

# Oxidized plant sterols in human serum and lipid infusions as measured by combined gas-liquid chromatography-mass spectrometry

Jogchum Plat,<sup>1,\*</sup> Harald Brzezinka,<sup>†</sup> Dieter Lütjohann,<sup>†</sup> Ronald P. Mensink,<sup>\*</sup> and Klaus von Bergmann<sup>†</sup>

Maastricht University,<sup>\*</sup> Department of Human Biology, P.O. Box 616, 6200 MD, Maastricht, The Netherlands; and University of Bonn,<sup>†</sup> Department of Clinical Pharmacology, Bonn, Germany

**Abstract** Some oxidized forms of cholesterol (oxysterols) are thought to be atherogenic and cytotoxic. Because plant sterols are structurally related to cholesterol, we examined whether oxidized plant sterols (oxyphytosterols) could be identified in human serum and soy-based lipid emulsions. We first prepared both deuterated and nondeuterated reference compounds. We then analyzed by gas-liquid chromatography-mass spectrometry the oxyphytosterol concentrations in serum from patients with phytosterolemia or cerebrotendinous xanthomatosis, in a pool serum and in two lipid emulsions. 7-Ketositosterol, 7 $\beta$ -hydroxysitosterol, 5 $\alpha$ , 6 $\alpha$ -epoxysitosterol, 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -sitostanetriol, and probably also 7 $\alpha$ -hydroxysitosterol were present in markedly elevated concentrations in serum from phytosterolemic patients only. Also, campesterol oxidation products such as 7 $\alpha$ -hydroxycampesterol and 7 $\beta$ -hydroxycampesterol were found. Interestingly, sitosterol was oxidized for approximately 1.4% in phytosterolemic serum, which is rather high compared with the approximate 0.01% oxidatively modified cholesterol normally seen in human serum. The same oxyphytosterols were also found in two lipid emulsions in which the ratio of oxidized sitosterol to sitosterol varied between 0.038 and 0.041. In conclusion, we have shown that oxidized forms of plant sterols are present in serum from phytosterolemic patients and two frequently used soy-based lipid emulsions. Currently, it is unknown whether oxyphytosterols affect health, as has been suggested for oxysterols. However, 7 $\beta$ -hydroxycholesterol may be one of the more harmful oxysterols, and both sitosterol and campesterol were oxidized into 7 $\beta$ -hydroxysitosterol and 7 $\beta$ -hydroxycampesterol. The relevance of these findings therefore deserves further exploration.—Plat, J., H. Brzezinka, D. Lütjohann, R. P. Mensink, and K. von Bergmann. Oxidized plant sterols in human serum and lipid infusions as measured by combined gas-liquid chromatography-mass spectrometry. *J. Lipid Res.* 2001. 42: 2030–2038.

**Supplementary key words** atherosclerosis • phytosterols • oxysterols • phytosterolemia • cerebrotendinous xanthomatosis • soy-based lipid emulsions

Plant sterols are nonnutritive compounds that differ from cholesterol only by an additional ethyl ( $\beta$ -sitosterol)

or methyl (campesterol) group at the 24-carbon atom of the sterol side chain. Western diets provide about 160–360 mg/day of plant sterols, which consist of approximately 80%  $\beta$ -sitosterol, some campesterol and stigmasterol, minor amounts of brassicasterol, and only traces of delta-5 saturated plant stanols (1).

Cholesterol is susceptible to oxidation, and cholesterol oxidation products, called oxysterols, can be formed by physical processes such as heating and radiation, by non-enzymatic processes involving reactive oxygen and free radical species (2), or enzymatically, by specific cytochrome P450 (CYP450) monooxygenases (3). Consequently, cholesterol oxidation products, as present in the human body, may be derived from absorption of oxidized sterols present in the food, as well as from endogenous origin. The nuclear B-ring of the cholesterol molecule is mainly oxidized by nonenzymatic processes. In this way, 7 $\alpha$ -hydroxycholesterol (7 $\alpha$ -OH-Chol), 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH-Chol), 7-ketocholesterol (7=O-Chol), 5 $\alpha$ ,6 $\alpha$ -epoxycholesterol (5 $\alpha$ ,6 $\alpha$ -

Abbreviations: BHT, butylated hydroxy toluene; CTX, cerebrotendinous xanthomatosis; CYP450, cytochrome P450; d-tri-hydroxy-Camp, deuterated tri-hydroxy-Camp; d-tri-hydroxy-Sit, deuterated tri-hydroxy-Sit; d-7=O-Sit, deuterated 7-ketositosterol; 5 $\alpha$ ,6 $\alpha$ -epoxy-Camp, 5 $\alpha$ ,6 $\alpha$ -epoxycampesterol; 5 $\beta$ ,6 $\beta$ -epoxy-Camp, 5 $\beta$ ,6 $\beta$ -epoxycampesterol; 5 $\alpha$ ,6 $\alpha$ -epoxy-Chol, 5 $\alpha$ ,6 $\alpha$ -epoxycholesterol; 5 $\beta$ ,6 $\beta$ -epoxy-Chol, 5 $\beta$ ,6 $\beta$ -epoxycholesterol; 5 $\alpha$ ,6 $\alpha$ -epoxy-Sit, 5 $\alpha$ ,6 $\alpha$ -epoxysitosterol; 5 $\beta$ ,6 $\beta$ -epoxy-Sit, 5 $\beta$ ,6 $\beta$ -epoxysitosterol; GC-MS, gas-liquid chromatography-mass spectrometry; 7=O-Camp, 7-ketocampesterol; 7=O-Chol, 7-ketocholesterol; 7 $\alpha$ -OH-Camp, 7 $\alpha$ -hydroxycampesterol; 7 $\beta$ -OH-Camp, 7 $\beta$ -hydroxycampesterol; 7 $\alpha$ -OH-Chol, 7 $\alpha$ -hydroxycholesterol; 7 $\beta$ -OH-Chol, 7 $\beta$ -hydroxycholesterol; 7 $\alpha$ -OH-Sit, 7 $\alpha$ -hydroxysitosterol; 7 $\beta$ -OH-Sit, 7 $\beta$ -hydroxysitosterol; 7 $\alpha$ -OH-stigmasterol, 7 $\alpha$ -hydroxystigmasterol; 7 $\beta$ -OH-stigmasterol, 7 $\beta$ -hydroxystigmasterol; 7 $\alpha$ -OOH-Sit, 7 $\alpha$ -hydroperoxy sitosterol; 7 $\beta$ -OOH-Sit, 7 $\beta$ -hydroperoxy sitosterol; 7=O-Sit, 7-ketositosterol; oxyphytosterols, oxidized plant sterols; oxysterols, oxidized forms of cholesterol; SIM, single ion monitoring; TMS, trimethylchlorosilane; 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Camp, 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxycampesterol; 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Chol, 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxycholesterol; 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Sit, 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -sitostanetriol.

<sup>1</sup> To whom correspondence should be addressed.  
e-mail: J.Plat@HB.UNIMAAS.NL

epoxy-Chol), 5 $\beta$ ,6 $\beta$ -epoxycholesterol (5 $\beta$ ,6 $\beta$ -epoxy-Chol), and 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-cholesterol (3 $\beta$ ,5 $\alpha$ , 6 $\beta$ -tri-hydroxy-Chol) can be formed. The side chain, on the other hand, is mainly oxidized by CYP450-specific enzymes, and various hydroxy derivatives of cholesterol are then formed (24S-OH-Chol and 27-OH-Chol) (4). The difference between the products formed by nonenzymatic and enzymatic oxidation is clearly illustrated by findings that during *in vitro* copper-catalyzed LDL oxidation, mainly oxysterols oxidized at C7 and C5-C6 are identified (5, 6), whereas 24S-OH-Chol and 27-OH-Chol are not found (6).

