In vivo studies of sterol and squalene secretion by human skin

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Abstract This work was aimed at studying the quantity and composition of sterols and squalene secreted by the human skin. Lipids secreted by the entire skin were recovered by Soxhlet extraction of the clothing worn by a patient for 24 hr with a chloroform–methanol azeotrope and by extracting the water of a shower taken by the patient at the end of the 24-hr period. Squalene and sterols were quantified by gas–liquid chromatography. Plant sterols were separated from total sterols by thin-layer chromatography. Free and esterified cholesterol were separated by digitonin precipitation. In eight adults, seven of them with hyperlipoproteinemia, the total skin secretion of cholesterol ranged from 59 to 108 mg/day, with a mean of 88 ± 17 (SD) mg/day. There was no difference in cholesterol secretion between the normocholesterolemic individual and the hypercholesterolemic ones, nor were there any differences according to type of hyperlipoproteinemia. Free cholesterol amounted to 54 ± 5% of the total cholesterol. The secretion of squalene ranged from 125 to 475 mg/day in five patients. The secretion of both squalene and cholesterol was quite constant for any individual on a given diet. Cholesterol constituted 95.6 ± 0.5% of the digitonin-precipitable total body surface sterols of eight patients, and lathosterol, the next largest fraction, 3.4 ± 0.4%. Total plant sterols formed only 0.65 ± 0.38% and β-sitosterol 0.35 ± 0.23% of the skin surface sterols in six patients whose dietary β-sitosterol intake ranged from 230 to 3400 mg/day.

Supplementary key words skin surface lipids · cholesterol · plant sterols · β-sitosterol

Human skin surface lipids contain 6–14% squalene and 2–20% sterols (1, 2), most of which is cholesterol. Skin surface sterols are synthesized locally by the epidermis and sebaceous glands (3) but are also derived from the plasma[2] (4), whereas squalene on the skin originates mainly from the sebaceous glands (3). The quantity of cholesterol lost from the entire skin surface to the environment is difficult to measure. Recently, Bhattacharyya, Connor, and Spector (4) approached this problem by summing the sterols adhering to hospital pajamas worn by patients plus those extractable with acetone from the total skin surface. Most of the sterol was cholesterol (83 mg/day), but appreciable amounts of plant sterols (8% of all sterols), mainly β-sitosterol, were also found.

The study to be reported here was designed to elucidate (*a*) normal composition of human skin surface sterols and the effect of dietary factors on it and (*b*) the rate of removal of squalene and sterols from the total body surface to the environment. The usual way in which lipid is removed from the skin surface is by absorption into clothing and by bathing with soap and water. Methods of collection were designed to mimic these removal mechanisms as closely as possible.

MATERIALS AND METHODS

Patients (Table 1)

The studies were carried out on the senior author and on nine patients hospitalized for long periods on a metabolic ward at the Rockefeller University Hospital. The age, sex, caloric intake required for energy balance, and clinical diagnosis of each person are listed in **Table 1.** Two were normocholesterolemic, three had type II hyperlipoproteinemia (classified according to Fredrickson, Levy, and Lees, Ref. 5), one had type III, and three had type IV. None had any skin disorder or history of skin disease.

Diets (Table 2)

The senior author (TN) ate a diet of normal American food, the composition of which was not monitored. For 30 days he was given 5.5 g of mixed plant sterols (Cytellin: 65% β-sitosterol, 30% campesterol, 5% stigmasterol; Eli Lilly and Co.) divided into three doses per day; during the next 15 days he ingested 3.7 g of mixed plant sterols daily.

The food intake of the other nine patients consisted of orally administered liquid formula feedings (6), which contained 15% of their calories as protein; the remaining

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TABLE 1. Clinical data

Patient No.	Initials	Age	Sex	Height	Weight	Calories[a]	Diagnosis
		yr		*cm*	*kg*		
1	TN	38	M	180	76	ad lib.	No disease
2	TB	22	F	164	64	1900	Hyperlipoproteinemia (type IIb)[b]
3	FG	62	F	157	67	1800	Hyperlipoproteinemia (type IV), IHD[c]
4	ThN	39	M	180	85	2700	Hyperlipoproteinemia (type IIa), tendinous xanthomatosis
5	JG	55	M	176	63	2560	Hyperlipoproteinemia (type IIa), IHD, xanthelasma, hypertension, osteoarthritis
6	RH	42	M	175	89	3250	Hyperlipoproteinemia (type III)
7	HS	56	M	169	83	2650	Hyperlipoproteinemia (type IV), IHD, hypertension
8	AG	53	M	169	72	2350	Hyperlipoproteinemia (type IV), generalized atherosclerotic vascular disease, xanthomatosis
9	AJ	72	M	172	63	1700	Normocholesterolemia, cerebrovascular disease, chronic obstructive pulmonary disease
10	SH	46	M	178	75	2100	Hyperlipoproteinemia (type V), gouty arthritis, thoracic spondylosis

[a] Calories required to maintain constant body weight.
[b] Typing of hyperlipoproteinemia according to Fredrickson et al. (5).
[c] IHD, ischemic heart disease.

calories were supplied by fat and carbohydrate, the relative amounts of which varied reciprocally. Caloric intake was adjusted to maintain total body weight at a constant level throughout the study; adequate amounts of vitamins and minerals were included in the diets. **Table 2** shows the dietary fats used in the formula diets in the present study.

Collection of the skin surface lipids

All articles of clothing, and toweling, washcloths, swabs, etc., were made lipid-free by overnight extraction in a Soxhlet apparatus (20 cycles) with a chloroform–methanol azeotrope (88:12, v/v).

Samples from small areas of skin (e.g., the forehead) were obtained using acetone swabs in the following way. A cotton swab on a wooden stick was moistened with acetone and used for wiping a given area of the skin, no special treatment of the skin having been applied previously.

TABLE 2. Formula diets and their sterol contents

Diet	Dietary Fat and Percentage of Total Caloric Intake	Sterol Content of Diet		
		Cholesterol	Total Plant Sterols	β-Sitosterol
		mg/100 g formula[a]		
I	Corn oil, 70%	2.1	92	64
II	Cottonseed oil, 70%	1.9	32	28
III	Corn oil,[b] 20%	2.3	101	64
IV	Cottonseed oil,[b] 20%	2.0	34	30
V	Lard,[b] 40%	8.1	100	89
VI	Olive oil, 45%	1.1	23	21
VII	Fat free[c]	1.6	0	0

[a] Each 100 g of formula contained 125 kcal.
[b] Plant sterols were added to the formula prior to homogenization.
[c] Plant sterols, 25 mg/100 g of formula, were given in capsular form as 90% β-sitosterol, 10% campesterol–stigmasterol.

