

Effect of dietary n-3 versus n-6 polyunsaturated fatty acids on hepatic excretion of cholesterol in the hamster¹

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Abstract Dietary polyunsaturated fatty acids of the n-6 and the n-3 class show differing effects on serum lipids and hepatic lipoprotein metabolism, which could be induced by alterations in hepatocellular cholesterol balance. As both fatty acid classes exert parallel effects on lipoprotein uptake and synthesis of cholesterol in the liver, we studied whether they have differing effects on the excretory pathways for cholesterol. Male Syrian hamsters were fed for 3 weeks low-cholesterol diets supplemented (9% w/w) with either saturated (coconut fat), n-6 unsaturated (safflower oil) or n-3 unsaturated fatty acids (fish oil), which shifted the serum lipid levels. N-6 unsaturated fatty acids increased both the synthesis of cholic acid (+57%; $P = 0.05$) and, in fistula bile, the secretion of cholesterol (+37%; $P < 0.05$ vs. saturated fatty acids). By contrast, n-3 unsaturated fatty acids did not enhance synthesis of cholic acid or biliary secretion of cholesterol (-30%, NS). The fatty acid pattern of biliary phospholipids was modified according to the major unsaturated fatty acids in the diet. The alterations both in phospholipid fatty acid composition and in secretory ratio of cholesterol to phospholipids and bile acids persisted during controlled secretion of taurocholic acid at increasing rates. In conclusion, hepatic excretion of cholesterol is increased on dietary n-6 unsaturated fatty acids, and low on n-3 unsaturated fatty acids. These two dietary fatty acid classes change differently the fatty acid composition of biliary phospholipids and the secretory ratio of cholesterol to phospholipids and bile acids in bile.—Berr, F., A. Goetz, E. Schreiber, and G. Paumgartner. Effect of dietary n-3 versus n-6 polyunsaturated fatty acids on hepatic excretion of cholesterol in the hamster. *J. Lipid Res.* 1993. **34**: 1275–1284.

Supplementary key words polyunsaturated fatty acids (n-3, n-6) • bile acid synthesis • bile cholesterol secretion • bile phospholipids

The major classes of fatty acids in dietary fat have received much attention because of their differing effects on lipid metabolism and atherogenesis (1-7). The three major families of unsaturated fatty acids, n-9 mono-unsaturated, n-6 polyunsaturated, and n-3 polyunsaturated fatty acids, are formed in plants and phytoplankton and, once delivered to vertebrates by the food chain, are not further interconverted; n-6 and n-3 unsaturated fatty acids are not synthesized in the body, whereas n-9 un-

saturated fatty acids can be synthesized from saturated fatty acids (2, 8). Accordingly, the fatty acid classes in the diet can modify the fatty acid composition of triglycerides and phospholipids in serum (9, 10) and cells (10, 11).

Dietary triglycerides composed either of saturated, or n-6 polyunsaturated, or n-3 polyunsaturated fatty acids (PUFA) differ in their effects on serum lipid levels. Saturated fat, the major class of dietary fat for many humans, raises both cholesterol, especially in the LDL fraction, and triglycerides (3, 12-14); n-6 polyunsaturated fatty acids lower cholesterol, but not triglycerides (3, 6, 7, 12-15); and n-3 polyunsaturated fatty acids lower cholesterol, especially in VLDL, and even more triglyceride levels in serum of rat (15-17) and humans (3-7, 18). These shifts of serum lipid concentration have been explained by changes in hepatic secretion of VLDL and subsequent production of LDL, and in receptor-mediated clearance of LDL by the liver (17, 19-23). However, the influences on hepatic cholesterol balance, which in part could trigger these effects, are not entirely known. N-6 and n-3 unsaturated fatty acids both decrease cholesterol synthesis (17) and increase LDL receptor activity in the liver (17). Whereas n-6 unsaturated fatty acids stimulate secretion of VLDL (19), and thus efflux of cholesterol from the liver, n-3 unsaturated fatty acids decrease secretion of VLDL (11, 18). However, it is not well understood which effects, if any, the two classes of dietary polyunsaturated fatty acids exert on hepatic excretion of cholesterol.

Therefore we studied in the hamster, a rodent with far-reaching analogies to man in hepatic cholesterol and bile

Abbreviations: FTR, fractional turnover rate; GLC, gas-liquid chromatography; HDL, high density lipoproteins; LDL, low density lipoproteins; PUFA, polyunsaturated fatty acids; VLDL, very low density lipoproteins.

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acid metabolism (24–26), whether isocaloric diets supplemented with either saturated or n-6 polyunsaturated or n-3 polyunsaturated fatty acids exert a differential effect on hepatic excretion of cholesterol. The intake of cholesterol was maintained low. This is important in species with a low rate of hepatic synthesis of cholesterol, such as hamster or humans (24), because increased hepatic influx of absorbed cholesterol in chylomicron remnants could not be compensated in these species by down-regulation of cholesterol synthesis, but may alter hepatic cholesterol excretion. We studied specifically whether dietary n-3 or n-6 polyunsaturated fatty acids increase excretion of cholesterol by either stimulating its conversion to bile acids or enhancing the biliary secretion of cholesterol.

