

# Effect of different dietary triglycerides on $7\alpha$ -hydroxylation of cholesterol and other mixed-function oxidations

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**Abstract** The effect of a diet containing triglycerides of different fatty acid composition on hepatic  $7\alpha$ -hydroxylation of cholesterol was studied.  $7\alpha$ -Hydroxylation of exogenous as well as endogenous cholesterol was significantly lower in the liver of rats fed trilinolein and triolein than in those fed tripalmitin and trierucin. The concentration of cytochrome P-450 in liver microsomes was significantly lower in the rats fed tripalmitin and trierucin than in those fed triolein and trilinolein. The inhibitory effect of triolein and trilinolein on  $7\alpha$ -hydroxylation of cholesterol and the stimulatory effect of these triglycerides on the concentration of cytochrome P-450 was not due to the small amounts of peroxides present in the unsaturated triglycerides. Thus addition of the antioxidant butylated hydroxyanisole did not change the general pattern with respect to  $7\alpha$ -hydroxylation and concentration of cytochrome P-450. However, a diet consisting of peroxidized linoleic acid further decreased  $7\alpha$ -hydroxylation of cholesterol. The difference between the effect obtained with triolein and trilinolein on the one hand and trierucin and tripalmitin on the other was observed also in experiments with lower concentrations of fat in the diet and in experiments with different lighting conditions and feeding patterns. The inverse relation between cytochrome P-450 and  $7\alpha$ -hydroxylation of cholesterol, as well as results obtained with substrates for mixed-function oxidation other than cholesterol suggest that most of the changes observed due to the different diets are specific for  $7\alpha$ -hydroxylation of cholesterol. The level of cholesterol  $7\alpha$ -hydroxylase activity was found to be better related to the degree of absorption of fat than to total amount of absorbed fat or degree of unsaturation of the fat. The results are discussed in relation to previous knowledge concerning mechanisms regulating biosynthesis of bile acid.

**Supplementary key words** bile acids · lipid peroxides · fat absorption · mass fragmentography

The rate-limiting step in bile acid biosynthesis,  $7\alpha$ -hydroxylation of cholesterol, is known to be influenced by bile acids, hormones, vitamins, starvation, and dietary cholesterol (1). The major regulation of activity is most probably related to the inhibitory ef-

fect of the bile acids reabsorbed from the intestine during the enterohepatic circulation.

The effects of lipids other than cholesterol on the enzyme activity is unclear. A diet rich in unsaturated fatty acids is known to decrease the cholesterol concentration in plasma (2). It has been suggested that this effect might be due to increased formation of bile acids from cholesterol. Different results have, however, been obtained in studies concerned with the effect of unsaturated dietary lipids on bile acid biosynthesis and metabolism. In some studies (3–12) a small or moderate increase in bile acid biosynthesis has been found. In other studies (13–16) either no effect on bile acid biosynthesis has been found or different effects in different individuals have been observed. In most studies, the unsaturated diet has been found to increase accumulation of cholesterol in liver. This increase has been ascribed either to an increased biosynthesis of cholesterol (3) or to a redistribution between plasma and tissue pools of cholesterol (13, 14).

Many of the studies referred to above are difficult to compare due to differences in species and composition of dietary fat, as well as experimental technique. Bile acid biosynthesis has either been calculated from balance studies or from kinetic studies with labeled bile acids.

In some recent work, the direct effect of a diet rich in unsaturated fatty acids on cholesterol  $7\alpha$ -hydroxylation has been determined in animal experiments. Mayer and Mayer (16) found that feeding of a polyunsaturated diet to rats for 14 days increased  $7\alpha$ -hydroxylation of cholesterol, whereas feeding of a saturated diet decreased the activity. The differences between the two types of diet were somewhat different in the dark period and in the light period. In contrast, Kritchevsky, Tepper, and Story (17) found

Abbreviations: BHA, butylated hydroxyanisole.

that a diet rich in unsaturated lipids decreased  $7\alpha$ -hydroxylation of cholesterol, at least when calculated as pmol converted/liver. The difference between the results by Mayer and Mayer (16) and by Kritchevsky et al. (17) might be due to differences in composition of the fat, or to presence of different amounts of lipid peroxides in the unsaturated fat, or to differences in the incubation procedures used.

In the present work,  $7\alpha$ -hydroxylation of cholesterol has been determined in livers of rats fed a semi-synthetic diet together with synthetic tripalmitin, trierucin, triolein, or a safflower oil with a high concentration of trilinolein. These diets had a low and defined amount of peroxides. The effect of adding antioxidants and lipid peroxides has also been studied. For comparison, several other microsomal mixed-function oxidases were assayed in rats under different dietary conditions.

## EXPERIMENTAL PROCEDURE

### Materials

[4- $^{14}$ C]-Cholesterol (53 Ci/mol) was obtained from Radiochemical Centre, Amersham, England, and was purified by chromatography on a column of aluminium oxide, grade III (Woelm, Eschwege, West Germany) prior to use. [1- $^{14}$ C]Lauric acid was obtained from Radiochemical Centre and was diluted with inactive lauric acid to give a specific radioactivity of 3 Ci/mol.  $7\alpha$ -Hydroxy-4-[6 $\beta$ - $^3$ H]cholesten-3-one (2.7 Ci/mol) and 5 $\beta$ -[7 $\beta$ - $^3$ H]cholestane-3 $\alpha$ ,7 $\alpha$ -diol (6.5 Ci/mol) were prepared as described previously (18, 19).

Tween 80 and cofactors were obtained from Sigma Chemical Co. (St. Louis, MO). Tripalmitin, triolein, trimyristin, and trilaurin were obtained from Fluka, FRG. Trierucin and safflower oil with a high concentration of trilinolein were obtained from Karlshamns Oljefabriker, Karlshamn, Sweden. The fatty acid composition of the different fats and their concentrations of peroxides are shown in **Table 1**. Butylated hydroxyanisol (BHA) was obtained from Fluka. In some experiments peroxidized safflower oil was used (cf. below). Peroxidation of the oil was performed as described by Andrews, Griffith, Mead, and Stein (20).