The lipid absorbed by the swab was eluted by pouring acetone over the cotton tip into a beaker. These wipes were repeated two or three more times in order to obtain one sample. The pooled extract was filtered and stored at 4°C for further analysis.

For analysis of lipids from larger skin areas and for the determination of sterol secretion of the total body surface, collections were made as follows. Prior to each collection period (usually 24 hr) the patient took a shower and washed the whole body (including the hair) twice with Ivory soap, using a cotton washcloth. Immediately after drying himself with a towel, the patient donned cotton underwear (long-sleeved shirt, long-legged bottoms, hospital socks; no elastic bands). To protect from possible outside contamination, the underwear was covered with hospital pajamas and socks. During the collection period the patient was ambulatory. In order to recover the lipid secreted by the exposed parts of the head, the patient was asked to wipe the skin of these parts every 1–2 hr with pieces of cotton cloth; these cloths were saved for analysis. During the night the patient used an all-cotton surgical cap and slept on a treated pillow cover.

After the collection period was over, all articles of clothing and bed linen contacted by the patient were extracted in a Soxhlet apparatus as described below. In order to recover the lipid secreted onto the skin surface but not absorbed by the clothing, the patient took a shower similar to the one at the beginning of the collection; a second person assisted. The water used for rinsing soap off the body was collected in a bathtub, the walls of which had been hydrophobized with Siliclad (Clay-Adams, Parsippany, N.J.). Immediately after the shower, the water was sucked out of the tub with continuous stirring, its volume was measured, and two aliquots of 800 ml were

taken for analysis of squalene and sterols (see below); these duplicates checked within the analytical error of the methods used. The amount of water collected in the tub was 19.9 ± 7.6 (SD) l (n = 12), and the average soap consumption was 7.6 ± 2.2 g/shower. The cholesterol content of the soap was 42 μg/g; there was no squalene in the soap.

Extraction of lipids from clothing

Prior to use, the underwear was extracted for at least 2 days (more than 50 cycles) in a Soxhlet apparatus of 3-l volume with the chloroform–methanol azeotrope; this lengthy extraction was required to render the underwear nearly lipid-free. The plant sterols, most of which was β-sitosterol, were the most difficult to remove. Acetone proved much less effective than the chloroform–methanol azeotrope. Extraction with acetone according to Bhattacharyya et al. (4) yielded only 70 μg of plant sterols, whereas subsequent reextraction of the same garments with the azeotrope yielded a further 3.4 mg of plant sterols. The quantity of plant sterols released from the clean clothing gradually decreased as the clothing was reused. Therefore, the same underwear was used by several patients; after each patient's use it was laundered and extracted with the azeotrope overnight.

The skin surface lipid absorbed by the underwear was extracted in the Soxhlet apparatus overnight (about 20 cycles) with the azeotrope. The extract was evaporated down to 400–700 ml, chloroform was added to adjust the volume ratio of chloroform and methanol to 2:1, and the extract was partitioned according to Folch, Lees, and Sloane Stanley (7). The washed chloroform extract was evaporated to dryness in a rotating vacuum evaporator at 30–40°C. The residue was sonicated for 0.5–1 min with several small volumes of *n*-hexane (to a total of 30 ml) (Branson Sonifier, Heat Systems-Ultrasonsics, Inc., Plainview, N.Y.); the insoluble residue was centrifuged off at 2000 rpm for 5 min. The extract was evaporated in a tared, glass-stoppered flask. After weighing, the residue was transferred with *n*-hexane into a graduated glass-stoppered tube and stored under nitrogen at 4°C.

To check the recovery using this procedure, a known amount of previously extracted skin surface lipid containing ^{14}C- or ^{3}H-labeled cholesterol in both free and esterified forms was added to the underwear to be extracted. The recovery of radioactivity was 100.1 ± 0.9% (n = 4).

Extraction of lipids from the shower water

An aliquot of 800 ml of the shower water was mixed with 800 ml of ethanol and 40 ml of 10 N NaOH in a 2-l graduated cylinder. The solution was extracted four times with 200 ml of petroleum ether (30–60°C), which was evaporated under reduced pressure in a rotary evaporator. The residue was dissolved in hexane by sonication (0.5–1 min), and the solutions were stored at 4°C.

Completeness of extraction was checked by adding ethanol solutions of tracer amounts of free [^{14}C]cholesterol to the shower water prior to extraction. The recovery was 101.4 ± 1.1% (n = 4). In another experiment, shower water of a patient who had received [^{14}C]cholesterol intravenously was extracted using the above procedure. Of the total radioactivity extracted, 93.2% was in the first petroleum ether extract, 3.4% in the second, 2.3% in the third, and 1.1% in the fourth. No more radioactivity could be extracted after refluxing the lower phase for 2 hr, nor was any radioactivity detected when aliquots of the lower phase were counted directly in a phosphor that dissolves water (Aquasol; New England Nuclear, Boston, Mass.).

