

Effect of esterified 4-desmethylsterols and -stanols or 4,4'-dimethylsterols on cholesterol and bile acid metabolism in hamsters

Elke A. Trautwein¹*, Claudia Schulz², Dörte Rieckhoff², Angelika Kunath-Rau²,
Helmut F. Erbersdobler², W. Arjan de Groot¹ and Gert W. Meijer³

¹Unilever Research, Vlaardingen, The Netherlands

²University of Kiel, Institute of Human Nutrition and Food Science, Kiel, Germany

³Lipton, 800 Sylvan Ave, Englewood Cliffs, New Jersey, USA

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4-Desmethylsterols and -stanols reduce plasma total cholesterol (TC) and LDL cholesterol by inhibition of intestinal cholesterol absorption, while the cholesterol-lowering potential of 4,4'-dimethylsterols is less well defined. The present study aimed to compare the effects of 4-desmethylsterols, -stanols, and 4,4'-dimethylsterols on plasma and hepatic cholesterol, sterol excretion and bile acid metabolism. Male golden Syrian hamsters were fed diets containing 13 g/100 g fat, 0.08 g/100 g cholesterol and 0 (control), 0.24 or 0.48 % (w/w) esterified 4-desmethylsterols (sterols) and esterified hydrogenated 4-desmethylsterols (stanols) from common vegetable oils or esterified 4,4'-dimethylsterols from rice bran oil for 5 weeks. Sterol and stanol esters at the dose of 0.24 % were equally effective and significantly ($P < 0.05$) lowered TC by 15 %, while 0.24 % 4,4-dimethylsterols reduced TC by 10 %. Liver total and esterified cholesterol concentrations were significantly ($P < 0.05$) lowered by 40, 22, 43 and 31 % in hamsters fed 0.48 % sterols, 0.24 % stanols, 0.48 % stanols or 0.48 % dimethylsterols, respectively. Daily faecal bile acid excretion and hepatic cholesterol 7 α -hydroxylase activity were not altered, indicating that sterols, stanols and dimethylsterols had no effect on the intestinal re-absorption of bile acids or on hepatic bile acid synthesis. Daily excretion of cholesterol was significantly higher in hamsters fed esterified sterols and stanols, but was only slightly increased in those fed dimethylsterols. The results indicate that esterified sterols and stanols were equally effective in lowering plasma TC and LDL cholesterol, while dimethylsterol esters caused a weaker cholesterol-lowering effect. Sterols and stanols achieve their cholesterol-lowering effect by stimulating faecal cholesterol excretion through inhibiting intestinal cholesterol absorption, but do not affect bile acid excretion. Other mechanisms need to be considered to explain the effect on plasma and hepatic cholesterol of dimethylsterols.

**4-Desmethylsterols: 4-Desmethylstanols: 4,4'-Dimethylsterols: Plasma lipids: Bile acids:
Faecal sterol excretion: Hamster**

Plant sterols, also called phytosterols, are components of a normal diet, mainly coming from plant sources, e.g. vegetable oils, seeds, nuts and grain-based products. The typical consumption of plant sterols with Western diets ranges between 200 and 400 mg/d, whereas the intake of phytosterols, the saturated form of plant sterols, is negligible (Jones *et al.* 1997). The most common plant sterols are the 4-desmethylsterols β -sitosterol, campesterol and stigmasterol; their saturated counterparts are sitostanol and campestanol.

The hypocholesterolaemic action of 4-desmethylsterols, i.e. of the predominant β -sitosterol, has been recognised since the early 1950s (Pollak & Kritchevsky, 1981; Ling & Jones, 1995a; Jones *et al.* 1997). Recently, interest has focused more on the cholesterol-lowering effect of plant stanols (Miettinen & Vanhanen, 1994; Vanhanen *et al.* 1994; Gylling *et al.* 1995; Ling & Jones, 1995b; Miettinen *et al.* 1995; Gylling & Miettinen, 1996; Ntanios & Jones, 1998; Nguyen *et al.* 1999). From data from animal studies, it was believed that sitostanol was more effective in

Abbreviations: apo, apolipoprotein; BC, biliary cholesterol; EC, esterified cholesterol; FC, free cholesterol; PL, phospholipid; TC, total cholesterol; TG, triacylglycerol.

* **Corresponding author:** Elke A. Trautwein, fax +31 10 460 5993, email elke.trautwein@unilever.com

lowering plasma cholesterol compared to sitosterol (Sugano *et al.* 1976, 1977; Ikeda *et al.* 1981). Recently, three human trials comparing directly esterified plant sterols *v.* plant stanols have clearly demonstrated that sterol esters are equally as effective as stanol esters in lowering total and LDL cholesterol in man (Weststrate & Meijer, 1998; Hallikainen *et al.* 2000; Jones *et al.* 2000).

Sterols with other structures, e.g. 4,4'-dimethylsterols, may differ in their potential to lower plasma cholesterol concentrations. Rice bran oil contains 4,4'-dimethylsterols such as cycloartenol and 24-methylene cycloartenol as ferulic acid esters (oryzanol). Results from animal and some human studies suggest that these components may contribute to the hypocholesterolaemic effect of rice bran oil (DeDeckere & Korver, 1996; Sugano & Tsuji, 1997). However, results from two recent human studies on the hypocholesterolaemic effect of rice bran oil sterols are conflicting (Weststrate & Meijer, 1998; Vissers *et al.* 2000).

