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Acute effect of dietary stanyl ester dose on post-absorptive α-tocopherol, β-carotene, retinol and retinyl palmitate concentrations

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Stanyl esters dissolved in margarine inhibit cholesterol absorption, lower sterol absorption in general, and lower serum total cholesterol, LDL-cholesterol and plant sterol levels. To find out whether stanyl esters inhibit absorption of fat-soluble vitamins and β-carotene in acute experiments, we performed two fat-tolerance tests fortified with vitamins (retinol 0·9–3·7 mg, α-tocopherol 70–581 mg), β-carotene (25–150 mg) and squalene (0·5 g) with and without 1 g of stanyl ester added to the test meal in ten healthy men. The concentrations or areas under the curves (AUC) of cholesterol, triacylglycerols, squalene and α-tocopherol, β-carotene and retinyl palmitate showed typical postprandial changes in serum, chylomicrons, VLDL and VLDL infranatant (intermediate-density lipoproteins, LDL and HDL) over 24 h after the test meal without stanyl esters, and they were not affected by the addition of stanyl esters. The post-absorptive serum campesterol concentration and campesterol : cholesterol were significantly lowered at 6–9 h by stanyl ester supplementation, reflecting reduced sterol absorption efficiency. Changes in vitamin and β-carotene AUC did not correlate with the given doses. In conclusion, the present study shows that stanyl esters dissolved in margarine do not detectably interfere in a short-term study with the absorption of α-tocopherol, β-carotene or retinol measured by a 24 h oral fat-load test.

Stanyl esters: Fat-soluble vitamins: β-Carotene: Squalene

Dietary plant sterols have been shown to reduce serum cholesterol levels in human subjects since the 1950s (Pollak, 1953). Sitostanol, a 5α-saturated sitosterol derivative, reduces the intestinal absorption of cholesterol more effectively than sitosterol, and is less well absorbed from the intestine (Sugano *et al.* 1977; Ikeda & Sugano, 1978; Heinemann *et al.* 1986, 1991; Ikeda *et al.* 1989; Becker *et al.* 1993). Sitostanyl ester, a fat-soluble form of sitostanol, has been shown to reduce serum total cholesterol and LDL-cholesterol levels, and serum plant sterols (campesterol and sitosterol) when added to a daily meal (Miettinen *et al.* 1995).

The exact cholesterol-lowering mechanism of stanyl esters, however, is not yet known. It has been shown in rats that the concentration of sitostanol-containing micelles in the jejunum is the important factor in suppression of cholesterol absorption (Hassan & Rampone, 1980; Ikeda *et al.* 1989). Sitostanol reduces the concentration of cholesterol in micelles both *in vitro* and *in vivo*, and reduces the micellar solubility of cholesterol *in vivo* (Ikeda *et al.* 1989). Plant sterols have been shown to interfere with cholesterol absorption more effectively when administered simultaneously with cholesterol (Mattson *et al.* 1982). As the absorption of α-tocopherol (Hollander *et al.* 1975), β-carotene (Hollander & Ruble, 1978) and retinol (Hollander, 1981) are passive diffusion processes taking place with the intestinal lipids, their absorption is facilitated by simultaneous ingestion of fat. Accordingly, dietary stanyl esters might interfere with the absorption of fat-soluble vitamins. In fact, it has been shown previously in a long-term study (Gylling *et al.* 1999*a*) that sitostanyl ester reduced the serum level of β-carotene, even in relation to cholesterol, but not that of retinol, which is a more polar compound than β-carotene.

Dietary retinyl esters are hydrolysed to retinol in the intestinal lumen and thereafter absorbed into the enterocyte (Goodman *et al.* 1965) where they are re-esterified and transported in chylomicrons and chylomicron remnants to the liver (Kanai *et al.* 1968). It is estimated that up to 75 % of the dietary retinol is absorbed in the small intestine

Abbreviation: AUC, area under the curve.

