Early effects of dietary orotic acid upon liver lipid synthesis and bile cholesterol secretion in rats

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Abstract Dietary orotic acid is known to cause impaired fatty acid synthesis and increased cholesterol synthesis in rats. We found that the impaired fatty acid synthesis occurs during the first day of orotic acid feeding and, in studies with albumin-bound [1-^{14}C]palmitic acid, an associated decrease in the rate of esterification of this fatty acid into triacylglycerol, phospholipid, and cholesteryl ester was observed. These changes may result from the known decreases in liver levels of adenine nucleotides or, as reported here, from decreased liver CoASH levels in orotic acid-fed rats. The increase in hepatic cholesterol synthesis occurred during the second day of orotic acid feeding. It was detected by increased incorporation of [1,2-^{14}C]acetate into cholesterol by liver slices and by a 7-fold increase in HMG-CoA reductase activity. At the same time the biliary output of cholesterol was increased 2-fold and studies using $^{3}H_2O$ revealed that the output of newly synthesized cholesterol in bile was increased 5-fold. The content of cholesteryl ester in hepatic microsomes decreased during orotic acid feeding but free cholesterol was unchanged. The findings are interpreted to suggest that the increased bile cholesterol secretion caused by orotic acid is a result of impaired hepatic cholesterol esterification and that the increase in HMG-CoA reductase activity is a result of diminished negative feedback due to the depleted content of cholesteryl ester in the hepatic microsomes. — **Tokmakjian, S. D., and D. S. M. Haines.** Early effects of dietary orotic acid upon liver lipid synthesis and bile cholesterol secretion in rats. *J. Lipid Res.* 1985. **26:** 478–486.

Supplementary key words bile phospholipids • bile acids • liver triacylglycerol • coenzyme A • liver free and esterified cholesterol • HMG-CoA reductase

When rats are fed a semisynthetic diet containing 1% orotic acid (OA), marked increases in the liver content of triacylglycerol and cholesteryl ester are present after 7 days (1), while the liver levels of purine nucleotides are decreased and pyrimidine nucleotides are increased after the first day (2). The increase in hepatic neutral lipids results from impaired secretion of lipoproteins (3–5). In addition to these changes, dietary OA exerts a paradoxical effect on liver lipid synthesis consisting of decreased synthesis of long chain fatty acids and increased synthesis of cholesterol (6). The experiments reported here were designed to examine the action of dietary OA on liver lipid synthesis to define *a*) the time of onset after commencement of OA-feeding; *b*) the fate of the increased liver cholesterol synthesized; and *c*) the possible mechanism responsible for these changes.

METHODS

Animals and diets

Male Wistar rats weighing 100–125 g were purchased from Woodlyn Farms Ltd., Guelph, Ontario. They were housed individually in metal screen-floored cages and were kept in a room with the temperature controlled at 22°C and illuminated from 0800–2000 hr. The semisynthetic cholesterol-free basal diet (7) had the following composition by weight: protein, 20%; carbohydrate, 64%; fat, 10%. The remainder consisted of vitamins including 0.5% choling chloride, minerals, and salts. The experimental diet contained 1.0 g of OA (Sigma Chemical Co.) per 100 g of diet. In some of the experiments, cholesterol (Fisher Scientific Co.) was added to both the basal diet and the 1% OA experimental diet in the amount of 2.0 g per 100 g of diet. The cholesterol was dissolved in ethyl alcohol which was thoroughly mixed with the diet and then evaporated (8). The rats were allowed access to food and water at all times. In every experiment all of the rats were conditioned on the cholesterol-free diet for 4 days before the experimental diet was offered. In every case the control rats were fed the basal diet for the same number of days as the experimental groups received the OA-containing diet. No significant difference was observed as a result of the varying number of days the control rats received the basal diet. The presence of 1% OA and/or 2% cholesterol in the diet had no effect on weight gain or food intake, which were recorded daily.

Abbreviations: OA, orotic acid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; HPLC, high performance liquid chromatography; ACAT, acyl cholesterol acyltransferase.

Procedures

The experiments were routinely performed between 0800 and 1000 hr. The rats were killed by a blow on the head followed immediately by decapitation. For lipid analysis, the liver was excised and frozen in liquid nitrogen. For measurement of microsomal HMG-CoA reductase activity, the two anterior lobes were removed and were kept chilled on ice and the remainder of the liver was frozen.