### Animals and their treatment

White male rats of the Sprague-Dawley strain weighing about 150 g at the start of the experiments were used (Anticimex, Stockholm, Sweden). In order to assay  $7\alpha$ -hydroxylation under optimal conditions (21, 22) the animals were subjected to reversed lighting periods (light was automatically switched on at 6 PM and off at 6 AM) in all experiments except in condition III (see below). In the latter experiment, the animals were subjected to a normal lighting period (lighting was switched on at 6 AM and off at 6 PM).

The animals were fed various semisynthetic diets that differed only in concentration and type of fat. The compositions of the diets were as follows: fat, 0, 6, or 20%; corn starch, 50, 44, or 30%; casein, 26%; cellulose powder, 4%; dextrose, 10%; CaCO<sub>3</sub>, 2%; NaH<sub>2</sub>PO<sub>4</sub>, 3%; salt mixture (mixture number 7, method 400, Astra Ewos AB, Södertälje, Sweden),

TABLE 1. Fatty acid composition<sup>a</sup> and concentration of peroxides in the different fats

Fatty Acid	Tripalmitin Diet	Trierucin Diet	Triolein Diet	Trilinolein Diet	Trilaurin Diet	Trimyristin Diet	Commercial Pellet Diet
12:0				0.5	96.3	0.6	
14:0	1.2		1.2	0.2	1.4	95.5	
14:1			0.2	0.7			
16:0	94.8		4.7	6.6		3.1	21.0
16:1			3.5	0.2			2.4
17:0			0.6				
18:0	4.0	0.8	1.1	2.4	2.3	0.8	3.3
18:1		0.7	79.1	12.3			25.0
18:2			9.0	77.1			41.2
18:3			0.6				4.3
20:1		1.8					
22:1		95.2					
20:4							2.1
22:6		1.5					0.7
Peroxide number <sup>b</sup>	1.1	2.4	57	57			0.6
Peroxide number <sup>b</sup> in presence of BHA	0	0	0.5	0			

<sup>a</sup> Percent (w/w).

<sup>b</sup> mmol/kg diet, 20% fat except in commercial diet.

4%; vitamin mixture (complete, for rat and mouse, Astra Ewos AB), 1%.

In all sets of experiments, one group of rats was fed a commercial pellet diet containing 4.5% fat (Standard feed for rats and mice, Astra Ewos AB). This diet was used only to ascertain that variations in hydroxylase capacity from time to time in the animals were tolerable. In spite of a highly standardized treatment of the animals and method of assay, however, the maximal difference between results of incubations of cholesterol with livers from animals fed the commercial diet under apparently identical conditions during the 2 yr of the study was about 50–60%. The variations within each period of 6 months were, however, never more than 25%.

The following major conditions were used in the animal experiments:

Condition I. Groups with six animals in each group were fed the semisynthetic diet containing 20% of the respective fat for 4 or 5 weeks. The rats were submitted to a reversed lighting period and were fasted overnight prior to being killed.

Condition II. Groups with six animals in each group were fed the semisynthetic diet containing 20% of the respective fat for 5 weeks. The rats were submitted to a reversed lighting period and had free access to food until they were killed.

Condition III. Groups with six animals in each group were fed the semisynthetic diet containing 20% of the respective fat for 5 weeks. The rats were submitted to a normal lighting period and were fasted overnight prior to being killed.

Condition IV. Groups with six animals in each group were fed the semisynthetic diet containing 6% of the respective fat for 5 weeks. The rats were submitted to a reversed lighting period and were fasted overnight prior to being killed.

In order to achieve approximately the same increase in body weight in the different groups of rats, the amount of food for each rat was restricted to 15 g per day. The rats had free access to drinking water. The rats were killed with a blow on the neck at about 9 AM on the day of the experiment.

#### Incubation procedure

The livers were removed immediately, chilled, and washed with ice cold 0.25 M sucrose. They were then weighed and minced into small pieces. Liver homogenate (20%, w/v) was prepared in 0.25 M sucrose containing 0.001 M EDTA (23). The homogenate was centrifuged at 20,000 g for 10 min and the microsomal fraction was isolated by centrifugation of the 20,000 g supernatant for 1 hr at 100,000 g. The microsomal

fraction was suspended in 0.1 M potassium phosphate buffer, pH 7.0, in a volume corresponding to that of the 20,000 g supernatant from which it had been isolated (23). In the case of microsomes used for incubation with [1-<sup>14</sup>C]laurate, 7 $\alpha$ -hydroxy-4-[6 $\beta$ -<sup>3</sup>H]cholesten-3-one, and 5 $\beta$ -[7 $\beta$ -<sup>3</sup>H]cholestane-3 $\alpha$ ,7 $\alpha$ -diol, the microsomal pellet was suspended in 0.1 M Tris-Cl buffer, pH 7.4.

In the case of incubation with [4-<sup>14</sup>C]cholesterol, incubations were carried out for 15 min at 37°C with 3 ml of microsomal fraction and 3  $\mu$ mol of NADPH in a total volume of 5 ml of 0.1 M potassium phosphate buffer, pH 7.0. The labeled cholesterol, 15 nmol, was added in 0.1 ml of a suspension of Tween in potassium phosphate buffer (24). Incubations were terminated by the addition of 20 volumes of chloroform–methanol 2:1 (v/v) and 3  $\mu$ g of 5-[3 $\alpha$ ,4 $\beta$ ,7 $\beta$ -<sup>2</sup>H<sub>3</sub>]cholestene-3 $\beta$ ,7 $\alpha$ -diol was added as internal standard (24–26). After filtration, 0.2 volumes of 0.9% (w/v) sodium chloride solution was added. The chloroform phase was collected and evaporated.