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(Biesalski, 1997). β-Carotene, a precursor of vitamin A, has a completely different method of absorption. Approximately 9–17 % of the dietary β-carotene, is absorbed via the lymphatic system (Goodman *et al.* 1966). In the enterocyte β-carotene is partly degraded to two retinaldehyde molecules and eventually into retinol (Goodman & Olson, 1969). Thus 60–70 % of the absorbed β-carotene is recovered as retinyl esters and about 15 % remains as intact β-carotene (Goodman *et al.* 1966). Postprandial maximal serum β-carotene concentration has been documented as much as 36 h after administration (Cornwell *et al.* 1962). Orally-administered α-tocopherol is absorbed in chylomicrons (Traber *et al.* 1990) and, similarly to other lipid-soluble compounds, requires bile acids for micellar formation (Gallo-Torres, 1970). Peak postprandial concentrations are detected 6–12 h after administration (Traber *et al.* 1988, 1990). About 45 % of an oral dose is absorbed into the lymph (Meydani, 1995).

The aim of the present study was to investigate the effect of a single stanyl ester dose in a test meal containing variable amounts of vitamins and squalene on postprandial cholesterol metabolism and absorption of α-tocopherol, β-carotene and retinol in healthy volunteers.

Experimental methods

Subjects

The study group consisted of ten healthy men with a mean age of 52 (SE 5) years. The subjects had no known history of renal, liver, thyroid or gastrointestinal diseases, or any hypolipidaemic medication. The study protocol was accepted by the Ethics Committee of our hospital.

Study design

The subjects visited the outpatient clinics twice, 2 weeks apart. A fat-load test was performed during both visits. The test was started at 08.00 hours after a 12 h fast. After the baseline blood samples were withdrawn from the forearm vein into light-protected 10 ml tubes (Venoject Autosep®; Terumo Europe, Leuven, Belgium), the subjects were given a fatty meal containing 90 g fat, 432 mg cholesterol and 0·5 g squalene. The test meals were supplemented with a mixture of vitamins (retinol 0·9–3·7 mg and α-tocopherol 70–581 mg) and β-carotene 25–150 mg (Table 1). The fat-soluble vitamins and β-carotene have markedly variable absorption percentages. To observe possible effects of dosage, two to three different dosages were used for vitamins and β-carotene. Margarine (8 g) with or without 1 g stanyl ester (Raisio Group, Raisio, Finland) was added to the fat load in random order. The stanyl ester mixture was mainly composed of sitostanyl ester (92 %). The contents of the major fatty acids in the margarine were (g/100 g): 16 : 0 16·7, 18 : 1 47·3, 18 : 2 17·7, 18 : 3 8·9. The total amount of *trans*-fatty acids was 0·5. The margarine did not have a vitamin supplement. The test meal was given as a cream–egg shake. After the test meal, the subjects fasted for 9 h before having their first normal meal of standard low-fat low-cholesterol hospital food. Postprandial blood samples were withdrawn after 3, 4, 6, 9, 12 and 24 h. The samples were stored at −20°C until analysed.

Table 1. Doses of vitamins and β-carotene (mg) given to each of the ten male subjects with their test meals

Subject no. …	1 and 2	3 and 4	5–7	8–10
α-Tocopherol	71	281	281	581
Retinol	0·9	3·7	3·7	3·7
β-Carotene	25	100	150	150

Analytical methods

Commercial kits were used to analyse enzymically serum total cholesterol and triacylglycerol and lipoprotein-cholesterol and -triacylglycerols (Roche Diagnostics; Hoffman-La Roche Ltd, Basel, Switzerland). Lipoproteins from fasting serum samples were separated by ultracentrifugation in a fixed-angle type 50·4 Ti rotor (Beckman Instruments Inc., Fullerton, CA, USA) into the following densities (g/ml; Warnick & Alberts, 1982): VLDL <1·006, intermediate-density lipoproteins 1·006–1·019, LDL 1·019–1·063, HDL 1·063–1·21. Chylomicrons and VLDL were separated from postprandial samples as follows: 7.2 ml serum was overlayered with an NaCl solution of density 1·0063 g/ml and centrifuged at 34 873 ***g*** (18 000 rpm) for 30 min. Chylomicrons were isolated by aspirating the top 3·6 ml. To separate VLDL, the infranatant was mixed with an NaCl solution of density 1·0063 g/ml and centrifuged at 131 849 ***g*** (35 000 rpm) for 18 h. VLDL was aspirated as the top 3·6 ml. The bottom fraction contained the remaining intermediate-density lipoproteins, LDL and HDL (VLDL infranatant).