Liver slice incubations with [1,2-^{14}C]acetate

The liver was chilled in ice-cold 0.9% NaCl and was sliced within a few minutes using a Stadie-Riggs Microtome. The weighed slices (ca. 300 mg) were added to 2.0 ml of Krebs Ringer phosphate buffer, pH 7.2, containing 0.3 M glucose in a Warburg Flask fitted with a 1-cm^2 filter paper strip in the center well. The flasks were incubated at 37°C for 10 min prior to addition of 0.75 μCi (1.5 μmol) of [1,2-^{14}C]acetate (New England Nuclear) dissolved in 75 μl of 0.9% NaCl. The flasks were then stoppered and incubated for 60 min at 37°C in a shaking water bath oscillating at 36 strokes/min. The reaction was stopped by addition of 0.5 ml of 2 N H_2SO_4 to the medium, and 0.3 ml of 1 N KOH was added to the center well by puncture through the diaphragmed stopper. The flasks were re-incubated for 45 min. The paper strip was immersed in 10 ml of PCS scintillation fluid and counted to measure the $^{14}CO_2$ content. The flask contents (medium + tissue) were homogenized and the total lipids were extracted and the lipids were analyzed as described below.

Liver lipid synthesis in vivo

The rats were allowed unrestricted access to diet until 0800 hr when they were injected intraperitoneally with ^3H_2O (New England Nuclear, 4.2 mCi in 0.4 ml of 0.9% NaCl/100 g body weight). They received no additional water or food. They were decapitated after 60 min and the liver was removed for lipid analysis. This interval was chosen because labeling of hepatic sterol is linear during this time and transport of lipid to other tissues is minimal (9).

Metabolism of [1-^{14}C]palmitic acid

[1-^{14}C]Palmitic acid (New England Nuclear, 51 mCi/mmol) was complexed to bovine serum albumin (10). This solution was made isotonic with NaCl such that 4 μCi was contained in 0.5 ml. Rats weighing 200–225 g were fed a cholesterol-free diet and the experimental group received 1% OA in this diet for 1 day. The rats were briefly anesthetized with ether and 0.5 ml of the [1-^{14}C]palmitic acid solution was injected into the jugular vein. The rats were re-anesthetized and were exsanguinated via the aorta at 10, 20, 30, and 40 min following tracer administration. The liver was removed for lipid extraction. Triacylglycerol and cholesteryl ester were isolated by TLC and their radioactivities were counted.

Bile collection experiments

At 0800 hr the rats were injected intraperitoneally with ^3H_2O as above and after 120 min a PE 10 polyethylene tube (Clay Adams) was inserted into the bile duct under ether anesthesia. Bile was collected at 60-min intervals for 3 hr with the rat held in a restrainer. No water was provided. The amount of newly synthesized cholesterol in bile was calculated using the formula (11): dpm ^3H in cholesterol per ml bile × 1.45 ÷ (sp act plasma water × 18 × 1000).

Chemical procedures

To extract total lipids, the frozen liver was homogenized for about 1 min in 20 volumes of chloroform–methanol 2:1 using a Polytron homogenizer (Brinkmann Instruments). Bile total lipids were extracted using 20 volumes of the same solvent. Phase separation was achieved by addition of 0.25 volumes of water followed by centrifugation; the chloroform phase was washed twice with 0.25 volumes of water. Individual lipids were separated by TLC on Silica Gel Type 60 plates (0.25 mm, Merck, Germany). Neutral lipids were resolved using petroleum ether–diethyl ether–acetic acid 85:15:1 and phospholipids with chloroform–methanol–water 65:25:4. After visualization with I_2 vapour, the lipid-containing zones were scraped into conical centrifuge tubes and the lipid was extracted (12). Aliquots were taken for triacylglycerol and lipid phosphorus determinations as previously described (7). Following alkaline hydrolysis, the fatty acids of these lipids were counted in 5 ml of PCS (Amersham Corp.) using a Beckman LS 7200 liquid scintillation counter with automatic quench correction.

Cholesterol and cholesteryl esters were visualized on the plates after spraying with Rhodamine G and these spots were extracted and the cholesteryl esters were hydrolyzed (13). Cholesterol in total liver lipid and bile lipid extracts was measured without derivatization by GLC at 260°C with N_2 as carrier using a Hewlett Packard Model 5840 gas chromatograph fitted with a column of 1.5% OV-17 on Gas Chrom Q (Ultrapak, 6' span, 0.4 mm i.d., Terochem Laboratories, Rexdale, Ont.). The internal standard was 5α-cholestane (Serdary Research Laboratories, London, Ont). Cholesteryl esters were hydrolyzed in ethanolic KOH and the free cholesterol was assayed as above. The fatty acids of the hydrolyzed cholesteryl esters were extracted and the radioactivity was counted. For measurement of cholesterol and cholesteryl esters in liver cytosolar and microsomal fractions, the cytosol was quantitatively removed for lipid extraction and assayed as described above. The microsomal fraction