Because plant sterols have a great structural similarity to cholesterol, analogous oxidation products can be formed from plant sterols (7). The terminology for oxidized plant sterols (oxyphytosterols) is similar to that described for oxysterols. After stereospecific peroxidation of sitosterol to 7 $\alpha$ - and/or 7 $\beta$ -hydroperoxy sitosterol (7 $\alpha$ -OOH-Sit and 7 $\beta$ -OOH-Sit, respectively), reduction of these compounds results in the formation of 7 $\alpha$ - or 7 $\beta$ -hydroxysitosterol (7 $\alpha$ - or 7 $\beta$ -OH-Sit; Fig. 1), while dehydration of the hydroperoxide leads to the formation of 7-ketositosterol (7=O-Sit; Fig. 1). Further, 5 $\alpha$ ,6 $\alpha$ -epoxysitosterol (5 $\alpha$ ,6 $\alpha$ -epoxy-Sit) and 5 $\beta$ ,6 $\beta$ -epoxysitosterol (5 $\beta$ ,6 $\beta$ -epoxy-Sit) are formed by epoxidation of the double bond between the C5 and C6 atoms of sitosterol. Epoxysterols at C5-C6 are rapidly converted into their triol end products such as 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -sitostanetriol (3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Sit; Fig. 1). Comparable products can be formed from campesterol and other plant sterols. Plant sterols may be oxidized even more easily than cholesterol (8). However, only little information is available on the presence of oxyphytosterols in food, but small amounts have been found in coffee, fried potatoes, wheat flour, and vegetable oils (9, 10). Recently, it has been shown in rats that oxyphytosterols [7=O-Sit, 7-ketocampesterol (7=O-Camp), 5 $\alpha$ ,6 $\alpha$ -epoxy-Sit, 5 $\beta$ ,6 $\beta$ -epoxy-Sit, 5 $\alpha$ ,6 $\alpha$ -epoxycampesterol (5 $\alpha$ ,6 $\alpha$ -epoxy-Camp), and

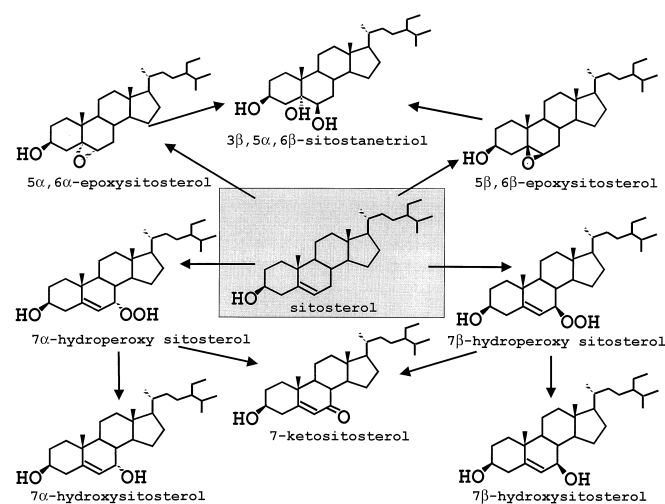
5 $\beta$ ,6 $\beta$ -epoxycampesterol (5 $\beta$ ,6 $\beta$ -epoxy-Camp)] are absorbed from the diet and incorporated into mesenteric lymph (11). Information on endogenous formation of oxyphytosterols, either enzymatically or nonenzymatically, and information on the presence of oxyphytosterols in the human circulation is scanty (12). Expanding our knowledge on the presence of oxyphytosterols in the circulation is of great importance, in particular because oxysterols, in general, may be atherogenic. Further, it was recently found that oxysterols and oxyphytosterols showed similar cytotoxic effects in cultured macrophages (13). In this way, oxidation of plant sterols and the presence of these oxyphytosterols in the circulation might have implications for health.

Phytosterolemia is a rare inherited sterol storage disease characterized by highly elevated serum plant sterol concentrations of up to 65 mg/dl (1.57 mM) (14), tendon and tuberous xanthomas, and by a strong predisposition to premature coronary atherosclerosis (15). Therefore, the primary aim of this study was to examine whether and what kinds of oxyphytosterols are present at all in serum of phytosterolemic patients. In addition, we analyzed serum from patients suffering from cerebrotendinous xanthomatosis (CTX). Patients suffering from CTX, a rare autosomal recessive inherited disease, have decreased concentrations of 27-OH-Chol caused by a genetic absence or restriction of the CYP450-dependent 27-hydroxylase. As a consequence, these patients have a reduced formation of normal C24 bile acids. Moreover, there is a negative feedback from bile acids on cholesterol 7 $\alpha$ -hydroxylase (CYP7A). Consequently, CTX patients have extremely high concentrations of 7 $\alpha$ -hydroxylated bile acid precursors because they lack the feedback on cholesterol 7 $\alpha$ -hydroxylase due to the absence of bile acids (15). It is therefore interesting to examine whether an increased activity of cholesterol 7 $\alpha$ -hydroxylase will result in the formation of endogenous 7 $\alpha$ -OH-Sit. We therefore hypothesized that oxyphytosterols, when formed in relevant concentrations and present in the circulation, should be found in serum of phytosterolemic and CTX patients. In addition, two different soybean oil-based lipid emulsions known to be rich in plant sterols and frequently used as parenteral infusion for short- and long-term nutritive therapy were analyzed for the presence of oxyphytosterols. To address these issues, we first developed a sensitive gas-liquid chromatography-mass spectrometry (GC-MS) method and synthesized all necessary reference compounds and deuterated internal standards.

## METHODS

### Materials

All reagents and solvents used were of analytical or HPLC grade. We analyzed by GC-MS a plant sterol mixture purchased from Sigma-Aldrich (Steinheim, Germany) that consisted of 60.19% sitosterol, 36.09% campesterol, and traces (2.72%) of stigmasterol.



**Fig. 1.** Possible *in vivo* oxidation of plant sterols, either by enzymatic or nonenzymatic mechanisms. In addition to the structures depicted for oxidation of sitosterol, similar products can be formed from campesterol and other plant sterols.

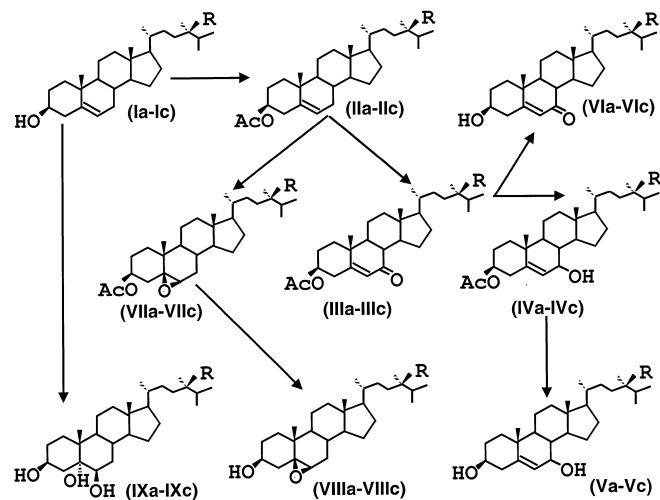
## Chemical synthesis of oxyphytosterols

Chemical synthesis of oxyphytosterols was based on the methods as described for oxygenation of cholesterol by Li et al. (16), with some minor modifications. Several oxygenated plant sterols were synthesized from the plant sterol mixture. In this way, a mixture of 7 $\alpha$ - and 7 $\beta$ -OH-Sit, 7 $\alpha$ - and 7 $\beta$ -hydroxycampesterol (7 $\alpha$ - and 7 $\beta$ -OH-Camp, respectively), and 7 $\alpha$ - and 7 $\beta$ -hydroxystigmasterol (7 $\alpha$ - and 7 $\beta$ -OH-stigmasterol, respectively), a mixture of 7=O-Sit/-Camp/-stigmasterol, a mixture of 5 $\alpha$ ,6 $\alpha$ -epoxy and 5 $\beta$ ,6 $\beta$ -epoxy-Sit/-Camp/-stigmasterol, and a mixture of 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Sit, 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-campesterol (3 $\beta$ , 5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Camp), and 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-stigmasterol were prepared (Fig. 2). We did not separate sitosterol oxidation products from campesterol oxidation products, as they are always present as a mixture in the circulation and can be sufficiently separated by GC-MS. Tentative identification of the synthesized oxyphytosterols and interpretation of the mass spectra was performed by comparison of the spectra with a standard oxyphytosterol mixture, which was kindly provided by Dr. André Grandgirard (INRA, Unité de Nutrition Lipidique, Dijon, France).