MATERIALS AND METHODS

Animal treatment

Male Syrian hamsters (Fa. Ivanovas, Kislegg, FRG) weighing 90–110 g were used for the experiments. The protocol complied with national guidelines for the care and use of laboratory animals and was approved by an official veterinary commission. All hamsters were maintained with alternating cycles of light (7:00 AM to 7:00 PM) and darkness and had free access to chow and water.

Litter-matched animals ($n = 16$ per group) were assigned to one of the following three dietary regimens for 18–21 days prior to study: Altromin Standard chow 1310® (Altromin GmbH, 4937 Lage, FRG) supplemented (8–9% wt/wt) with either coconut fat (saturated fatty acids), safflower oil (n-6 PUFA), or fish oil (n-3 PUFA). The chow was freshly prepared every 3 days by soaking pelleted Altromin in the pure triglyceride oils in sealed containers under nitrogen. Marine fish oil (MaxEPA®),

safflower oil, and coconut fat of high grade purity were a generous gift from R. P. Scherer GmbH, Eberbach, FRG; they all contained as antioxidant 2 mg vitamin E per gram. The final composition of the diets is detailed in **Table 1**. The chow in the animal cages was changed every other day and daily consumption of chow was determined by weighing. Daily intake of chow (6.3 ± 0.7 g/d; $n = 48$, mean \pm SD) and total weight gain (13.1 ± 7.1 g) during pretreatment, final body weight (111 ± 8 g), liver weight (4.3 ± 0.7 g; $n = 33$) and liver/body weight ratio ($3.8 \pm 0.4\%$) were nearly the same in all three groups. All animals were fasted for 18 h prior to study of serum lipids, cholesterol absorption, or biliary secretion; nevertheless their stomach was filled with food by the time of study due to chow supplied out of the cheek pouches. Surgery and bile collections were performed during pentobarbital anesthesia (26). Blood was collected through the abdominal aorta under ether anesthesia for the determination of serum lipids and for the assay of intestinal absorption of cholesterol. Liver weight was determined after exsanguination.

Cholesterol absorption studies

Intestinal absorption of cholesterol was estimated by the dual-isotope plasma ratio method of Zilversmit and Hughes (27). A bolus (0.5 ml) of 20% soybean triglyceride emulsion (Intralipid, Kabi-Vitrum AB, Stockholm, Sweden) containing 10^6 dpm [$4\text{-}^{14}\text{C}$]cholesterol (60 mCi/mmol; New England Nuclear, Dreieich, FRG) and 2 mg of unlabeled cholesterol was administered by gavage into the stomach of ether-anesthetized animals that had been fed ad libitum. Another 0.5 ml bolus of Intralipid containing 2×10^6 dpm [$1,2\text{-}^3\text{H}$]cholesterol (45 Ci/mmol; New England Nuclear) and 20 μg unlabeled cholesterol was simultaneously injected through a jugular vein catheter hidden in a subcutaneous pouch in the neck. After 72 h,

TABLE 1. Composition of experimental diets

Content	Standard Chow (Altromin 1310)® with		
	Coconut Fat	Safflower Oil	Fish Oil
Energy (kcal/g)	3.77 ± 0.03	3.72 ± 0.04	3.76 ± 0.05
Cholesterol (% w/w)	0.02	0.02	0.04
Fat (% w/w)	9.4 ± 0.3	8.4 ± 0.5	8.8 ± 0.5
Fatty acid composition (%) ^a			
Palmitic (16:0)	49.0	7.6	27.8
Palmitoleic (16:1, n-9)	0	0	9.1
Stearic (18:0)	13.7	6.1	11.6
Oleic (18:1, n-9)	30.5	9.9	21.2
Linoleic (18:2, n-6)	6.7	70.6	0
Linolenic (18:3, n-3)	0	0	0
Arachidonic (20:4, n-6)	0	1.0	0
Eicosapentaenoic (20:5, n-3)	0	1.7	18.5
Docosahexaenoic (22:6, n-3)	0	0	11.9

^aDetermination by chloroform-methanol extraction and gas-liquid chromatography (36).

when the $^3\text{H}/^{14}\text{C}$ plasma isotope ratio remained constant, serum was obtained by exsanguination. Cholesteryl esters in serum were saponified (27) and steroids were extracted into hexane. ^3H and ^{14}C radioactivity of the hexane extracts and of the initial doses dissolved in Intralipid were assayed in a liquid scintillation spectrometer (LSC BF-5000/300, Berthold, Karlsruhe, FRG) using Aquasolv (New England Nuclear, Boston, MA) as scintillation cocktail. Cholesterol concentration of the extracts was determined with commercial photometric kits (Boehringer, Mannheim, FRG). ^3H and ^{14}C radioactivity in serum were expressed as the fraction of the intravenously administered dose of [^3H]cholesterol radioactivity and as the fraction of the orally administered dose of [^{14}C]cholesterol radioactivity, respectively. Fractional absorption of cholesterol (%) was calculated as serum fraction of the oral ^{14}C dose divided by the serum fraction of the intravenous ^3H dose times 100 according to Zilversmit (27). The [^3H]cholesterol tested according to Davidson et al. (28) was radiochemically reliable, i.e., the $^3\text{H}/^{14}\text{C}$ radioactivity ratio did not decay in vivo.