was re-suspended and re-centrifuged twice at 100,000 *g* for 60 min prior to lipid extraction. The cholesterol and cholesteryl esters were separated by TLC; following hydrolysis of cholesteryl esters, the cholesterol was derivatized as the silyl ether with *t*-butyldimethylchlorosilane imidazole (Applied Science Division, Milton Roy Co.) prior to assay by GLC. Stigmasterol (Serdary Research Laboratories, London, Ont.) was used as internal standard. Free and conjugated bile acids were extracted from bile (14) and were assayed enzymatically (15) using purified hydroxysteroid dehydrogenase (Sigma Chemical Co.). Microsomal HMG-CoA reductase was assayed by the method of Shapiro et al. (16). Total protein was measured by a dye-binding procedure (17). Liver free coenzyme A (CoASH) was extracted by homogenization of the quick-frozen liver in 5% perchloric acid and was measured by HPLC (18) using a Beckman Model 420 high performance liquid chromatograph.

RESULTS

Liver total lipid levels in rats fed 1% OA

The effect of feeding a cholesterol-free diet containing 1% OA for 1, 2, 3, 5, and 8 days on the liver levels of triacylglycerol, phosphatidylcholine, phosphatidylethanolamine, free and esterified cholesterol is shown in **Table 1.** During the first day of OA feeding, the liver level of triacylglycerol was decreased to about 50% of the control value. On the 3rd day this level had returned to the control level and then it progressively increased to produce the typical OA-induced fatty liver. The level of esterified cholesterol in the liver decreased progressively to about 50% of the control value during the first 3 days of OA feeding and subsequently rose such that at the fifth day, it was increased about 2.5-fold; at 8 days it was about 8-fold higher than the control value. Liver phosphatidylcholine and phosphatidylethanolamine levels were both slightly increased above the control after the first day of OA feeding and these increases persisted unchanged throughout the entire 8-day OA feeding period. The liver level of free cholesterol was not different from the control value at any time in rats fed the cholesterol-free diet containing 1% OA.

In rats fed the control diet containing 2% cholesterol for 2 days, the liver level of triacylglycerol was slightly but significantly increased compared to rats fed the cholesterol-free control diet. Addition of 1% OA to the cholesterol-containing diet caused the liver triacylglycerol level to be decreased 50% below the control.

The presence of 2% cholesterol in the diet for 2 days caused a marked increase (5.5-fold in the liver content of cholesteryl ester and a much smaller increase in the liver free cholesterol level. When 1% OA was included for 2 days in the cholesterol-containing diet, there was no significant difference in liver esterified cholesterol content as compared to the rats fed this diet without OA.

Cholesterol levels in liver microsomal and cytosolic fractions

The levels of free cholesterol in liver microsomes and cytosol were unchanged up to 5 days of OA-feeding (**Table 2**). The cholesteryl ester level in hepatic microsomes decreased by about 30% during the first day and further marked decreases occurred during the second and subsequent days of OA-feeding. Cholesteryl ester in the cytosol fell abruptly after 1 and 2 days of OA feeding but was significantly increased above the control value after the fifth day.

Liver levels of CoASH in rats fed OA

The liver level of CoASH decreased to about 60% of the control value during the first day of OA-feeding and persisted at this level throughout the 8-day period (**Fig. 1**).

TABLE 1. Levels of liver lipids in rats fed the 1% OA diet for various lengths of time

Diet	n	Tiracylglycerol	Phosphatidylcholine	Phosphatidyl-ethanolamine	Cholesterol (Free)	Cholesterol (Esterified)
			μmol/g liver (mean ± SD)			
Cholesterol-free						
Control	82	11.1 ± 3.6	18.9 ± 2.0	7.4 ± 1.1	3.9 ± 0.4	1.6 ± 0.4
1 day OA	27	5.4 ± 1.7	20.5 ± 2.5	9.0 ± 1.2	3.9 ± 0.4[a]	1.1 ± 0.3
2 days OA	36	5.2 ± 1.5	21.0 ± 2.7	10.6 ± 2.4	3.8 ± 0.3[a]	0.8 ± 0.3
3 days OA	17	10.7 ± 3.2[a]	21.0 ± 2.2	9.8 ± 1.9	3.8 ± 0.4[a]	0.9 ± 0.3
5 days OA	4	21.1 ± 6.6	22.2 ± 1.1	9.7 ± 1.0	3.9 ± 0.1[a]	3.6 ± 1.3
8 days OA	7	70.1 ± 18.4	24.3 ± 2.0	9.6 ± 0.7	3.8 ± 0.5[a]	11.5 ± 3.5
2% Cholesterol						
Control	6	14.57 ± 4.6	18.30 ± 1.4	7.3 ± 0.1	5.7 ± 0.5	8.8 ± 3.2
2 days OA	8	7.28 ± 1.6	18.80 ± 1.3[a]	8.0 ± 0.7	5.0 ± 0.5	6.5 ± 2.1

[a]These values are not significantly different ($P > 0.05$) from the corresponding control value. For all other values, $P < 0.01$.