It is generally assumed that plant sterols lower plasma cholesterol by inhibition of intestinal absorption of exogenous (dietary) and endogenous (biliary) cholesterol, although the exact mechanisms of action remain unclear. Whether plant sterols further influence the conversion of cholesterol into bile acids, e.g. via stimulation of cholesterol 7 α -hydroxylase activity, the key enzyme in bile acid synthesis is uncertain. Available data are conflicting, depending on whether sterols are infused intravenously or administered orally (Shefer *et al.* 1973, 1994; Boberg *et al.* 1989; Ling & Jones, 1995a). In addition, little is known about the effects of 4-desmethylsterols and 4,4'-dimethylsterols on biliary lipid composition and the bile acid profile. The present study examined the effects of three different types of plant sterols, i.e. esterified 4-desmethylsterols, esterified hydrogenated 4-desmethylsterols (stanols) and esterified 4,4'-dimethylsterols, on cholesterol concentrations, and more especially on bile acid metabolism. This included bile acid composition, synthesis and excretion in cholesterol-fed hamsters and aimed to determine whether the structural differences of these sterols affect their cholesterol-lowering action. To our knowledge, this is the first time that esterified sterols, stanols and dimethylsterols have been directly compared. The Syrian golden hamster (*Mesocricetus auratus*) was chosen because of its well-established similarities to human cholesterol and bile metabolism (Spady *et al.* 1985, 1986; Imray *et al.* 1992).

Materials and methods

Animals

A total of 96 male Syrian golden hamsters (SASCO, Omaha, NE, USA) weighing 71 (SD 4) g were randomly assigned to eight diet groups (twelve per group). Hamsters were housed in groups of four per cage in a temperature-controlled environment under a 12 h light–dark cycle (lights on 18.00 hours). The experimental protocol and procedures were approved by the Animal Care and Use Committee of the University of Kiel, Germany.

Diets and feeding procedures

Animals were fed semipurified diets for 5 weeks. The diets contained 130 g/kg fat (30 % fat based on total energy) and 0 % (cholesterol-free control diet) or 0.8 g/kg cholesterol (cholesterol-rich control diet and all plant sterol-supplemented diets). The basal composition of the diet was as follows (g/kg dry weight): casein 200, wheat starch 358, glucose 150, cellulose 100, palm oil 59.8, olive oil 32.2, butter 26, sunflower oil 13, mineral mix 46, vitamin mix 12, choline chloride 3 and cholesterol 0.8. The composition of the Ausman–Hayes mineral mix (F8530 BioServ, Frenchtown, NJ, USA) and the Hayes–Cathcart vitamin mix have been detailed previously (Hayes *et al.* 1989). The fatty acid composition of the diets resembled a typical Western fat intake with 13.8 % saturated fatty acids, 12.2 % monounsaturated fatty acids and 4.0 % polyunsaturated fatty acids with polyunsaturated:saturated fatty acids of 0.33. In the enriched diets, two doses, 2.4 or 4.8 g/kg, of (1) esterified 4-desmethylsterols from commonly used edible oil (mainly soyabean oil) distillates (Henkel Corporation, LaGrange, IL, USA), (2) esterified hydrogenated 4-desmethylsterols from the same source or (3) esterified 4,4'-dimethylsterols isolated by preparative chromatography after saponification from Oryzanol from rice bran oil (Tsuno, Wakayama, Japan) were added. All sterols had been esterified with fatty acids from rapeseed oil to an esterification degree of >92 % (Unilever Research Vlaardingen, The Netherlands). The 4-desmethylsterol, 4-desmethylstanol and 4,4'-dimethylsterol esters as well as the cholesterol were incorporated into the fat blend. By melting the sterol esters and the fat to approximately 60°C, mixing and then cooling, a homogeneous mixture was obtained.

Hamsters were given free access to food and water and the food consumption per cage was recorded daily. Body weights were monitored on a weekly basis.

Sample collection

During week 4, faecal samples were collected over a 3 d period from eight randomly selected hamsters per diet to measure faecal neutral sterols and bile acid excretion. After 5 weeks, all hamsters were housed individually in wire-bottomed cages and deprived of food for 18 h and then exsanguinated under anaesthesia using a gaseous mixture of CO₂:O₂ (50:50). Blood samples were drawn into EDTA-wetted syringes by cardiac puncture and the liver, small intestine and caecum were excised, blotted and weighed. A portion of the liver was removed and frozen for hepatic cholesterol analysis. From eight randomly selected hamsters per diet group (hamsters fed the 0.48 % 4,4'-dimethylsterol diet were not included, owing to lack of available rotor space), hepatic microsomes were isolated to measure microsomal cholesterol 7 α -hydroxylase activity. Gallbladder bile was aspirated, weighed and analysed for biliary lipids, biliary sterols and bile acid composition.

Plasma lipid and lipoprotein analysis

Plasma total cholesterol (TC) and triacylglycerol (TG) concentrations were determined by enzymic assays (Sigma

Chemicals, Deisenhofen, Germany). For lipoprotein isolation, plasma (1.6 ml) was either taken from a single hamster or pooled from two hamsters from the same dietary group with similar TC concentrations. Plasma lipoproteins were isolated by sequential ultracentrifugation (Havel *et al.* 1955). A preservation solution (final concentration in plasma: 1 mmol/l benzamidine, 0.04 % EDTA, 0.005 % gentamycin sulfate, 0.05 % NaN₃) was added to protect lipoproteins from enzymic degradation. Three fractions were isolated based on the following densities (d): VLDL (d<1.006 kg/l), LDL (1.006<d<1.055 kg/l), HDL (1.055<d<1.21 kg/l). With the exception of VLDL, lipoprotein fractions were dialysed against 0.15 mol/l NaCl, 0.04 % EDTA and 0.05 % NaN₃ at 4°C for 24–36 h. TC, free cholesterol (FC), TG and phospholipid (PL) concentrations were determined using enzymic assays (for TC and TG, kits from Sigma Chemicals; for FC and PL, kits from Wako Chemicals, Düsseldorf, Germany). Protein concentration was determined by a modification of the Lowry procedure (Markwell *et al.* 1978). The density cut-off points were verified in previous experiments by checking for cross-contamination; LDL and HDL apolipoproteins (apo) were separated by gradient SDS–PAGE (4–20 %) and stained with Coomassie Brilliant Blue. No traces of apo B₁₀₀ were present in the HDL fractions, while LDL fractions contained traces of apo A₁. A previous characterisation of apo B- and A₁-containing lipoprotein fractions of the hamster demonstrated that dense LDL-like particles overlapped apo A₁-containing HDL particles in the density range from 1.039 to 1.074 (Goulinet & Chapman, 1993).

