fats. Olive oil-fed males did not have high serum cholesterols, but did have high liver lipids and cholesterol. An attempt to duplicate the effect of olive oil by adding equivalent amounts of commercial squalene to diets containing 10% cottonseed oil and 10% coconut oil is reported. The squalene-fed females on diets with added cholesterol had higher serum cholesterols than did those fed cholesterol only. Values were not nearly so high as in the rats fed olive oil. Both males and females fed squalene with cholesterol and coconut oil had significantly higher liver lipids and cholesterol than did littermates fed only coconut oil and cholesterol in the same amounts. Little or no effect attributable to squalene was observed in castrates. The possible effect of squalene as a stimulant of secretion of gonadal hormones is discussed.

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Phosphate Metabolism in Nutritional Muscular Dystrophy and Hyperthyroidism.* (24572)

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Impairment of phosphorylation of creatine has been suggested as the cause of creatinuria of both hyperthyroidism and Vit. E deficiency (1,2). In hyperthyroidism it is possible to demonstrate *in vitro* the uncoupling of oxidative phosphorylation in mitochondria(3), but recent evidence indicates that this may be due to a primary effect on the mitochondrial membrane(4). A method for measuring oxidative phosphorylation *in vivo* is not available, but it is possible to determine the entry of radioactive phosphorus into tissue phosphates. This approach is used in the following experiments to measure phosphorylation of creatine.

Methods. Vit. E deficiency was produced in white male New Zealand rabbits, weighing approximately 500 g, and in weanling Sprague-Dawley rats of both sexes by feeding previously described purified diets deficient only in Vit. E(5,6). Oral supplementation of control animals with Vit. E and general handling of animals were also the same as in previous studies(5,6). Rabbits on the deficient diet exhibited signs of muscular dystrophy after 3 to 4 weeks, then were used in experiments. The group of rats given Vit. E-deficient diet exhibited marked creatinuria and significant decrease in growth rate when compared to normal rats, and there was microscopic evidence of muscular dystrophy after 7 months of feeding, then chosen for the experiments. Two other groups of weanling Sprague-Dawley rats of both sexes were placed on regular laboratory chow diet. One group served as controls while hyperthyroidism was induced in the other group by daily subcutaneous injections of 0.5 mg of sodium thyroxinate for 21 days. The thyroxine-injected rats exhibited usual signs of hyperthyroidism when used in experiments. All animals were given 0.4 mc of $P^{32}O_4$ in dilute HCl/kilo body weight. Specific activity of the $P^{32}O_4$ was approximately 100,000 mc/g. Control and Vit. E-deficient rabbits were given P^{32} intravenously and killed 30 minutes later by large intravenous doses of pentobarbital. Immediately after death the animals were transferred to the cold room where samples of blood and skeletal muscle were obtained for analysis. The rats were handled in the same manner except that injections were given intraperitoneally. Control and Vit. E-deficient rats were killed 30 minutes after injection of P^{32} and control and hyperthyroid rats were killed one hour following injection of P^{32}. The scheme of fractional hydrolysis and precipitation of tissue phosphates devised by Sacks(7) was used to isolate serum inorganic phosphate, skeletal muscle inorganic phosphate, and the phosphate of creatine phosphate and adenosinetriphosphate (ATP). Portions of each specimen were used for phosphorus determination by the method of Fiske and SubbaRow and for counting with an end window Geiger counter.

Results. The skeletal muscle inorganic phosphate concentrations were in good agreement with the values given by Threlfall(8) and no differences were observed between the

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