TABLE 2. Free and esterified cholesterol levels in liver cytosol and microsomes from rats fed the 1% OA diet

	n	Cytosol		Microsomes	
		Free Cholesterol	Esterified Cholesterol	Free Cholesterol	Esterified Cholesterol
		nmol/mg protein (mean ± SD)			
Control	6	4.78 ± 0.86	31.8 ± 5.9	48.6 ± 3.3	14.9 ± 4.0
1 day OA	4	4.45 ± 2.09	9.3 ± 1.7[a]	49.6 ± 2.3	10.1 ± 3.0[b]
2 days OA	3	4.30 ± 0.78	13.7 ± 4.3[a]	46.6 ± 2.9	3.6 ± 2.8[a]
5 days OA	4	5.67 ± 1.27	53.9 ± 17.2[a]	48.9 ± 1.9	1.8 ± 0.8[a]

[a]Statistical significance of difference from control value $P < 0.01$.
[b]Statistical significance of difference from control value $P < 0.05$.

Metabolism of [^{14}C]acetate by liver slices of rats fed OA

The results of the studies in which liver slices from control rats and from rats fed the cholesterol-free diet containing 1% OA for 1, 2, 3, and 4 days were incubated in the presence of [1,2-^{14}C]acetate are given in **Table 3.**

There was no difference in any of the groups in the amount of radioactivity recovered as $^{14}CO_2$ during the incubation. Significantly decreased amounts of radioactivity were recovered in triacylglycerol in the liver slice preparations from rats fed OA for 1 day and this decrease persisted in rats fed OA for 2, 3, and 4 days. The radioactivity incorporated into liver free cholesterol was not significantly changed in slices prepared from rats fed OA for 1 day, but in rats fed OA for 2, 3, and 4 days, the radioactivity recovered in liver free cholesterol was increased about 3-fold over the control value. No significant radioactivity was detected in liver cholesteryl ester in any of the groups.

Incorporation of ^3H_3O into liver lipids

The radioactivity levels in liver lipid fatty acids and liver free cholesterol following injection of ^3H_2O intraperitoneally to rats fed 1% OA for 1, 2, and 8 days and in control rats are shown in **Table 4.** There were marked decreases in the labeling of the fatty acids of triacylglycerol, phosphatidylcholine, phosphatidylethanolamine, and esterified cholesterol in all of the OA-fed groups. OA had no effect on the amount of tritium incorporated into liver free cholesterol in any of the groups and no significant radioactivity was recovered in the sterol portion of liver cholesteryl esters.

Incorporation of [1-^{14}C]palmitic acid into liver lipids

The radioactivity levels in liver triacylglycerol and cholesteryl ester at 10, 20, 30, and 40 min following intrajugular injection of 4 μCi of [1-^{14}C]palmitic acid to rats fed 1% OA for 1 day and to control rats are shown in **Fig. 2.** The radioactivity recovered in both of these liver lipids was markedly decreased in the OA-fed groups.

Levels of biliary lipids in rats fed OA

These results are shown in **Table 5.** In rats fed the cholesterol-free diet, there was no change in the level of cholesterol in bile after 1 day of OA feeding; but after the second day, the biliary cholesterol level was increased 2-fold above the control. After 8 days of OA feeding, the bile cholesterol remained increased although the extent of the increase was significantly less than it had been after the second day. Dietary OA had no effect on the bile content of phospholipids or of free and conjugated total bile acids.

The presence of 2% cholesterol in the control diet had no effect on the biliary content of cholesterol, total phospholipids, or free and conjugated bile acids, but when 1% OA was included in the diet containing 2% cholesterol for 2 days, the bile cholesterol was significantly increased

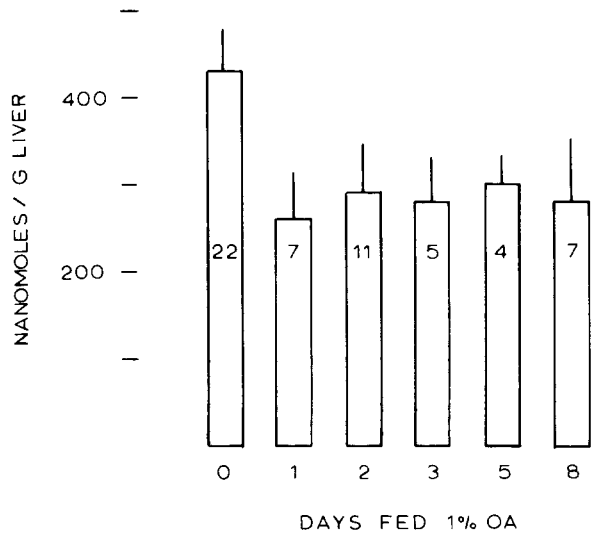

Fig. 1 Liver levels of CoASH in control rats and in rats fed a cholesterol-free diet containing 1% OA for 1, 2, 3, 5, and 8 days. The number of rats in each group is shown within each column; means ± SD. The differences among the OA-fed groups were not significant but they all were significantly lower than the control value ($P < 0.001$).