Hepatic cholesterol analysis

Cholesterol concentrations were analysed following the procedure described in detail previously (Trautwein *et al.* 1993b). TC was determined with an enzymic assay and FC

was analysed by HPLC. Esterified cholesterol (EC) concentrations were calculated as the difference between TC and FC.

Bile analysis

Biliary cholesterol (BC) and PL were determined using an enzymic assay (Wako Chemicals). Bile acids were analysed in an aliquot of the methanol/KCl phase after Folch extraction as taurine- and glycine-conjugated bile acids by HPLC (Rossi *et al.* 1987). Total bile acid concentration was calculated as the sum of individual bile acids (taurine and glycine conjugates of cholate, chenodeoxycholate, deoxycholate and lithocholate).

Hepatic cholesterol 7 α -hydroxylase activity

Hepatic microsomes were isolated by ultracentrifugation as previously described (Hylemon *et al.* 1989) and stored at –80°C. Microsomal protein was determined using a modified Lowry procedure (Markwell *et al.* 1978). Hepatic cholesterol 7 α -hydroxylase activity was determined by HPLC after enzymic conversion to 7 α -hydroxy-4-cholesten-3-one using cholesterol oxidase (Chiang, 1991).

Determination of faecal bile acids and sterols

Faecal sterols and total bile acid concentrations were analysed in an oven-dried faecal sample using a modification of the method of Suckling *et al.* (1991). Faecal total bile acid concentration was determined using an enzymic assay (Sigma bile acid kit; Sigma Chemicals) with some modifications. Faecal sterols including plant sterols, stanols and dimethylsterols and neutral sterols (cholesterol plus breakdown products) were analysed by GC as free (unsilylated) sterols as previously detailed (Trautwein *et al.* 1997). A dried sample of each diet was

Table 1. Plasma cholesterol and triacylglycerol concentrations of hamsters fed on diets containing 0% cholesterol and 0% plant sterols or 0.08% cholesterol plus 0, 0.24 or 0.48% 4-desmethylsterols, hydrogenated 4-desmethylsterols (stanols) or 4,4'-dimethylsterols for 5 weeks† (Mean values and standard deviations of twelve hamsters per diet for TC and TG and of five to six hamsters per diet for VLDL, LDL and HDL)

Diet ...	Cholesterol (C) (mmol/l)								Triacylglycerol (TG) (mmol/l)			
	TC		VLDL-C		LDL-C		HDL-C		TG		VLDL-TG	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0% cholesterol												
0% sterols*	4.77	0.37	1.03	0.26	0.40	0.03	3.31	0.27	3.42	1.02	2.91	0.74
0.08% cholesterol												
0% sterols	6.26 ^c	0.65	1.57	0.42	0.70 ^b	0.20	3.99	0.28	4.99	1.90	3.86 ^{abc}	0.93
0.24% sterols	5.34 ^{ab}	0.57	1.09	0.14	0.44 ^a	0.08	3.60	0.22	4.06	1.56	2.69 ^a	0.37
0.48% sterols	5.70 ^{ab}	0.52	1.43	0.14	0.51 ^{ab}	0.14	3.68	0.25	4.82	2.21	4.16 ^{bc}	0.74
0.24% stanols	5.29 ^a	0.43	1.21	0.34	0.46 ^a	0.07	3.63	0.32	3.48	1.29	2.91 ^{ab}	0.86
0.48% stanols	5.32 ^{ab}	0.41	1.18	0.34	0.53 ^{ab}	0.09	3.85	0.25	3.74	1.24	3.25 ^{abc}	0.91
0.24% 4,4'-dimethylsterols	5.62 ^{ab}	0.52	1.47	0.30	0.55 ^{ab}	0.05	4.00	0.17	3.48	1.18	3.65 ^{abc}	0.75
0.48% 4,4'-dimethylsterols	5.95 ^{bc}	0.52	1.58	0.25	0.60 ^{ab}	0.06	3.81	0.27	3.72	1.12	4.34 ^c	0.71

TC, total cholesterol.

^{a,b,c} Values in a column with unlike superscripts were significantly different ($P<0.05$) as determined by one-way ANOVA and the Student–Neuman–Keuls *post hoc* test.

* This group was not included in the statistical analysis.

† For details of diets see p. 228.

analysed for cholesterol and phytosterol concentration applying the same method as described for the faecal samples.

Statistical analysis

All results are expressed as mean and standard deviation. Statistical differences were calculated using ANOVA followed by the Student–Neuman–Keuls *post hoc* test. The group receiving the cholesterol-free control diet was not included in the statistical analysis. Differences between diet groups were considered significant at $P < 0.05$. All statistical analyses were performed using the Super ANOVA statistical software package (Abacus Concepts, Berkeley, CA, USA).

Results

Food intake, body weight gain and organ weights

Food intake, final body weights, body weight gain and relative weights of the small intestine and the caecum were similar between groups (data not shown). Absolute liver weights were also similar between groups, but relative liver weights of hamsters fed the diets supplemented with 0.24% sterols, 0.24% stanols and 0.48% stanols were significantly lower (4.3 (SD 0.3), 4.3 (SD 0.2), 4.3 (SD 0.2) g/100 g body weight, respectively) than those of the cholesterol-fed control animals (4.7 (SD 0.5) g/100 g body weight).

Plasma lipids and lipoproteins

After 5 weeks plasma TC concentrations were significantly lower (–15%) in hamsters fed 0.24% plant sterols, 0.24% and 0.48% plant stanols compared to those fed the cholesterol-containing control diet (Table 1). TC was 9%

lower in hamsters fed 0.48% plant sterols compared to controls, but this was not statistically significant. The cholesterol-lowering effect was not dose-dependent. The lower dose (0.24%) of 4,4'-dimethylsterols significantly lowered TC compared with controls (–10%), while doubling the dose to 0.48% did not lower TC significantly. Plasma TG concentrations were up to 30% lower in hamsters fed the sterol-, stanol- and dimethylsterol-supplemented diets, but owing to a large intra-individual variation the effect did not reach the level of statistical significance ($P = 0.095$) (Table 1).