In the case of incubations with [1-<sup>14</sup>C]laurate, incubations were carried out for 15 min with 1 ml of microsomal fraction in a total volume of 3 ml of 0.1 M Tris-Cl buffer pH 7.4 at 37°C together with a NADPH-generating system (27). The labeled fatty acid, 100  $\mu$ g, was added dissolved in 50  $\mu$ l of acetone. The incubations were terminated by addition of ethanol. The incubation mixtures were then diluted with water, acidified, and extracted with ether.

5 $\beta$ -[7 $\beta$ -<sup>3</sup>H]cholestane-3 $\alpha$ ,7 $\alpha$ -diol, 250  $\mu$ g, dissolved in 50  $\mu$ l of acetone, was incubated under the same conditions as [1-<sup>14</sup>C]laurate, with the exception that the incubation time was 20 min (19). The incubations were terminated and extracted as were the incubations with cholesterol. 7 $\alpha$ -Hydroxy-4-[6 $\beta$ -<sup>3</sup>H]cholesten-3-one, 200  $\mu$ g, dissolved in 50  $\mu$ l of acetone, was incubated under the same conditions as 5 $\beta$ -[7 $\beta$ -<sup>3</sup>H]cholestane-3 $\alpha$ ,7 $\alpha$ -diol, with the exception that 1.5 ml of microsomal suspension was used. The incubations were terminated and extracted as were the incubations with cholesterol.

The conditions used for assay of all the hydroxylase activities were optimal. Thus the conversion was linear with incubation time and, with the exception of incubation with [4-<sup>14</sup>C]cholesterol, also with the amount of microsomal fraction. With the exception of incubations with [4-<sup>14</sup>C]cholesterol, enzyme activity was assayed at substrate saturation.

#### Analysis of incubations

The residue of the chloroform extract of incubations with [4-<sup>14</sup>C]cholesterol was subjected to thin-

layer chromatography with benzene-ethyl acetate 2:3 (v/v) as solvent. The conversion of labeled cholesterol into 7 $\alpha$ -hydroxycholesterol was determined by scanning the chromatoplates with a radioscaner (Berthold, Wildbad, FRG). The silica gel zones corresponding to 7 $\alpha$ -hydroxycholesterol were collected, eluted with methanol, and converted into trimethylsilyl ethers. The mass of 7 $\alpha$ -hydroxycholesterol was determined by mass fragmentography, using an LKB 9000 instrument equipped with a MID (multiple ion detector) and a 1.5% Se-30 column (24, 26). The first channel of the MID was focused on the ion at *m/e* 456 and the second on the ion at *m/e* 459, corresponding to the peak at M - 90 of the trimethylsilyl ether of unlabeled and trideuterated 7 $\alpha$ -hydroxycholesterol, respectively.

The residue of the ether extract of the incubations with [1-<sup>14</sup>C]laurate was subjected to thin-layer chromatography using diethyl ether-hexane-acetic acid 2:18:1 (v/v/v) as solvent. The extent of conversion into 11- and 12-hydroxylated lauric acid was assayed by radioscaning of the chromatoplate followed by radio-gas-liquid chromatography of methyl ester of the material eluted from the appropriate chromatographic zone (26, 27).

The residue of the chloroform extract of the incubations with 5 $\beta$ -[7 $\beta$ -<sup>3</sup>H]cholestane-3 $\alpha$ ,7 $\alpha$ -diol was subjected to thin-layer chromatography using ethyl acetate as solvent. The extent of conversion was assayed by radioscaning followed by radio-gas-liquid chromatography of the trimethylsilyl ether of material eluted from the chromatographic zone containing the 12- and 26-hydroxylated product (19).

The residue of the chloroform extract of the incubation with 7 $\alpha$ -hydroxy-4-[6 $\beta$ -<sup>3</sup>H]cholesten-3-one was subjected to thin-layer chromatography using benzene-ethyl acetate 1:1 (v/v) as solvent. The extent of conversion into the 12-hydroxylated product was assayed by radioscaning of the chromatoplates.

#### Determination of protein, cholesterol, fatty acids, cytochrome P-450, and lipid peroxides

Protein was determined according to Lowry et al. (28). The protein content of the microsomal suspension in general was between 3 and 6 mg/ml. Cholesterol content in whole liver and in liver microsomes was assayed with a mass fragmentographic method using deuterated cholesterol as standard (29). The concentrations of cholesterol, triglycerides, and glucose in serum were assayed with enzymatic methods (30-32). The accuracy of these methods has been established (33). Cytochrome P-450 was assayed from the absorbance of the carbon monoxide-cytochrome P-450 complex after reduction with sodium dithionite

using an extinction coefficient of 91 cm<sup>-1</sup> nM<sup>-1</sup> (34). Fatty acids were assayed as methyl esters with a gas-liquid chromatographic method (35). Peroxide values were determined according to a modified Wheeler method (36).

## RESULTS

### Composition of the various fats

The different diets were extracted and the lipids obtained were analyzed with respect to composition of fatty acids and content of lipid peroxides (Table 1).

Hydrolysis of the extract from the tripalmitin diet showed that palmitic acid made up about 95% of the fatty acids. There were no detectable amounts of unsaturated fatty acids. The trierucin diet contained about 95% erucic acid and small amounts of other unsaturated fatty acids. The triolein diet contained about 78% oleic acid, about 9% linoleic acid, and about 7% saturated fatty acids. The trilinolein diet contained about 77% linoleic acid, about 12% oleic acid and about 11% saturated fatty acids (Table 1).

Analysis for peroxides gave very low values in the case of tripalmitin and trierucin and about 60 mmol/kg diet in the case of triolein and trilinolein. After addition of the antioxidant BHA to the diet, no detectable peroxides were found in tripalmitin, trierucin, or trilinolein and only very small amounts in triolein (Table 1).