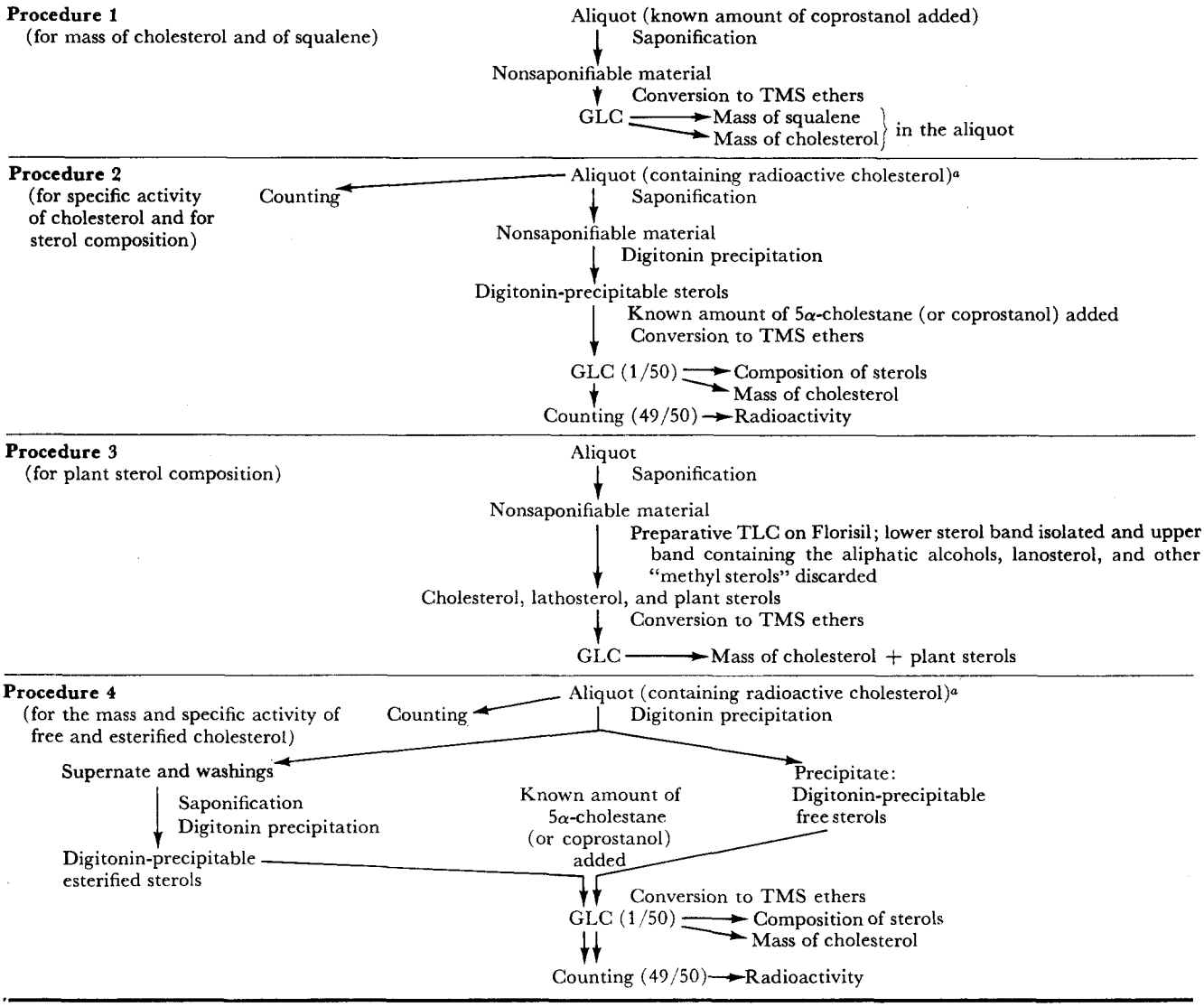
Analysis of the skin surface lipid extracts (Table 3)

Table 3 summarizes the four procedures used for the analysis of squalene and sterols in the skin surface lipids. Procedure 2 was the standard method that gave both the mass and specific radioactivity of cholesterol as well as the composition of the sterols. In order to obtain the mass of squalene, procedure 1 was used; it also gave the mass of cholesterol, but it could not be used for studies of the composition of the sterols because small amounts of nonsterol material contaminated the sterol peaks in GLC. Procedure 3 was designed specifically for the determination of the mass and composition of the plant sterols. Procedure 4 was the method used for isolation of free and esterified cholesterol. The paragraphs below describe and validate the specific steps that were common to the four procedures shown in Table 3.

Saponification. Aliquots of the skin surface lipid extracts (up to about 1 mg of cholesterol) were transferred to 15-ml glass-stoppered tubes. After evaporation of the solvent, 3 ml of absolute ethanol and 0.3 ml of 10 N NaOH were added and the tubes were refluxed for 1 hr (8). After the addition of 2 ml of water, the nonsaponifiable material was extracted by vigorous shaking twice with 5 ml and once with 2 ml of *n*-hexane. The hexane was evaporated and the nonsaponifiable material was dissolved in a suitable solvent for further analyses. The recovery of added free [^{14}C]cholesterol through the saponification and extraction steps was 99.7 ± 0.6% (n = 4).

Digitonin precipitation. The lipid was dissolved in 2.0 ml of acetone–ethanol 1:1 (v/v), and 1.0 ml of 0.5% digitonin (Merck) in ethanol–water 1:1 (v/v) was added (9). Digitonides were collected by centrifugation, repeatedly washed, dissociated by treatment with pyridine (10), and recovered in ethyl ether, all according to standard procedures. The ethereal solutions were evaporated and then analyzed by TLC and GLC. The recovery through saponification and digitonin precipitation of free [^{14}C]cholesterol (previously purified by digitonin precipitation and TLC) was 86.7 ± 1.9% (n = 4).

TABLE 3. Procedures used for analysis of skin surface lipids



[a] Radioactive cholesterol present as recovery standard, either added in vitro or when sample was derived from labeled patient.

Thin-layer chromatography of this nonsaponifiable material was performed on either 0.25-mm layers of silica gel H (Merck) or 0.5-mm layers of Florisil (<200 mesh; Floridin Co., Berkeley Springs, W.Va.) that had been activated at 110°C for 1 hr. The plates were developed with ethyl ether–heptane 40:60 (v/v) and stained with rhodamine 6G. Bands were collected by suction (11) and solutes were eluted three times with 5 ml of ethyl ether.

Gas–liquid chromatography of the silylated sterols was performed in a 6-ft glass column containing 1% DC 560 on Gas-Chrom Q (Applied Science Laboratories, State College, Pa.) maintained at 230°C in a Packard model 804 (flame ionization) gas chromatograph (Packard Instrument Co., Downers Grove, Ill.) equipped with a model 878 electrometer/voltage supply and a Hewlett-Packard model 3370B electronic digital integrator (Hewlett-Packard, Loveland, Colo.). From 1 to 3 μl of the pyridine solution of silylating reagents (0.5–5 μg of sterols) was usually chromatographed. Linearity of response within the sample range used was checked using appropriate standards; this and details on silylation have been described by Grundy, Ahrens, and Miettinen (12).

Measurement of radioactivity. The aliquot to be counted was evaporated in a counting vial; solute was dissolved in 10 ml of toluene–phosphor (Liquifluor; New England Nuclear). Counting was performed in a Tri-Carb model 3380 liquid scintillation spectrometer equipped with an Absolute Activity Analyzer (model 544). The samples were usually no more quenched than the background vial. The efficiency for ^{14}C was 63% and for ^{3}H was 38%.