Serum squalene and non-cholesterol sterols as non-saponifiable lipids were quantified using GLC with a 50 m HP Ultra 1 SE-30 capillary column (Hewlett-Packard Co., Wilmington, DE, USA; Miettinen & Koivisto, 1983; Miettinen, 1988). Squalene is a cholesterol precursor, which reflects postprandial chylomicron remnant transport (Gylling & Miettinen, 1994). Serum cholesterol-precursor sterol levels reflect cholesterol synthesis, and those of serum plant sterols (campesterol and sitosterol) reflect the absorption efficiency of cholesterol (Tilvis & Miettinen, 1986). The analyses of α-tocopherol, β-carotene, retinol and retinyl palmitate were carried out using reverse-phase HPLC (Schäfer Elinder & Walldius, 1992) on a 20 cm ODS Hypersil column (Hewlett-Packard Co.) using α-tocopheryl acetate as internal standard. The HPLC system comprised a 2150 HPLC Pump, 2152 LC Controller, 2157 Autosampler (Pharmacia LKB, Uppsala, Sweden), and a HP 1050 Diode Array Detector (Hewlett-Packard Co.). Serum or lipoprotein samples (0·4 ml) were extracted into heptane, dried under N_2 and reconstituted in 0·1 ml mobile phase, which consisted of acetonitrile–water–tetrahydrofuran (81·3:5·7:13, by vol.); the flow rate was 0·4 ml/min. The column temperature was maintained at 40°C. Injected samples (5 μl) were separated in the mobile phase on a 20 cm ODS Hypersil column (Hewlett-Packard Co.). The detection wavelengths (nm) were: α-tocopheryl acetate 285, α-tocopherol 292, retinol and retinol palmitate 326, β-carotene 450. All samples obtained from an individual

subject were analysed on the same day. The between-day CV varied between 9·2 and 11·9 %, and the within-day CV varied between 2·2 and 8·0 %.

Calculations

Postprandial triacylglycerol, retinyl palmitate and squalene are expressed as concentrations or are given as incremental values calculated by subtracting the respective fasting value from each postprandial value. Postprandial responses were also quantified by calculating the area under the curve (AUC) and the incremental area between the zero level and the 24 h concentration curve for each subject. For triacylglycerols and cholesterol, incremental areas under curves were calculated from their respective 9 h concentration curves, since after 9 h some of the values were negative. As the baseline concentrations of the compounds studied were similar for both fat loads, and due to frequent negative incremental values, we preferred to use AUC rather than incremental area under the curve in the following.

Statistical analysis

The data are expressed as means with their standard errors. The level of statistical significance was taken as $P < 0{\cdot}05$. ANOVA for repeated measures was used to test the stanyl ester effects and time × stanyl ester interaction for postprandial triacylglycerol, cholesterol, squalene and campesterol : cholesterol concentration curves. Follow-up comparisons were made by one-way ANOVA at each time point. In addition, Student's two-sided *t* test and paired *t* test were used when appropriate. Due to the different doses of vitamins used, non-parametric statistics (Fisher's exact probability test and the Wilcoxon signed-rank test) were used to test the differences of vitamin responses. Logarithmic transformations were used for skewed distributions.

Results

Fasting serum total and lipoprotein lipid values and serum vitamin and β-carotene levels are shown in Table 2. α-Tocopherol and β-carotene were carried mainly in the VLDL infranatant, and there were no differences between the fasting and postprandial states, whereas 45–55 % of the retinyl palmitate and squalene were carried in chylomicrons and VLDL 9 h after the test meal (Table 3). The recovery of retinol in the VLDL infranatant was 100 % both in the fasting state and postprandially (data not shown).

Stanyl ester supplementation had no effect on postprandial serum total, chylomicron (Fig. 1) or VLDL-triacylglycerol, -cholesterol and -squalene concentrations or their respective AUC.

In the following analyses, non-parametric tests were used due to the different doses of vitamins and β-carotene administered. Serum β-carotene AUC decreased in five subjects and increased in five subjects (P 0·34), α-tocopherol AUC decreased in six and increased in four subjects (P 0·24) and retinyl palmitate AUC increased in six, decreased in two and remained unchanged in two subjects (P 0·06; Fig. 2). The percentage changes in AUC were not significant: β-carotene −2·94 (SE 11·8); α-tocopherol +1·8 (SE 11·3); retinyl palmitate +16·9 (SE 9·0). The different doses of vitamins and β-carotene did not correlate with the changes in AUC due to supplementation with stanyl esters (data not shown). Fig. 2 shows that the presence of stanyl ester in the test meal had no consistent effect on the AUC for β-carotene, α-tocopherol or retinyl palmitate. Stanyl esters had no effect on peak postprandial serum concentration times of the compounds studied (Table 4).

