Critical role of ring structure in the differential uptake of cholesterol and plant sterols by membrane preparations in vitro

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Abstract To determine the role of the ring structure in the differential absorption of sterols, we have used rat jejunal brush border vesicles and erythrocytes to examine the uptake of cholesterol, campesterol, and sitosterol following successive chemical degradations of rings A and B. The cell and membrane preparations were incubated with the sterols and sterol analogues (about 30 micromolar each) dissolved in 7 mM sodium taurocholate and 0.6 mM egg phospholipid. The uptake of the analogues was analyzed by high performance liquid chromatography and capillary gas–liquid chromatography. In both membrane preparations, the uptake of the 7-dehydro analogues of cholesterol, campesterol, and sitosterol was linear with time. 7-Dehydrocholesterol was absorbed 4–5 times faster than 7-dehydrositosterol by both preparations. The uptake of the campesterol analogue was intermediate between that of the analogues of cholesterol and sitosterol at all time points. Following conversion of the 7-dehydrosterols to their calciferol derivatives, the 27-carbon sterols were absorbed only 1.9 and 1.4 times faster than those of the 29-carbon sterols by the erythrocyte and brush border membranes, respectively. A similar degree of selectivity was expressed in the erythrocytes during the uptake of a steroid series possessing keto-4-ene ring system. Complete oxidation of the calciferol derivatives to the des-AB-8-ones resulted in a total loss of discrimination among the various side-chain homologues during absorption from micellar solutions. It is concluded that the selective absorption of animal and plant sterols depends upon the presence of a ring system having the bulk of the cholestane nucleus, although not necessarily a rigid or planar one containing a hydroxyl group.—**Child, P., and A. Kuksis.** Critical role of ring structure in the differential uptake of cholesterol and plant sterols by membrane preparations in vitro. *J. Lipid Res.* 1983. **24:** 1196–1209.

Supplementary key words membranes • mixed micelles • brush border vesicles • erythrocytes • side chain • sterol absorption • sitosterol

The plant sterols, campesterol and sitosterol, are not absorbed by the mammalian intestine as readily as cholesterol (1–3). Although the additional alkyl groups in the side chain of the plant sterols obviously influence their absorption, it is not clear whether other features of the sterol molecule are required for the effects of the side chain to be expressed. It is important to establish these features in view of the ability of sitosterol to interfere with the intestinal absorption of cholesterol and to serve as an effective hypocholesterolemic agent in man (1–3).

We have previously reported (4, 5) that the 3–5-fold greater uptake of cholesterol over sitosterol that is found in the intestinal wall of the rat after feeding radioactive sterols (6–8) can be duplicated in rat erythrocytes and purified brush border membranes by incubating them with the sterols in solutions containing sodium taurocholate and egg yolk phospholipid. In later experiments (4), the 7-dehydro analogues of these sterols were used to facilitate their analysis by HPLC using ultraviolet detection. These derivatives reflected the absorptive behavior of the parent sterols and also possessed the functional groups necessary for further chemical degradation of the ring system. In the present study we have compared the selectivity of absorption between the 7-dehydro derivatives of cholesterol, campesterol, and sitosterol and the corresponding derivatives with modified ring structures, as an indication of the relative importance of the ring system in sterol uptake in vitro. It is demonstrated that partial fracture of ring B or oxidation of the 3β-hydroxyl group has little effect on the selectivity, but a complete removal of rings A and B fully suppresses the absorptive influence of the side-chain alkylation.

METHODS AND MATERIALS

Egg yolk phospholipid was fractionated by TLC to yield separate fractions for PC, PE, lyso-PC, and sphin-

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography, PC, phosphatidylcholine; PE, phosphatidylethanolamine; GLC, gas–liquid chromatography; TMS, trimethylsilyl; MS, mass spectrometry.

gomyelin. These were recombined to give the lipid class ratios indicated in the legends to the figures and tables. Cholest-4-en-3-one and 7-dehydrocholesteryl acetate were obtained from Steraloids (Wilton, NH). Vitamin D_3 was purchased from Sigma Chemical Co. (St. Louis, MO) and methyl heptadecanoate was from Supelco Inc. (Bellefonte, PA). All steroidal materials were found to be better than 95% pure by HPLC using a detector wavelength of 265 nm and by GLC on short OV-1 columns using flame ionization detection. All biochemicals and cell preparation supplies were as previously described (5, 9). A 1:1 mixture of campesterol and sitosterol was recovered by acetone recrystallization from a crude soy sterol mixture supplied by Distillation Products (Rochester, NY). Cholesterol was supplied by British Drug Houses (Toronto, Canada).

Preparation of 7-dehydrosterols

The 7-dehydrosterols were prepared from cholesterol and the purified mixture of soy sterols. Following conversion of the precursor cholesterol and the plant sterols to the benzoate esters, the additional double bond was introduced according to the method of Neville and DeLuca (10). The free sterols were regenerated from the benzoates by brief saponification with 0.5 N KOH in methanol, under a nitrogen atmosphere.

Preparation of the 3-oxo,4-ene derivatives

The parent sterols were oxidized using CrO_3 as reported by Djerassi, Engle, and Bowers (11). Following oxidation, water was added to the mixture and the sterols were recovered by filtration. The crude filtrate was taken up in dilute methanolic KOH and heated at 80°C for 5 min to isomerize the double bond to the 4,5 position. The mixture was subsequently diluted with water, neutralized with acetic acid, and extracted with chloroform. The extracts were purified on silica gel H plates developed with chloroform–methanol 100:1. The purity of the compounds was monitored by GLC and reverse phase HPLC, and the identity was established by mass spectrometry and ultraviolet spectrophotometry, and by comparison of the chromatographic mobility of the synthetic product with that of authentic cholest-4-en-3-one.

Preparation of the calciferol derivatives

The calciferol derivatives were prepared by irradiation of a solution of the 7-dehydrosterols (1 mg/ml) in a quartz photolysis tube (11). The free sterols were taken up in fresh, cold ether and irradiated for 15 min in a Rayonet photolysis unit (Southern NE Ultraviolet Co., Middletown, CT) containing 11 Rayonet photochemical reactor lamps (300 nm wavelength). The reaction mixture was not stirred during the irradiation. The optimal irradiation time was determined during a trial run with 7-dehydrocholesterol. Samples taken at various times were assessed for their content of the calciferol product by reversed phase HPLC using a detector wavelength of 265 nm. The reaction mixture was evaporated to dryness at room temperature, taken up in a small volume of iso-octane, and heated to 80°C for 2 hr to bring about a thermal equilibration of the precalciferol and calciferol derivatives. The calciferol derivatives were isolated by TLC, using silica gel H plates and benzene–acetone 9:1. The calciferol bands were located under ultraviolet light and recovered from the gel by elution with chloroform–methanol using a small column. The yield of vitamin D was estimated to be about 25% (from 7-dehydrosterol). The purity of the final preparation was judged to be greater than 90% by HPLC and GLC. The compounds were characterized by mass spectrometry, ultraviolet absorption, and by comparison of the chromatographic behavior to that of an authentic vitamin D_3 standard. All of the steroid derivatives were stored in ethanol under nitrogen and were purified by TLC before use.