**Acetylation of plant sterols.** All plant sterols were acetylated prior to further use for synthesis of all oxyphytosterols, except for the triols. To this end, 2 g of the plant sterol mixture (Fig. 2, Ia-Ic) was acetylated in toluene after addition of 20 ml acetic anhydride (Fluka Chemika, Buchs, Switzerland), which finally resulted in the acetylated products (Fig. 2, IIa-IIc) as white crystals.

**7-Hydroxyphytosterols.** Two hundred milligram molecular sieve 0.4 nm (Merck, Darmstadt, Germany) and 3.2 g pyridinium chlorochromate (Sigma-Aldrich) were added to a solution of 340 mg acetylated plant sterols (Fig. 2, IIa-IIc) in 200 ml benzene and heated under reflux. The white crystals that formed (Fig. 2, IIIa-IIIc) were used for further synthesis of the two isomeric 7-hydroxyphytosterols.

One gram of lithium aluminium hydride (Sigma-Aldrich) was added to a solution of 100 mg of IIIa-IIIc crystals (Fig. 2) dissolved in 100 ml di-ethyl ether, and was stirred overnight under



**Fig. 2.** In vitro synthesis of oxyphytosterols. Oxyphytosterols were synthesized from a mixture consisting of sitosterol, campesterol, and traces of stigmasterol (sitosterol:campesterol 60:40) as described in the Methods section. Therefore, all oxidized plant sterol standards contained isoforms from sitosterol, campesterol, and stigmasterol, all approximately in the same percentages as the plant sterol distribution of the original plant sterol mixture. Campesterol (a): R = -CH<sub>3</sub>; sitosterol (b): R = -C<sub>2</sub>H<sub>5</sub>; stigmasterol (c): R = -C<sub>2</sub>H<sub>5</sub> (plus a double bond between C22 and C23).

nitrogen at room temperature. Next, the acetylated compound IVa-IVc (Fig. 2) was saponified to result in the formation of white to light-yellow crystals (Fig. 2, Va-Vc). The purity of the synthesized 7-hydroxy plant sterols was checked by TLC. TLC showed that in addition to hydroxy sterols, nonhydroxylated plant sterols were present. Therefore, the 7-hydroxylated plant sterols were purified by using a silica column (25 cm, diameter 2 cm). The fractions containing pure 7-hydroxy plant sterols (a mixture of alpha and beta isomers) were combined and crystallized in n-hexane. The GC-MS spectra showed a pure mixture of 7 $\alpha$ -OH-Sit, 7 $\alpha$ -OH-Camp, 7 $\beta$ -OH-Sit, and 7 $\beta$ -OH-Camp, and traces of 7-OH-stigmasterol. The ratio of 7 $\alpha$ -OH-Sit to 7 $\alpha$ -OH-Camp was 69:31, whereas the ratio of 7 $\beta$ -OH-Sit to 7 $\beta$ -OH-Camp was 70:30. This indicates that both sitosterol and campesterol from the initial plant sterol mixture were equally converted into 7-hydroxy plant sterols. Retention times and *m/z* used for analysis of 7-hydroxy sterols are shown in Table 1.

**7-Keto plant sterols.** 7-Keto plant sterols were formed from IIIa-IIIc (Fig. 2) via saponification, and resulted in the formation of white crystals (Fig. 2, VIa-VIc). The synthesized 7-keto plant sterols were also contaminated with free plant sterols, which were removed similarly as described for the hydroxy plant sterols. The purified 7-keto plant sterols were crystallized in n-hexane. GC-MS spectra showed a pure mixture of 7=O-Sit and 7=O-Camp. The ratio of 7=O-Sit to 7=O-Camp was 67:33. Retention times and *m/z* used for analysis of 7-keto sterols are shown in Table 1.

**TABLE 1.** Retention times and *m/z* values for the detection of plant sterols, oxyphytosterols, cholesterol, and oxysterols as TMS derivatives

Plant Sterols	RT	<i>m/z</i>	M <sup>+</sup>	B <sup>+</sup>
7=O-Sit	25.66	500	x	
7=O-Camp	24.12	486	x	
7 $\alpha$ -OH-Sit	19.80	484		x
7 $\beta$ -OH-Sit	21.51	484		x
7 $\alpha$ -OH-Camp	18.92	470		x
7 $\beta$ -OH-Camp	20.55	470		x
5 $\beta$ ,6 $\beta$ -epoxy-Sit	22.63	412		x
5 $\alpha$ ,6 $\alpha$ -epoxy-Sit	22.97	412		x
5 $\beta$ ,6 $\beta$ -epoxy-Camp	21.47	398		x
5 $\alpha$ ,6 $\alpha$ -epoxy-Camp	21.80	398		x
3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Sit	23.81	484		x
3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Camp	22.52	470		x
2,2,4,4,6-d <sub>5</sub> -3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Sit	23.72	488		x
2,2,4,4,6-d <sub>5</sub> -3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Camp	22.42	474	x	
2,2,4,4,6-d <sub>5</sub> -7=O-Sit	25.55	504	x	
2,2,4,4,6-d <sub>5</sub> -7=O-Camp	24.09	490	x	
Sitosterol	20.85	486	x	
Campesterol	19.89	472	x	
Brassicasterol	19.11	470	x	
Stigmasterol	20.23	484	x	
Cholesterol	18.74	458	x	
26,26,26,27,27-d <sub>6</sub> -Chol	18.63	464	x	
26,26,26,27,27-d <sub>6</sub> -7=O-Chol	22.18	478	x	
26,26,26,27,27-d <sub>6</sub> -7 $\alpha$ -OH-Chol	17.81	462		x
26,26,26,27,27-d <sub>6</sub> -7 $\beta$ -OH-Chol	19.29	462		x
26,26,26,27,27-d <sub>6</sub> -3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Chol	21.01	462		x
26,26,26,27,27-d <sub>6</sub> -5 $\beta$ ,6 $\beta$ -epoxy-Chol	20.11	390		x
26,26,26,27,27-d <sub>6</sub> -5 $\alpha$ ,6 $\alpha$ -epoxy-Chol	20.43	390		x
7=O-Chol	22.44	472		x
7 $\alpha$ -OH-Chol	17.90	456		x
7 $\beta$ -OH-Chol	19.38	456		x
3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Chol	21.07	456		x
5 $\beta$ ,6 $\beta$ -epoxy-Chol	20.15	384		x
5 $\alpha$ ,6 $\alpha$ -epoxy-Chol	20.45	384		x

RT, retention time; M<sup>+</sup>, molecular ion; B<sup>+</sup>, base peak.

**5,6-Epoxy plant sterols.** Acetylated phytosterols (Fig. 2, IIa-IIc) were dissolved in chloroform, and by adding *m*-chloroperoxybenzoic acid (Sigma-Aldrich) and potassium bicarbonate, white crystals (Fig. 2, VIIa-VIIc) were formed, which were saponified as described for the hydroxy plant sterols (Fig. 2, VIIIa-VIIIc). GC-MS spectra showed a pure mixture of 5 $\alpha$ ,6 $\alpha$ -epoxy-Sit, 5 $\alpha$ ,6 $\alpha$ -epoxy-Camp, 5 $\beta$ ,6 $\beta$ -epoxy-Sit, and 5 $\beta$ ,6 $\beta$ -epoxy-Camp, and traces of 5 $\beta$ ,6 $\beta$ -epoxy-stigmasterol. The ratio of 5 $\alpha$ ,6 $\alpha$ -epoxy-Sit to 5 $\alpha$ ,6 $\alpha$ -epoxy-Camp was 63:37, whereas the ratio of 5 $\beta$ ,6 $\beta$ -epoxy-Sit to 5 $\beta$ ,6 $\beta$ -epoxy-Camp was 59:41. Mainly the 5 $\alpha$ ,6 $\alpha$ -epoxy forms were formed during synthesis, as the ratio of 5 $\alpha$ ,6 $\alpha$ -epoxy-Sit to 5 $\beta$ ,6 $\beta$ -epoxy-Sit was 79:21 and the ratio of 5 $\alpha$ ,6 $\alpha$ -epoxy-Camp to 5 $\beta$ ,6 $\beta$ -epoxy-Camp was 76:24. Retention times and *m/z* used for analysis of epoxy sterols are shown in Table 1.