Table 2. Baseline characteristics of healthy male volunteers participating in the study*

(Mean values with their standard errors and ranges for ten men)

Characteristics	Mean	SE	Range
Age (years)	52·3	5·3	26–67
BMI (kg/m^2)	23·8	1·4	16·6–33·6
Cholesterol (mmol/l):			
Total serum	5·0	0·3	3·8–6·2
VLDL	0·50	0·12	0·13–1·40
IDL	0·14	0·02	0·07–0·24
LDL	2·45	0·22	1·80–4·00
HDL	1·28	0·10	0·85–1·83
Triacylglycerol (mmol/l):			
Total serum	1·43	0·28	0·64–3·49
VLDL	0·98	0·26	0·28–2·81
IDL	0·07	0·01	0·04–0·11
LDL	0·18	0·02	0·13–0·30
HDL	0·14	0·02	0·10–0·25
Serum: α-Tocopherol (μmol/l)	34·8	3·0	21·9–49·4
β-Carotene (μmol/l)	1·00	0·24	0·26–2·91
Retinol (μmol/l)	3·46	0·38	1·74–5·35
Campesterol (μg/l)	4206	516	2477–7112
Sitosterol (μg/l)	2239	262	1239–3917

IDL, intermediate-density lipoprotein.
* For details of procedures, see p. 142.

Table 3. Distribution (%) of α-tocopherol, β-carotene, retinyl palmitate and squalene in chylomicrons, VLDL and VLDL infranatant in the fasting state and 9 h after the test meal in healthy male volunteers*

(Mean values with their standard errors for ten men)

	Fasting		Postprandial	
	Mean	SE	Mean	SE
α-Tocopherol				
Chylomicron	5·4	1·3	6·0	1·0
VLDL	11·6	3·8	10·7	5·2
VLDL infranatant†	83·0	4·4	83·3	5·8
β-Carotene				
Chylomicron	0·0	0·0	0·0	0·0
VLDL	2·0	1·1	4·7	3·2
VLDL infranatant†	98·0	1·1	95·3	3·2
Retinyl palmitate				
Chylomicron	0·0	0·0	18·9	6·0
VLDL	32·5	14·5	27·0	7·5
VLDL infranatant†	67·5	14·5	54·1	11·1
Squalene				
Chylomicron	7·7	1·2	24·3	2·3
VLDL	27·0	2·7	33·4	2·9
VLDL infranatant†	65·3	2·5	42·3	4·2

* For details of procedures, see p. 142.
† Comprised intermediate-density lipoproteins, LDL and HDL.

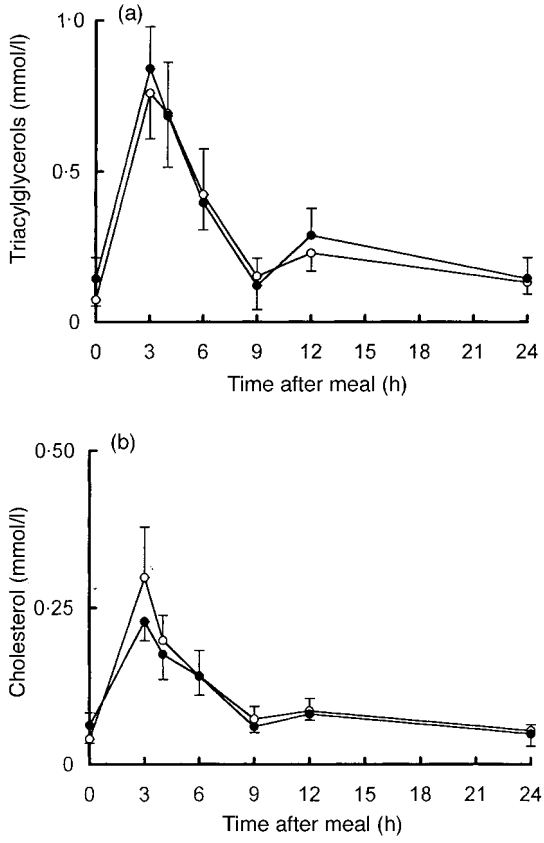

Fig. 1. Postprandial chylomicron concentrations of (a) triacylglycerols and (b) cholesterol in healthy men after consumption of a test meal with (○) and without (●) stanyl ester supplementation. Values are means with their standard errors represented by vertical bars for ten men. For details of procedures, see p. 142.