Preparation of the des-AB,8-one derivatives

These compounds were obtained from the calciferol derivatives using a modification of the permanganate-periodate oxidation method of ApSimon et al. (12). Cholecalciferol and the calciferol analogues of the 1:1 plant sterol mixture (100 mg) were separately taken up in warm t-butanol (25 ml) and added to 18 molar equivalents of $NaIO_4$ and 9 mg of $KMnO_4$ in 50 ml of water. The pH was adjusted to 8 with 5% aqueous potassium carbonate. TLC of a sample taken following 1.5 hr of oxidation showed that none of the starting material remained. The reaction mixture was then extracted three times with ether, and the pooled organic phases were backwashed with 0.9% saline. The extracts were applied to a 20% $AgNO_3$-silica gel G plate and developed with chloroform–methanol 100:1. The main band (R_f 0.90) was eluted with chloroform–methanol and backwashed with dilute ammonia to remove the fluorescein used for visualization of the TLC plate. GLC on a 90 cm OV-1 column gave a single peak for the cholesterol derivative and a doublet of peaks for the plant sterol derivatives. The identity of the compounds was verified by mass spectrometry.

Preparation of rat jejunal brush border vesicles

Brush border membranes were prepared from fresh jejunal scrapings using the method of Kessler et al. (13). For sterol uptake studies, the final brush border pellet was resuspended (about 1 mg/ml) in Hank's balanced salt solution (HBSS) containing 15 mM HEPES buffer, 5 mM EGTA, and 4% fatty acid-free BSA complexed

(14) with oleic acid (4 mol per mol of protein). The brush border suspension was kept on ice until used.

Isolation of rat erythrocytes

Rat erythrocytes were isolated from fresh EDTA-treated blood as reported earlier (5). The cells were stored at 30% hematocrit in HBSS + Hepes (phenol red omitted) at room temperature. The blood was left in contact with the EDTA for not longer than 30 min and the washed cells were used within 90 min of their isolation.

Preparation of micellar solutions

These were prepared by mixing the lipid components in amounts sufficient to give 6.6 mM sodium taurocholate, 0.6 mM phospholipid, and 1–30 μM of each sterol derivative in the desired volume. Following evaporation of the solvents under nitrogen, the required volume of HBSS + Hepes buffer was added and the suspension was filtered under light plunger pressure through a Milex filter disc (0.2 micron pore size, Millipore Corp., Mississauga, Ont.) attached to a disposable syringe. The pH of all solutions was adjusted to 7.4. At the levels of sterol used, the micellar mixtures were water-clear before and after filtration. The proportions of the neutral lipids in the final filtered solutions were assessed following total lipid extraction by HPLC or GLC using the TMS derivatives (15). The actual compositions of the solutions are indicated in the legends to the figures.

Measurements of sterol uptake

Incubations were initiated by the addition of 1 ml of the cell or membrane suspension to 4 ml of the micellar solution prewarmed to 37°C, and were continued for various periods of time. Following incubation (100 cycles/min in a Dubnoff shaker bath), samples were withdrawn and the intestinal cell preparations were released into 5 ml of iced saline (0.9% w/v) containing 7 mM sodium taurocholate. Erythrocytes were released into iced saline. To determine the erythrocyte pellet volumes following the first centrifugation, calibrated conical centrifuge tubes were used for the cell washes. After centrifugation and aspiration of the supernatant, the cell material was again washed with 10 ml of the appropriate solution. Prior to extraction of the resultant pellets, a known amount of internal standard was added for quantitation. 7-Dehydrocholesteryl acetate was used as the standard in experiments with the 7-dehydro, calciferol, and 3-oxo,4-ene derivatives. In experiments with des-AB,8-one compounds, methyl heptadecanoate was the internal standard. Cell pellets were extracted with chloroform–methanol 2:1 as described earlier (5, 9). With the brush border preparations, protein determinations were carried out on the aqueous phase from the lipid extraction procedure using the biuret reaction as outlined by Hoffman and Kuksis (16). Total lipid extracts containing UV-absorbing steroids were assayed by reversed phase HPLC using a Supelco LC-18 column installed in a Hewlett-Packard (Model 5880) liquid chromatograph equipped with a variable wavelength detector. The sterols were eluted with isocratic methanol–acetonitrile 1:1 at a flow rate of 1.5 ml/min and an oven temperature of 30°C. A detector wavelength of 265 nm was used for the 7-dehydro and calciferol derivatives while the 3-oxo,4-ene compounds were monitored at 241 nm.

The des-AB,8-one analogues were assayed by GLC. The total lipid extracts were first evaporated to a small volume and applied to silica gel H plates. Following development with chloroform–methanol 100:1, bands corresponding to des-AB-cholestan-8-one and the methyl heptadecanoate were scraped off, recombined, and eluted with chloroform–methanol 2:1. The extracts were chromatographed on a 10 m SP-2230 capillary column (Supelco Inc., Bellefonte, PA) installed in a Hewlett-Packard (Model 5880) gas–liquid chromatograph as described by Myher and Kuksis (17). In this system, the des-AB,8-one analogues of cholesterol, campesterol, and sitosterol elute separately in the C_{20-22} fatty acid methyl ester region.

Other methods

Ultraviolet absorption spectra were obtained in ethanol using a Cary 1111 spectrophotometer, and in methanol–acetonitrile. using the variable wavelength detector of the liquid chromatograph. Electron impact GLC–MS of the sterols and sterol derivatives was done with a Hewlett-Packard (Model 5985) quadrupole mass spectrometer fitted with a short OV-1 column as described previously for bile acids (18). With the calciferol derivatives, separate spectra were obtained for the pyro- and isopyrocalciferol isomers generated by thermal isomerization (19) in the injector port.