Internal standards. [4-^{14}C]Cholesterol (New England Nuclear) was purified by TLC and with digitonin precipitation. It was added as an internal standard to nonra-

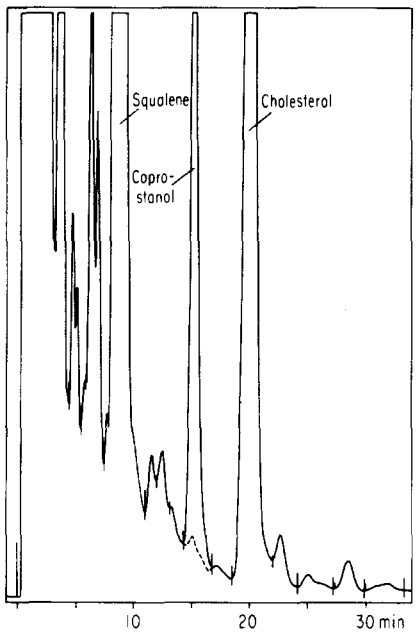


Fig. 1. GLC pattern of skin surface total nonsaponifiable lipid after conversion of the sterols and other alcohols to their TMS ethers (6-ft column of 1% DC 560 at 230°C). Sample: extract from clothing worn by patient TN, who ate regular American food plus 5.5 g of plant sterols/day for 1 month. Internal standard added; broken line shows tracing without added coprostanol.

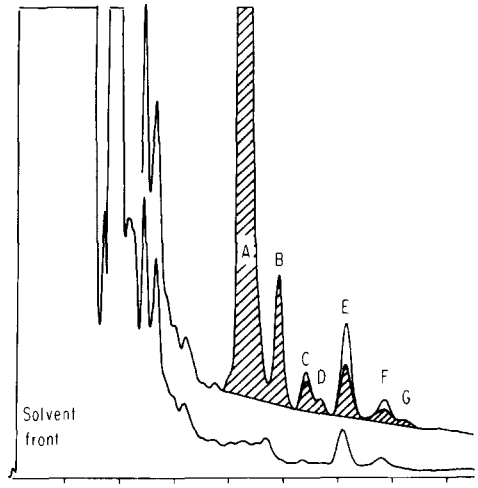


Fig. 2. GLC of skin surface nonsaponifiable lipid, analyzed as TMS ethers on 1% DC 560. The sample was an aliquot of a pool of skin surface lipid obtained from the foreheads of six normal persons. The *upper curve* was obtained from the total nonsaponifiable fraction; the hatched area shows the digitonin-precipitable sterols. The *lower curve* was obtained from the supernate remaining after overnight digitonin precipitation; the small peaks in the sterol area were not identified. The retention times of the fractions indicated with letters *A–G* were similar to those of the following reference compounds: *A*, cholesterol; *B*, lathosterol; *D*, campesterol; *E*, dihydrolanosterol and stigmasterol; *F*, lanosterol; *G*, β-sitosterol.

dioactive specimens before saponification. Radioactive cholesterol secreted by the skin of labeled patients served as its own internal standard: aliquots of original extracts were counted and, after determining the specific radioactivity of cholesterol at the end of the procedure, the mass of sterol originally present could be calculated. Prior to GLC, 5α-cholestane (Mann Research Laboratories, New York) was added to sterol samples that had undergone digitonin precipitation. Its GLC response was 97% of that of an equivalent mass of coprostanol (see below) or cholesterol (Applied Science Laboratories). Since coprostanol occupies a "window" in the gas chromatogram of the total nonsaponifiable fraction of human skin surface lipids (**Fig. 1**), it was added to one of two smilar aliquots of the skin surface lipid sample; the other aliquot was used as a control (that is, for quantification of the very small amount of material lying under the coprostanol peak). Coprostanol (Applied Science Laboratories) was 100% pure on a weight basis when compared with cholesterol by GLC and was used for quantification of both cholesterol and squalene (Fig. 1). It could not be used as an internal standard when digitonin precipitation was involved because the recovery of its digitonide was appreciably lower than that of any other sterol.

Analysis of sterols in plasma

Plasma cholesterol was measured by the method of Block, Jarret, and Levine (13) in an AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N.Y.). For more specific studies of the composition of plasma sterols, an aliquot of 300 μl of plasma was saponified as described above, and the total nonsaponifiable material was studied by GLC; prior to saponification, coprostanol was added as an internal standard.

RESULTS

Identification of squalene and sterols

Fig. 1 shows a typical chromatogram of the nonsaponifiable fraction of the skin surface lipid in man, using procedure 1 (Table 3). With the aid of reference compounds, squalene and cholesterol peaks were easily identified; the smaller peaks on both sides of squalene represent aliphatic monohydric alcohols. In 10 samples squalene and the alcohols were separated from each other by TLC, and the fractions were analyzed by GLC on 1% DC 560 and 1% QF-1 columns: the squalene peak as shown in Fig. 1 was found to be homogeneous and contaminated with aliphatic alcohols by less than 2%. Thus, its quantification from a chromatogram of the total nonsaponifiable material by procedure 1 was considered to be sufficiently accurate for present purposes.

To study the composition of sterols, they were precipitated with digitonin according to procedure 2 (Table 3), and the fractions obtained were studied by GLC (**Fig. 2**). Since the cholesterol peak did not contain any significant amount of nonsterol material, its quantification from the

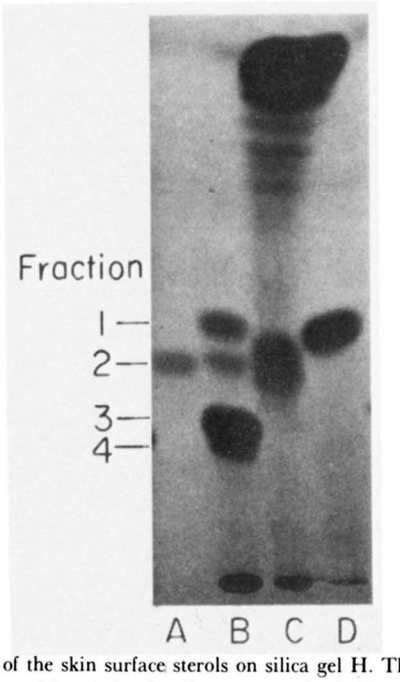


Fig. 3. TLC of the skin surface sterols on silica gel H. The plate was developed twice with ethyl ether–heptane 40:60 (v/v). *A*, reference coprostanol; *B*, digitonin-precipitable skin surface sterols; *C*, supernate after digitonin precipitation; *D*, reference lanosterol. Fractions 1–4 of sample *B* were isolated by preparative TLC and analyzed by GLC (Fig. 4).