Bile acid pool size and synthesis rate of cholic acid

To estimate cholic acid pool size as validated in hamsters (26), [$24\text{-}^{14}\text{C}$]cholate (60 mCi/mol; New England Nuclear) (75–90 nCi in 50 μl 5% ethanol) was injected into the jugular vein and after 2 h the common duct was cannulated, the gallbladder was emptied, and the cystic duct was ligated (26). Gallbladder bile and hepatic output of bile during the first hour of depletion of the bile acid pool were analyzed for cholic acid mass and ^{14}C radioactivity to calculate cholic acid pool size (26). As the recovery of [^{14}C]cholic acid was decreased by 46% in fish oil-fed hamsters as compared to hamsters on the other diets, turnover and size of the cholic acid pool were further studied by a dual-label technique. Bile acid pool composition (29) and the kinetics of cholic acid were determined in five hamsters on each dietary regimen using the double-label single-isotope ratio principle (30) with $^2\text{H}_4$ -labeled and ^{13}C -labeled cholic acid (Merck, Sharp & Dohme, Montreal, Canada). To this end, hamsters were injected 0.25 mg [$2,2,4,4\text{-}^2\text{H}_4$]cholic acid at time zero and 0.25 mg [$24\text{-}^{13}\text{C}$]cholic acid 18 h later through a subcutaneously placed jugular vein catheter. At 36 h the animals were exsanguinated and liver, biliary tract, and small intestine were excised en bloc; the liver weight was determined. All the tissue was minced in a 100-ml Erlenmeyer flask, and total steroids were extracted into ethanol using tauro-[$24\text{-}^{13}\text{C}$]chenodeoxycholic acid (50 mCi/mmol; New England Nuclear) as recovery standard (29). Appropriate aliquots of the ethanol extract were added to 2.5 μg hyodeoxycholic acid as internal standard, washed 2 times with 10-fold excess of hexane, and then subjected to enzymatic hydrolysis, methylation, and silylation, and analyzed by GLC for the concentrations of

individual bile acids (26, 31). The masses of individual and total bile acids in the tissue extract represent the total bile acid pool (without any fraction of the pool reabsorbed from the colon) (29) and its bile acid composition. The same samples were analyzed by mass spectrometry for $^2\text{H}_4/^{13}\text{C}$ isotope ratio of cholic acid, which was measured by gas-liquid chromatography-mass spectrometry-single ion monitoring of cholic acid at m/z ratios of 462 and 458 mass units by previously established methods (31). The fractional turnover rate of the cholic acid pool was calculated according to Vantrappen, Rutgeerts, and Ghoois (30).

Rates and linkage coefficients of biliary lipid output

Output of biliary lipids was determined in the bile collected during the first hour after interruption of the enterohepatic circulation (basal secretory rate). Linkage coefficients, defined as nmoles secretory increment per 1 nmole increment of bile salt secreted (26, 32), were determined after 90 min interruption of the enterohepatic circulation during a subsequent graded infusion of taurocholate (Calbiochem-Behring GmbH, Frankfurt, Germany), which was given for 130 min (26). The infusion of taurocholate was started at a rate of 6 $\mu\text{mol h}^{-1}$ 100 g^{-1} body weight 80 min after insertion of the bile fistula and was stepwise increased to 12, 24, and 36 $\mu\text{mol h}^{-1}$ 100 g^{-1} . Each infusion rate was maintained for 30 min, allowing for a 10-min equilibration and a 20-min bile collection. Secreted bile acids consisted of 90–94% taurocholate. The slopes of the linear regression lines of biliary outputs (nmol min^{-1} g^{-1} liver) of cholesterol versus bile salt, of phospholipid versus bile salt, and of cholesterol versus phospholipid yielded the respective linkage coefficients (26).

Chemical analyses

Concentrations of cholesterol and bile acids in bile samples were determined by capillary gas-liquid chromatography, total phospholipid concentration by the photometric assay of Bartlett (33), and output rates of biliary lipids were calculated from bile flow rates and lipid concentrations as described (26). The cholesterol saturation index according to Carey (34) was computed from the actual concentrations of cholesterol, phospholipids, and total bile salts in bile (35). Concentrations of cholesterol, HDL cholesterol, and triglycerides in serum were assayed using commercial kits of Boehringer GmbH, Mannheim, FRG. Total lipids were extracted from serum, which had been pooled from six animals per dietary group; fatty acid patterns of these serum lipid extracts as well as of lipid extracts of bile were determined by capillary gas-liquid chromatography after acidic transmethylation (9, 36).