It was shown that less than 0.2% of the lipid extract was sterols in any of the diets.  $\beta$ -Sitosterol was the predominant sterol of those present.

### Weight increase on the different diets

Fig. 1 and Table 2 show results obtained with the semisynthetic diet containing 20% fat (Condition I). The rats kept on trilinolein diet and triolein diet gained weight faster than the rats kept on tripalmitin

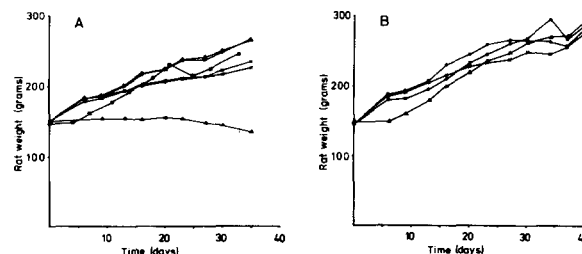


Fig. 1. Growth of rats on different diets. A, Diet containing 20% fat; B, diet containing 6% fat. Each value represents mean of six rats.  $\times$ , Tripalmitin diet;  $\bullet$ , trierucin diet;  $\circ$ , triolein diet;  $\Delta$ , trilinolein diet;  $\blacktriangle$ , peroxidized trilinolein diet;  $\square$ , fat-free diet.

TABLE 2. Effect of different diets on body weight, serum cholesterol, serum triglycerides, serum glucose, liver cholesterol<sup>a</sup>

	Body Weight	Serum Cholesterol	Serum Triglycerides	Serum Glucose	Liver Cholesterol (total)	Liver Microsomal Cholesterol (total)	Liver Microsomal Cholesterol (free)
	g	mmol/l	mmol/l	mmol/l	mg/g	nmol/mg prot.	nmol/mg prot.
Tripalmitin diet, 20% (condition I)	278 ± 10	2.08 ± 0.10	0.81 ± 0.09	7.00 ± 0.22	1.54 ± 0.05	55.6 ± 1.3	55.6 ± 1.6
Trierucin diet, 20% (condition I)	267 ± 5	2.02 ± 0.17	0.54 ± 0.07	8.12 ± 0.37	2.45 ± 0.05	69.8 ± 1.5	60.4 ± 1.5
Triolein diet, 20% (condition I)	292 ± 8	2.24 ± 0.15	0.40 ± 0.08	8.12 ± 0.26	2.41 ± 0.12	62.9 ± 2.4	52.4 ± 2.3
Trilinolein diet (sufflower oil, 20% (condition I)	275 ± 7	1.95 ± 0.15	0.41 ± 0.07	8.00 ± 0.28	2.02 ± 0.07	54.0 ± 2.3	50.3 ± 2.4
Fat free diet (condition I)	283 ± 10	1.54 ± 0.13				64.4 ± 1.1	58.4 ± 1.3
Trilinolein diet, peroxidized (condition I)	135 ± 6	1.08 ± 0.12	0.16 ± 0.03		1.87 ± 0.05		

<sup>a</sup> Values given are mean ± SEM of results from six animals in each group of rats under condition I treatment.

diet, trierucin diet, and fat-free diet. The rats kept on peroxidized trilinolein diet decreased markedly in weight during the 5 weeks on the diet. There were only small differences in weight gain of rats kept on a semisynthetic diet containing 6% fat (Fig. 1).

### Serum cholesterol, serum triglycerides, serum glucose, and content of cholesterol in the liver

Serum cholesterol levels were not significantly different in the different groups of animals kept on the 20% fat diet (Table 2). Significantly lower values were however obtained in the groups of rats kept on the fat-free diet and the peroxidized diet. Serum triglycerides were higher in the groups of rats kept on the tripalmitin diet than in those on trierucin, triolein, and trilinolein. Very low serum triglycerides were found in the rats on the peroxidized diet. Serum glucose was lower in the rats on the tripalmitin diet than in the other rats. Liver cholesterol was significantly lower in rats on the tripalmitin diet than in those on the trierucin, triolein, and trilinolein diets. The cholesterol content of the microsomal fraction was higher in rats on the trierucin and triolein diet than in those on the tripalmitin and trilinolein diet. The cholesterol present in the liver microsomes of animals fed tripalmitin was almost exclusively in the free form, whereas 7–16% of the cholesterol in the liver microsomes of the other animals was esterified (Table 2).

### Effect of the different diets on 7 $\alpha$ -hydroxylation of cholesterol, 11- and 12-hydroxylation of laurate, 12 $\alpha$ - and 26-hydroxylation of 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ -diol, 12 $\alpha$ -hydroxylation of 7 $\alpha$ -hydroxy-4-cholesten-3-one, and concentration of microsomal cytochrome P-450

Fig. 2 summarizes the results obtained with rats on the various diets containing 20% fat (Condition I).

7 $\alpha$ -Hydroxylation of exogenous as well as endog-

enous cholesterol was high in the microsomal fraction of liver of rats fed tripalmitin, lower in rats fed trierucin, and lowest in rats fed trilinolein.