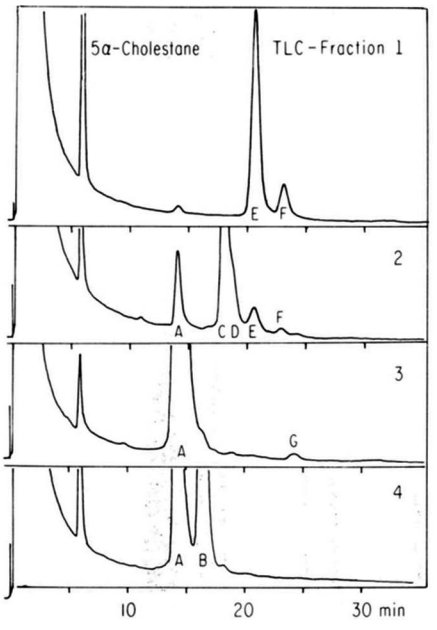


Fig. 4. GLC patterns of the fractions isolated by TLC from the digitonin-precipitable skin surface sterols (sample *B* of Fig. 3). The letters *A–G* refer to the GLC fractions of the unfractionated sample (Fig. 2): *A*, cholesterol; *B*, lathosterol; *C*, "methyl sterols"; *D*, "methyl sterols" (+ campesterol); *E*, dihydrolanosterol (+ stigmasterol); *F*, lanosterol; *G*, β-sitosterol.

chromatogram of the total nonsaponifiable material (as in procedure 1, Table 3) appeared justified. The quality of the small amount of material in the sterol region that was not precipitated with digitonin (Fig. 2) was not studied. No attempts were made to find out whether the cholesterol peak contained any cholestanol.

For further identification, the digitonin-precipitable sterols were fractionated by TLC into four fractions (sample B in **Fig. 3**). GLC analyses of each of these four fractions (**Fig. 4**) showed the presence of cholesterol, lathosterol, dihydrolanosterol, lanosterol, and β-sitosterol. On the basis of their TLC mobility, the GLC peaks *C* and *D* were assumed to contain C_{28-29} precursors of cholesterol ("methyl sterols," Ref. 14). No detectable amounts of campesterol (*D*) or stigmasterol (*E*) were found in the plant sterol–cholesterol fraction (fraction *3* of Fig. 4) of this pooled sample; in certain other samples small amounts of campesterol and stigmasterol were identified and included in peaks *D* and *E*, respectively.

Recovery of cholesterol from the skin surface

To determine how much lipid on the skin surface is actually removed by the procedures used for their collection, [^{14}C]cholesterol was applied to the skin, and its recovery in clothing and shower water was measured. In the first experiment, 98% of the radioactive free and esterified cholesterol with carrier applied to the back of a patient was recovered in the shirts worn during the following 45 hr. In the second experiment, 91% of a tracer amount of free cholesterol applied to the back of the patient was recovered in the shower water 5 min later, and 2% of the radioactivity was absorbed by the shirt that was worn subsequently for 24 hr. In this second experiment, 7% of the cholesterol applied to the skin was not accounted for.

Amount of cholesterol and other sterols secreted (Table 4)

The average secretion of cholesterol by the skin of the eight patients studied was 88 ± 17 mg (n = 12), and that of the total digitonin-precipitable sterols was 92 ± 18 mg (n = 12) (**Table 4**); variations among patients were almost twofold. There was no difference in the cholesterol secretion between the normocholesterolemic individual (TN) and the hypercholesterolemic ones nor among the three type II and three type IV patients.

For sake of brevity, the distribution of sterols found in the separate garments of the 12 experiments described in Table 4 are not listed. The data not shown can be summarized as follows: daily excretion of cholesterol on shirts was 18.9 ± 8.0 mg (n = 12); on bottoms, 10.0 ± 2.6 (n = 10); on socks, 2.0 ± 0.71 (n = 10); on caps, face cloths, and pillows, 7.4 ± 4.1 (n = 10); and in shower water, 47 ± 12 mg (n = 12). The proportion of total skin surface cholesterol recovered on articles of clothing was 45%, and in shower water 55% (Table 4).

TABLE 4. Amount of lipids excreted on the surface of the total body (mg/24 hr) (according to procedures 1 and 2, Table 3)

Patient	TN		TB			FG	ThN	JG	RH	HS	AG		
Age	38		22			62	39	55	42	56	53		
Sex	M		F			F	M	M	M	M	M		
Type[a]	Normal		IIb			IV	IIa	IIa	III	IV	IV		
Diet[b]	Reg. 1	Reg. 2	I	III	V	IV	IV	IV	IV	IV	VI	VII	
Days on diet		30	30	31	27	9[c]	33	37	86	54	43	21	Mean ± SD (n)
Total sterol excretion													
Cholesterol	108	106	83	97	98	73	94	59	59	79	89	107	88 ± 17 (12)
Lathosterol	3.3	3.5	3.1	3.1	3.2	3.0	3.4	1.9	2.5	3.0	3.2	4.2	3.1 ± 0.54 (12)
β-Sitosterol[d]	0.08	0.14	0.27	0.26	0.22	0.14	0.27	0.16	0.24	0.34	0.43	0.82[e]	0.28 ± 0.19 (12)
β-Sitosterol[f]	0.17	0.20									0.33		0.23 ± 0.08 (3)
Total sterols[g]	113	111	87	101	102	77	98	61	62	82	94	113	92 ± 18 (12)
Percentage recovery of cholesterol in:													
Clothing etc.	53	55	50	42	37	27	30	49	45	37	58	59	45 ± 11 (12)
Shower water	47	45	50	58	63[h]	73	70	51[i]	55	63	42	41	55 ± 11 (12)
Total lipids excreted from clothing	1968	2110	1669	1296	1074	435	1308	939	693	715	1772	1681	1305 ± 543 (12)

[a] Type of hyperlipoproteinemia according to Fredrickson et al. (5).
[b] Diets (see Table 2): Reg. 1, regular American food; Reg. 2, regular American food + 5.5 g of plant sterols/day; I, 70% corn oil formula; II, 70% cottonseed oil formula ; III, 20% corn oil formula; IV, 20% cottonseed oil formula; V, 40% lard formula; VI, 45% olive oil formula; VII, fat-free formula. Diets I, III, and V contained three times or greater amounts of plant sterols than diets II, IV, VI, and VII (see Table 2).
[c] Prior to this the patient was on diet II for 89 days.
[d] The amount of β-sitosterol was determined by GLC of the total digitonin-precipitable sterols; the figure includes lanosterol.
[e] Most of this (72%) was obtained from the shirt; only 9% was in the shower water.
[f] The figure for β-sitosterol was obtained after its separation from lanosterol by procedure 3 (Table 3).
[g] Digitonin-precipitable sterols.
[h] 38% was obtained from the head, mostly from the hair; 25% from the rest of body.
[i] 24% was obtained from the head, mostly from the hair; 27% from the rest of body.