Plasma VLDL and HDL cholesterol concentrations did not differ among the groups, while LDL cholesterol concentrations were significantly lower in hamsters fed 0.24% plant sterols and 0.24% stanols (Table 1). When expressed as a percentage of TC, no differences in the relative distribution of VLDL, LDL and HDL cholesterol was found. Most cholesterol was present as HDL cholesterol (64–70%). Compared to cholesterol-fed controls, VLDL TG concentrations tended to be lower in hamsters fed 0.24% sterols or 0.24% stanols (Table 1). No relevant changes were observed in the composition of VLDL, LDL and HDL (data not shown).

Hepatic cholesterol

TC and EC concentrations were significantly lower in livers of hamsters fed 0.48% plant sterols, 0.24% and 0.48% stanols and 0.48% 4,4'-dimethylsterols compared with cholesterol-fed control hamsters, while 0.24% sterols and 0.24% 4,4'-dimethylsterols decreased liver TC accumulation non-significantly (Table 2). The effect produced by 0.48% plant sterols and 0.48% stanols was comparable. Hepatic FC concentrations did not differ between groups.

Table 2. Hepatic cholesterol concentrations ($\mu\text{mol/g}$ liver) of hamsters fed on diets containing 0% cholesterol and 0% plant sterols or 0.08% cholesterol plus 0, 0.24 or 0.48% 4-desmethylsterols, hydrogenated 4-desmethylsterols (stanols) or 4,4'-dimethylsterols for 5 weeks†

(Mean values and standard deviations of twelve hamsters per diet)

Diet ...	Total cholesterol (TC)		Free cholesterol		% TC		Esterified cholesterol		% TC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0% cholesterol										
0% sterols*	14.84	3.67	7.12	0.77	51 ^d	14	7.71	3.80	49	13
0.08% cholesterol										
0% sterols	67.01 ^d	9.62	8.08	1.04	12 ^a	1	58.93 ^d	8.96	88 ^d	1
0.24% sterols	60.90 ^{cd}	11.77	8.21	1.41	14 ^{ab}	2	52.69 ^{cd}	11.07	86 ^{cd}	2
0.48% sterols	40.19 ^a	9.24	8.09	1.31	21 ^d	5	32.10 ^a	9.20	79 ^a	5
0.24% stanols	52.45 ^{bc}	12.51	8.23	0.94	16 ^{bc}	3	44.22 ^{bc}	12.38	84 ^{bc}	3
0.48% stanols	38.32 ^a	6.20	8.18	1.10	22 ^d	3	30.15 ^a	5.62	78 ^a	3
0.24% 4,4'-dimethylsterols	58.53 ^{cd}	5.06	8.58	1.38	15 ^{ab}	3	49.94 ^c	5.40	85 ^{cd}	3
0.48% 4,4'-dimethylsterols	46.27 ^{ab}	8.58	8.45	1.02	19 ^{cd}	3	37.82 ^{ab}	8.27	81 ^{ab}	3

^{a,b,c,d} Values in a column with unlike superscripts were significantly different ($P < 0.05$) as determined by one-way ANOVA and the Student–Neuman–Keuls *post hoc* test.

* This group was not included in the statistical analysis.

† For details of diets see p. 228.

Biliary lipids and bile acid profile

The total lipid concentrations in bile did not differ among groups (data not shown). Neither the concentrations (mmol/l), nor the molar percentages (mol/100 mol total lipids) of biliary bile acids and PL were altered in hamsters fed on diets enriched with different types of plant sterol esters compared with controls (Table 3). However, the molar percentage of BC was lower in hamsters fed 0.24 and 0.48 % stanols (−19 and −10 %, respectively) than in those fed the cholesterol-rich control diet, although this was statistically significant only for the lower dose (Table 3). No differences in the concentrations of individual bile acids in gallbladder bile were found in hamsters fed the different diets (data not shown). In addition, no major alterations were observed in the relative molar percentages of individual bile acids (Table 4). However, the molar percentage of total cholate (sum of tauro- and glycocholic acid) was significantly higher in bile from hamsters fed on diets supplemented with the two doses (0.24 and 0.48 %) of plant sterols, stanols and 4,4'-dimethylsterols. In contrast, the molar percentage of total chenodeoxycholate (sum of tauro- and glycochenodeoxycholic acid) was lower, but the decrease was statistically significant only in hamsters fed 0.48 % sterols or stanols (Table 4). As a result of these changes in the bile acid profile, cholate:chenodeoxycholate was significantly higher in hamsters fed 0.48 % plant sterols, 0.24 and 0.48 % stanols and 0.24 % 4,4'-dimethylsterols (Table 4). The glycine:taurine conjunction ratio as well as the ratio of primary:secondary bile acids did not differ among groups (Table 4).

Activities of cholesterol 7 α -hydroxylase

No difference in the activity of microsomal cholesterol 7 α -hydroxylase was observed between hamsters fed the

cholesterol-free and cholesterol-containing control diets (22.1 (SD 10.0) and 18.2 (SD 7.4) pmol/h and mg protein, respectively). Further cholesterol 7 α -hydroxylase activity was not significantly different in animals fed the two doses (0.24 and 0.48 %) of plant sterol and stanol esters or 0.24 % 4,4'-dimethylsterol esters (17.5 (SD 5.1), 19.4 (SD 6.9), 18.6 (SD 6.1), 23.2 (SD 6.8), 22.8 (SD 9.8) pmol/h and mg protein, respectively).

Faecal output and faecal bile acid excretion

The daily faecal output (g dry faeces) was non-significantly higher (15–40 %) in hamsters fed plant sterols or stanols compared to cholesterol-fed control animals. Hamsters fed 0.48 % 4,4'-dimethylsterols had a significantly higher faecal output than the cholesterol-fed controls (Table 5). The faecal concentration of bile acids (μ mol/g) was 13–28 % lower in hamsters fed diets with different types of plant sterol esters compared to cholesterol-fed controls, but this was statistically significant only in hamsters fed 0.24 % sterols, 0.48 % stanols as well as 0.24 and 0.48 % 4,4'-dimethylsterols (Table 5). Daily bile acid excretion, however, did not differ among the groups (Table 5).