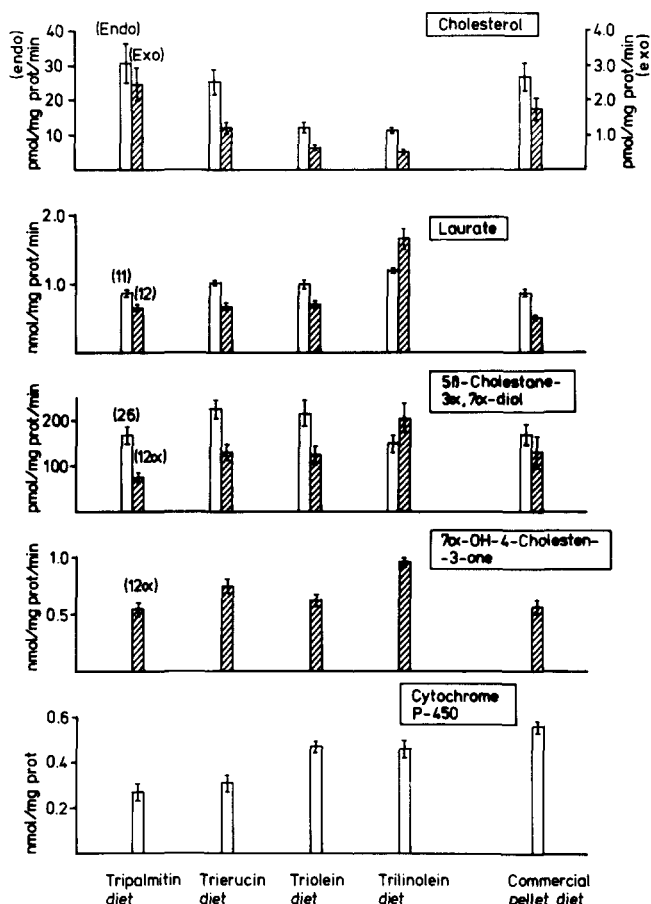
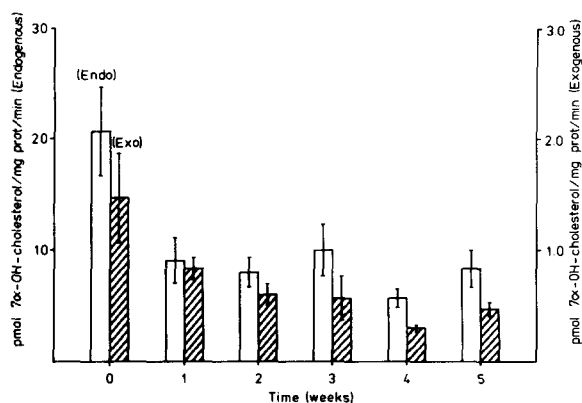


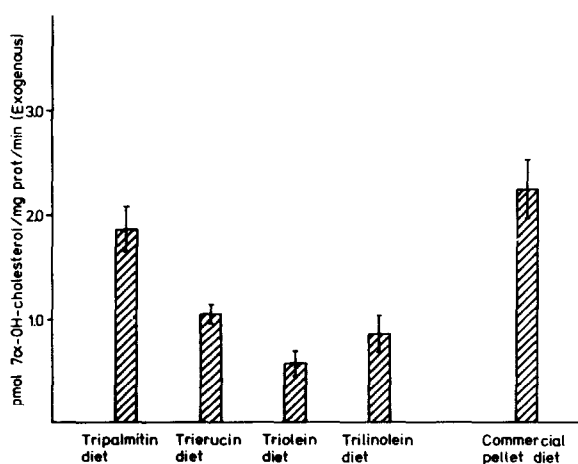
Fig. 2. Effect of different diets containing 20% fat on 7 $\alpha$ -hydroxylation of cholesterol, 11- and 12-hydroxylation of laurate, 12 $\alpha$ - and 26-hydroxylation of 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ -diol, 12 $\alpha$ -hydroxylation of 7 $\alpha$ -hydroxy-4-cholesten-3-one, and concentration of microsomal cytochrome P-450. The rats were submitted to reversed lighting periods and were fasted overnight prior to being killed (Condition I). Vertical bars represent the SEM of the measurements made on six rats.



**Fig. 3.** Effect of feeding 20% trilinolein diet for different times on the cholesterol  $7\alpha$ -hydroxylase activity in rat liver microsomes. The rats were submitted to reversed lighting periods and were fasted overnight prior to being killed (Condition I). Vertical bars represent the SEM of the measurements made on four rats.

The differences obtained with the different diets were most marked in the case of  $7\alpha$ -hydroxylation of exogenous cholesterol (almost fivefold).

In contrast, 11- and 12-hydroxylation of laurate as well as  $12\alpha$ -hydroxylation of  $5\beta$ -cholestane- $3\alpha,7\alpha$ -diol and  $7\alpha$ -hydroxy-4-cholesten-3-one were highest in livers of rats fed tripalmitin and lowest in the rats fed trilinolein and lowest in the rats fed tripalmitin. The concentration of cytochrome P-450 roughly followed the same pattern as 11- and 12-hydroxylation of laurate and the  $12\alpha$ -hydroxylation of the two steroids. Thus the microsomal fraction of rats treated with trilinolein and triolein had significantly higher concentrations of cytochrome P-450 than had that of rats treated with tripalmitin and trierucin.



**Fig. 4.** Effect of different diets containing 20% fat on the cholesterol  $7\alpha$ -hydroxylase activity in rat liver microsomes. The rats were submitted to reversed lighting periods and had free access to food until they were killed (Condition II). Vertical bars represent the SEM of the measurements made on six rats.

It is evident that the most marked effects were obtained on  $7\alpha$ -hydroxylation of cholesterol. The mechanism behind this effect was further studied by variation of the time factor, feeding pattern, lighting period, concentration of fat, and concentration of lipid peroxides.

### Time required for development of inhibitory effect of $7\alpha$ -hydroxylation of cholesterol by trilinolein diet

**Fig. 3** summarizes results of experiments designed to determine the time required for development of an inhibitory effect of trilinolein on  $7\alpha$ -hydroxylation of cholesterol in rats previously fed commercial pellet diet. Groups of four rats in each group were maintained for different times on the trilinolein diet (20%, Condition I). From the results obtained, it is evident that the inhibitory effect appeared already after 1 week on the diet and remained about constant during the 3rd, 4th, and 5th weeks.