Statistical analysis

All results, unless otherwise stated, are expressed as means \pm SD. Means of the three experimental groups

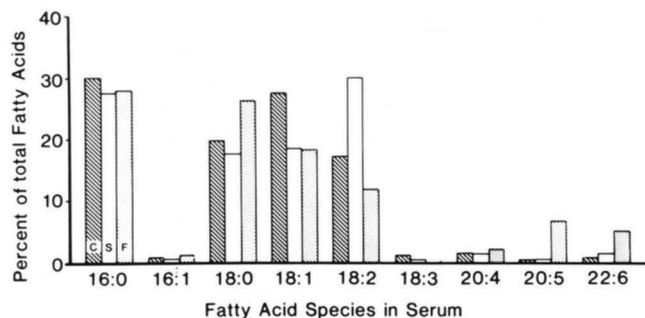


Fig. 1. Average distribution of fatty acids in total lipids of serum (pooled from six hamsters of each group) after 18–21 days intake of chow supplemented with coconut fat (■), safflower oil (□), or marine fish oil (▨). Differences were induced by the fatty acid composition of the diet (compare Table 1, also for abbreviation of fatty acids).

were compared by one-way analysis of variance; differences in means were tested using the Student's *t* test at the $P < 0.05$ level (37).

RESULTS

Serum lipids

When male hamsters had been maintained for 3 weeks on isocaloric diets differing in fatty acid composition (Table 1), the fatty acid pattern of total serum lipids was accordingly modified (Fig. 1). The safflower oil diet (70.5% linoleate) raised the fraction of linoleate (18:2, *n*-6) and largely displaced *n*-3 and *n*-9 unsaturated fatty acids. Fish oil diet (30.4% *n*-3 PUFA, 21% oleate) increased the fractions of eicosapentaenoic (20:5, *n*-3) and docosahexaenoic acid (22:6, *n*-3) and reduced that of linoleate. The coconut fat diet (63% saturated fatty acids, 30.5% oleate) raised oleate (18:1, *n*-9) and decreased *n*-3 and *n*-6 PUFA content of total serum lipids.

As compared with the unsupplemented, low-cholesterol diet (Altromin 1310®) serum triglycerides were raised by 33% ($P < 0.05$) on the coconut fat diet, unchanged

(–6%) on the safflower oil diet, and lowered by 28% ($P < 0.05$) on the fish oil diet (Table 2); total serum cholesterol was increased by 30% ($P < 0.05$) on the coconut fat diet and not significantly changed on the safflower oil diet (+11%) and on the fish oil diet (–7%). Total serum cholesterol levels were lower (–28%, $P < 0.01$) on fish oil than on the coconut fat diet, but not significantly lower on safflower oil (–15%) than on the coconut fat diet.

Dietary intake and intestinal absorption of cholesterol

Daily intake of chow (6.2 ± 0.6 ; 6.5 ± 0.7 ; 6.1 ± 0.3 g/day) was comparable on coconut fat, safflower oil, and fish oil diets, which all were low in cholesterol content (Table 1). Average daily intake of cholesterol, therefore, was low in the three groups, i.e., 1.2 mg/d on coconut fat, 1.1 mg/d on safflower oil, and 2.1 mg/d on fish oil diet. As estimated by the double-isotope labeling technique (27), fractional absorption of cholesterol (administered in soybean triglyceride emulsion) averaged $61 \pm 12\%$, $66 \pm 3\%$, and $60 \pm 9\%$ ($n = 5$; mean \pm SD) on coconut fat, safflower oil, and fish oil diet, respectively.

Bile acid pool size and synthesis rate of cholic acid

Hamsters fed the fish oil diet had the total bile salt pool reduced by 48% on average, and the cholic acid pool by 47% as compared with animals fed the safflower oil diet (Fig. 2). Synthesis of cholic acid was higher (+57%; +67%) on the safflower oil diet than on the fish oil or coconut fat diets and was not different between hamsters fed the fish oil or the coconut fat diet (Table 3). As the synthesis rate was normal, the reduced size of the cholic acid pool during intake of fish oil was explained by its increased fractional turnover (Table 3), which represents enhanced intestinal loss of cholic acid. The pool size of deoxycholic acid and the fraction of lithocholic acid in the bile salt pool were also clearly reduced by the fish oil diet, while the pool of chenodeoxycholic acid was not different (Fig. 2).

When determined by isotope dilution with [^{14}C]cholic acid during collection of basal output of bile, similar esti-

TABLE 2. Effect of diets on serum lipid concentrations

Serum Lipid Concentration	n	Dietary Pretreatment with 9% (wt/wt)		
		Coconut Fat	Safflower Oil	Fish Oil
		mg/dl, mean \pm SD		
Triglycerides ^a	10	210 \pm 38	149 \pm 27 ^b	114 \pm 30 ^c
Cholesterol ^a	10	153 \pm 26	131 \pm 12	110 \pm 19 ^c
HDL cholesterol	6	71 \pm 15	61 \pm 7	45 \pm 10 ^{c,d}

^aControl hamsters ($n = 10$) on unsupplemented diet (Altromin 1310®) had serum triglyceride levels of 158 ± 34 mg/dl and cholesterol levels of 118 ± 25 mg/dl.

^b $P < 0.05$ vs. hamsters on coconut fat diet.

^c $P < 0.01$ vs. hamsters on coconut fat diet.

^d $P < 0.01$ vs. hamsters on safflower oil diet.