RESULTS

Synthesis and characterization of the steroid derivatives

The structures of the steroid derivatives used in these experiments and their relationship to the parent, 5-ene sterols are shown in **Fig. 1.**

3-Oxo,4-ene steroids

The 3-oxo,4-ene structure was confirmed by GLC–MS, ultraviolet spectrophotometry, and HPLC on reversed phase columns. The oxidation of the hydroxyl

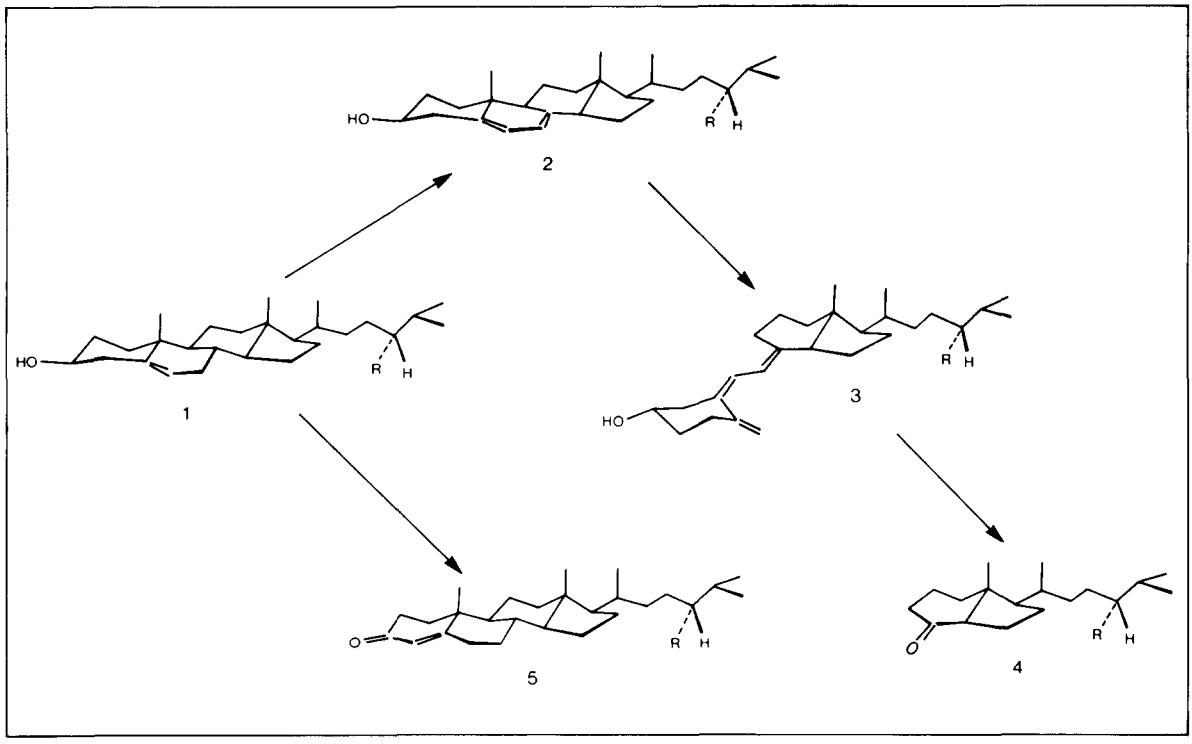

Fig. 1. Interconversions of sterols. *1*, 24-R, cholest-5-en-3-ol; *2*, 24-R, cholesta-5,7-dien-3-ol; *3*, 24-R, 9,10-seco-cholestan-3β-ol; *4*, 24-R, des-AB,cholestan-8-one; *5*, 24-R, cholest-4-en-3-one. Sterol interconversions were performed as described under Materials and Methods.

group was indicated by an increase in TLC mobility (R_f 0.3 to 0.75) using the solvent system chloroform–methanol 100:1. The R_f value of the synthetic compounds corresponded to that of authentic cholest-4-en-3-one. GLC–MS showed the presence of molecular ions at m/z 384, 398, and 412 in the electron impact spectra of the cholesterol, campesterol, and sitosterol oxidation products. The presence of the 3-oxo-4-ene unit in each compound was established by the UV absorption maximum at 241 nm, which was identical to that in the authentic cholest-4-en-3-one and as predicted by Woodward's rules (20).

The 27-, 28-, and 29-carbon derivatives were well resolved from each other by reversed phase HPLC using methanol–acetonitrile 1:1 as the eluting solvent. From the capacity factors in **Table 1,** it is evident that the 3-oxo, 4-ene derivatives are also well resolved from the parent, 5-ene sterols. A similar elution order for cholest-4-en-3-one and cholesterol has been reported (21) with acetonitrile–water 9:1 as the solvents. The detector response at 241 nm was found to be linear in the range 0.1–5 nmol and the detection limit was estimated to be about 50 pmol (20 ng).

7-Dehydrosterols

The synthesis and characterization of the 7-dehydro analogues of cholesterol, campesterol, and sitosterol have been previously described (4). The identification is based on the presence of the appropriate molecular ions (m/z 488, 502, 516) and the m/z 253 ion [M^+-side chain-H_2O] in the spectra of the benzoates of 7-dehydrocholesterol, 7-dehydrocampesterol, and 7-dehydrositosterol, respectively. The 5,7-diene system was established by the presence of characteristic ultraviolet absorption maxima (10) at 262, 271, 282, and 294 nm in the spectra of each. The spectra of the free sterols and benzoates of each compound were identical in every respect to those shown by Neville and DeLuca (10). The

TABLE 1. HPLC capacity factors for the steroid derivatives

Sterol Derivatives	k'[a]			
	Δ^5,3β-ol	3-one,4-ene	$\Delta^{5,7}$,3β-ol	Calciferol
C_{27}	8.35	6.94	6.50	4.84
C_{28}	9.22	7.92	7.37	5.45
C_{29}	10.37	8.97	8.33	6.20
C_{27}-acetate			10.09	

HPLC was performed on C_{18} reversed-phase columns using isocratic acetonitrile–methanol 1:1 as the developing solvent. The flow rate was 1–1.5 ml/minute. The detector wavelength was 265 nm for the 7-dehydro and calciferol derivatives, 241 nm for the 3-oxo,4-ene derivatives, and 210 nm for cholesterol, campesterol, and sitosterol.

[a] $k' = \frac{\text{elution volume (ml)} - \text{void volume (ml)}}{\text{void volume (ml)}}$.

absence of an absorption maximum at 236 nm confirmed that the 5,7-diene product was not significantly contaminated with the 4,6 isomer. The melting point of synthetic 7-dehydrocholesterol benzoate (141–143°C) compared closely with the benzoate prepared from authentic 7-dehydrocholesterol (138–140°C). The melting point of the 7-dehydro plant sterol benzoate mixture was 146–148°C.

As with the keto steroids, the 7-dehydro analogues of cholesterol, campesterol, and sitosterol were well separated from each other by HPLC (Table 1) and emerged from the column much earlier than the parent sterols. 7-Dehydrositosterol was found to overlap with cholesterol under these conditions. A resolution of 7-dehydrocholesterol from cholesterol, similar to that seen in Table 1, has been reported (22) using a C_{18} reversed phase column and 100% acetonitrile as the solvent. The detector response at 265 nm was found to be linear in the range 1–10 nmol with a lower detection limit of about 60 pmol/injection (about 25 ng).

Calciferol sterols

Conversion of the 7-dehydrosterols to the calciferol derivatives was carried out in about 25% yield using ultraviolet radiation centered at 300 nm. Following purification by TLC, single peaks were obtained for each of the three homologues by reversed phase HPLC. A clear doublet, representing pyrocalciferol and isopyrocalciferol formed in the injection port (19), was found for each homologue during GLC. Based on GLC peak areas, the purity of the compounds was estimated to be greater than 90%.