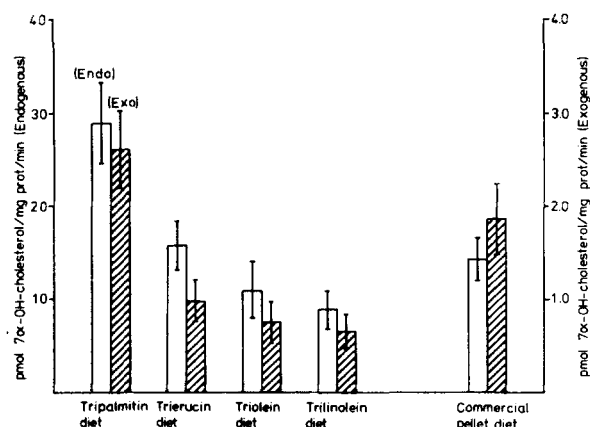
### Effect of feeding pattern on $7\alpha$ -hydroxylation of cholesterol

$7\alpha$ -Hydroxylation is known to be affected by starvation (37). It was shown in the present work, however, that fasting of the rats overnight in general gave less variation in activity of  $7\alpha$ -hydroxylase than when the rats had free access to food up to the time of killing. Therefore, in all experiments except those shown in **Fig. 4**, the rats were fasted overnight prior to being killed.

The results of the experiments summarized in **Fig. 4** show that the relation between  $7\alpha$ -hydroxylase activity of the rats on the tripalmitin diet and that of the rats on the trierucin diet was affected to some extent when the rats had free access to food up to the time of killing (Condition II). With this exception, the pattern was similar to that obtained with rats fasted overnight. Thus, the rats fed tripalmitin had significantly higher  $7\alpha$ -hydroxylase activity than those fed with triolein and trilinolein.

### Effects of lighting period on $7\alpha$ -hydroxylation of cholesterol

**Fig. 5** shows the results of experiments with rats subjected to a normal lighting period (Condition III). The general pattern was the same as that obtained with reversed lighting. The activity of the  $7\alpha$ -hydroxylase towards endogenous cholesterol in livers of rats fed the commercial pellet diet was somewhat lower in this experiment than in the experiments with reversed lighting (21, 22). Surprisingly, the level



**Fig. 5.** Effect of different diets containing 20% fat on the cholesterol 7 $\alpha$ -hydroxylase activity in rat liver microsomes. The rats were submitted to normal lighting periods and were fasted overnight prior to being killed (Condition III). Vertical bars represent the SEM of the measurements made on six rats.

of activity of the 7 $\alpha$ -hydroxylase in livers of rats fed the different semisynthetic diets was similar to that obtained in experiments with reversed lighting.

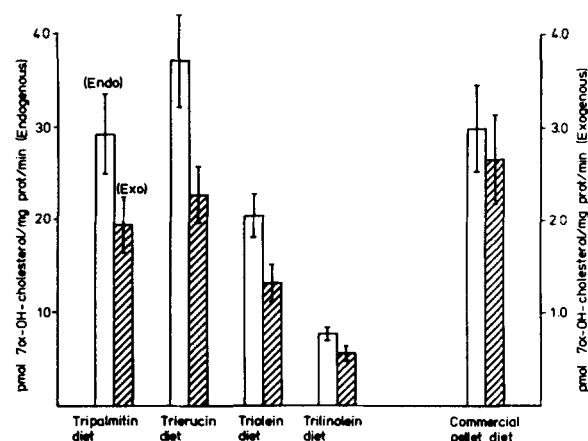
#### Effect of 6% fat in diet on 7 $\alpha$ -hydroxylation of cholesterol

**Fig. 6** shows the results obtained with rats treated with 6% fat in the diet (Condition IV). The 7 $\alpha$ -hydroxylase activity was higher in the rats treated with trierucin and triolein as compared to experiments with 20% fat. The difference between 7 $\alpha$ -hydroxylase activity in rats treated with tripalmitin and rats treated with trilinolein diet was however of the same magnitude as that obtained in experiments with 20% fat.

#### Effect of lipid peroxides on 7 $\alpha$ -hydroxylation of cholesterol

**Fig. 7** shows the results of experiments with rats treated with 20% fat to which the antioxidant BHA had been added (Condition I). These diets were practically free from peroxides (Table 1). As is evident, the general pattern with respect to 7 $\alpha$ -hydroxylation of cholesterol was about the same as in experiments without BHA.

In a separate experiment, parallel groups of rats were fed trilinolein, trilinolein + BHA, and peroxidized trilinolein with defined amounts of lipid peroxides. There was no significant difference between the rats fed trilinolein and trilinolein + BHA with respect to 7 $\alpha$ -hydroxylation of cholesterol (Table 3). The rats fed the peroxidized diet, however, had significantly lower 7 $\alpha$ -hydroxylase activity.

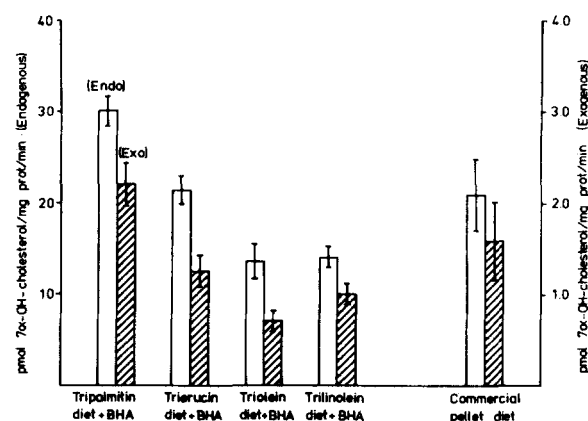


**Fig. 6.** Effect of different diets containing 6% fat on the cholesterol 7 $\alpha$ -hydroxylase activity in rat liver microsomes. The rats were submitted to reversed lighting periods and were fasted overnight prior to being killed (Condition IV). Vertical bars represent the SEM of the measurements made on six rats.