Table 4. Peak serum concentration times (h) of α-tocopherol, β-carotene, retinyl palmitate, squalene, triacylglycerols and cholesterol after consumption of a test meal with and without stanyl ester supplementation in healthy male volunteers‡

(Mean values with their standard errors for ten men)

	With stanyl ester§		Without stanyl ester	
	Mean	SE	Mean	SE
α-Tocopherol*	15·6	2·4	13·8	2·5
β-Carotene*	22·0	2·0	22·8	1·0
Retinyl palmitate†	4·8	0·4	6·4	1·0
Squalene†	6·7	0·8	7·2	0·9
Triacylglycerols	3·5	0·3	4·1	0·9
Cholesterol	5·8	2·1	4·5	0·9

Mean values were significantly different from the other peak times: *$P < 0·05$.
Mean values were significantly different from those for triacylglycerols: †$P < 0·05$.
‡ For details of procedures, see p. 142.
§ Stanyl ester supplementation had no significant effect on peak serum concentration times.

Serum cholesterol precursor : cholesterol were similar and practically unchanged from the baseline in both fat-load tests (data not shown). However, for postprandial serum campesterol : cholesterol there was a significant time × stanyl ester interaction for 0–12 h ($P = 0·01$, ANOVA for repeated measures). In the follow-up comparisons by one-way ANOVA, campesterol : cholesterol was significantly lower at 6 ($P = 0·046$) and 9 ($P = 0·009$) h after the addition of stanyl esters to the test meals (Fig. 3). The decrease in the ratio was due to a net decrease in serum campesterol concentration after the stanyl ester-supplemented meal. Serum sitosterol : cholesterol values were not consistently changed by stanyl esters (data not shown).