TABLE 3. Incorporation of [1,2-^{14}C]acetate into lipids and into CO_2 by liver slices of rats fed the 1% OA diet

Diet	n	CO_2	Triacylglycerol	Free Cholesterol
		dpm × 10⁻³/g per hr (mean ± SD)		
Cholesterol-free				
Control	10	130 ± 30	14.7 ± 5.5	21.0 ± 8.1
1 day OA	4	111 ± 7	8.1 ± 2.2[a]	28.8 ± 7.2
2 days OA	6	149 ± 44	8.5 ± 2.1[a]	66.6 ± 19.0[b]
3 days OA	5	135 ± 15	8.0 ± 2.3[a]	61.2 ± 21.0[b]
4 days OA	3	136 ± 20	7.8 ± 1.8[a]	69.3 ± 37.0[b]

[a]Statistical significance of difference from the control value $P < 0.05$.
[b]Statistical significance of difference from the control value $P < 0.001$.

(1.7-fold) above the corresponding control value. The presence of 1% OA in the 2% cholesterol-containing diet resulted in no change in the biliary content of total phospholipids or of free and conjugated bile acids.

Liver HMG-CoA reductase activity

The hepatic microsomal HMG-CoA reductase activities are shown in **Fig. 3.** No statistically significant change was present in the microsomal HMG-CoA reductase activity after 1 day of OA feeding to rats receiving the cholesterol-free diet, but after the second and subsequent days the activity was increased about 8-fold.

The presence of 2% cholesterol in the diet caused a marked decrease in the HMG-CoA reductase activity in the control group and no increase in HMG-CoA reductase activity occurred as a result of feeding 1% OA in this diet for 2 days.

Radioactivity of biliary cholesterol

The total radioactivities and the specific radioactivities of biliary cholesterol following administration of ^3H_2O are given in **Table 6.** After the first day of OA feeding, the total radioactivity in biliary cholesterol was slightly increased ($P = 0.05$) and the specific radioactivity of biliary cholesterol was slightly but significantly higher than the control value. After the second day of OA feeding, the total radioactivity in bile cholesterol was markedly increased (4.2-fold) over the control value and the specific radioactivity of the biliary cholesterol was almost twice that of the control groups. These increases in total and specific radioactivities of biliary cholesterol were present also after 8 days of OA feeding.

In rats fed the diet containing 2% cholesterol, both the total and the specific radioactivity of bile cholesterol were markedly decreased compared to the values obtained with cholesterol-free diet; the addition of 1% OA to the cholesterol-containing diet had no effect on either the total or the specific radioactivity of bile cholesterol.

Biliary cholesterol synthesis rates

The biliary content of newly synthesized cholesterol was slightly elevated after the first day of OA feeding but after the second day there was a marked (4.8-fold) increase (Table 6). This decreased slightly after 8 days of OA feeding but remained 3-fold higher than the controls.

The secretion of newly synthesized cholesterol in bile was much decreased in the rats fed the diet containing 2% cholesterol and OA-feeding for 2 days had no significant effect on this.

DISCUSSION

Decreased hepatic synthesis of long-chain fatty acids was the earliest detectable metabolic effect of OA-feeding.

TABLE 4. Incorporation of ^3H_2O in vivo into liver lipid fatty acids and free cholesterol of rats fed the 1% OA diet

Diet	n	Triacylglycerol	Phosphatidylcholine	Phosphatidylethanolamine	Cholesterol	
					Esterified[a]	Free
		dpm × 10⁻²/g liver (mean ± SD)				
Cholesterol-free						
Control	6	97.4 ± 28.0	92.4 ± 32.4	23.7 ± 5.6	5.7 ± 0.4	30.0 ± 9.0
1 day OA	3	14.0 ± 6.5	37.0 ± 11.9	12.4 ± 3.3	1.1 ± 0.2	28.0 ± 3.0[b]
2 days OA	6	19.8 ± 11.8	27.7 ± 8.5	10.3 ± 4.2	1.0 ± 0.1	39.0 ± 12.0[b]
8 days OA	3	32.4 ± 14.4	24.4 ± 6.8	10.6 ± 2.0	1.2 ± 0.3	38.0 ± 8.0[b]

[a]Cholesteryl ester radioactivity was almost entirely in the fatty acid portion.
[b]These values are not significantly different from the control values; For all others, $P < 0.01$.