**3 $\alpha$ ,5 $\beta$ ,6 $\alpha$ -triols.** For plant sterols (Fig. 2; Ia-Ic) dissolved in formic acid (95%) and worked up to the triols (Fig. 2, IXa-IXc), GC-MS spectra showed a pure mixture of 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Sit and 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Camp. The ratio of 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Sit to 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Camp was 67:33. Retention times and *m/z* used for analysis of tri-hydroxy sterols are shown in Table 1.

**Internal standard preparation [2,2,4,4,6-<sup>2</sup>H<sub>5</sub>]-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-phytosterols and [2,2,4,4,6-<sup>2</sup>H<sub>5</sub>]-7-keto-phytosterols.** As internal standards, we synthesized deuterated tri-hydroxy-phytosterols and deuterated 7-keto-phytosterols from a mixture of d<sub>5</sub>-2,2,4,4,6-sitosterol/-campesterol/-stigmasterol (Medical Isotopes Inc., Pelham, USA) by similar procedures as described for the unlabeled plant sterols. Identification of the synthesized triols revealed that both deuterated tri-hydroxy-Sit (d-tri-hydroxy-Sit) and deuterated tri-hydroxy-Camp (d-tri-hydroxy-Camp) were formed in a ratio 66:34, as well as were traces of stigmasterol. Moreover, analyzing the isotope differentiation (corrected for the natural background of <sup>13</sup>C) showed the deuterium distribution as d<sub>0</sub>-tri-hydroxy-Sit, 8.9%; d<sub>1</sub>-tri-hydroxy-Sit, 3.9%; d<sub>2</sub>-tri-hydroxy-Sit, 9.2%; d<sub>3</sub>-tri-hydroxy-Sit, 21.2%; d<sub>4</sub>-tri-hydroxy-Sit, 29.8%; d<sub>5</sub>-tri-hydroxy-Sit, 22.4%; d<sub>6</sub>-tri-hydroxy-Sit, 2.7%; and d<sub>7</sub>-tri-hydroxy-Sit, 1.9%. Because d<sub>4</sub>-tri-hydroxy-Sit was the most abundant isotope, we measured on *m/z* 488 for d<sub>4</sub>-tri-hydroxy-Sit as internal standard.

The deuterated 7-keto standards were formed in a 60:40 distribution, with some minor traces of stigmasterol as well. Analyzing the isotope differentiation (corrected for the natural background of <sup>13</sup>C) showed the deuterium distribution as d<sub>0</sub>-7=O-Sit, 6.5%; d<sub>1</sub>-7=O-Sit, 4.2%; d<sub>2</sub>-7=O-Sit, 7.3%; d<sub>3</sub>-7=O-Sit, 20.3%; d<sub>4</sub>-7=O-Sit, 31.9%; d<sub>5</sub>-7=O-Sit, 22.4%; d<sub>6</sub>-7=O-Sit, 5.7%; and d<sub>7</sub>-7=O-Sit, 1.8%. Because d<sub>4</sub>-7=O-Sit was the most abundant isotope, we measured on *m/z* 504 for d<sub>4</sub>-7=O-Sit as internal standard. The deuterated 7-keto standards were used as internal standard for the calculation of 7=O, 7-OH, and 5,6-epoxy plant sterols, and the deuterated tri-hydroxy standards were used to calculate tri-hydroxy sterols. Retention times and *m/z* used for analysis of both deuterated sterols are shown in Table 1.

### Oxyphytosterol analysis in serum

Butylated hydroxy toluene (BHT) and EDTA were added to the blood collection tube prior to sampling and to the serum just before analysis. Final amounts added corresponded with 10  $\mu$ l BHT (25 mg/ml in methanol) and 20  $\mu$ l EDTA (10 mg/ml in methanol) for 1 ml serum. Before saponification and extraction, 6  $\mu$ g each of each internal standard, [2,2,4,4,6-<sup>2</sup>H<sub>5</sub>]-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-phytosterol and [2,2,4,4,6-<sup>2</sup>H<sub>5</sub>]-7-keto-phytosterol, were added to the serum sample. Next, the sample was saponified in a closed tube under nitrogen for 2 h at room temperature by adding 10 ml of 0.35 M ethanolic KOH solution. In addition, prior to saponification, the samples were extensively saturated

with nitrogen for removal of oxygen to minimize autoxidation. After saponification, 130  $\mu$ l phosphoric acid 50% in water (v/v) was added to neutralize the solution, followed by addition of 6 ml NaCl solution in water (9 mg/ml), according to procedures for oxysterol analysis as described previously (17). After extraction with 20 ml di-chloro-methane, the bottom layer was transferred into a round bottom flask and evaporated to dryness under vacuum at 50°C. The residue was dissolved in 5 ml ethanol and again evaporated under vacuum to remove all traces of water. The nonsaponifiable serum lipids were dissolved in 1 ml toluene. Silica cartridges (Bond Elut, bonded phase SI, 100 mg, 1 ml; Varian, Harbor City, CA) were equilibrated with cyclohexane before the toluene fractions were loaded. Neutral sterols (including cholesterol) were eluted from the column with 4 ml 0.5% ethyl-acetate-cyclohexane (v/v), whereas the absorbed oxysterols were eluted with 9 ml ethyl-acetate. The oxysterol fraction was dried under vacuum. Finally, the oxidized sterols were dissolved in 100  $\mu$ l cyclohexane and transferred to an injection vial. Sterols were silylated by addition of 100  $\mu$ l silylation reagent (dry pyridine-hexamethyl-disilazane-trimethylchlorosilane (TMS) 3:2:1 (v/v/v) and incubation for 1 h at 90°C for GC-MS analysis.

### GC-MS analysis

TMS derivatives of the oxyphytosterols were analyzed by GC-MS. For this, 2  $\mu$ l of the TMS derivatives in cyclohexane were injected via an AS2000 autosampler (Thermoquest CE Instruments, Egelsbach, Germany) on a Trace GC2000 (Thermoquest CE Instruments) gas chromatograph equipped with a RTX5MS column (30 m  $\times$  0.25 mm internal diameter, 0.25  $\mu$ m film thickness) coupled to a GCQ-plus ion trap (Thermoquest CE Instruments). Analysis was carried out in single ion monitoring (SIM) mode, making *m/z* the primary resolving parameter other than retention time. The injector temperature was set at 280°C. Helium was used as carrier gas (constant flow 1 ml/min). The oven temperature gradient was programmed for 150 s at 150°C, then increased by 10°C/min toward 290°C, and then increased by 7°C/min toward 320°C and kept there for 20 min. Thus, one analytical run lasted approximately 42 min. The ions and retention times of all individual compounds are given in Table 1. In addition to plant sterols and oxyphytosterols, we also indicated retention times and *m/z* of cholesterol oxidation products (and also of d<sub>6</sub>-26,26,27,27-cholesterol oxidation products), which illustrates that interference of oxysterols and oxyphytosterols in GC-MS identification is not a problem.

### Patients and lipid emulsions

Serum sampled at three different time points from a phyto-sterolemic patient was used for analysis of oxyphytosterols. The patient in 1998 was a 12-year-old girl being treated for her illness with cholestyramine (4 g/day). Two serum samples that were obtained at different time points in 1998 and stored at -20°C with BHT added as well as a recent fresh blood sample from the year 2000 obtained on the day of analysis were used. Furthermore, serum from two CTX patients not treated at the moment of blood sampling was used: The blood samples were taken in 1998 and were stored at -20°C with BHT since that time. In addition, serum from 15 patients not suffering from any sterol metabolism-related disease was pooled and analyzed.