State of esterification of cholesterol in skin surface lipids (Table 5)

The percentage of free cholesterol in total cholesterol was 54 ± 5.4% (n = 5) in the total body extract (**Table 5**). The ratios found at any given site did not vary greatly from patient to patient, but from site to site the variations were large: the percentage of free cholesterol was low in areas rich in sebaceous glands (28% on the forehead) and high in areas poor in sebaceous glands (77% on the feet). These data are presented in detail because they do not agree with those of Bhattacharyya et al. (4).

Amount of squalene secreted (Table 6)

The secretion rate of squalene varied from patient to patient much more than that of cholesterol, i.e., over a fourfold range of from 125 mg to 475 mg per day in the five individuals studied (**Table 6**). The inclusion of 5.5 g of plant sterols daily in the diet of subject TN did not have any effect on the secretion of squalene, and in patient AG the change from a 45% olive oil formula (containing 580 mg of squalene per day) to fat-free formula was followed by a trivial decrease in squalene secretion.

The mass ratio of squalene to cholesterol varied greatly, depending on the body site studied. It was high in areas rich in sebaceous glands (up to 11:1 on the foreheads of some patients not included in the present study) and low in skin areas poor in sebaceous glands (down to 1:16 on feet). The percentage of skin surface squalene removed by showering (53 ± 14; n = 7) was quite similar to that for cholesterol (55 ± 11%; n = 12) (Table 4).

Composition of skin surface sterols (Table 7)

Cholesterol constituted 93% of skin surface sterols from the forehead of six normal males (**Table 7**) on ad lib.

TABLE 5. Percentage of free cholesterol in total cholesterol in samples of skin surface lipids in seven patients[a] (according to procedure 4, Table 3)

	Mean ± SD (n)
Acetone swabs	
Forehead	28 ± 6.1 (4)
Back	47 (1)
Arm	60 (2)
Clothing	
Shirt	47 ± 8.6 (5)
Bottoms	62 ± 7.4 (4)
Socks	77 ± 6.1 (3)
Caps etc.	32 ± 2.4 (4)
Shower	
Body, Head	57 ± 5.9 (5)
Total body[b]	54 ± 5.4 (5)

[a] Patients 1, 3, 5, 6, and 8–10.
[b] Calculated with the aid of the figures for mass in Table 3.

TABLE 6. Secretion of squalene onto the skin surface in five patients[a] (according to procedure 1, Table 3)

	Mean ± SD (n)[b]	Patient AG	
		VI[c]	VII[c]
Squalene secretion (mg/24 hr)	257 ± 138 (5)	302	285
Distribution of squalene (%)			
Clothing etc.	47 ± 14 (7)	55	63
Shower water	53 ± 14 (7)	45	37
Ratio of squalene to cholesterol			
Forehead	6.4 ± 1.2 (4)	6.7	
Feet	0.11 ± 0.05 (5)	0.10	0.19
Total body	3.3 ± 1.0 (7)	3.4	2.7

[a] Patients 1, 3, 5, 6, and 8.
[b] Mean ± SD, where n = number of tests made in five patients on one or more diet regimens.
[c] Diets (see Tables 2 and 4). Diet VI (45% olive oil) in patient AG furnished a daily intake of 580 mg of squalene, whereas all other formula diets were essentially squalene-free.

TABLE 7. Percentage composition of digitonin-precipitable sterols in acetone swabs of forehead in six normal males on ad lib. diets (according to procedure 2, Table 3)

Sterol	First Swab[a]	Second Swab[a]
	mean ± SD	
Cholesterol	92.90 ± 1.50	93.05 ± 1.80
Lathosterol	3.19 ± 1.01	2.97 ± 0.92
"Methyl sterols"	0.92 ± 0.35	1.26 ± 0.55
Campesterol and/or "methyl sterols"	0.33 ± 0.16	0.37 ± 0.18
Dihydrolanosterol and/or stigmasterol	1.97 ± 0.50	1.77 ± 0.63
Lanosterol[b]	0.50 ± 0.42	0.38 ± 0.30
β-Sitosterol[b]	0.24 ± 0.19	0.19 ± 0.10

[a] The first acetone swab was made without prior cleaning of the forehead. The second swab was taken 4 hr after the first; during this time the forehead remained uncovered.
[b] Lanosterol and β-sitosterol peaks were quantified separately by cutting and weighing the gas chromatogram.

diets and the next largest fraction, lathosterol, 3%. Only about 0.2% of the total sterols was β-sitosterol. The sterol composition of the sebum secreted in 4 hr was not significantly different from that present on the skin at the time of the first sampling.

The composition of the digitonin-precipitable sterols of the total body surface in eight subjects on controlled diets did not differ greatly from the values discussed above. Cholesterol constituted 96% of the sterols and lathosterol 3.4%. β-Sitosterol was not quantified separately, but together with lanosterol it represented only 0.31% of total skin sterols.

Dietary plant sterols in skin surface lipids (Table 8)

Daily intake of 5.5 g of mixed plant sterols (Cytellin) (equivalent to 3400 mg/day of β-sitosterol) by subject TN for 1 month did not alter the amount of cholesterol or β-sitosterol appearing daily on the skin surface (Table 4). Table 4 also indicates that in the seven patients on formula feedings containing known amounts of sterols the β-sitosterol + lanosterol content of total skin surface sterols averaged 0.28 mg/24 hr, with a range of 0.08–0.82. Diets I, III, and V contained two to three times as much β-sitosterol as diets II, IV, VI, and VII, yet there were no systematic differences in plant sterol excretion by patients ingesting higher or lower amounts of β-sitosterol.

The question of the relationship among β-sitosterol secretion by the skin, dietary intake, and plasma levels of this sterol was examined in greater detail in **Table 8.** This table shows only some of many analyses (plasma, forehead, individual garments, shower water, and total body skin surface sterols) carried out at different times after starting diets containing known amounts of β-sitosterol. Considering only the data obtained in extraction of shirts (where β-sitosterol recoveries were the highest of all garments), β-sitosterol made up 0.36 ± 0.25% (n = 10) and total plant sterols 0.70 ± 0.38% (n = 10) of the "cholesterol" band (fraction 3 in Figs. 3 and 4) when dietary β-sitosterol intake differed from 230 to 3400 mg/day. The highest β-sitosterol content recorded by us in any skin surface sterol sample was 0.86% (in patient RH, Table 8).