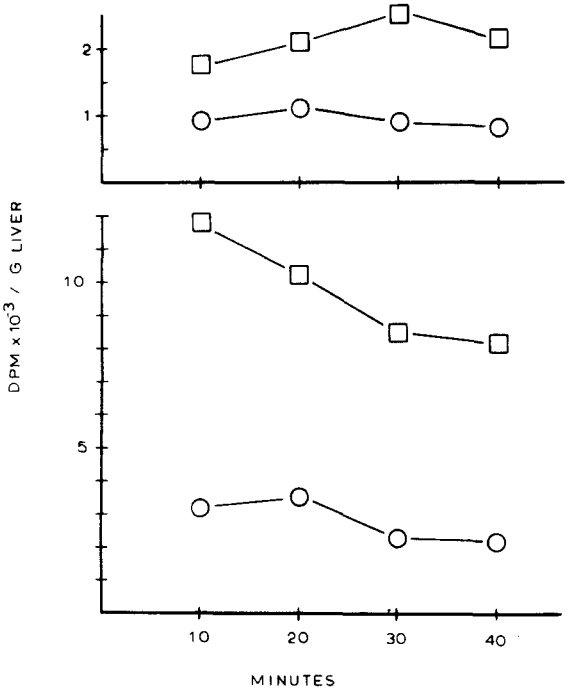

Fig. 2 Radioactivities in liver cholesteryl esters (upper section) and triacylglycerol (lower section) in control rats (□) and in rats fed 1% OA for 1 day (○) at various times following intrajugular injection of 4 μCi of [1-^{14}C]palmitic acid. Each point is the average of the results from two livers.

This was followed by increased hepatic synthesis of cholesterol. Both of these changes confirm the earlier findings of Windmueller and Spaeth (6). The present findings show also that OA markedly increases the secretion of cholesterol into bile. When cholesterol synthesis was studied in vivo, the experiments were carried out 60 min after ^3H_2O administration to minimize the effect of transport of newly synthesized lipid to and from the liver. Therefore the observation that the ^{3}H in bile cholesterol was increased after the second day of OA-feeding whereas the ^{3}H in liver cholesterol was not indicates that the excess newly synthesized hepatic cholesterol resulting from OA-feeding was secreted rapidly and quantitatively into bile. It also indicates that the excess cholesterol synthesized from [1,2-^{14}C]acetate in liver slices from OA-fed rats must have been biliary cholesterol which accumulated during the incubation.

In spite of the marked increase in newly synthesized bile cholesterol in rats fed the basal diet containing OA, newly synthesized cholesterol comprised only 12.4% of the total bile cholesterol in these rats as compared to 5.4% in the controls. Thus the majority of the increase in bile cholesterol secretion caused by OA consisted of preformed cholesterol. The observation that newly synthesized cholesterol comprises only a minor portion of bile cholesterol agrees with the findings of Turley and Dietschy (11).

Bile cholesterol is almost totally unesterified and is derived from a "metabolically active free cholesterol pool" (11). Hepatic free cholesterol may be secreted into bile, incorporated into cell membranes and lipoproteins, or esterified by ACAT which is located in the hepatic microsomes (19, 20). The resultant cholesteryl esters may be incorporated into lipoproteins or stored largely in the cytoplasm. The finding of decreased cholesteryl ester levels in both the cytosol and microsomes of rats fed OA for 1 and 2 days suggests that OA impaired the hepatic synthesis of cholesteryl ester. This is supported also by the finding of decreased incorporation of [1-^{14}C]palmitate into cholesteryl ester in livers of rats fed OA for 1 day. OA is known to decrease hepatic ATP (2) and, as reported here, there is a 40% decrease in liver CoASH after 1 day of OA-feeding. Since both of these co-factors are required for the activation of long chain fatty acids (21), their depletion could be responsible for the decreased hepatic esterification of cholesterol. We have examined the fatty acid composition of liver cholesteryl esters in OA-fed rats and have found it to be unchanged (Haines, D. S. M., and S. Tokmakjian, unpublished data). Impaired synthesis of

TABLE 5. Bile flow rate and levels of biliary compounds in rats fed the 1% OA diet