Two frequently used soybean oil-based lipid emulsions in total parenteral nutrition protocols were analyzed for oxyphytosterols by the same procedures as described for serum samples.

### 7 $\alpha$ -OH-Chol and plant sterol analysis

7 $\alpha$ -OH-Chol (18) and plant sterol concentrations (19) were analyzed as described before.

TABLE 2. Serum plant sterol and oxidized sitosterol concentrations in a phytosterolemic patient treated with cholestyramine (4 g/day)

	1998	1998	2000
Plant sterols (mg/dl) <sup>a</sup>	47.3	44.5	40.3
Oxidized sitosterol forms (μg/ml)			
7=O-Sit	0.82	0.99	0.94
7β-OH-Sit	0.82	0.93	ND
5α,6α-epoxy-Sit	2.77	2.23	1.90
3β,5α,6β-tri-hydroxy-Sit	0.08	0.15	1.38
Total oxidized sitosterol	4.49	4.30	4.21

Serum from a phytosterolemic patient at three different time points was used for analysis of oxidized plant sterols. Two serum samples were obtained at different time points in 1998, stored at -20°C with BHT added, and analyzed together with a fresh blood sample from the year 2000, obtained on the day of analysis. ND = not detectable.

<sup>a</sup> Plant sterols are calculated as the sum of sitosterol and campesterol.

## RESULTS

### Plant sterols and 7α-OH-Chol

Serum plant sterol concentrations from the phytosterolemic patient contained large amounts of plant sterols at all time points (Table 2). Serum plant sterol concentrations (sitosterol plus campesterol) from the phytosterolemic patient were 47.3, 44.5, and 40.3 mg/dl compared with 1.3 mg/dl in the pool serum of nonphytosterolemic patients (Table 3). Because esterified plant sterols are transported in the circulation in lipoproteins, concentrations are usually expressed relative to those of cholesterol. However, because serum cholesterol concentrations were not increased to the same extent as plant sterol concentrations, cholesterol-standardized sitosterol and campesterol concentrations were also still extremely high. Sitosterol concentrations were  $19,714 \times 10^2$ ,  $18,007 \times 10^2$ , and  $16,714 \times 10^2$  μmol/mmol cholesterol, whereas campesterol concentrations were  $9,200 \times 10^2$ ,  $8,289 \times 10^2$ , and  $8,909 \times 10^2$  μmol/mmol cholesterol. Compared with concentrations from the pool serum of nonphytosterolemic patients, cholesterol-standardized plant sterol concentrations were approximately 60 to 70 times increased.

Serum concentrations of 7α-OH-Chol were approxi-

mately 83.3 ng/ml in the phytosterolemic patient receiving 4 g/day of cholestyramine. This concentration was comparable to the value of 76.3 ng/ml, as observed in pool serum (Table 3). As expected, CTX patients had very high concentrations of 7α-OH-Chol, about 10 to 15 times higher than pool- and phytosterolemic serum.

### GC-MS assay for oxyphytosterols

For the identification and quantification of oxyphytosterols in serum, we first developed a GC-MS assay, as described in the Methods section. First, standard curves of all individual compounds were made after preparing a standard mixture with the same procedures as described for the serum samples. For this, crystals of all synthesized standards were dissolved in ethanol in a final concentration of 10 mg/100 ml. Correlation coefficients for the different standards, as measured in a range of 0–4 μg/ml, were at least 0.96. The detection limits, as calculated according DIN32645 procedures, were 0.41 μg/ml for 7=O-Sit, 0.48 μg/ml for 7α-OH-Sit, 0.67 μg/ml for 7β-OH-Sit, 0.54 μg/ml for 5α,6α-epoxy-Sit, 0.83 μg/ml for 5β,6β-epoxy-Sit, and 0.65 μg/ml for 3β,5α,6β-tri-hydroxy-Sit.

### Artificial ex vivo oxidation

As indicated above, we were extremely careful to inhibit artificial oxidation of plant sterols during work-up. For example, BHT was added in excess both during serum sampling and again before work-up procedures. Further, EDTA was also added before work-up, and fluids were saturated with nitrogen to remove oxygen before being used. Finally, oxysterols were separated from nonoxidized sterols as quickly as possible. To examine the possibility of ex vivo oxidation during sample preparation, we worked up four different amounts of the plant sterol mixture corresponding to the range of plant sterol concentrations as can be present in phytosterolemic serum (10–40 mg/dl), according the same procedures as described for the serum samples. As shown in Fig. 3, a small amount of 7=O-Sit, 7α-OH-Sit, 5α,6α-epoxy-Sit, and 3β,5α,6β-tri-hydroxy-Sit was present in all samples. These amounts, however, were independent of the initial sitosterol concentration. For 7=O-Sit and 3β,5α,6β-tri-hydroxy-Sit, these amounts were derived from d<sub>0</sub>-7=O-Sit and d<sub>0</sub>-3β,5α,6β-tri-hydroxy-Sit as present in the deuterated internal standards. This finding means that the formation of these specific oxyphytosterols in our assay is independent of the sitosterol concentration of the sample. We therefore corrected the serum samples of the patients and the lipid emulsions by subtracting the base amounts of these oxyphytosterols. As shown in Fig. 3, the amount of 7β-OH-Sit increased linearly in relation to the sitosterol concentration of the sample. The correction for ex vivo oxidation for 7β-OH-Sit was therefore based on the sitosterol concentration present in the serum samples of the patients. For campesterol, 7α-OH-Camp and 7β-OH-Camp formation were also related to the campesterol concentration in the sample.

### Oxyphytosterols

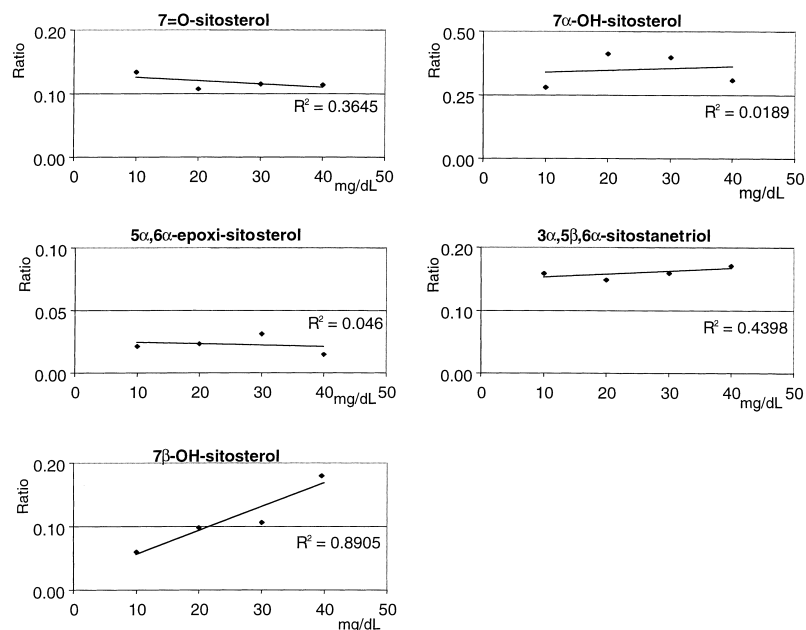
Serum from the phytosterolemic patient contained significant amounts of oxyphytosterols at all time points (Table

TABLE 3. Serum plant sterol and oxidized sitosterol concentrations in three samples of a phytosterolemic patient versus two CTX patients and pool serum

	Control Serum	Phytosterolemia	CTX
Plant sterols (mg/dl) <sup>a</sup>	1.3	44.0 ± 3.5	1.2 and 1.4
Oxidized sitosterol forms (μg/ml)			
7=O-Sit	ND	0.92 ± 0.09	0.04 and ND
7β-OH-Sit	ND	0.88 ± 0.08	0.01 and ND
5α,6α-epoxy-Sit	ND	2.33 ± 0.44	ND and ND
3β,5α,6β-tri-hydroxy-Sit	ND	0.54 ± 0.73	ND and ND
7α-OH-Chol (ng/ml)	76.3	83.3 ± 36.9	1,221 and 671

Values are mean ± SD. ND = not detectable.