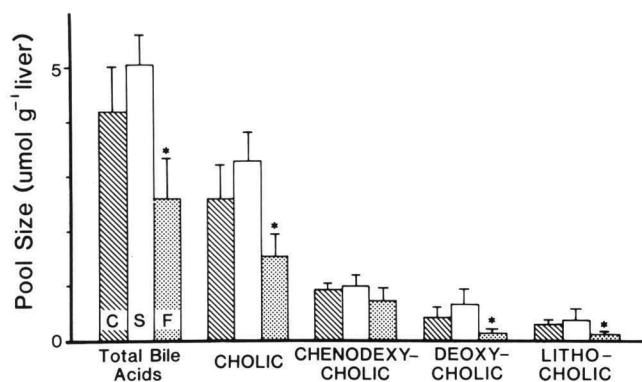


Fig. 2. Size and composition of the bile acid pool in hamsters ($n = 5$; mean \pm SD) after 21 days intake of low-cholesterol diet supplemented with coconut fat (▨), safflower oil (□), or fish oil (▤). Pool size was determined by extraction and GLC analysis of the bile acids from liver, biliary tract, and intestine of these hamsters (26, 29). * $P < 0.05$ vs. safflower oil and coconut fat.

mates as with chemical determination (Table 3) were obtained for cholic acid pool size on the safflower oil diet (3.4 ± 0.8 nmol g^{-1} liver; $n = 6$; $P < 0.02$ vs. coconut fat) and on the coconut fat diet (2.3 ± 0.3 nmol/ g^{-1} liver; $n = 6$); the latter compare well with cholic acid pools (1.97 ± 0.71 nmol g^{-1} liver) previously determined in untreated female control hamsters (26). By contrast, in fish oil-fed hamsters the pool size of cholic acid could not be estimated by radioisotope dilution, because biliary recovery of [^{14}C]cholic acid ($40.9 \pm 10.5\%$; $n = 6$) was much lower than in the hamsters fed coconut fat ($64.1 \pm 12.1\%$; $n = 6$) or safflower oil ($69.0 \pm 8.0\%$; $n = 6$). This, too, is explained by increased intestinal loss of cholic acid during intake of the fish oil diet.

Biliary lipid output

Basal secretion. Bile flow and output of bile salts and phospholipids were not significantly different among the three groups during acute bile fistula drainage for 1 h.

Hamsters fed the safflower oil diet showed increased output of cholesterol in bile ($+95\%$; $P < 0.01$) as compared with hamsters on the fish oil diet. On the coconut fat diet, biliary output of cholesterol was intermediate (Table 4). Cholesterol saturation of bile was increased ($P < 0.01$) on the safflower oil diet as compared with the fish oil and coconut fat diets.

The pattern of bile acids secreted during basal output of bile showed a moderately higher fraction of deoxycholic acid on the safflower oil diet ($11.3 \pm 3.1\%$) than on the fish oil ($5.4 \pm 3.5\%$; $P < 0.01$) or coconut fat diets ($6.8 \pm 2.0\%$; $P < 0.05$), while the fractions of cholic acid ($53 \pm 5\%$; $54 \pm 8\%$; $58 \pm 6\%$), chenodeoxycholic acid ($33 \pm 2\%$; $27 \pm 5\%$; $29 \pm 4\%$), and lithocholic acid ($3 \pm 2\%$) were similar in all three dietary groups (coconut fat, safflower oil, fish oil, respectively).

The composition of dietary fat modified the pattern of unsaturated fatty acids of biliary phospholipids (Fig. 3). The coconut fat diet increased the fraction of oleic acid, and safflower oil diet that of linoleic acid. The fish oil diet led to the highest fraction of $n-3$ unsaturated fatty acids and to the lowest linoleate fraction of biliary phospholipids. All three diets contained very little arachidonic acid. Therefore, the content of arachidonic acid in biliary phospholipids was low and only slightly increased on the safflower oil diet. These diet-induced patterns of phospholipid fatty acids (Fig. 3) nearly persisted during taurocholate infusion after 240 min interruption of the enterohepatic circulation; only the linoleate fraction decreased by 8% ($P < 0.02$) in the safflower oil group and by 5% (NS) in the coconut fat group.

Biliary secretion during taurocholate infusion. Comparable bile flow and biliary output of bile salts, mainly taurocholate, and phospholipids were attained in all three experimental groups during intravenous infusion up to the maximum rate of taurocholate (Table 4). During this well-defined, increasing biliary secretion of taurocholate, cholesterol output in bile was elevated on the safflower oil

TABLE 3. Kinetic analysis of cholic acid metabolism

Hamster Number	Pool Size			Fractional Turnover Rate			Synthesis Rate		
	Coco	Saff	Fish	Coco	Saff	Fish	Coco	Saff	Fish
	$\mu\text{mol } g^{-1}$			d^{-1}			$\mu\text{mol } g^{-1} d^{-1}$		
1	3.15	3.90	1.80	-0.48	-1.01	-1.05	1.51	3.94	1.88
2	2.05	3.30	1.91	-0.86	-0.60	-0.65	1.75	1.98	1.24
3	1.92	2.45	1.03	-0.51	-0.71	-1.29	0.98	1.73	1.33
4	2.55	3.54	1.52	-0.62	-0.68	-1.22	1.58	2.41	1.85
5	3.28	3.42	1.34	-0.45	-0.63	-1.09	1.47	2.15	1.46
Mean	2.59	3.32	1.52	-0.58	-0.73	-1.06	1.46	2.44	1.55
\pm SD	$\pm 0.62^a$	$\pm 0.54^a$	± 0.35	$\pm 0.17^a$	± 0.16	$\pm 0.25^b$	$\pm 0.29^b$	± 0.87	$\pm 0.30^c$

Abbreviations: Coco, coconut fat; Saff, safflower oil; Fish, fish oil.