Bhattacharyya et al. (4) recorded much higher contents of β-sitosterol in the skin surface lipids of 11 patients on measured diets containing plant sterols. Since they used acetone washes for the extraction of lipids from the entire skin surface, we tested in patient TN whether rubbing the skin with acetone would increase the permeability of the skin to β-sitosterol. Immediately after each of two regular collections, when 108 and 106 mg of cholesterol were recovered from the skin, respectively (Table 4), the entire skin surface was rubbed four times with a cotton bathcloth moistened with acetone; about 1 l of acetone was used on both occasions. While the amount of additional cholesterol recovered in the acetone rubbings was 8.3 and 5.0 mg, respectively, the rubbings contained no more β-sitosterol (0.13 and 0.11% of the sterols, respectively) than the extracts shown in Table 8, obtained by conventional means. To test the effect of longer-term acetone extraction on the permeability of skin to the plant sterols, the upper part of the body of patient TN was rubbed thoroughly four times with acetone twice daily for 2 wk; during this time his β-sitosterol intake was 2.3 g/day. However, the sterol compositions of the skin surface lipids obtained from his shirt and from the last acetone rubbing did not differ from each other (β-sitosterol content was 0.10% of sterols) or from data obtained previously by our usual procedure.

TABLE 8. Sterols of plasma and skin surface secretions in six patients on diets containing β-sitosterol (according to procedure 3, Table 3)

Patient	Dietary β-Sitosterol: No. of Days Fed	Dietary β-Sitosterol: Intake	Plasma β-Sitosterol	Skin Surface Sterols: Cholesterol	Skin Surface Sterols: Lathosterol	Skin Surface Sterols: Total Plant Sterols	Skin Surface Sterols: β-Sitosterol
		mg/day	*mg/dl plasma*	*% composition*[a]			
TN	ad lib. diet 0–30 days	NM[b]	0.09	96.21*a*	3.40	0.37	0.14
	30–60	3400	0.16	96.10*a*	3.52	0.35	0.17
	60–75	2300	NM	95.61	4.03	0.33	0.09
FG	99–109	300–470	0.11	95.72	3.70	0.55	0.31
ThN	91–97	230–650	0.61	95.86	3.26	0.89	0.58
JG	76–93	530–600	0.86	96.22	3.23	0.52	0.32
	101–107		0.43	96.52	2.96	0.50	0.29
RH	58–62	570–780	0.41	93.65	4.69	1.63	0.86
	107–111		0.74	94.64	4.54	0.79	0.36
AG	126–127	380–500	0.34	95.47*a*	3.96	0.57	0.35

[a] Except when the composition of total body surface sterols was available (values marked with *a*), the sterol composition was analyzed from the extracts of shirts, which usually contained the highest percentages of β-sitosterol compared with all other skin surface lipid samples.
[b] NM, not measured.

Cholesterol secretion and dietary fat content

With this small number of patients, it was not possible to obtain sufficient data to answer the question of whether isocaloric substitution of dietary carbohydrate for dietary fat had a predictable effect on total daily cholesterol secretion by the skin. Table 4 presents data obtained from patients TB and AG that suggest that a low-fat diet may lead to a small decrease in cholesterol secretion: in patient TB the change from a 70% to a 20% corn oil diet resulted in an increase of 17% (from 83 to 97 mg/day), and in patient AG there was a 20% increase (from 89 to 107 mg/day) on moving from a 45% olive oil to a fat-free formula. Clearly, these findings must be greatly extended before they can be considered to be meaningful.

DISCUSSION

Methods

The methods used in the present study for the collection of the skin surface lipids gave satisfactory recoveries. This was shown (*a*) by measuring the amount of radioactive cholesterol recovered from the skin after applying it there and (*b*) by experiments in which the total skin surface of a patient was rubbed thoroughly with acetone after the collection by regular methods had been completed. Despite the fact that rubbing was performed vigorously enough to cause discomfort, the amounts of cholesterol and squalene subsequently extracted represented only 5–8% and 2–4%, respectively, of the quantities of these compounds previously collected. *However, our methods were not designed to see how much lipid can be removed from the skin surface, but to recover and quantify the lipid that is being lost to the environment during everyday life,* and so the general washes with acetone were not made a part of our standard procedure.

Cholesterol secretion

The figure for cholesterol secretion of the skin recorded in the present study (88 ± 17 mg/day) is similar to that obtained by Bhattacharyya et al. (4) for six normal persons (82.6 ± 21.5 mg/day) and for five type II hyperlipoproteinemic patients (82.7 ± 6.5 mg/day). These recoveries are strikingly similar despite marked differences in methods of collection: Bhattacharyya et al. (4) used uncovered hospital pajamas in place of our covered underwear and acetone swabbing instead of our shower. However, in their studies about 90% of the sterols was collected by the clothing and only 10% was obtained through the acetone washes, whereas in our patients only 45% of the cholesterol and 47% of the squalene were absorbed by the underclothing, and the rest by showering. This discrepancy is difficult to explain, especially when one considers that the underwear worn by our patients fitted tightly to the surface of the skin and was more likely to absorb any lipid secreted by the skin than the loosely fitting hospital pajamas used by Bhattacharyya et al. (4). An appreciable proportion (24–38%; see footnotes *h* and *i* to Table 4) of the total skin surface cholesterol is contributed by the scalp because the hair functions as a lipid reservoir and prevents the loss of lipid to the cap, from which only about 2% of the total skin surface cholesterol was collected. Considerable error results if the hair is not sampled for cholesterol determination; this was the case in four out

of five hyperlipoproteinemic patients studied by Bhattacharyya et al. (4).

Free and esterified cholesterol

Another unexplained discrepancy between our results and those of Bhattacharyya et al. (4) is their finding that 87 ± 2.5% of the skin surface cholesterol was in esterified form, whereas in our studies the percentage of esterified cholesterol in the total skin surface lipids was only 46 ± 5.4% (Table 5). Yet, the method used for the separation of free and esterified cholesterol was by digitonin precipitation in both laboratories. In our patients the percentage of free in total cholesterol depended greatly on the skin site studied: it was low on the forehead (22–34%) and high on the feet (70–81%). Thus, there appeared to be an inverse correlation between the percentage of free cholesterol and the density of the sebaceous glands, as noted also by Greene et al. (2). They found that the ratio of free cholesterol to cholesteryl esters (the mass of fatty acids included) was 0.41 on forehead, 0.50 on chest, and 1.1 on arms. According to Windhorst and Foster (15), the percentage of free in total cholesterol in scalp fat was 47% on the average, which corresponds well with our value of 46% obtained by showering the head of patient JG (Table 5). The average percentage of free cholesterol in epidermal lipids is known to be high, 80% according to Reinertson and Wheatley (16), which is in accordance with the average of 77% found by us on the feet.