<sup>a</sup> Plant sterols are calculated as the sum of sitosterol and campesterol.



**Fig. 3.** To determine ex vivo oxidation and to create correction factors for our further calculations, we worked up four different amounts of a plant sterol mixture. A constant amount of 7=O-Sit, 7 $\alpha$ -OH-Sit, 5 $\alpha$ ,6 $\alpha$ -epoxy-Sit, and 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Sit was present in all samples, which was independent of the sitosterol concentration. This amount was subtracted from the values found in all samples. The 7 $\beta$ -OH-Sit concentration, however, increased in relation to the sitosterol concentration. Therefore, the correction for ex vivo oxidation for 7 $\beta$ -OH-Sit was based on the individual sitosterol concentration present in the serum samples of the patients. The concentration of sitosterol in the sample (mg/dl) on the *x*-axis is plotted versus the ratio of the peak area oxyphytosterol/peak area internal standard (*y*-axis).

2). 7 $\alpha$ -OH-Sit was the most abundant oxidized plant sterol in serum, followed by 5 $\alpha$ ,6 $\alpha$ -epoxy-Sit, 7=O-Sit, 7 $\beta$ -OH-Sit, and 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Sit. 5 $\beta$ ,6 $\beta$ -Epoxy-Sit could not be detected. Concentrations of 7 $\alpha$ -OH-Sit in all phytosterolemic serum samples and in both lipid emulsions were rather high, as we cannot exclude the possibility of autoxidation of 7 $\alpha$ -OH-Sit. However, based on the corrections for autoxidation as described above, there was no indication of artificial oxidation of sitosterol during work-up of our samples. Further, most of the nonoxyphytosterols were separated from oxidized sterols during elution on the Bond Elut silica cartridges, making oxidation of plant sterols after this separation impossible. However, because we are not entirely sure that the analyzed 7 $\alpha$ -OH-Sit concentration is a real reflection of the in vivo situation, we decided not to use 7 $\alpha$ -OH-Sit in the calculations. The percentage of oxidized sitosterol in serum of phytosterolemic patients was around 1.4%. If at least a part of the 7 $\alpha$ -OH-Sit signal is from nonartificial 7 $\alpha$ -OH-Sit formation, then this figure is even an underestimation. Also, 7 $\alpha$ -OH-Camp and 7 $\beta$ -OH-Camp could be identified in serum, whereas no 7=O-Camp, epoxy-Camp, or tri-hydroxy-Camp could be detected. Although a positive identification of oxidized campesterol molecules (7 $\alpha$ -OH-Camp and 7 $\beta$ -OH-Camp) was possible, exact concentrations could not be calculated because of difficulties in analyzing the campesterol internal standards. If we used the sitosterol internal standards for these calculations, it appeared that the percentage of oxidized campesterol in serum of phytosterolemic patients was similar to sitosterol.

To examine whether 7 $\alpha$ -OH-Sit could be formed endogenously, we analyzed serum from CTX patients. These CTX patients are characterized by a very high 7 $\alpha$ -hydroxylase activity, which may also lead to the endogenous formation of 7 $\alpha$ -OH-Sit. In these two CTX patients, serum plant sterol concentrations were 1.2 and 1.4 mg/dl, which was comparable to concentrations in pool serum (Table 3).

As expected, 7 $\alpha$ -OH-Chol concentrations were increased. We could not, however, identify 7 $\alpha$ -OH-Sit in serum from CTX patients. In fact, none of the oxyphytosterols was present in CTX serum in amounts above our detection limits. This might indicate that no enzymatic formation of 7 $\alpha$ -OH-Sit occurs in vivo in humans, even in a situation of high 7 $\alpha$ -hydroxylase activity. However, it is also possible that serum-oxidized plant sterol concentrations in CTX patients are below our detection limits.

### Lipid emulsions

Analysis of two different frequently used soybean oil-based lipid emulsions showed that emulsion A contained less plant sterols compared with emulsion B and also contained more cholesterol than emulsion B (Table 4). Oxidized plant sterol concentrations were somewhat higher in emulsion B. The ratios of oxidized sitosterol to sitosterol, however, were comparable: 0.038 in emulsion A; 0.041 in emulsion B. This suggests that the higher oxyphytosterol concentrations in emulsion B were simply due to

TABLE 4. Serum plant sterol and oxidized sitosterol concentrations in lipid emulsions

	Emulsion A	Emulsion B
Cholesterol (mg/dl)	29.8	17.8
Plant sterols (mg/dl) <sup>a</sup>	11.3	27.7
Sitosterol (mg/dl)	8.6	20.1
Campesterol (mg/dl)	2.7	7.6
Oxidized sitosterol forms ( $\mu$ g/ml)		
7=O-Sit	0.70	1.26
7 $\beta$ -OH-Sit	0.65	0.37
5 $\alpha$ ,6 $\alpha$ -epoxy-Sit	2.73	9.24
3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Sit	0.16	0.49
7 $\alpha$ -OH-Chol (ng/ml)	19.5	9.7

<sup>a</sup> Plant sterols are calculated as the sum of sitosterol and campesterol.

higher plant sterol concentrations. 7 $\alpha$ -OH-Chol concentrations in emulsion A were twice as high as those in emulsion B.

## DISCUSSION

Feeding LDL receptor (-/-) and apolipoprotein E (-/-) mice oxysterols (a mixture of 7–10% 7 $\alpha$ -OH-Chol, 15–20% 7 $\beta$ -OH-Chol, 15–20% 5 $\beta$ ,6 $\beta$ -epoxy-Chol, 10–15% 5 $\alpha$ ,6 $\alpha$ -epoxy-Chol, 40–45% 7=O-Chol, and traces of 25-OH-Chol) accelerated fatty streak lesion formation when compared with cholesterol feeding (20). This confirms earlier ideas that oxysterols are atherogenic and may play a role in plaque development in humans (4). Most information about their possible atherogenicity, however, is derived from *in vitro* studies showing that oxysterols are cytotoxic to several cell types (21) including endothelial cells (22, 23), U937 monocytes (24), and HepG2 liver cells (24), and may induce apoptosis (24, 25, 26). For humans, however, no causal relation so far has been established between oxysterols and lesion formation, but several findings are suggestive. For example, the highest oxysterol/cholesterol ratio was found in the most atherogenic LDL subfractions (LDL<sup>-</sup>) (27). Furthermore, the ratio of oxysterol to cholesterol is higher in foam cells isolated from atherosclerotic plaques than in plasma (28). Foam cells play a crucial role in early fatty streak formation. Therefore, expanding our knowledge about the presence of oxysterols in the circulation is of great importance not only because of their potential atherogenicity, but also because oxysterols have several other biological effects such as the regulation of gene expression (28). Whether oxysterols also possess atherogenic and/or gene regulatory characteristics needs further study.

It is very difficult to distinguish between oxysterols derived from the diet and those endogenously formed (29). Breuer and Björkhem (30) showed that 7=O-Chol, 7 $\beta$ -OH-Chol, 24-OH-Chol, and 25-OH-Chol are formed endogenously, in contrast to epoxides. In our study, we have did not try to trace the origin of the oxysterols. From the literature, there was only limited evidence on the presence of oxysterols in the circulation (12). We therefore decided first to set up a sensitive GC-MS assay, which enabled us to accurately analyze many different oxysterols in human serum. We showed that similar oxidation products to those formed from cholesterol were present in serum from phytosterolemic patients. We were able to identify 7=O-Sit, 7 $\beta$ -OH-Sit, 5 $\alpha$ ,6 $\alpha$ -epoxy-Sit, 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -tri-hydroxy-Sit, and most probably 7 $\alpha$ -OH-Sit as well. 7 $\alpha$ -OH-Camp and 7 $\beta$ -OH-Camp, two campesterol oxidation products, were also found. A recent study found that plant sterol oxides and cholesterol oxides showed the same cytotoxic effects in a cultured macrophage-derived cell line (13).