^a $P < 0.05$ vs. hamsters on fish oil diet.

^b $P < 0.05$ vs. hamsters on safflower oil diet.

^c $P = 0.06$ vs. hamsters on safflower oil diet.

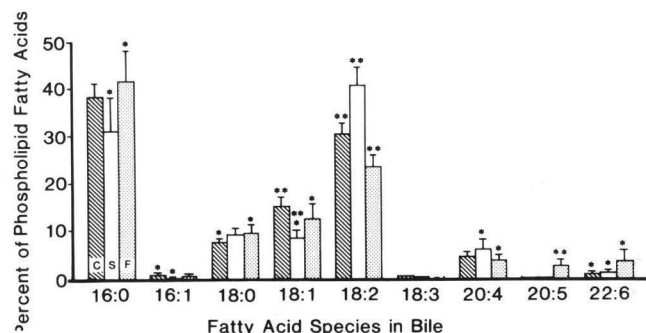


Fig. 3. Pattern of phospholipid fatty acids during basal secretion of bile in hamsters ($n = 6$; mean \pm SD) fed for 21 days a low-cholesterol diet supplemented with coconut fat (■), safflower oil (□), or fish oil (▨). Differences were induced by fatty acid composition of the diet (compare Table 1). Minor fatty acid species are not shown; they comprised $2.2 \pm 0.4\%$ on coconut fat, $3.4 \pm 1.5\%$ on safflower oil, and $2.9 \pm 2.1\%$ on fish oil. * $P < 0.05$; ** $P < 0.01$.

diet as compared with the two other diets. The linkage coefficients indicated that safflower oil diet significantly increased the secretory coupling of cholesterol to bile salts (+90% vs. coconut fat, +73% vs. fish oil diet) and of cholesterol to phospholipids (+53% vs. fish oil diet). The secretory coupling of phospholipids to bile salts was simi-

lar in the three experimental groups. Also, the y-intercepts of the regression lines were very similar for all three experimental groups (data not shown). Thus, the different diets had caused persistent changes in the fatty acid composition of biliary phospholipids and in the secretory coupling of cholesterol to phospholipids and to bile salts.

DISCUSSION

Ingestion for 3 weeks of isocaloric, low-cholesterol diets that differed in the composition of fatty acid classes (Table 1) exerted different effects on the hepatic excretion of cholesterol in this hamster model. The fish oil diet enriched in n-3 polyunsaturated fatty acids yielded the lowest rate of biliary secretion of cholesterol and a low conversion rate of cholesterol to cholic acid. Dietary n-6 polyunsaturated fatty acids in safflower oil diet increased both hepatic synthesis of cholic acid and, most of all, biliary secretion of cholesterol. Coconut fat consisting mainly of saturated and n-9 monounsaturated fatty acids had an intermediate effect on biliary secretion of cholesterol and none on cholic acid synthesis. Furthermore, the differences in biliary secretion of cholesterol were associated with diet-induced alterations of the fatty acid pattern of the phospholipids secreted in bile.

TABLE 4. Biliary secretion of lipids

Variable	Dietary Pretreatment with 9% (wt/wt)		
	Coconut Fat	Safflower Oil	Fish Oil
A. Basal output of bile ($\text{min}^{-1}\text{g}^{-1}$)^a			
Flow (μl)	1.63 ± 0.50	1.66 ± 0.50	1.24 ± 0.37
Bile salt (nmol)	24.9 ± 7.1	30.3 ± 6.3	24.1 ± 7.1
Phospholipid (nmol)	4.94 ± 1.01	4.90 ± 1.00	3.57 ± 0.61
Cholesterol (nmol)	0.84 ± 0.27^b	1.15 ± 0.17^c	0.59 ± 0.15
Cholesterol saturation index ^d	0.85 ± 0.14	1.06 ± 0.08^e	0.72 ± 0.17
B. Taurocholate-stimulated output of bile: maximum output ($\text{min}^{-1}\text{g}^{-1}$)^a			
Flow (μl)	3.74 ± 0.91	4.02 ± 0.97	3.46 ± 0.93
Bile salt (nmol)	62.3 ± 13.3	60.2 ± 9.0	53.4 ± 7.5
Taurocholate (nmol)	58.2 ± 12.2	55.7 ± 7.9	49.9 ± 6.8
Phospholipid (nmol)	6.92 ± 1.90	7.10 ± 1.26	6.74 ± 1.35
Cholesterol (nmol)	0.92 ± 0.17	1.42 ± 0.33^e	0.87 ± 0.19
Cholesterol saturation index ^d	0.41 ± 0.09	0.54 ± 0.10^e	0.37 ± 0.10
Linkage coefficients^f			
Phospholipid vs. bile salt	0.064 ± 0.031	0.082 ± 0.018	0.084 ± 0.040
Cholesterol vs. bile salt	0.010 ± 0.003	0.019 ± 0.005^g	0.011 ± 0.004
Cholesterol vs. phospholipid	0.165 ± 0.042	0.207 ± 0.036^e	0.135 ± 0.029
Bile salt-dependent flow ($\mu\text{l}/\mu\text{mol}$)	0.033 ± 0.011	0.037 ± 0.009	0.037 ± 0.015
Bile salt-independent flow ^h	1.26 ± 0.49	0.99 ± 0.36	0.92 ± 0.42