Faecal sterol excretion

The daily faecal excretion of neutral sterols (cholesterol, coprostanol, cholestanol and coprostanone) was significantly higher in hamsters fed the sterol- and stanol-supplemented diets, but not in those fed 4,4'-dimethylsterols compared to cholesterol-fed controls (Fig. 1). This was mainly the result of the significantly higher faecal cholesterol concentration while coprostanol concentrations were unchanged (data not shown). Cholesterol and

Table 3. Biliary lipid concentration (bile acids, phospholipids and biliary free cholesterol) of hamsters fed diets containing 0 % cholesterol and 0 % plant sterols or 0.08 % cholesterol plus 0, 0.24 or 0.48 % 4-desmethylsterols, hydrogenated 4-desmethylsterols (stanols) or 4,4'-dimethylsterols for 5 weeks†

(Mean values and standard deviations of twelve hamsters per diet)

Diet ...	Bile acids				Phospholipids				Cholesterol			
	mmol/l		mol % [‡]		mmol/l		mol %		mmol/l		mol % [‡]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0 % cholesterol												
0 % sterols*	185	28	88.4	1.7	20.8	3.8	9.9	1.4	3.4	0.7	1.6	0.3
0.08 % cholesterol												
0 % sterols	179	42	88.2	1.4	19.7	5.4	9.7	1.2	4.3 ^{bc}	1.2	2.1 ^{bc}	0.3
0.24 % sterols	180	28	87.4	1.6	21.1	4.0	10.3	1.4	4.8 ^{bc}	1.1	2.3 ^c	0.3
0.48 % sterols	180	35	87.9	2.5	19.7	4.2	9.9	2.3	4.6 ^{bc}	0.9	2.2 ^c	0.3
0.24 % stanols	168	46	88.4	1.6	18.8	5.6	9.9	1.5	3.2 ^a	1.1	1.7 ^a	0.4
0.48 % stanols	187	34	88.7	1.1	19.7	3.6	9.4	0.9	3.9 ^{ab}	1.0	1.9 ^{ab}	0.4
0.24 % 4,4'-dimethylsterols	165	32	87.5	1.8	19.2	4.3	10.2	1.6	4.3 ^{bc}	0.9	2.3 ^c	0.3
0.48 % 4,4'-dimethylsterols	189	20	87.2	1.9	22.7	4.3	10.4	1.7	5.2 ^c	0.9	2.4 ^c	0.3

^{a,b,c} Values in a column with unlike superscripts were significantly different ($P < 0.05$) as determined by one-way ANOVA and the Student–Neuman–Keuls *post hoc* test.

* This group was not included in the statistical analysis.

† For details of diets see p. 228.

‡ mol %, mol/100 mol total biliary lipids.

Table 4. Bile acid profile of gallbladder bile (mol/100 mol total bile acids, and molar ratios) from hamsters fed on diets containing 0% cholesterol and 0% plant sterols or 0.08% cholesterol plus 0, 0.24 or 0.48% 4-desmethylsterols, hydrogenated 4-desmethylsterols (stanols) or 4,4'-dimethylsterols for 5 weeks†
(Mean values and standard deviations of twelve hamsters per diet)

Diet...	TCA		GCA		Total cholate		TCDCA		GCDCA		Total cheno		TDCA		GDCA		Cholate: cheno		Glycine: taurine conjugation		Primary: secondary	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0% cholesterol	31.1	6.0	31.5	5.8	62.6	1.6	15.8	3.1	12.3	2.7	28.1	2.1	6.2	2.1	2.7	1.4	2.24	0.16	0.92	0.35	10.9	4.1
0% sterols*	26.6	4.3	28.7	5.5	55.3 ^a	3.6	18.1 ^{ab}	3.4	17.1	5.6	35.2 ^c	4.5	5.9	1.7	2.7	0.8	1.60 ^a	0.26	1.00	0.35	10.3	3.3
0.08% cholesterol	27.5	6.4	29.9	6.0	57.3 ^b	1.7	17.7 ^{ab}	4.1	15.5	4.5	33.2 ^{bc}	2.1	5.8	1.4	2.9	1.4	1.73 ^{ab}	0.15	1.05	0.64	9.9	1.6
0.24% sterols	30.5	4.4	29.5	4.0	60.0 ^b	2.3	16.7 ^{ab}	2.6	12.7	2.0	29.5 ^a	2.0	6.5	0.6	2.9	1.2	2.05 ^b	0.20	0.85	0.24	8.7	1.4
0.48% sterols	26.9	3.3	31.3	2.5	58.2 ^b	2.3	16.7 ^{ab}	1.3	15.5	2.9	32.2 ^{abc}	2.9	5.8	1.4	3.1	0.9	1.83 ^b	0.22	1.01	0.18	9.9	2.4
0.24% stanols	27.6	5.2	31.3	5.0	58.9 ^b	1.7	16.3 ^{ab}	3.1	14.4	2.5	30.7 ^{ab}	1.9	6.3	1.2	3.4	1.3	1.93 ^{bc}	0.14	1.00	0.28	9.1	2.7
0.48% stanols	24.5	5.7	33.0	5.7	57.5 ^b	1.4	15.1 ^a	3.1	16.1	3.5	31.2 ^{ab}	1.2	6.4	1.3	4.0	1.2	1.85 ^b	0.09	1.22	0.47	8.0	1.5
dimethylsterols																						
0.48%, 4,4'-dimethylsterols	27.8	4.4	29.6	4.4	57.4 ^b	2.8	19.3 ^b	3.6	14.4	2.6	33.6 ^{bc}	3.5	5.4	1.4	3.0	0.9	1.73 ^{ab}	0.25	0.91	0.26	10.6	2.4

a,b,c Values in a column with unlike superscripts were significantly different ($P < 0.05$) as determined by one-way ANOVA and the Student–Neuman–Keuls *post hoc* test.