It is generally accepted that 7 $\alpha$ -OH-Chol is derived from both enzymatic and nonenzymatic oxidation, whereas 7 $\beta$ -OH-Chol is entirely derived from nonenzymatic origin (4). Therefore, 7 $\beta$ -OH-Chol may be a better marker for


free radical-related lipid peroxidation than 7 $\alpha$ -OH-Chol. Two separate studies have now shown that in humans, especially 7 $\beta$ -OH-Chol is associated with the risk for atherosclerosis (31, 32). Interestingly, the *in vitro* toxicity of 7 $\beta$ -OH-Chol could not be counteracted by antioxidants (33). Rabbit studies also showed no reduction in serum 7 $\beta$ -OH-Chol concentrations after probucol treatment (34), vitamin E supplementation (35), or vitamin E plus vitamin C supplementation (36). In human diabetic patients, however, vitamin E supplementation lowered both 7 $\beta$ -OH-Chol, 7=O-Chol, and triols (37). However, because antioxidant supplementation may lower the risk for atherosclerosis, these findings indicate that a causal relation between 7 $\beta$ -OH-Chol with coronary heart disease is still controversial. Because 7 $\beta$ -OH-Sit and 7 $\beta$ -OH-Camp are also present in serum from phytosterolemic patients, it seems relevant to determine whether these oxysterols are also atherogenic *in vivo*, and whether antioxidants affect oxysterol concentrations and toxicity.

It has been suggested that sitosterol inhibits cholesterol 7 $\alpha$ -hydroxylase activity (38, 39), possibly by stereochemical hindrance of the ethyl group at C24 in sitosterol. Indeed, patients with phytosterolemia have a reduced cholesterol 7 $\alpha$ -hydroxylase activity (40) and, consequently, a lower bile acid synthesis (19). However, in cholesterol-free microsomes, 7 $\alpha$ -OH-Sit was formed, suggesting that at a relatively high ratio of sitosterol to cholesterol, some conversion of sitosterol may occur (41). Because this ratio is increased in serum from phytosterolemic patients, 7 $\alpha$ -OH-Sit might be formed in phytosterolemic patients. This might also explain why Boberg, Einarsson, and Björkhem (38) could not show any conversion of sitosterol into 7 $\alpha$ -OH-Sit in human liver microsomes isolated from nonphytosterolemic patients. The fact that we were able to identify 7 $\alpha$ -OH-Camp strengthens our assumption that 7 $\alpha$ -OH-Sit may be formed to some extent in phytosterolemic subjects.

Interestingly, approximately 1.4% of the sitosterol molecules in phytosterolemic serum was oxidized, which is rather high compared with the approximate 0.01% oxidatively modified cholesterol (37, 42) normally present in human serum. This could indicate that plant sterols are more susceptible to oxidation, as suggested before (8). Another explanation might be that in contrast to cholesterol, plant sterols cannot be converted into bile acids. In pool serum from nonphytosterolemic patients and in CTX patients, no oxysterols could be detected. This suggests that with our assay, rather high serum concentrations are needed to find oxidized forms of these plant sterols. It should be mentioned that others recently reported oxysterol concentrations of 0.3  $\mu$ g/ml, but no details on the type of analysis and the origin of the serum were provided (12). It therefore seems worthwhile to lower the detection limits of our assay, for example, by using Cl-negative-ion-GC-MS.

Soy-based lipid emulsion used in parenteral nutrition protocols may be associated with a higher prevalence of cholestasis, especially in children (43, 44). Indeed, a 3-year-old patient with total parenteral nutrition-associated cholestasis showed a high serum plant sterol concentra-

tion (45). It therefore is tempting to suggest that the intravenous administration resulted in accumulation of plant sterols (44). We found that two different soybean oil-based lipid emulsions contain not only plant sterols, but that a part of these plant sterols is also present in its oxidized form. It would be interesting to examine the role of oxyphytosterols in the pathology described.

In conclusion, this study showed that oxidized forms of plant sterols are present in serum from phytosterolemic patients and in two frequently used lipid emulsions. However, it is unknown whether oxyphytosterols also affect health, as has been suggested for oxysterols. One of the most atherogenic oxysterols is 7 $\beta$ -OH-Chol, and we found both 7 $\beta$ -OH-Sit and 7 $\beta$ -OH-Camp in serum from phytosterolemic patients. The relevance of these findings needs further investigation. 

We would like to thank Dr. André Grandgirard (INRA, Unité de Nutrition Lipidique, Dijon, France) for kindly providing the standard oxyphytosterol mixture. This research was financially supported by the Netherlands Organization for Scientific Research (NWO; R98-148) and by a research grant from the Bundesministerium fuer Bildung, Forschung Wissenschaft und Technologie (01EC9402).

Manuscript received 26 March 2001 and in revised form 9 August 2001.