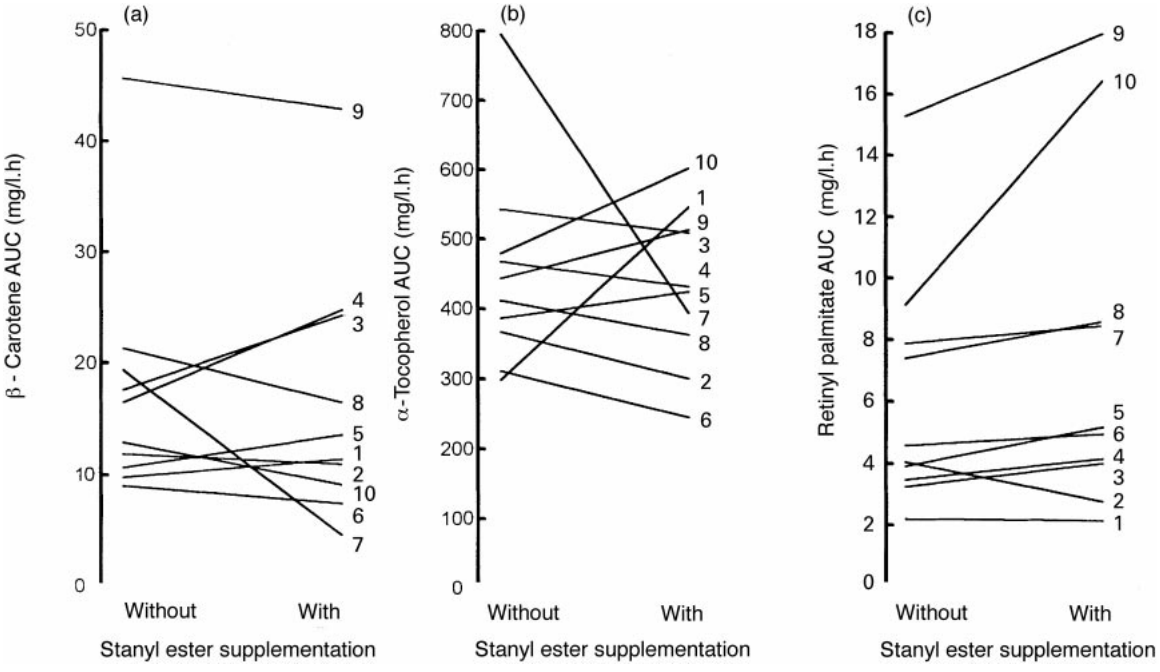

Fig. 2. Effect of stanyl ester supplementation on the area under the curve (AUC) for postprandial β-carotene (a), α-tocopherol (b) and retinyl palmitate (c) concentrations in ten healthy men. (—), AUC values for individual subjects (nos. 1–10) without and with stanyl ester in the test meal. For details of procedures, see p. 142.

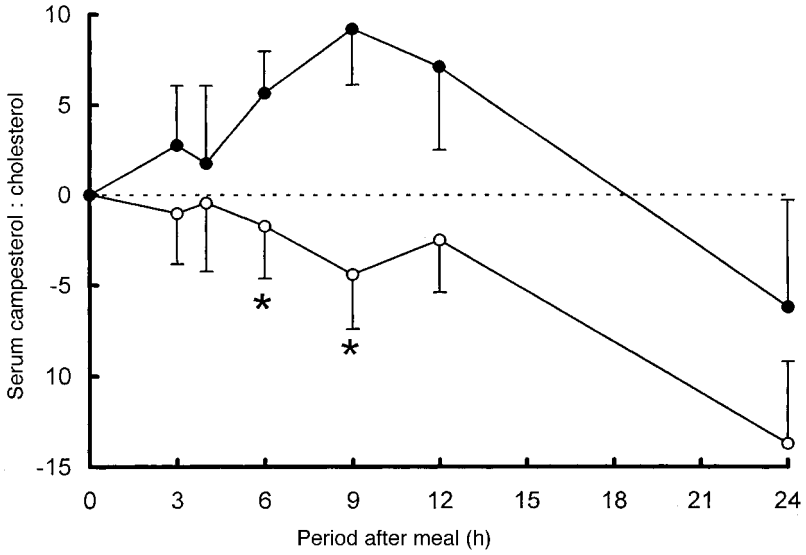

Fig. 3. Postprandial change in serum campesterol : cholesterol in healthy men after consumption of a test meal with (○) and without (●) stanyl ester supplementation. Values are means with their standard errors represented by vertical bars for ten men. Mean values were significantly different from those without stanyl ester supplementation: $^{*}P < 0{\cdot}05$. For details of procedures, see p. 142.

Discussion

Stanyl esters, which are supposed to compete with cholesterol in micelles (Hassan & Rampone, 1980; Ikeda *et al.* 1989), could theoretically interfere also with the absorption of fat-soluble vitamins. However, long-term (12 months) treatment with stanyl ester-containing margarine did not lower serum retinol and vitamin D concentrations or α-tocopherol : cholesterol; only the serum β-carotene ratios were reduced (Gylling *et al.* 1999*a*). However, treatment for 8–20 weeks did not lower β-carotene levels or ratios (Hallikainen & Uusitupa, 1999; Hallikainen *et al.* 2000). The present study showed for the first time that a single dose of 1 g dietary stanyl, consumed as its esters in margarine, did not detectably interfere with the serum or lipoprotein responses of simultaneously-ingested cholesterol, triacylglycerol, α-tocopherol, β-carotene, retinol and retinyl palmitate for 24 h, whereas it lowered campesterol: cholesterol after 6–9 h reflecting reduced sterol absorption efficiency.

Intestinal absorption of α-tocopherol, β-carotene and retinol is variable, ranging from 9 to 75 % (Goodman *et al.* 1966; Meydani, 1995; Biesalski, 1997). In addition, the individual variation in plasma levels following the same oral dose is large (Borel *et al.* 1998). Accordingly, we decided to use variable vitamin and β-carotene doses in order to observe any effects of dosage on changes in absorption. The smallest doses were similar to physiological daily intakes. Since different doses of vitamins and β-carotene were used, the post-absorptive responses could not be compared in aggregate. However, there were no consistent effects of stanyl esters on AUC for α-tocopherol, β-carotene or retinyl palmitate, and no relation to the dosage was observed.