TCA, taurocholic acid; GCA, glycocholic acid; TCDCA, taurochenodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; TDCA, taurodeoxycholic acid; GDCA, glycodeoxycholic acid; cheno, chenodeoxycholate.

* This group was not included in the statistical analysis.

† For details of diets see p. 228.

coprostanol were the two major sterols excreted in the faeces.

Plant sterol, stanol and 4,4'-dimethylsterol concentrations measured in the faecal sample reflected the sterol pattern of the supplemented diets. As expected, daily faecal sterol, stanol and 4,4'-dimethylsterol excretion was significantly higher in hamsters fed the enriched diets than in controls (Fig. 2). Daily excretion of sterols, stanols or 4,4'-dimethylsterols was significantly higher in hamsters fed the 0.48 v. 0.24% dose, although there was a large intra-animal variation (Fig. 1).

Discussion

The present study examined for the first time the effects of three different types of plant sterols (at two doses of 0.24 v. 0.48%), i.e. esterified 4-desmethylsterols (sterols), hydrogenated 4-desmethylsterols (stanols) and 4,4'-dimethylsterols on plasma cholesterol concentrations. In addition, aspects of bile acid metabolism, in particular bile acid pattern, synthesis and excretion, were assessed and data on bile acid profile in gallbladder bile are presented.

In the present study with hamsters fed on cholesterol-enriched diets (0.08% dietary cholesterol) esters of 4-desmethylsterols and -stanols significantly ($P < 0.05$) lowered TC and LDL cholesterol. Both had equal cholesterol-lowering effects resulting in a 15% reduction in TC with a dose of 0.24%. 4,4'-Dimethylsterol esters at a dose of 0.24% significantly ($P < 0.05$) lowered TC by 10%, but were altogether less effective in lowering TC compared to desmethylsterol esters. This finding is similar to previous findings in human trials (Weststrate & Meijer, 1998; Sierksma *et al.* 1999; Vissers *et al.* 2000).

With regard to hepatic cholesterol accumulation, esterified dimethylsterols significantly ($P < 0.05$) lowered TC and EC concentrations in the liver. However, the effect was again less than seen with desmethylsterols or -stanols. The weaker effect of 4,4'-dimethylsterols could possibly relate to their chemical structure. 4-Desmethylsterols such as β -sitosterol and campesterol are similar to cholesterol, differing only in a side-chain substitution, while 4,4'-dimethylsterols such as cycloartenol also have two additional methyl groups at the site adjacent to the hydroxyl group. Despite the reductions seen in plasma and liver cholesterol, faecal cholesterol excretion was only moderately enhanced in hamsters fed the dimethylsterol-enriched diets. Similar to the present findings, Ikeda *et al.* (1985) found in rats that hepatic cholesterol accumulation was mitigated by cycloartenol, while no effect on TC was seen. Further, cycloartenol was also less effective than β -sitosterol in enhancing faecal sterol excretion, similar to the observations in the present hamster study. Interestingly, cycloartenol was absorbed at a 4-fold higher rate than β -sitosterol (Ikeda *et al.* 1985). Since 4,4'-dimethylsterols had only minor effects on faecal cholesterol excretion, it is unlikely that the primary mechanism for the cholesterol-lowering actions of these sterols is a diminished intestinal cholesterol absorption via interference with micellar solubilisation of cholesterol, as suggested by others (Nagao *et al.* 2001). Therefore, as suggested by Ikeda *et al.* (1985), post-absorptive, secondary effects such as an

Table 5. Faecal excretion, faecal bile and concentration and daily faecal bile acid excretion in hamsters fed on diets containing 0% cholesterol and 0% plant sterols or 0.08% cholesterol plus 0, 0.24 or 0.48% 4-desmethylsterols, hydrogenated 4-desmethylsterols (stanols) or 4,4'-dimethylsterols for 5 weeks.† Faecal samples were collected over a 3 d period during week 4. Hamsters were randomly selected and housed individually in wire-bottomed cages during faecal collection

(Mean values and standard deviations of eight hamsters per diet)

Diet ...	Faecal excretion (g/d (dry wt))		Bile acid concentration ($\mu\text{mol/g}$ dry faeces)		Daily bile acid excretion ($\mu\text{mol/d}$)	
	Mean	SD	Mean	SD	Mean	SD
0% cholesterol 0% sterols*	0.76	0.19	2.65	0.40	2.06	0.69
0.08% cholesterol 0% sterols	0.75 ^a	0.15	3.42 ^b	0.71	2.51	0.57
0.24% sterols	0.87 ^{ab}	0.13	2.46 ^a	0.43	2.12	0.32
0.48% sterols	0.96 ^{ab}	0.14	2.96 ^{ab}	0.33	2.83	0.46
0.24% stanols	0.86 ^{ab}	0.18	2.87 ^{ab}	0.25	2.48	0.55
0.48% stanols	0.95 ^{ab}	0.14	2.68 ^a	0.34	2.55	0.40
0.24% 4,4'-dimethylsterols	0.90 ^{ab}	0.11	2.62 ^a	0.48	2.37	0.50
0.48% 4,4'-dimethylsterols	1.06 ^b	0.24	2.73 ^a	0.62	2.87	0.98

^{a,b,c} Values in a column with unlike superscripts were significantly different ($P < 0.05$) as determined by one-way ANOVA and the Student–Neuman–Keuls *post hoc* test.

* This group was not included in the statistical analysis.

† For details of diets see p. 228.