## REFERENCES

- Ling, W. H., and P. J. H. Jones. 1995. Minireview dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sciences*. **57**: 195–206.
- Smith, L. L. 1996. Review of progress in sterol oxidations: 1987–1995. *Lipids*. **31**: 453–487.
- Russell, D. W., and K. D. Setchell. 1992. Bile acid biosynthesis. *Biochemistry*. **31**: 4737–4749.
- Brown, A. J., and W. Jessup. 1999. Oxysterols and atherosclerosis. *Atherosclerosis*. **142**: 1–28.
- Chang, Y. H., D. S. P. Abdalla, and A. Sevanian. 1997. Characterization of cholesterol oxidation products formed by oxidative modification of low density lipoprotein. *Free Rad. Biol. Med.* **23**: 202–214.
- Patel, R. P., U. Diczfalusy, S. Dzeletovic, M. T. Wilson, and V. M. Darley-Usmar. 1996. Formation of oxysterols during oxidation of low density lipoprotein by peroxynitrite, myoglobin, and copper. *J. Lipid Res.* **37**: 2361–2371.
- Daly, G. G., E. T. Finocchiaro, and T. Richardson. 1983. Characterization of some oxidation products of sitosterol. *J. Agric. Food Chem.* **31**: 46–50.
- Li, W., and R. Przybylski. 1995. Oxidation products formed from phytosterols. *INFORM*. **6**: 499–500.
- Dutta, P. C., and L. Appelqvist. 1997. Studies on phytosterol oxides I: effect of storage on the content in potato chips prepared in different vegetable oils. *JAOCS*. **74**: 647–657.
- Dutta, P. C. 1997. Studies on phytosterol oxides. II: content in some vegetable oils and in French fries prepared in these oils. *JAOCS*. **74**: 659–666.
- Grandgirard, A., J. P. Sergiel, M. Nour, J. Demaison-Meloche, and C. Ginies. 1999. Lymphatic absorption of phytosterol oxides in rats. *Lipids*. **34**: 563–570.
- Grandgirard, A., L. Bretilon, L. Martine, and B. Beaufre. 1999. Oxyphytosterols in human plasma. *Chem. Phys. Lipids*. **101**: 198.
- Adcox, C., L. Boyd, L. Oehrl, J. Allen, and G. Fenner. 2001. Comparative effects of phytosterol oxides and cholesterol oxides in cultures macrophage-derived cell lines. *J. Agric. Food Chem.* **49**: 2090–2095.
- Salen, G., I. Horak, M. Rothkopf, J. L. Cohen, J. Speck, G. S. Tint, V. Shore, B. Dayal, T. Chen, and S. Shefer. 1985. Lethal atherosclerosis associated with abnormal plasma and tissue sterol composition in sitosterolemia with xanthomatosis. *J. Lipid Res.* **26**: 1126–1133.
- Björkhem, I., and K. M. Boberg. 1994. Inborn errors in bile acid biosynthesis and storage of sterols other than cholesterol. In *The Metabolic Basis of Inherited Diseases*. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw Hill, New York. 2073–2100.
- Li, S., J. Pang, W. K. Wilson, and G. J. Schroeffer. 1999. Sterol synthesis. Preparation and characterization of fluorinated and deuterated analogs of oxygenated derivatives of cholesterol. *Chem. Phys. Lipids*. **99**: 33–71.
- Lütjohann, D., A. Papassotiropoulos, I. Björkhem, S. Locatelli, M. Bagli, R. D. Oehring, U. Schlegel, F. Jessen, M. L. Rao, K. von Bergmann, and R. Heun. 2000. Plasma 24S-hydroxycholesterol (cerebrosterol) is increased in Alzheimer and vascular demented patients. *J. Lipid Res.* **41**: 195–198.
- Hahn, C., C. Reichel, and K. von Bergmann. 1995. Serum concentration of 7 $\alpha$ -hydroxycholesterol as an indicator of bile acid synthesis in humans. *J. Lipid Res.* **36**: 2059–2066.
- Lütjohann, D., I. Björkhem, U. F. Beil, and K von Bergmann. 1995. Sterol absorption and sterol balance in phytosterolemia evaluated by deuterium-labeled sterols: effect of sitostanol treatment. *J. Lipid Res.* **36**: 1763–1773.
- Staprans, I., X. M. Pan, J. H. Rapp, C. Grunfeld, and K. R. Feingold. 2000. Oxidized cholesterol in the diet accelerates the development of atherosclerosis in LDL receptor- and apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol.* **20**: 708–714.
- Smith, L. L., and B. H. Johnson. 1989. Biological activities of oxysterols. *Free Rad. Biol. Med.* **7**: 285–332.
- Zhou, Q., E. Wasowicz, B. Handler, L. Fleischer, and K. A. Kummerow. 2000. An excess concentration of oxysterols in the plasma is cytotoxic to cultures endothelial cells. *Atherosclerosis*. **149**: 191–197.
- Petersen, J. S., K. M. Boberg, A. Stabursvik, and H. Prydz. 1991. Toxicity of oxygenated cholesterol derivatives towards cultured human umbilical vein endothelial cells. *Arterioscler. Thromb.* **11**: 423–428.
- O'Callaghan, Y. C., J. A. Woods, and N. M. O'Brien. 1999. Oxysterol induced cell death in U937 and HepG2 cells at reduced and normal serum concentrations. *Eur. J. Nutr.* **38**: 255–262.
- Nishio, E., and Y. Watanabe. 1996. Oxysterols induced apoptosis in cultured smooth muscle cells through CPP32 protease activation and bcl-2 protein down regulation. *Biochem. Biophys. Res. Com.* **226**: 928–934.
- Harada, K., S. Ishibashi, T. Miyashita, J. Osuga, H. Yagyu, K. Ohashi, Y. Yazaki, and N. Yamada. 1997. Bcl-2 protein inhibits oxysterol-induced apoptosis through suppressing CPP32-mediated pathway. *FEBS Letters*. **411**: 63–66.
- Sevanian, A., G. Bittolo-Bon, G. Cazzolato, H. Hodis, J. Hwang, A. Zamburlini, M. Maiorino, and F. Ursini. 1997. LDL- is a lipid hydroperoxide-enriched circulating protein. *J. Lipid Res.* **38**: 419–428.
- Mattson-Hulten, L., H. Lindmark, U. Diczfalusy, I. Björkhem, M. Ottosson, Y. Liu, G. Bondjers, and O. Wiklund. 1996. Oxysterols present in atherosclerotic tissue decrease the expression of lipoprotein lipase messenger RNA in human monocyte-derived macrophages. *J. Clin. Invest.* **97**: 461–468.
- Sevanian, A., R. Seraglia, P. Traldi, P. Rossato, F. Ursini, and H. Hodis. 1994. Analysis of plasma cholesterol oxidation products using gas and high performance liquid chromatography/mass spectrometry. *Free Rad. Biol. Med.* **17**: 397–409.
- Breuer, O., and I. Björkhem. 1995. Use of an <sup>18</sup>O<sub>2</sub> inhalation technique and isotopomer distribution analysis to study oxygenation of cholesterol in rat: evidence for in vivo formation of 7-oxo-, 7 $\beta$ -hydroxy-, 24-hydroxy- and 25-hydroxycholesterol. *J. Biol. Chem.* **270**: 20278–20284.
- Salonen, J. T., K. Nyyssonen, R. Salonen, E. Porkkala-Sarataho, T. Tuomainen, U. Diczfalusy, and I. Björkhem. 1997. Lipoprotein oxidation and progression of carotid atherosclerosis. *Circulation*. **95**: 840–845.
- Zieden, B., and U. Diczfalusy. 1997. Higher 7 $\beta$ -hydroxycholesterol in high cardiovascular risk population. *Atherosclerosis*. **134**: 172.
- Colles, S. M., K. C. Irwin, and G. M. Chisolm. 1996. Roles of multiple oxidized LDL lipids in cellular injury: dominance of 7 $\beta$ -hydroperoxycholesterol. *J. Lipid Res.* **37**: 2018–2028.
- Stalenhoef, A. F. H., H. A. Kleinveld, T. G. Kosmeijer-Schuil, P. N. M.



- Demacker, and M. B. Katan. 1993. In vivo oxidized cholesterol in atherosclerosis. *Atherosclerosis*. **98**: 113–114.
35. Wiseman, S. A., M. A. P. van den Boom, N. J. de Fouw, M. G. Wasink, J. A. F. op den Kamp, and L. B. M. Tijburg. 1995. Comparison of the effects of dietary vitamin E on in vivo and in vitro parameters of lipid peroxidation in the rabbit. *Free Rad. Biol. Med* **19**: 617–627.
36. Mahfouz, M. M., H. Kawano, and F. A. Kummerow. 1997. Effect of cholesterol-rich diets with and without added vitamins E and C on the severity of atherosclerosis in rabbits. *Am. J. Clin. Nutr.* **66**: 1240–1249.
37. Mol, M. J. T. M., Y. B. de Rijke, P. N. M. Demacker, and A. F. H. Stalenhoef. 1997. Plasma levels of lipid and cholesterol oxidation products and cytokines in diabetes mellitus and cigarette smoking: effects of vitamin E treatment. *Atherosclerosis*. **129**: 169–176.
38. Boberg, K. M., K. Einarsson, and I. Björkhem. 1990. Apparent lack of conversion of sitosterol into C24-bile acids in humans. *J. Lipid Res.* **31**: 1083–1088.
39. Boberg, K. M., J. E. Akerlund, and I. Björkhem. 1989. Effect of sitosterol on rate limiting enzymes in cholesterol synthesis and degradation. *Lipids*. **24**: 9–12.
40. Shefer, S., S. Hauser, V. Lapar, and E. H. Mosbach. 1973. Regulatory effects of sterols and bile acids on hepatic hydroxy-3-methylglutaryl CoA reductase and cholesterol 7 $\alpha$ -hydroxylase in the rat. *J. Lipid Res.* **14**: 573–580.
41. Shefer, S., G. Salen, L. Nguyen, A. K. Batta, V. Packin, G. S. Tint, and S. Hauser. 1988. Competitive inhibition of bile acid synthesis by endogenous cholestanol and sitosterol in sitosterolemia with xanthomatosis; effect on cholesterol 7 $\alpha$ -hydroxylase. *J. Clin. Invest.* **82**: 1833–1839.
42. Murakami, H., N. Tamasawa, J. Matsui, M. Yasujima, and T. Suda. 2000. Plasma oxysterols and tocopherol in patients with diabetes mellitus and hyperlipidemia. *Lipids*. **35**: 333–338.
43. Moss, R. L., and L. A. Amii. 1999. New approaches to understanding the etiology and treatment of total parenteral nutrition-associated cholestasis. *Semin. Pediatr. Surg.* **8**: 140–147.
44. Bindl, L., D. Lütjohann, S. Buderus, M. J. Lentze, and K. von Bergmann. 2000. High plasma levels of phytosterols in patients on parenteral nutrition: a marker of liver dysfunction. *J. Pediatr. Gastroenterol. Nutr.* **31**: 313–316.
45. Clayton, P. T., P. Whitfield, and K. Lyer. 1998. The role of phytosterols in the pathogenesis of liver complications of pediatric parenteral nutrition. *Nutrition*. **14**: 158–164.