#### Effects of trimyrustin, trilaurin, and fat-free diet on 7 $\alpha$ -hydroxylation of cholesterol

**Fig. 8** shows the effect of a semisynthetic diet containing 20% trimyrustin or 20% trilaurin (Condition I). The level of 7 $\alpha$ -hydroxylase activity was lower in the control experiment with commercial pellet diet as compared to most other experiments (cf. below). The level of 7 $\alpha$ -hydroxylase activity obtained in the rats treated with trimyrustin was about the same as that obtained with the control diet, which corresponds to the level usually obtained with the tripalmitin diet (cf. below).

The level of 7 $\alpha$ -hydroxylase activity obtained with the trilaurin diet was only about half of that obtained with trimyrustin. The level of 7 $\alpha$ -hydroxylase



**Fig. 7.** Effect of different diets containing 20% fat and the antioxidant BHA on the cholesterol 7 $\alpha$ -hydroxylase activity in rat liver microsomes. The rats were submitted to reversed lighting periods and were fasted overnight prior to being killed (Condition I). Vertical bars represent the SEM of the measurements made on six rats.

TABLE 3. Effect of lipid peroxides in trilinolein diet on  $7\alpha$ -hydroxylation of cholesterol and concentration of cytochrome P-450

	Trilinolein Diet + BHA	Trilinolein Diet	Peroxidized Trilinolein Diet
Cytochrome P-450 nmol/mg protein	$0.49 \pm 0.01$	$0.51 \pm 0.01$	$0.49 \pm 0.03$
$7\alpha$ -Hydroxylation of endogenous cholesterol pmol/mg prot/min	$14.0 \pm 1.1$	$14.1 \pm 1.1$	$6.3 \pm 1.2$
$7\alpha$ -Hydroxylation of exogenous cholesterol pmol/mg prot/min	$0.99 \pm 0.11$	$0.85 \pm 0.06$	$0.34 \pm 0.08$

The diets contained 0, 25, and 90 mmol of lipid peroxides/kg. Values given in the table are mean of six experiments  $\pm$  SEM.

activity obtained with a fat-free diet was about the same as that obtained with the trilaurin diet (Fig. 8).

## DISCUSSION

In most previous studies the effect of diets containing triglycerides consisting of long chain fatty acids of varying degrees of unsaturation has been compared with the effect of diets containing triglycerides consisting of medium chain saturated fatty acids. A reason for using triglycerides consisting of medium chain saturated fatty acids in studies on the effect of saturated fat is the fact that these fatty acids are absorbed from the intestine about as efficiently as long chain unsaturated fatty acids. On the other hand, many important natural fats contain relatively high concentrations of fatty acids that are absorbed less readily, such as long chain saturated fatty acids and erucic acid. In the present work, almost homogeneous triglycerides were used in most of the experiments and this homogeneity in

combination with the different degree of absorption may explain the rather marked effects obtained as compared to previous studies.

In view of the more efficient absorption (38), it can be expected that the rats kept on a constant amount of triolein diet or trilinolein diet should gain weight faster than the rats kept on a constant amount of trierucin diet and tripalmitin diet. This was also observed although the differences were relatively small. No significant differences in gain of weight were observed in the groups of rats fed 6% fat in the diet. Since the most important differences between the different fats with respect to effect on  $7\alpha$ -hydroxylation of cholesterol were found also in the experiments with only 6% fat in diet, and since the level of  $7\alpha$ -hydroxylase activity in these experiments was similar to that observed with 20% fat in the diet, differences in caloric intake per se seem to be of little importance. In addition, a reduced caloric intake, such as that obtained in the experiments with 20% tripalmitate, should result in a *decreased* rate of  $7\alpha$ -hydroxylation (37, 39) rather than the *increased* rate that was observed. Isocaloric feeding would therefore, if anything, tend to increase the differences between the different diets. The possibility of varying the fat content of the diet in such a way that the amount of fat absorbed would be similar with all diets (cf. ref. 40) was considered; however, such an experimental approach is difficult and the very different amounts of fat excreted in feces might complicate the evaluation (cf. below).

The decreasing weight of the animals kept on a peroxidized trilinolein diet is in agreement with a study by Andrews et al. (20), in which evidence was presented that the toxic action of the peroxides mainly was due to a specific effect on the intestinal enzymes and that only very small amounts of the lipid peroxides were absorbed as such.

The rats kept on a 20% trilinolein diet did not have lower cholesterol concentration in serum than the other rats. Failure to reduce serum cholesterol in rats by feeding polyunsaturated fat for less than

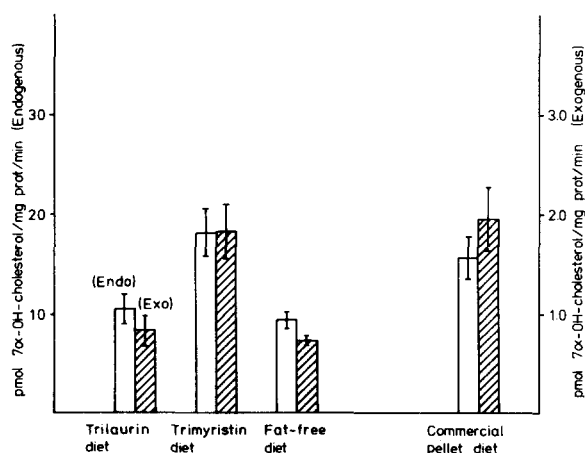


Fig. 8. Effect of 20% trilaurin, 20% trimyristin, and fat-free diet on the cholesterol  $7\alpha$ -hydroxylase activity in rat liver microsomes. The rats were submitted to reversed lighting periods and were fasted overnight prior to being killed (Condition I). Vertical bars represent the SEM of the measurements made on six rats.



4 weeks has been previously reported (13). In accordance with previous work, the rats fed unsaturated fat had a higher cholesterol concentration in liver than the rats fed saturated fat (3, 11). The concentrations of cholesteryl esters were higher in liver of rats fed the unsaturated lipid diets (3, 11).