Post-absorptive α-tocopherol concentrations have previously been observed to decline in chylomicrons after 12 h, but concentrations in LDL and HDL remain elevated for much longer, up to 24–48 h after oral administration (Traber *et al.* 1998). Similarly, in the present study, peak α-tocopherol concentrations occurred later than those of retinyl palmitate and triacylglycerols, and were not affected by stanyl esters.

β-carotene is absorbed by different pathways compared with retinol (Cornwell *et al.* 1962; Goodman *et al.* 1966; Goodman & Olson, 1969; Hollander & Ruble, 1978), involving the portal vein system in addition to the lymphatic system (Johnson & Russell, 1992). Serum concentrations rose steadily towards the end of the 24 h study period, which is in concordance with findings of earlier studies (Cornwell *et al.* 1962; Johnson *et al.* 1997). It has been assumed that β-carotene is released from the mucosal cells more slowly than its retinoid metabolites (Hollander & Ruble, 1978), probably due to its lower polarity when compared with the fat-soluble vitamins. The large inter-individual variation in β-carotene responses in the present study can partly explain the lack of dose–response, since the intra-individual responses of ingested β-carotene have been shown to be small (O'Neill & Thurnham, 1998).

Serum retinol concentrations were unchanged after retinol ingestion, a finding observed previously (Rasmussen *et al.* 1991). Retinyl palmitate concentrations rose markedly and similarly in chylomicrons after both test meals, which together with the unaltered triacylglycerol and cholesterol concentrations and AUC suggest that chylomicron formation is not altered by stanyl esters. In addition, postprandial squalene curves, which have previously been shown to reflect postprandial chylomicron remnants (Gylling & Miettinen, 1994), were almost identical to those of retinyl palmitate and were not affected by stanyl esters.

Postprandial cholesterol was not altered by a single dose of stanyl esters. It is apparent that both lowered serum cholesterol (Miettinen *et al.* 1995) and β-carotene concentrations in long-term feeding (Gylling *et al.* 1999*a*) are a result of chronic stanyl ester consumption. The mechanism is related to a diminished intestinal pool of cholesterol and a compensatory alteration in whole-body cholesterol metabolism. Our earlier observation of reduced postprandial VLDL-squalene and VLDL-retinyl palmitate concentrations after 1–2 weeks of stanyl ester consumption is obviously related to long-term stanyl ester feeding, which is associated with enhanced postprandial lipoprotein clearance (Relas *et al.* 2000). Thus, the cholesterol-lowering effect of stanyl esters also seems to be based on mechanisms that take place during regular long-term consumption (Miettinen *et al.* 1995) as well as the potential ability of stanyl esters to interfere with the absorption of biliary cholesterol. On the other hand, squalene, a cholesterol precursor administered with the fat load, might have contributed to intestinal cholesterol synthesis and thus increased cholesterol concentrations in chylomicrons and VLDL, thereby counteracting the effect of stanyl esters on post-absorptive cholesterol values.

Serum plant sterol : cholesterol decreases and cholesterol precursor : sterol increases during long-term stanyl ester feeding (Gylling *et al.* 1999*b*), suggesting that cholesterol absorption efficiency is decreased followed by a compensatory increase in cholesterol synthesis. The present study showed that ingestion of 1 g stanyl esters acutely reduced serum campesterol concentration and campesterol : cholesterol up to 12 h after ingestion. Sterols, including plant sterols campesterol and sitosterol, are in equilibrium with the exchangeable sterol pools in lipoproteins and in tissues, and are ultimately eliminated from the body via bile. Thus, it can be assumed that the drop in concentration of campesterol is caused by inhibited absorption from the intestinal pool of campesterol. Accordingly, the reduced postprandial campesterol ratio after 3–12 h reflected reduced sterol (including cholesterol) absorption efficiency, even though no changes were observed in postprandial cholesterol concentrations. It is possible that no consistent changes in sitosterol were observed as sitosterol is less well absorbed (4–5 %) than campesterol (10 %; Heinemann *et al.* 1993).

In conclusion, stanyl esters which are known to inhibit cholesterol absorption and reduce serum total and LDL-cholesterol levels do not detectably interfere with the absorption of simultaneously-ingested α-tocopherol, β-carotene or retinol during a 24 h follow-up. The mechanism of lowered β-carotene concentrations related to chronic feeding remains to be further investigated.

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