^aBasal biliary output was collected from 0 min to 60 min after cannulation of the common bile duct and ligation of the cystic duct. Maximum output was subsequently determined during a graded intravenous infusion of taurocholate (see Methods). Data are calculated per g liver weight. Values are given as means \pm SD; $n = 6$.

^b $P = 0.05$ vs. hamsters on fish oil diet.

^c $P < 0.01$ vs. hamsters on fish oil diet.

^dAccording to Carey (34).

^e $P \leq 0.02$ vs. hamsters on coconut fat and on fish oil diets.

^fDefined as secretory increment (nmol) per 1 nmol increment of bile salt secreted (calculated by linear regression analysis; the y-intercepts of the regression lines were very similar in the three dietary groups).

^hDefined as y-intercept of the regression of bile flow rate vs. bile salt secretion rate.

The design of this study was balanced to assure that the alterations in hepatic excretion of cholesterol on the three different dietary regimens were induced by the differences in degree of unsaturation and position of double bonds of dietary fatty acids. The cholesterol content of the fish oil diet was slightly higher than that of either of the other diets. But at a level of 40 mg/100 g, it was very low even in the fish oil diet and below the lowest levels (60–100 mg/100 g) that influence LDL metabolism (20) and hepatic cholesterol content in hamsters (38). Furthermore, dietary n-6 and n-3 polyunsaturated fatty acids, as compared with saturated fatty acids, led to similar differences in serum cholesterol and HDL cholesterol concentrations in these hamsters as in the rat (15–17) and humans (3, 5, 18). However, in response to dietary saturated fatty acids, triglycerides had greater increases in serum of these hamsters than in fasting serum of rats (15, 17) or humans (2–4, 6, 39). This could be explained by alimentary triglyceridemia which differs after intake of saturated or polyunsaturated fatty acids (39), as even after 18 h withdrawal of food the stomachs of these hamsters were still filled with food that obviously had been supplied from the cheek-pouches. Thus, it is reasonable to assume that the differential dietary effects of these three fatty acid classes on hepatic lipoprotein metabolism had been achieved by the dietary pretreatment in this study.

Controversy exists whether n-6 and n-3 polyunsaturated fatty acids interfere with intestinal absorption of cholesterol. We have not observed differences in fractional absorption of cholesterol when the tracer was administered in soybean triglyceride emulsion (20%, 0.5 ml) into the stomach that was filled with approximately 2–3 g of the respective chow. The consistently high fraction (48–68%) of absorbed cholesterol suggests that the mucosal ability to absorb cholesterol was not impaired by chronic ingestion of polyunsaturated fatty acids. However, because the cholesterol tracer was administered in an emulsion of soybean triglycerides (n-6 PUFA), the data do not exclude the possibility that dietary n-3 polyunsaturated fatty acids could interfere with the luminal events required for cholesterol absorption. Thus, in lymph-canulated rats, fractional absorption of cholesterol from the small intestine was reported to be impaired by n-3 polyunsaturated fatty acids. Given as large intraduodenal boluses (280 and 170 mg, respectively) the emulsions of corn oil (n-6 PUFA-rich triglycerides) and even more so of salmon oil (n-3 PUFA-rich triglycerides) led to malabsorption of fat and cholesterol as compared to an emulsion of unesterified oleic acid (40, 41). Most likely, however, this was caused by poor digestion of the acute triglyceride loads by pancreatic enzymes, because cholesterol had been well absorbed in the duodenum from emulsions of the free fatty acids such as eicosapentaenoic (n-3), arachidonic (n-6), or oleic acid (n-9) (41). The effect that chronic ingestion of fish oil versus lard (i.e.,

saturated fatty acids) exerts on cholesterol absorption has been studied in African green monkeys (42); cholesterol absorption had been determined by the continuous isotope feeding method (43) from the ratio of cholesterol tracer to sitosterol tracer in feces. This study showed that the fractional absorption of cholesterol is high and not impaired by n-3 or n-6 polyunsaturated fatty acids, as long as the cholesterol content of the diet is low (42). This is in agreement with the present study, in which the estimated dietary intake (≤ 2.1 mg/d) of cholesterol also was low, even less than the estimated amount of cholesterol daily excreted in bile (≤ 2.75 mg d⁻¹ 100 g⁻¹ on the safflower oil diet, calculated as basal hourly output times 24).