These findings suggest that esterified cholesterol originates preferentially from the sebaceous glands, whereas free cholesterol is preferentially produced by the epidermis (for further discussion of the role of the epidermis and sebaceous glands in sterol synthesis see Refs. 17 and 18). Recently, Prottey, Hartop, and Ferguson (19) reported that the epidermis of the rat preferentially synthesizes free sterols, whereas the sterols of the sebaceous glands are mainly in esterified form. However, the sterol content of sebum is very low, whereas that of the skin surface lipid originating mainly from the epidermal cells is much higher (1). In our studies the content of cholesterol was 1.5 ± 0.2% (n = 8) of the total lipids on the forehead, 2.5 ± 0.6% (n = 19) of the lipid on the whole face, 2.9 ± 0.8% (n = 43) of lipids collected by a shirt, and 8.8 ± 3.5% (n = 19) on feet.

Squalene secretion

Squalene is synthesized mainly by the sebaceous glands (3), and thus its percentage from the total skin surface lipids (1, 2) and the squalene/cholesterol ratios (see Tables 4 and 6) depend much on the skin site, being high on the head and low on the feet. The total secretion of squalene by human skin, not previously reported, was found to be quite variable from one patient to another, namely, from 125 to 475 mg/day. Searching for an explanation for these variations, we noted that in two patients switched from a high fat to a high carbohydrate diet there was an increase in cholesterol secretion (Table 4), while in one patient (AG) there was a slight decrease in squalene secretion (Table 6). Following up this lead, we measured the squalene/cholesterol ratio in the forehead lipids serially: in three of five patients there was a clear-cut fall in this ratio on changing from high fat to high carbohydrate feeding. More studies are needed to confirm these preliminary findings and to explain the meaning of this ratio.

Plant sterol secretion

Plant sterols constituted about 8% of total sterols in the skin surface lipids collected by Bhattacharyya et al. (4); the total secretion of plant sterols was estimated to be about 7 mg/day, most of which was β-sitosterol. In no patient did our recoveries of plant sterols from the skin surface approach the proportions noted by Bhattacharyya et al. (4). To account for the much lower percentage (an average of 0.2–0.4%, Tables 7 and 8) and secretion rate (an average of 0.3 mg/day, Table 4) of skin surface β-sitosterol in our patients, we have considered several possibilities:

(*a*) A different dietary intake of β-sitosterol. However, the β-sitosterol intake of the patients of Bhattacharyya et al. (4) was 350–400 mg/day, which was generally lower than the intake of our patients (Table 8). Bhattacharyya et al. (4) reported that in one patient the plant sterols disappeared from the skin surface when the patient was put on sterol-free liquid formula diet. Since our basal level of β-sitosterol was already so low, we attempted to raise it by feeding large amounts of plant sterols: patient TN ate 5.5 g of plant sterols/day for 1 month, but there was no significant increase in the small percentage of β-sitosterol in the total skin surface sterols (0.14 and 0.17%, respectively, Table 8), nor in absolute amounts (0.17 and 0.20 mg/24 hr, respectively, Table 4).

(*b*) All but one of our patients were on liquid formula diets, whereas the patients of Bhattacharyya et al. (4) apparently were on solid food diets. However, the β-sitosterol secretion of TN, our only patient eating solid food, also was very low (0.17–0.2 mg/day, Table 4).

(*c*) The possibility that swabbing with acetone might increase the permeability of the skin for plant sterols was excluded by two experiments designed to test this point.

(*d*) Bhattacharyya et al. (4) extracted the hospital pajamas by boiling with 2- to 3-l portions of acetone three times. They claimed that only trace quantities of cholesterol and β-sitosterol were present in the clothing after this treatment. In our experiments the cotton underclothing was found to be extremely difficult to free from β-sitosterol, some of which could still be extracted after 2 days' extraction (more than 50 Soxhlet cycles) with hot chloroform–methanol. Yet, this solvent azeotrope is a more powerful extractant of β-sitosterol than acetone, as shown by its ability to remove more than 3 mg of β-sitos-

terol from clothing previously extracted seven times with hot acetone. On the other hand, Bhattacharyya's used hospital pajamas may have been easier to extract free from plant sterols than the new underclothing employed by us.

(*e*) The pajamas worn by the patients of Bhattacharyya et al. (4) were not protected against outside contamination, whereas our patients had their garments well protected by lipid-free pajamas worn over the underclothing. Fecal or food soiling of the pajamas (especially the shirts) worn by Bhattacharyya's patients eating β-sitosterol-containing diets is the only explanation we can offer for the variations in findings between our two laboratories. In our studies the garment that was least protected against outside contamination was the shirt; it regularly contained the highest proportion of β-sitosterol (less than 1% of total sterols, however). On the other hand, shower water contained extremely small percentages of the plant sterols. However, Bhattacharyya et al. (4) reported that radioactive β-sitosterol given orally to one patient "was detected by about 21 days in the skin surface sterols." If, indeed, there was no detectable radioactive β-sitosterol in the skin surface sterols *until* that late time, then food and fecal soiling of pajamas appears to be ruled out as an explanation for Bhattacharyya's findings: maximum radioactivity ought then to have been detected in the first few days after oral dosage, because 95% of dietary β-sitosterol is not absorbed in normal individuals (20).

We are not satisfied, then, with any of these explanations for the discrepancies between our β-sitosterol data and those of Bhattacharyya et al. (4), providing that they sought and did not find radioactive β-sitosterol in the skin surface sterols soon after giving it orally. As they commented, "33% of the daily absorbed β-sitosterol was excreted in the lipids of the skin" when only 5% of the ingested plant sterol was presumably absorbed (18): we find it difficult to conceive that the intestinal mucosa selectively excludes β-sitosterol whereas the skin selectively secretes it.

Kinetic aspects

We have a second paper in preparation that will discuss certain kinetic aspects of the movement of cholesterol through the skin and will differentiate the quantitative aspects of the origin of skin surface cholesterol, whether from plasma or de novo synthesis by the skin. These studies were carried out in the same patients discussed in the present report.

We are grateful to Miss Susan Turner for her excellent technical assistance.

This study was supported in part by U.S. Public Health Service grant HL-06222 from the National Heart and Lung Institute, and by U.S. Public Health Service grant FR-00102 from the General Clinical Research Centers Branch of the Division of Research Resources.

Manuscript received 2 October 1973 and in revised form 26 April 1974; accepted 23 July 1974.

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