GLC–MS spectra of the 27-, 28-, and 29-carbon calciferol (pyrocalciferol) analogues were characterized by ions at 384, 398, and 412 $[M]^+$ and at 351, 365, and 379 $[M\text{-}H_2O\text{-}CH_3]^+$, respectively. The spectra of the underivatized compounds (**Fig. 2**) show that the fragmentation pattern is similar in each case but with increments of 14 mass units in the ions containing the side chain. The spectrum of the cholesterol analogue, vitamin D_3, is identical to that of a purified commercial

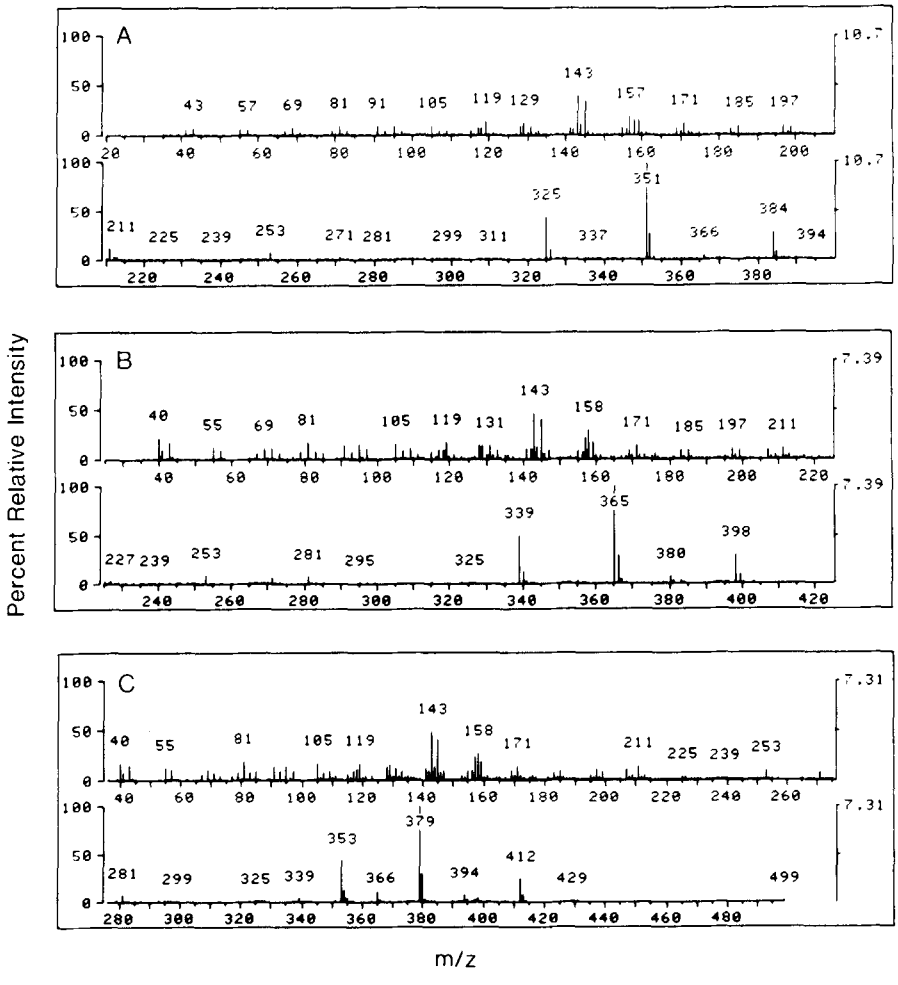

Fig. 2. Electron impact mass spectra of the calciferol analogues of cholesterol (A), campesterol (B), and sitosterol (C). GLC–MS spectra of the underivatized sterols were obtained as indicated under Methods.

product. The presence of an absorption maximum at 265 nm with an extinction coefficient identical to that of the commercial vitamin D_3 confirmed the 9,10-seco-5,7,10-triene structure in each case. The ultraviolet absorption spectra of the synthetic products were identical to those presented by Neville and DeLuca (10) and the similarity in the UV absorption spectra taken at several points during the elution of the calciferols from the HPLC indicated the homogeneity of the compounds. A separation of vitamin D compounds from precalciferol$_3$ and tachysterol$_3$ by C_{18} reversed phase HPLC has been previously reported (23) using methanol–isopropanol–water 20:4:1. The latter two side products were not found to be contaminants of the present synthetic preparations. Lumisterol formed during the irradiation is separated from the vitamin D at the TLC stage (24).

The calciferol derivatives were eluted much earlier from the reversed phase HPLC column than any of the other synthetic sterols (Table 1). The k′ value for the synthetic vitamin D_3 matched that of the authentic standard. Under the conditions used, the calciferol analogue of sitosterol was only partially resolved from 7-dehydrocholesterol.

Des-AB,8-one derivatives

The 9,10-seco-5,7,10(18)-triene structure of the calciferols was further confirmed by their conversion to compounds consistent with the des-AB,8-one structure (Fig. 1) following oxidation with permanganate–periodate. Purification by $AgNO_3$-TLC gave a product of better than 95% purity. The presence of a keto function was demonstrated by the formation of the semicarbazone. The semicarbazone of the cholesterol derivative had a melting point of 126–128°C after recrystallization from methanol–water. This is somewhat higher than that reported (25) for the same derivative of des-AB,cholestan-8-one formed by the ozonolysis of vitamin D_3 (mp 115–116°C, recrystallized from methanol).

As shown in **Fig. 3,** the electron impact spectra contained molecular ions of m/z 264, 278, and 292 for the cholesterol, campesterol, and sitosterol derivatives, re-