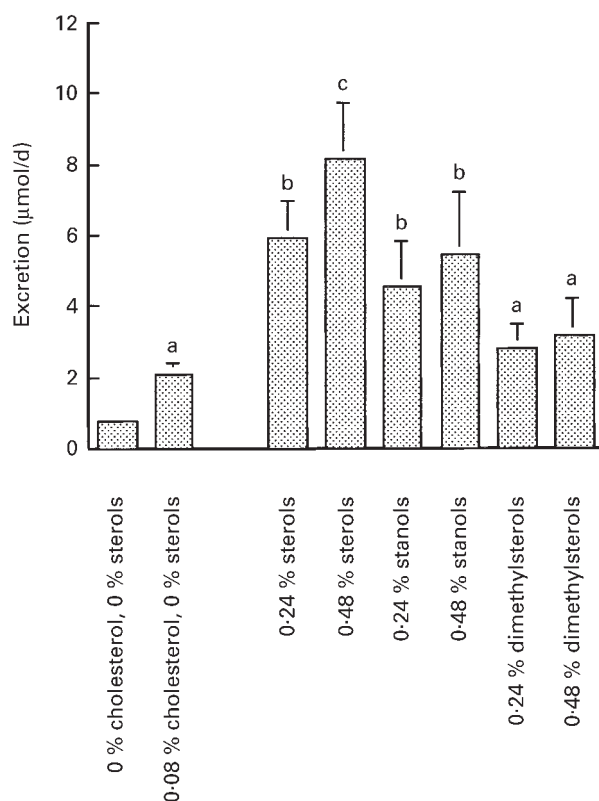


Fig. 1. Daily excretion of neutral sterols (cholesterol plus coprostanol, cholestanol and coprostanone) of hamsters fed diets containing 0% cholesterol and 0% plant sterols or 0.08% cholesterol plus 0, 0.24 or 0.48% 4-desmethylsterols, hydrogenated 4-desmethylsterols (stanols) or 4,4'-dimethylsterols. Values are means with their standard deviations represented by vertical bars (n 8). Values with unlike superscript letters were significantly different ($P > 0.05$) as determined by the Student–Neuman–Keuls *post hoc* test after ANOVA. The group containing no cholesterol (control group) was not included in the statistical analysis.

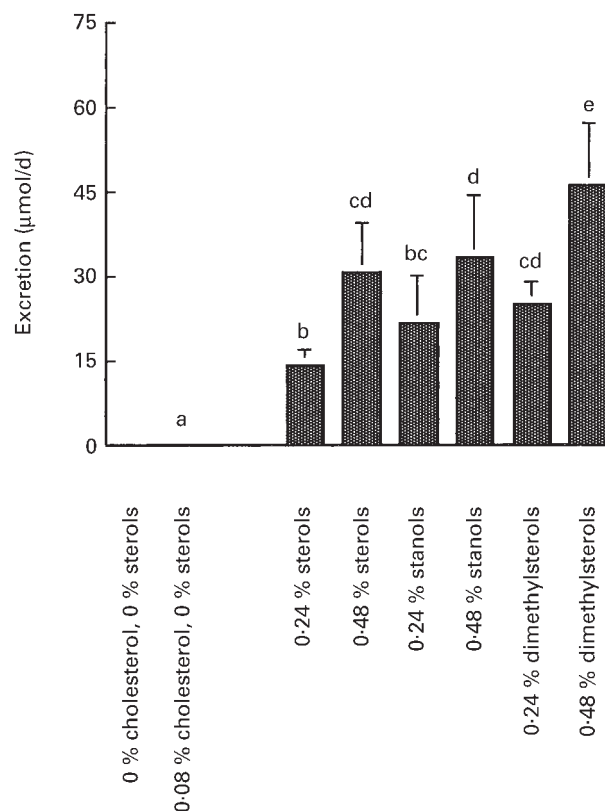


Fig. 2. Daily excretion of phytosterols, -stanols or dimethylsterols of hamsters fed diets containing 0% cholesterol and 0% plant sterols or 0.08% cholesterol plus 0, 0.24 or 0.48% 4-desmethylsterols, hydrogenated 4-desmethylsterols (stanols) or 4,4'-dimethylsterols. Values are means with their standard deviations represented by vertical bars (n 8). Values with unlike superscript letters were significantly different ($P > 0.05$) as determined by the Student–Neuman–Keuls *post hoc* test after ANOVA. The group containing no cholesterol (control group) was not included in the statistical analysis.

inhibitory effect on cholesterol biosynthesis or a hormone-like effect might explain the cholesterol-lowering action of cycloartenol and other 4,4'-dimethylsterols. Recently, Nagao *et al.* (2001) found a reduction in serum and liver cholesterol and a significant increase in faecal cholesterol excretion in rats fed on rice bran oil or unsaponifiable compounds prepared from rice bran oil. In addition, no significant effects on the mRNA abundances for various cholesterol metabolism-related proteins (3-hydroxy-3-methylglutaryl CoA reductase, LDL receptor or cholesterol 7 α -hydroxylase) were observed, and it was concluded that interrupting the intestinal absorption of cholesterol was the main mechanism for the cholesterol-lowering action of rice bran oil sterols (Nagao *et al.* 2001). However, rice bran oil and the unsaponifiable compounds of rice bran oil used in that study contained mainly desmethylsterols (mostly in the form of β -sitosterol) and only minor amounts of dimethylsterols. Therefore, the observed effects seem more attributable to desmethylsterols rather than dimethylsterols.

The differences between 0.24 and 0.48% plant sterol and stanol ester diets on plasma cholesterol concentrations were not significant ($P > 0.05$). The differences were significant ($P < 0.05$) for hepatic cholesterol, for which a dose-dependent reduction was seen. The lack of a striking dose-response effect is not likely to be linked to the animal model, the cholesterol level of the diet or the duration of the study. The hamster is a suitable animal model to study effects on cholesterol metabolism and a 5-week feeding period is sufficient to reach a new metabolic steady stage condition (Kris-Etherton & Dietschy, 1997). Moreover, 0.08% dietary cholesterol is equivalent to the endogenous cholesterol synthesis rate and resulted in hypercholesterolaemia, in contrast to the cholesterol-free diet. However, we cannot completely rule out the suggestion that the statistical power to detect a significant lowering of LDL cholesterol was too low, and therefore no clear dose-response effect was detectable. Another conceivable explanation could be that the hamster may be less sensitive and may respond less well than certain other species, including man. So far, only a few human studies have directly evaluated a dose-response relationship between sterol and stanol intake and cholesterol-lowering efficacy. They did not demonstrate a strong dose dependency (Miettinen & Vanhanen, 1994; Hendriks *et al.* 1999; Nguyen *et al.* 1999; Hallikainen *et al.* 2000). In these studies, a doubling of sterol or stanol intake did not result in a corresponding doubling of TC and LDL cholesterol reduction. Moreover, a previous hamster study also did not find significant differences in plasma cholesterol lowering by 0.01, 0.2 and 1% sitostanol (Ntanos & Jones, 1998). Taken together, this indicates that the relationship between doses of plant sterol or stanol ester intake and plasma cholesterol reduction may not be linear, at least at these levels of intake. A plausible explanation for the lack of a clear dose response is the compensatory increase in cholesterol synthesis with higher doses of sterol and stanol esters (Jones *et al.* 2000), which appears to reduce the lowering of LDL cholesterol. Although dose dependency may be shallow,