Diet	n	Flow Rate	Total Cholesterol	Total Phospholipids	Conjugated Bile Acids	Free Bile Acids
		ml/hr per 100 g (mean ± SD)		*μmol/ml (mean ± SD)*		
Cholesterol-free						
Control	8	0.25 ± 0.04	0.81 ± 0.16	7.10 ± 0.50	24.20 ± 4.90	9.00 ± 2.20
1 day OA	7	0.23 ± 0.05	0.86 ± 0.26	7.90 ± 1.50	25.90 ± 4.30	8.00 ± 2.10
2 days OA	8	0.32 ± 0.06[a]	1.70 ± 0.24[a]	6.90 ± 1.10	24.30 ± 3.90	8.20 ± 1.70
8 days OA	3	0.34 ± 0.06[a]	1.20 ± 0.10[a]	6.70 ± 0.70	19.10 ± 5.00	8.00 ± 2.10
2% Cholesterol						
Control	6	0.24 ± 0.06	0.70 ± 0.13	7.81 ± 1.40	29.70 ± 7.20	10.72 ± 3.09
2 days OA	8	0.29 ± 0.07[b]	1.20 ± 0.27[b]	8.07 ± 1.56	26.30 ± 3.70	11.03 ± 3.75

[a]Significance of difference from the corresponding control value: $P < 0.025$.
[b]Significance of difference from corresponding control group: $P < 0.01$.

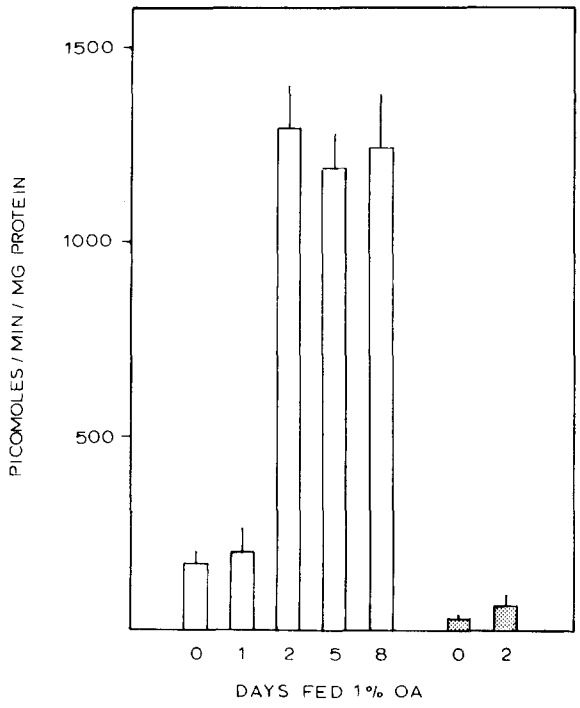

Fig. 3 Liver microsomal HMG-CoA reductase activities in control rats and in rats fed 1% OA contained in cholesterol-free diet (open bars) and diet containing 2% cholesterol (stippled bars); means ± SD, seven rats per group.

cholesteryl ester would be expected to increase the amount of hepatic free cholesterol available for biliary secretion and, since the liver total free cholesterol and the microsomal free cholesterol levels were not increased as a result of OA-feeding, the increase in bile free cholesterol output could be a mechanism whereby excess free cholesterol resulting from the impairment of cholesterol esterification is removed from the liver.

A similar conclusion was reached by Erickson et al. (22) on the basis of measurements of ACAT activity resulting from alterations in hepatic cholesterol concentration. ACAT is located predominantly on the cytoplasmic surface of RNA-rich microsomes, while microsomal cholesteryl esters are localized in RNA-poor microsomes remote from their sites of synthesis (20). This could explain the observation that the cytosolic level of cholesteryl ester fell during the first day of OA-feeding, whereas the majority of the decline in microsomal cholesteryl ester occurred only during the second day.

The OA-induced increase in bile cholesterol was not accompanied by increases in bile phospholipids or bile acids so that the molar pecentage of bile cholesterol, which was 1.9% in control rats receiving the basal diet, rose to 4.1% after 2 days of OA-feeding. Thus OA caused an uncoupling of the normal relationship between biliary cholesterol, phospholipid, and bile acids. This has been suggested as the regulatory step in bile cholesterol secretion (23). The finding that bile cholesterol output can be doubled without increases in bile phospholipids or bile acids indicates that coupling of the biliary lipids is not an essential step in the process of bile cholesterol secretion and is therefore not likely to be rate-limiting.