Dietary n-6 polyunsaturated fatty acids enhanced both pathways for hepatic excretion of cholesterol, i.e., biliary secretion and bile acid synthesis. They stimulated synthesis of cholic acid and expanded the size of the cholic acid and of the total bile acid pool (Table 3; Fig. 2) as compared to the two other dietary regimens. The average amounts of cholesterol converted to cholic acid (calculated from Table 3) were 2.2, 3.7, and 2.3 mg d⁻¹ 100 g⁻¹ body weight on coconut fat, safflower oil, and fish oil diets, respectively. Six of eight studies in humans (13, 24, 44–49) and one study in the rat (50) had also found higher fecal excretion of bile acids or acidic steroids as evidence of increased bile acid synthesis during intake of a diet rich in n-6 polyunsaturated fatty acids as compared to a diet rich in saturated fat. In contrast to n-6 polyunsaturated fatty acids, feeding of n-3 polyunsaturated fatty acids did not stimulate hepatic synthesis of cholic acid, even though it enhanced intestinal loss of cholic acid and thus reduced the cholic acid pool (Table 3, Fig. 2). There are only two studies of the effect of dietary n-3 polyunsaturated fatty acids on hepatic synthesis of bile acids (15, 44). Connor, Lin, and Harris (44) found that fecal excretion of bile acids, i.e., steady state synthesis of bile acids, increased in healthy subjects on a salmon oil diet as compared to a control diet with saturated fat. In rats, as in hamsters (Table 3), the synthesis of bile acids was not increased on a fish oil diet, when studied during 10–24 h interruption of the enterohepatic circulation (15). In our study, intake of n-3 versus n-6 polyunsaturated fatty acids clearly had different effects on the regulation of bile acid synthesis in the liver.

Biliary secretion of cholesterol was raised not only in the hamster (Table 4) but also in rat (15, 50) and humans (51) by chronic ingestion of n-6 polyunsaturated fatty acids. By contrast, the influence of dietary n-3 polyunsaturated fatty acids on biliary secretion of cholesterol is still controversial. Balasubramaniam et al. (15) found that secretion of cholesterol was increased threefold in rats fed n-3 polyunsaturated fatty acids as compared to others fed coconut fat. Cholesterol homeostasis, however, had been perturbed by 10–24 h biliary drainage in these rats. By contrast, biliary drainage for only 1 h probably did not

perturb cholesterol homeostasis in the hamsters of our study, in which dietary n-3 polyunsaturated fatty acids lowered output of cholesterol in bile. In a similar fashion, dietary n-3 polyunsaturated fatty acids decreased cholesterol saturation and thus the molar ratio of cholesterol to phospholipids and bile acids in bile of African green monkeys (52) and humans (53, 54).

The most interesting finding of our study is the opposite effect of n-3 and n-6 fatty acids on biliary secretion of cholesterol (Table 4). The associated shifts in the molar linkage coefficients of cholesterol secretion to bile acid and phospholipid secretion demonstrate that these different dietary fatty acids changed the secretory relationship between cholesterol and phospholipids as well as bile acids in bile. N-6 polyunsaturated fatty acids raised cholesterol output, n-3 polyunsaturated fatty acids lowered it, and mixed saturated and n-9 monounsaturated fatty acids ingested in coconut fat provided for intermediary output values of cholesterol in bile. Biliary secretion of cholesterol could be increased by deoxycholic acid, when it is the predominant bile acid in bile (55). However, the small differences in the fraction of deoxycholic acid in bile (Fig. 2) cannot account for the changes in cholesterol secretion observed with the three dietary regimens. Nor can the difference in cholesterol secretion be explained by different secretion rates of bile acids (Table 4), because it persisted even at comparable bile acid secretion rates that had been generated by intravenous infusion of taurocholate. At least two potential explanations should be further tested. *a*) A differential effect on hepatic synthesis of cholesterol could supply cholesterol at different rates for biliary secretion. Hepatic synthesis of cholesterol had been suppressed by n-3 fatty acids in the rat (17), but in contrast to this hypothesis, it had also been diminished almost to a similar extent by dietary n-6 fatty acids (17). *b*) The diet-induced changes in the composition of phosphatidylcholine species destined for secretion into bile (compare Fig. 3) could require different amounts of cholesterol for secretion in biliary phospholipid vesicles. The latter hypothesis seems supported by correlations between the cholesterol to phospholipid ratio in bile and the fatty acid composition of biliary phospholipids (36).

In summary, dietary n-3 and n-6 polyunsaturated fatty acids clearly differ in their effects on the excretory routes of cholesterol, i.e., bile acid synthesis and biliary secretion of cholesterol, in the hamster liver. To search for the intracellular signals that initiate these differential effects, further studies should *a*) assess hepatic cholesterol balance in a quantitative fashion to test for a putative regulatory pool of free cholesterol in the liver cells; and *b*) clarify whether the fatty acid composition of the phospholipid species influences the rate of bile acid synthesis or the secretory ratio of cholesterol to phospholipid in bile. ■

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