these data clearly demonstrate that sterol and stanol esters are effective in cholesterol lowering at these doses.

Faecal cholesterol excretion was significantly enhanced in hamsters fed on the diets enriched with sterol and stanol esters. This was possibly caused by diminished intestinal cholesterol absorption, which is thought to be the major mechanism for the cholesterol-lowering action of sterols and stanols, as reported by others (Ikeda *et al.* 1988; Miettinen & Vanhanen, 1994; Vanhanen *et al.* 1994; Weststrate *et al.* 1999). Previous findings suggest that intestinal cholesterol absorption is reduced, and subsequently cholesterol excretion is more effectively increased, by stanols compared to sterols (Sugano *et al.* 1977; Ikeda & Sugano, 1978; Heinemann *et al.* 1993). Recent human studies, however, did not find a difference in inhibition of cholesterol absorption (Jones *et al.* 2000; Normen *et al.* 2000). In this hamster study, the esterified sterols and stanols had a comparable effect on increasing daily faecal cholesterol excretion, suggesting that possibly cholesterol absorption was diminished to the same extent by sterols and stanols.

It has been suggested that sterols may also regulate cholesterol metabolism through directly affecting key enzymes that regulate cholesterol metabolism and excretion (Shefer *et al.* 1973; Ikeda & Sugano, 1983; Ling & Jones, 1995a). Therefore, a particular focus of the present hamster study was to address effects on bile acid metabolism, i.e. bile acid excretion, hepatic synthesis, biliary lipid and bile acid composition. Reported effects on bile acid synthesis are conflicting. In rats, infusion of β -sitosterol inhibited (Boberg *et al.* 1989; Shefer *et al.* 1994), while dietary administration increased the activity of cholesterol 7 α -hydroxylase (Shefer *et al.* 1973). In the present hamster study, cholesterol 7 α -hydroxylase activity did not differ among the dietary groups, suggesting no influence of sterols, stanols and dimethylsterols on bile acid synthesis in the liver. Moreover, faecal bile acid excretion was also not affected by these treatments. Previous animal and human studies have reported discrepant effects of dietary sterols or stanols on faecal excretion of bile acids, but most studies revealed only minor or no changes (Jones *et al.* 1997; Weststrate *et al.* 1999). Therefore, these findings indicate that sterols do not impair the enterohepatic circulation of bile acids. In addition, a previous human study demonstrated that plant sterol esters did not alter the faecal concentration of secondary bile acids (Weststrate *et al.* 1999). This is in line with our findings that the ratio of primary:secondary bile acids in gallbladder bile was not altered by sterols or stanols.

The effects on biliary lipids and the bile acid profile of gallbladder bile was also investigated in the present hamster study. Feeding hamsters with dietary cholesterol usually results in a reduction in the molar ratio of biliary lipids and in changes in the bile acid profile due to a marked increase in chenodeoxycholate (Trautwein *et al.* 1993b, 1997). This is associated with the high prevalence of cholesterol gallstones in the cholesterol-fed hamster model. The effect has been linked to an inhibition of the activity of 12 α -hydroxylase, a key enzyme in the synthesis of cholic acid (Kuroki *et al.* 1983). Apparently, feeding

sterols and stanols tended to reverse at least partly this cholesterol-induced effect, since cholate:chenodeoxycholate was improved. Similar findings, e.g. an increase in the molar percentage of cholate and a decrease in chenodeoxycholate, have been reported in mice fed β -sitosterol (Uchida *et al.* 1984). The observed changes in biliary cholate:chenodeoxycholate were small, but statistically significant (20–30% increase); however, their full physiological significance is not fully interpretable and remains to be further elucidated.

The molar percentages of biliary lipids, i.e. cholesterol, PL and bile acids, were mostly unchanged, except for a significant decrease in the molar percentage of BC found in gallbladder bile of hamsters fed the stanol-supplemented diets. Although not a primary aspect of this study, the improved cholate:chenodeoxycholate together with the lower molar percentage of BC seen in this hamster model could indicate a gallstone preventive effect. However, effects of plant sterols and stanols on BC saturation in man are inconsistent (Begemann *et al.* 1978; Tangedahl *et al.* 1979). Nevertheless, in a recent human study, bile acid composition and the molar percentage of BC were unchanged after consumption of plant stanol esters, indicating that potential gallstone formation is unlikely or might even be prevented by plant sterols or stanols (Miettinen *et al.* 2000).

In conclusion, esters of desmethylsterols were as effective as esters of desmethylstanols in lowering plasma TC and LDL cholesterol in cholesterol-fed hamsters without affecting HDL cholesterol, while 4,4'-dimethylsterols from rice bran oil were less effective. Enhanced faecal cholesterol excretion caused by an inhibition of intestinal cholesterol absorption appeared to be the main mechanism for the cholesterol-lowering action of desmethylsterols and -stanols, while enhanced faecal bile acid excretion and stimulation of bile acid synthesis did not appear to be involved. For 4,4'-dimethylsterols, other not yet fully evident mechanisms need to be considered to explain their modest cholesterol-lowering effect.

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