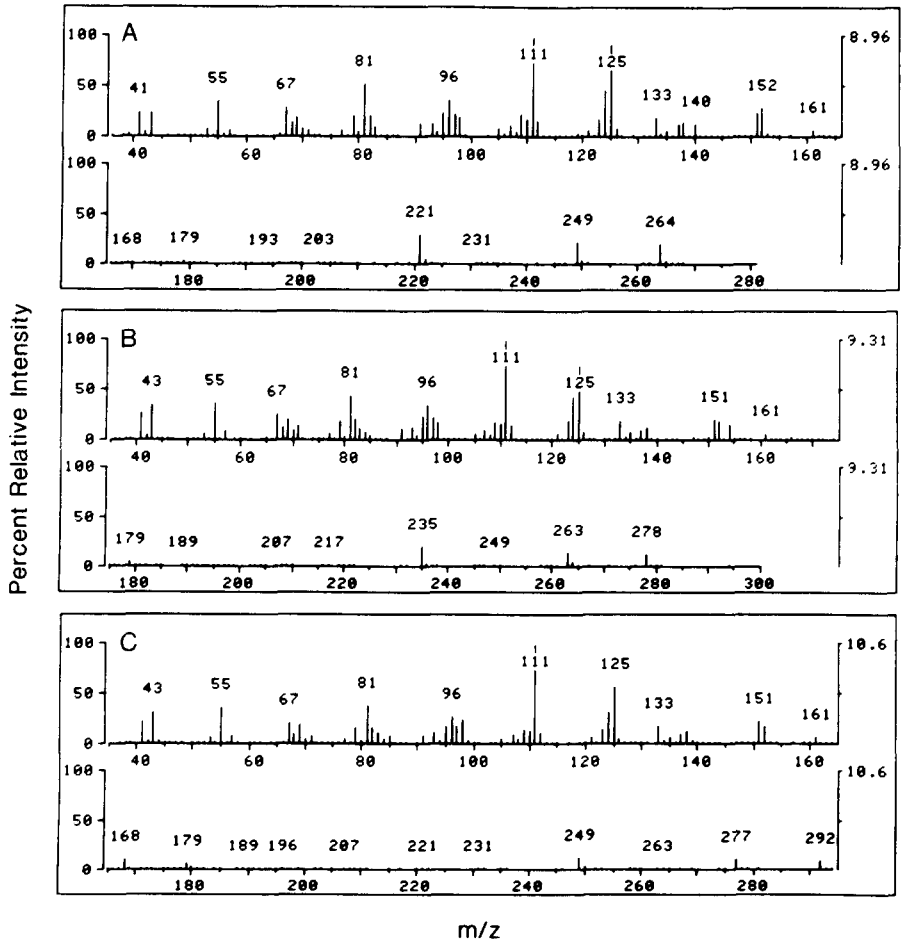

Fig. 3. Electron impact spectra of des-AB,8-one derivatives of cholesterol (A), campesterol (B), and sitosterol (C). GLC–MS spectra were obtained as indicated under Methods.

spectively. These molecular weights are anticipated for products containing the 8-one functional group and are different from those expected for products derived from the oxidation of isomers of vitamin D. Thus, using the permanganate–periodate treatment, the des-AB,8-one series was obtained with high purity and in about 40% yield from the calciferol starting materials.

Because of the low ultraviolet absorbance of the keto derivatives, the des-AB compounds were analyzed by capillary GLC. The profile of an approximately equimolar mixture of the three sterol analogues is illustrated in **Fig. 4.** The analogues were found to elute in the C_{20-22} fatty acid methyl ester region (17). It is evident from this figure that the peak representing the 28-carbon sterols is split. As the mass spectra of both peaks in the doublet are identical, it is likely that the split arises through a partial resolution of the C-24α, and C-24β-methyl derivatives which are known to be present in the C_{28} sterols from soybeans (26). Soybean sitosterol has been shown (26) to be exclusively the 24α-ethyl isomer.

Characterization of brush border and erythrocyte preparations

The enzymatic characterization of the brush border vesicles has been reported in detail (4). The distribution of the enzyme marker invertase was in essential agreement with that reported by Kessler et al. (13). A 20–25-fold enhancement in the specific activity of this enzyme indicated that the membrane fraction was predominantly of brush border origin but the presence of demonstrable monoacylglycerol acyltransferase activity suggested some contamination by endoplasmic reticulum. The absence of mitochondria was confirmed by transmission electron microscopy. By electron microscopy, the membranes were found to be closed vesicles with a uniform diameter of about 0.1 micron. The brush border membrane preparation consistently displayed a free cholesterol/phospholipid ratio of 1 or greater, a feature characteristic of brush border, but not of basolateral membranes (27). The brush border fraction was also found to contain a greater percentage of sphingomyelin than the total mucosal homogenate. This finding is also consistent with a preparation of plasma membrane origin.

Analysis of an extract of the brush border preparation by HPLC showed that the level of endogenous 7-dehydrocholesterol was less than 0.2 nmol/mg protein. This sterol was absent from the erythrocyte extracts. Vitamin D_3 or 3-oxo-4-ene steroids were not detected in either the brush border or the erythrocyte extracts. Analysis of the preparations by GLC showed that there were no endogenous des-AB,8-one derivatives in these membrane preparations.

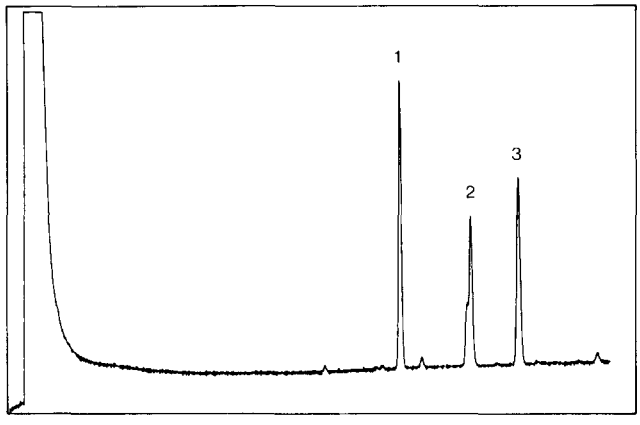

Fig. 4. Capillary GLC profile of a mixture of des-AB,8-one derivatives of cholesterol (1), campesterol (2), and sitosterol (3). The compounds were chromatographed on a 10 m SP-2330 column with temperature programming from 150 to 250°C.

Uptake of 7-dehydrosterols

The accumulation of the 7-dehydro derivatives of cholesterol, campesterol, and sitosterol was found to proceed linearly with time when the cell preparations were incubated in micellar solutions containing sodium taurocholate, egg phospholipid, and the sterols at levels ranging from 1–50 micromolar (4). In the erythrocyte, 50 nmol of 7-dehydrocholesterol was taken up in 30 min per ml of packed cells, while in the same time period 10–11 nmol was taken up per mg of brush border protein. In both membranes, the amount of 7-dehydrocholesterol incorporated represented 2–5% of the total membrane sterol. The linear sterol uptake in both cases was associated with a preferential absorption of 7-dehydrocholesterol over the 7-dehydro plant sterols. Following a 30-min incubation in an equimolar sterol solution, the ratio of 7-dehydrocholesterol/7-dehydrositosterol was 4.16 ± 0.41 ($n = 9$) in the erythrocytes and 3.72 ± 0.10 ($n = 3$) in the brush border membranes. The selective uptake is evident in **Fig. 5,** which gives the HPLC profiles of the lipid extracts of the two membranes and of the incubation medium. At all time points, the rate of uptake of 7-dehydrocampesterol was intermediate between that of the cholesterol and sitosterol analogues. The ratio of 7-dehydrocholesterol/7-dehydrositosterol increased with time in the erythrocyte and brush border preparations, as previously reported for the parent sterols: cholesterol and sitosterol (5). The degree of selectivity expressed by the membrane preparation was independent of the concentration of sterol in the medium. As can be seen in **Table 2,** there is a slight decrease in the ratio of 7-dehydrocholesterol/7-dehydrositosterol at the highest medium concentration, but the difference is minor.