In consonance with previous studies (17, 37), rats fed a semisynthetic fat-free diet had a lower cholesterol  $7\alpha$ -hydroxylase activity than rats fed a commercial pellet diet. Addition of 20% tripalmitin or 20% trierucin to the fat-free semisynthetic diet increased  $7\alpha$ -hydroxylation to a level similar to or above that obtained with the commercial pellet diet. In preliminary experiments, not shown in Results, the 20% trilinolein diet and the 20% triolein diet gave significantly lower  $7\alpha$ -hydroxylase activity than a semisynthetic fat-free diet. Addition of 20% trilaurin to the fat-free diet had little or no effect on  $7\alpha$ -hydroxylation of cholesterol. A direct comparison between levels of activity of cholesterol  $7\alpha$ -hydroxylase in livers of rats fed trilinolein and trilaurin was not performed. It is to be expected that the level of  $7\alpha$ -hydroxylase activity should be similar under such conditions or lower in rats fed trilinolein. This is in contrast to the results by Mayer and Mayer (16), who found that rats fed a semisynthetic diet containing medium chain saturated fatty acids had a lower cholesterol  $7\alpha$ -hydroxylase activity than rats fed a semisynthetic diet containing long chain unsaturated fatty acids. The present results are, at least in part, similar to the results of Kritchevsky et al. (17) who also studied the effect of a semipurified diet containing different fats on  $7\alpha$ -hydroxylation of cholesterol in rats. The highest level of  $7\alpha$ -hydroxylase activity was obtained in rats fed coconut oil, which contains a high concentration of lauric acid and myristic acid and only small concentrations of unsaturated fatty acids.

In the present work,  $7\alpha$ -hydroxylation of both exogenous and endogenous cholesterol were assayed. The effects of the different fats were similar on both conversions. This is in accordance with previous work from this laboratory in which it was shown that, provided the cholesterol content in the microsomal fraction of liver is similar, the two different techniques for assaying  $7\alpha$ -hydroxylase activity give similar results with respect to influences on the enzyme system (24–26).

In view of a possible inhibitory effect of auto-oxidized products on  $7\alpha$ -hydroxylation of cholesterol, the inhibitory effect of unsaturated lipids on  $7\alpha$ -hydroxylation of cholesterol might be explained by the presence of lipid peroxides. It was clearly shown that the diets containing triolein and trilinolein

contained far more lipid peroxides than the diets containing tripalmitin and trierucin. Addition of the antioxidant BHA, however, reduced the amount of lipid peroxides drastically without affecting the level of  $7\alpha$ -hydroxylase activity. It is evident that the peroxides normally present in the triolein and trilinolein had little or no effect on  $7\alpha$ -hydroxylation of cholesterol. Trilinolein with a high concentration of peroxides decreased the rate of  $7\alpha$ -hydroxylation of cholesterol. In view of the considerable loss of weight in this group of animals (cf. ref. 20) it is possible that the effect on  $7\alpha$ -hydroxylation of cholesterol is due to starvation of the animals.

The general difference in effect on  $7\alpha$ -hydroxylation of cholesterol by tripalmitin diet and trilinolein diet was retained under different feeding conditions, lighting conditions, and different concentrations of fat. This finding is to some extent in disagreement with the results of Mayer and Mayer (16), who found somewhat different effects of the diets depending upon whether cholesterol  $7\alpha$ -hydroxylase activity was assayed in the day or the night.

The effects obtained seem to be specific for  $7\alpha$ -hydroxylation of cholesterol. Thus several other mixed-function oxidations were affected in the opposite direction by the different fat diets and, in general, these activities followed the changes in concentration of cytochrome P-450. That the concentration of cytochrome P-450 in liver is higher after feeding unsaturated lipids than after feeding saturated lipids has been previously reported (41). There are several conditions in which the concentration of the bulk of cytochrome P-450 is increased and the activity of the cholesterol  $7\alpha$ -hydroxylase is decreased or vice versa (42).

It is evident that, under the specific conditions used in the present work, the major changes in  $7\alpha$ -hydroxylase activity are better related to the degree of absorption of the fat than to the degree of unsaturation of the fat or the chain-length of the fatty acids. Thus the lowest level of  $7\alpha$ -hydroxylase activity was obtained with fatty acids that are readily absorbed from the intestine, such as lauric acid, linoleic acid, and oleic acid. According to the literature, the degree of absorption of palmitic acid, erucic acid, oleic acid, and lauric acid from rat intestine is 48%, 52%, 84%, and 86%, respectively (38). Preliminary experiments in our laboratory have given similar results and, according to these experiments, linoleic acid is absorbed to about the same extent as lauric acid. Surprisingly, the total amount of fatty acids absorbed or excreted seems to be less important, for similar results were obtained with 6% fat as with 20% fat in the diet.

A possible explanation for the results is that a reduced absorption of some fatty acids is coupled with a reduced absorption of bile acids. The higher loss of bile acids in feces may then increase 7 $\alpha$ -hydroxylation of cholesterol due to a reduced feedback inhibition. Thus the effect of the tripalmitin diet should be similar to the effect of cholestyramine. Another possible explanation is that the number of enterohepatic circulations of each bile acid may be increased by the presence of fatty acids that are easily absorbed from the intestine. Such increased enterohepatic circulation may increase the feedback inhibition of the cholesterol 7 $\alpha$ -hydroxylase.

The above considerations are only valid if the rate of 7 $\alpha$ -hydroxylation of cholesterol really reflected the synthesis of bile acids under the conditions employed. Preliminary experiments in which the excretion of bile acids in feces of rats fed the different diets have been determined suggest that this is the case. In this connection, it may be mentioned that Mayer and Mayer (16), who arrived at different conclusions from their work, assayed 7 $\alpha$ -hydroxylation in a very crude system in which the soluble fraction of the cell was also present.

Whether or not the type of in vitro system used is of importance in studies on different influences on 7 $\alpha$ -hydroxylation of cholesterol is being studied at present in our laboratory. ■■

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