As has been previously reported (23), the presence of cholesterol in the diet in the absence of OA caused no change in bile cholesterol output. When OA was included in the cholesterol-containing diet, bile cholesterol output was increased but HGM-CoA reductase activity was not, indicating that the increased activity of this enzyme is not essential for the OA-induced increase in bile cholesterol secretion. There are several possible causes for the OA-induced increase in HMG-CoA reductase activity. Firstly, it could be a result of direct activation of the enzyme by either OA or its metabolites. This is considered unlikely because the increase occurred only during the second day of OA-feeding while liver nucleotide levels are changed during the first day (2). Moreover, OA does not increase

TABLE 6. Radioactivity levels and quantity of newly synthesized cholesterol in the bile of control rats and rats fed the 1% OA diet[a]

		Radioactivity in Bile Cholesterol		Newly Synthesized Bile Cholesterol	
Diet	n	dpm/ml × 10^{-4}	dpm/μmol × 10^{-4}	μmol/ml	% of Total Bile Cholesterol
Cholesterol-free					
Control	6	0.38 ± 0.14	0.52 ± 0.18	0.044 ± 0.017	6.1 ± 2.2
1 day OA	4	0.58 ± 0.15	0.75 ± 0.07[c]	0.068 ± 0.017[c]	8.7 ± 0.7[c]
2 days OA	4	1.60 ± 0.51[b]	0.99 ± 0.03[c]	0.21 ± 0.04[b]	13.4 ± 1.7[b]
8 days OA	3	1.08 ± 0.31[b]	0.95 ± 0.14[c]	0.13 ± 0.03[b]	11.4 ± 1.7[b]
2% Cholesterol					
Control	3	0.06 ± 0.01	0.082 ± 0.01	0.007 ± 0.001	0.95 ± 0.08
2 days OA	4	0.10 ± 0.04	0.082 ± 0.06	0.010 ± 0.007	0.89 ± 0.4

[a]Means ± SD.
[b]Statistically different from corresponding control value $P < 0.001$.
[c]Statistically different from corresponding control value $P < 0.025$.

cholesterol synthesis when incubated directly with liver slices (24). A second possible mechanism is that the increased loss of free cholesterol into bile results in diminished negative feed-back on the enzyme. Although no decrease in liver total free cholesterol or microsomal free cholesterol levels was present in the OA-fed rats, it is conceivable that small localized decreases were present within the microsomal membranes. Localized changes in microsomal free cholesterol levels are believed to play a role in regulation of HMG-CoA reductase (25–27). It has also been suggested that esterified cholesterol may play a similar role (28, 29). The decreased levels of cholesteryl ester in the hepatic microsomes of the OA-fed rats supports this as the most likely mechanism responsible for the increased HMG-CoA reductase activity. The fact that the major decrease in the microsomal cholesterol ester level occurred during the second day of OA-feeding simultaneous with the increase in HMG-CoA reductase further supports this conclusion.

The mechanism for the rapid decrease in long chain fatty acid synthesis may be related to the decreases in purine nucleotides which occur during the first day of OA feeding (2, 30). It has recently been shown that acetyl CoA carboxylase, which catalyzes the rate-limiting step in fatty acid synthesis, can be activated by coenzyme A (31) and by guanine nucleotides (32). Decreased liver levels of guanine nucleotides occur in OA-fed rats (30) and, as shown here, OA causes an abrupt decrease in the liver content of CoASH. The decreased synthesis of triacylglycerol and cholesteryl ester occur during the first day of OA-feeding, i.e., before the impaired secretion of lipoproteins is fully developed (4). This explains the early decreases in the total liver levels of these neutral lipids. With longer duration of OA-feeding, hepatic lipoprotein secretion is markedly impaired and liver triacylglycerol and cholesteryl ester levels increase. The OA-induced increase in cholesteryl ester was limited to the cytosol, which agrees with the observation that, in OA-fed rats, neutral lipid accumulates in cytoplasmic droplets (33). Intestinal mucosa actively synthesizes cholesteryl ester from lumenal cholesterol (34) and because it lacks orotate phosphoribosyltransferase, it is not affected by dietary OA (35). Thus the progressive accumulation of cholesteryl ester occurring after the third day of OA-feeding likely originated in intestinal mucosa and was transported to liver in chylomicrons.

The mechanism for the impaired lipoprotein secretion caused by OA is not known. It has been observed that the rate of hepatic secretion of triacylglycerol-rich lipoproteins appears to be linked to the rate of fatty acid synthesis in liver (36, 37). In view of the marked decrease in fatty acid synthesis caused by OA, it is conceivable that this could be related to the impairment in lipoprotein secretion.

In spite of the changes in liver lipid metabolism caused by dietary OA, it is interesting that oxidative metabolism as reflected by $^{14}CO_2$ production by liver slices incubated with [1,2-^{14}C]acetate was not affected. This agrees with the observation that rats can tolerate OA-feeding for long periods (38).

It is noteworthy that the liver levels of phosphatidylcholine and phosphatidylethanolamine are both increased from the first day of OA-feeding. Synthesis of phosphatidylcholine via the cytidine pathway has been shown to be increased in OA-fed rats, presumably as a consequence of elevated liver levels of cytidine nucleotides (39) which could explain the elevation of liver phosphatidylcholine. The cause of the increased liver phosphatidylethanolamine level may have a similar basis.

Manuscript received 25 January 1984.

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