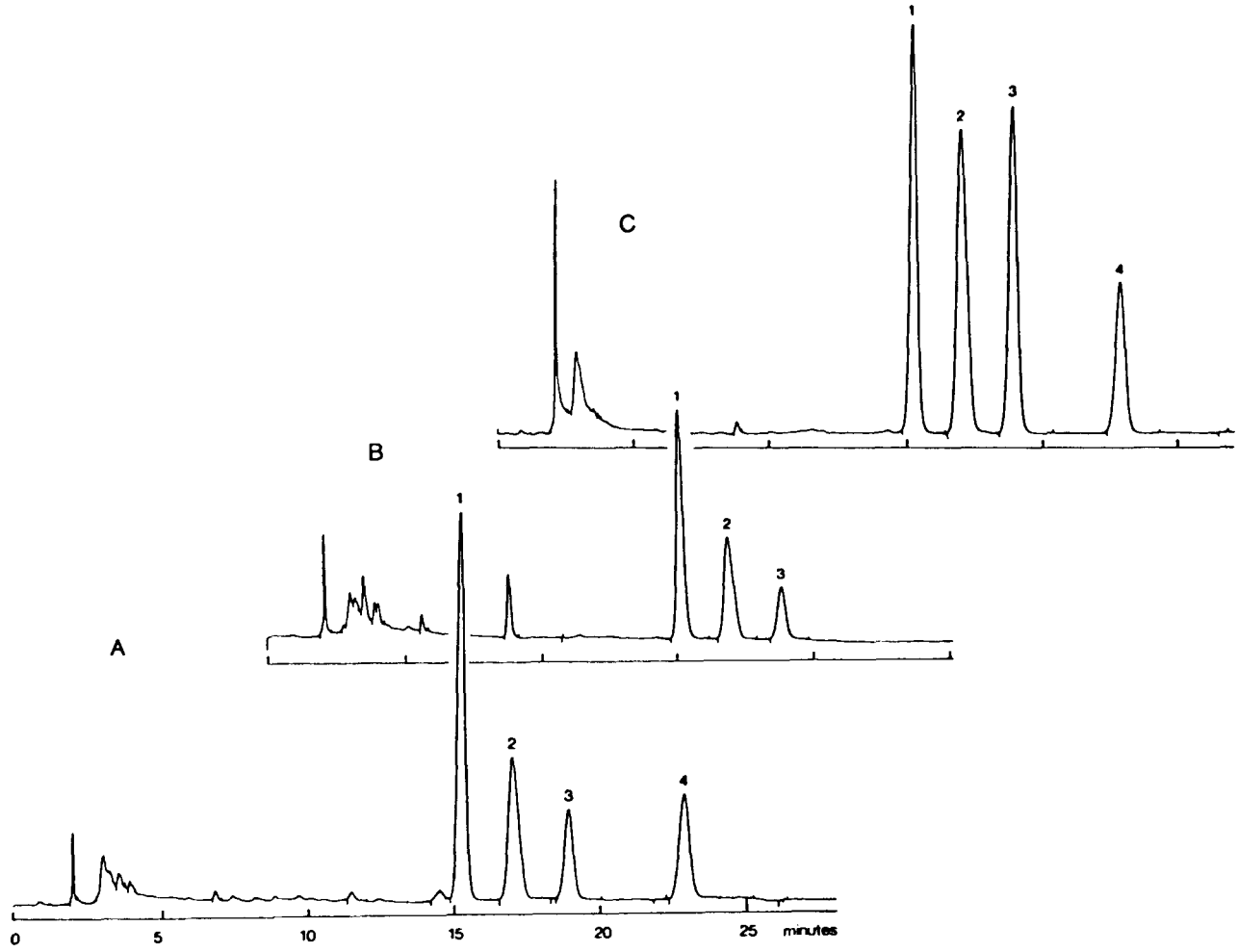

Fig. 5. HPLC profiles of total lipid extracts of brush border and erythrocyte membranes following incubation with 7-dehydrosterols in a mixed micellar medium. A, brush border extract; B, erythrocyte extract; C, extract of medium prior to incubation. Peak 1, 7-dehydrocholesterol; peak 2, 7-dehydrocampesterol; peak 3, 7-dehydrositosterol; peak 4, 7-dehydrocholesterol acetate (internal standard). Brush borders and erythrocytes were incubated for 30 min at 37°C in a medium containing 6.6 mM sodium taurocholate, 0.6 mM egg PC, and 30 μM each of the 7-dehydrosterols. HPLC was performed as described under Methods.

Uptake of calciferol derivatives

A preference for the side chain of cholesterol was also observed during the uptake of the calciferols, but, as shown in **Fig. 6,** the selectivity was greatly reduced. The ratio of C_{27}/C_{29} sterols was 1.88 ± 0.20 in the erythrocytes and 1.38 ± 0.08 in the brush borders following a 30-min incubation. Because of some erythro-

TABLE 2. Uptake and relative ratios of 7-dehydrosterols in rat erythrocytes

Media Concentration[a]	Cellular Uptake[b]			Uptake Ratio[c]	
	C_{27}	C_{28}	C_{29}	C_{27}/C_{29}	C_{28}/C_{29}
1.2	4.45 (0.44)	2.54	0.94	4.51 (0.31)	2.31 (0.20)
15	49 (6)	25 (3)	10 (2)	4.48 (0.26)	2.35 (0.13)
32	58 (13)	38 (8)	17 (4)	3.94 (0.16)	2.22 (0.10)

Rat erythrocytes were incubated for 30 min at 37°C in micellar media containing 6.6 mM taurocholate, 0.6 mM egg yolk PC, and the 7-dehydro derivatives of cholesterol (C_{27}), campesterol (C_{28}), and sitosterol (C_{29}) in the concentrations indicated. The cellular content of sterols was measured by HPLC as described in Methods and Materials. The values are expressed as the mean (standard deviation in parentheses) for three or more replicates.

[a] Media concentrations expressed in micromolar units for each sterol.

[b] Cellular uptake expressed as nmol sterol/ml packed cells.

[c] Ratios are adjusted for small deviations in the media ratio from unity.

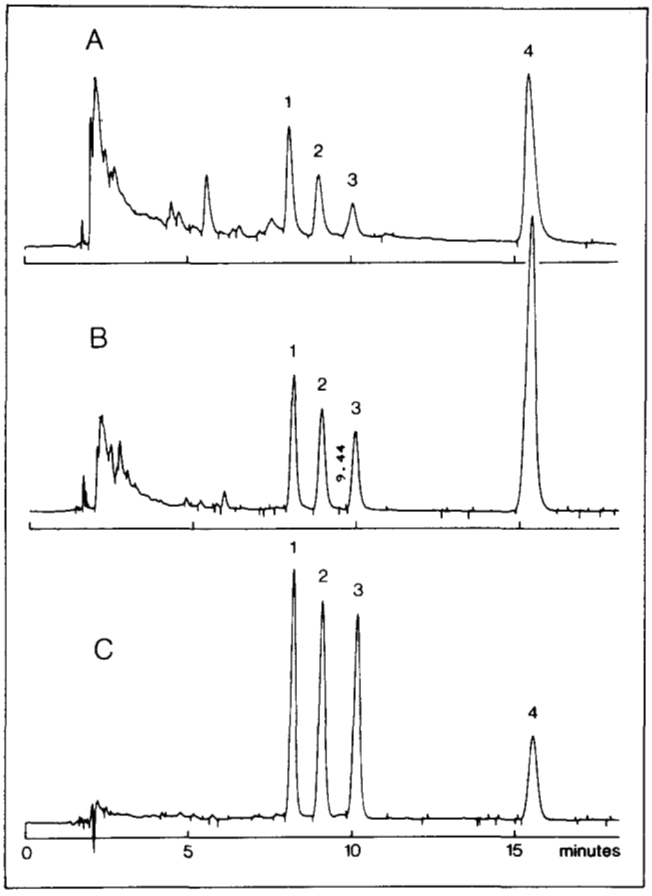

Fig. 6. HPLC profiles of brush border and erythrocyte extracts following incubation with the calciferol derivatives. A, extract of erythrocytes; B, extract of brush borders; C, extract of incubation medium. Peak 1, cholesterol analogue; peak 2, campesterol analogue; peak 3, sitosterol analogue; peak 4, 7-dehydrocholesteryl acetate (internal standard). Brush borders and erythrocytes were incubated for 30 min at 37°C in a medium containing 6.6 mM sodium taurocholate, 0.6 mM egg PC, and 20 μM each of the calciferols. HPLC conditions as given under Methods.

cyte lysis during incubations with the calciferol derivatives, it is possible that the reduced selectivity was due to a disruption of the erythrocyte and possibly brush border membranes. To examine this possibility, sterol uptake in the erythrocytes was monitored with time (**Fig. 7**). The uptake of the three sterol homologues is clearly nonlinear (20 μM sterol; 0 to 30 min) and the cellular C_{27}/C_{29} ratio decreases with time from 2.7 after 10 min of incubation to 1.9 at 30 min. This contrasts with the results of the 7-dehydrosterol experiments and is consistent with a gradual loss of the selectivity with time. The degree of selection expressed in the erythrocytes was not further influenced by an increased sterol concentration in the medium, although the rate of absorption increased proportionally. At all time points, the rate of uptake of the calciferol derivatives was 5–10 times less than that of the 7-dehydrosterols.

Uptake of des-AB,8-one derivatives

The GLC profiles of the des-AB,8-one derivatives absorbed by the erythrocytes and brush border membranes are shown in **Fig. 8.** The membrane preparations were unable to distinguish between these side-chain homologues and there was no apparent difference in the ratio of the peaks of the "campesterol" doublet following uptake. The rate of uptake of des-AB-cholestan,8-one was closely similar to that of 7-dehydrocholesterol in both membrane types.

Uptake of 3-oxo,4-ene derivatives

To ensure that the absence of the hydroxyl group in the des-AB compounds was not alone responsible for the lack of selectivity during uptake, erythrocytes were incubated with 3-oxo,4-ene steroids, which also lack a hydroxyl group. The corresponding HPLC profiles are presented in **Fig. 9.** The ratio of absorbed C_{27}/C_{29} steroid reached 1.81 ± 0.07 following a 30-min incubation. This level of selectivity, although less than that achieved with the 7-dehydrosterols, is in close agreement with the ratio found for the calciferols under similar conditions. The rate of uptake of cholest-4-en-3-one was similar to that of 7-dehydrocholesterol but greater than that of vitamin D_3. A summary of the uptake ratios obtained in erythrocytes and brush border membranes for each sterol type is presented in **Fig. 10.**

DISCUSSION

Synthesis and analysis of steroid derivatives

The 7-dehydro derivatives of cholesterol, campesterol, and sitosterol were chosen as the starting material

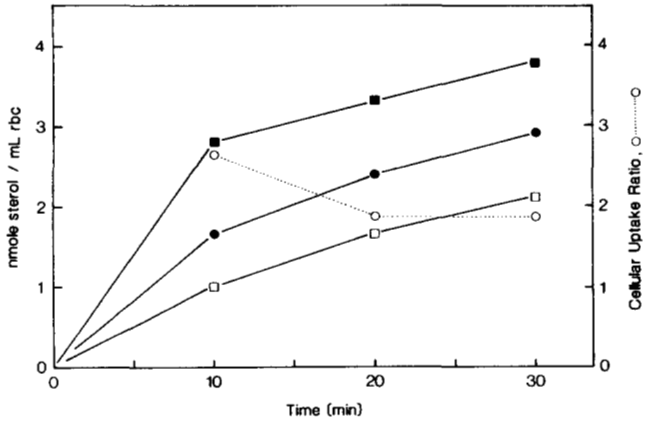

Fig. 7. Time course of uptake of the calciferol derivatives by erythrocytes. (■ —— ■), cholesterol analogue; (● —— ●), campesterol derivative; (□ —— □), sitosterol derivative; (○ · · · · ○), cellular ratio of C_{27} and C_{29} sterols. Data points represent means of three determinations. Incubation conditions as given in Fig. 6. Analytical conditions as given under Methods.

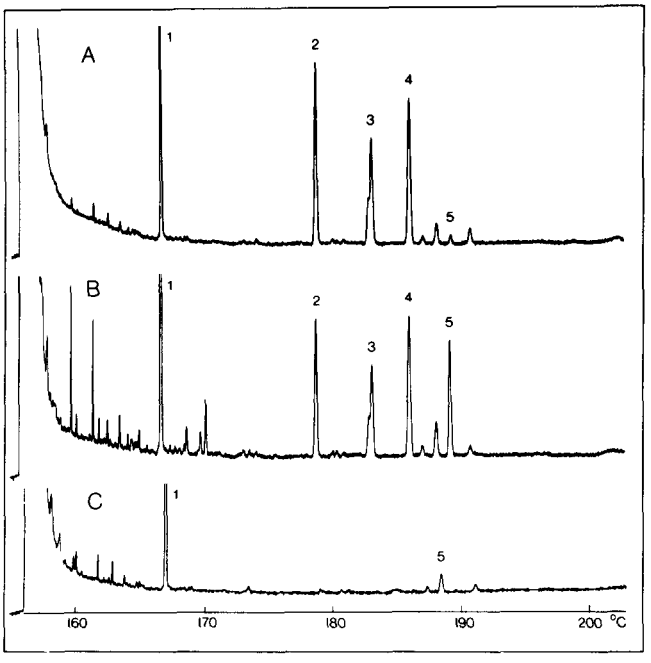

Fig. 8. Capillary GLC profiles of extracts of brush border and erythrocyte membranes following incubation with des-AB,8-one derivatives, A, erythrocytes; B, brush border membranes; C, brush border membranes before incubation. Peak 1, methyl heptadecanoate (internal standard); peak 2, cholesterol analogue; peak 3, campesterol analogue; peak 4, sitosterol analogue; peak 5, dibutyl phthalate (contaminant). Brush border vesicles and erythrocytes were incubated for 30 min at 37°C in a medium containing 6.6 mM sodium taurocholate, 0.6 mM egg PC, and the analogues of cholesterol (32 mM), campesterol (22 mM), and sitosterol (25 mM). The composition of the steroid mixture in the medium is illustrated in Fig. 4.

and reference point for several reasons. First, the use of nonendogenous sterols in the absorptive studies allowed a comparison of uptake of a homologous series of molecules without interference from endogenous pools of cholesterol. Second, the 7-dehydrosterols are the known precursors of ring B-disrupted calciferol derivatives which possess several sites for further oxidative cleavage. The ease of these chemical conversions allowed the preparation of products of greater than 90% purity in each case. Third, the strong ultraviolet absorbance of the derivatives, except for the des-AB,8-one series, allowed a sensitive assay of their uptake by HPLC, without prior isolation of the steroids. The des-AB compounds were analyzed by capillary GLC and required preliminary isolation.

In all cases, the three members of the homologous series were well resolved from each other. The "campesterol" peak was noticeably broader than those of the sitosterol or cholesterol derivatives in the HPLC profiles and a definite splitting was observed in the capillary GLC profiles of the des-AB compounds. Soybean campesterol is known to be composed of both epimers of 24-methylcholesterol (26) and the peak broadening is likely a result of their resolution. Based on earlier work (28), the front shoulder of the peak may be expected to be the 24β-epimer, and the trailing shoulder the 24α-methyl steroid. We were not able to resolve the epimers of the 24-methyl-7-dehydrosterols well enough to discern a preferential absorption of either isomer in this series.

Uptake of steroid derivatives by brush border membranes and erythrocytes

The greatest degree of selectivity was observed between 7-dehydrocholesterol and 7-dehydrositosterol in both membrane preparations. These derivatives closely mimic the parent, 5-ene sterols in rate of uptake and degree of selectivity. This is in keeping with the minor influence of the additional double bond on the overall shape of the molecule (29) and is also to be anticipated from the similar behavior of cholesterol and 7-dehydrocholesterol in liposomes of egg phospholipid, as determined by electron spin resonance (ESR) (30), and from the ability of the sterols to reduce the permeability of the bilayer to water-soluble markers (31, 32). The cellular ratio of C_{27}/C_{29}-sterol increased with time of absorption in this series and was not significantly altered by variations in the concentration of sterol in the medium. This reflects a continued selective partition of 7-dehydrocholesterol into the membrane and suggests that its uptake was not inhibited by accumulations of 7-dehydrositosterol in the cell or vesicle membranes. This was not the case in the calciferol series. With latter compounds, the C_{27}/C_{29} ratio in erythrocytes decreased with time as a result of a nonlinear uptake of the cholesterol analogue (vitamin D_3). This is consistent with an inhibition of absorption by the sitosterol analogue, but the absence of a concentration effect does not support this hypothesis. Disruption of the membranes by the vitamin D derivatives is an alternate explanation for the shape of the time-course curve. A disordering effect of vitamin D_3 on phospholipid bilayers has been detected by ESR using spin-labeled fatty acyl and cholestane probes (31) and, in contrast to most other sterols, vitamin D_3 increases the permeability of liposomes of egg lecithin to water-soluble solutes (32). It is not clear, therefore, whether the reduced selectivity in the microvillar vesicles compared to the erythrocytes is due to an increased sensitivity to the disruptive influence of vitamin D or to a greater susceptibility to an inhibitory influence of the sitosterol analogue.

It is of interest in this regard that the low rate of uptake of vitamin D_3 was the only marked deviation from the mean rate of absorption of the other cholesterol analogues in these experiments. In both brush borders and red blood cells, 7-dehydrocholesterol, cholest-4-en-3-one, and des-AB-cholestan-8-one were ab-

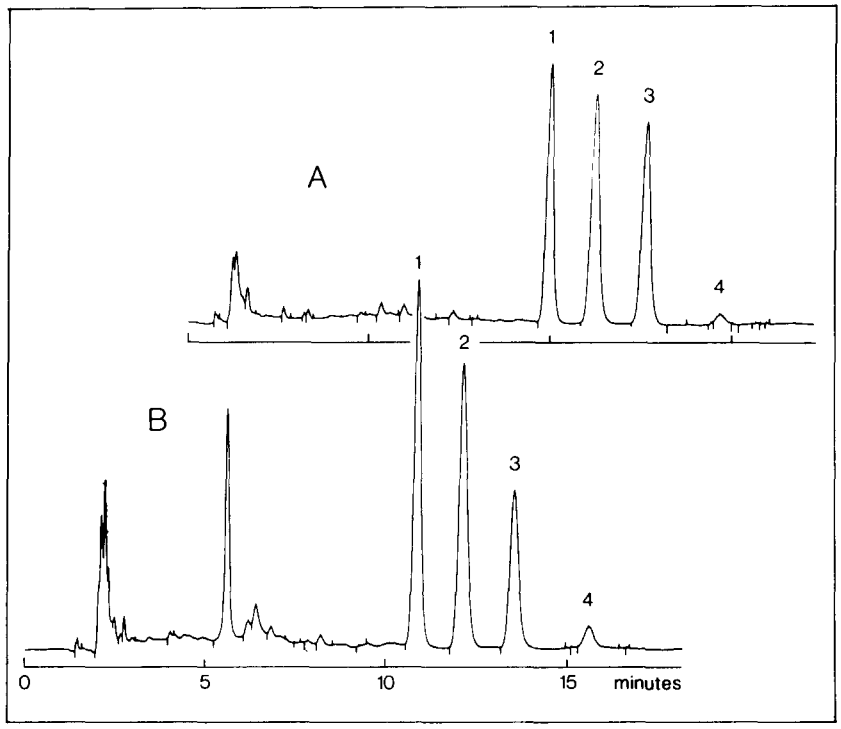

Fig. 9. HPLC profiles of erythrocyte membranes following incubation with the 3-oxo-4-ene derivatives. A, incubation medium; B, erythrocytes. Peak 1, cholesterol derivative; peak 2; campesterol derivative; peak 3, sitosterol derivative; peak 4, 7-dehydrocholesteryl acetate (internal standard). The erythrocytes were incubated for 30 min in a medium containing 6.6 mM sodium taurocholate, 0.6 mM egg PC, and the 3-oxo-4-ene analogues (35 μM each). Other conditions of incubation and of analysis are given under Methods.

sorbed at similar rates. The lesser rate of uptake in the calciferol series may reflect a shift in the distribution of the sterol between the aqueous and micellar phases. The greater solubility of the D vitamins in polar solvents (33) and a reduced interaction with phospholipid compared to sterols with an intact ring B, may cause them to prefer the aqueous phase, and thus reduce the partition into the membrane. The initial absorption of vitamin D_3 has been reported to be less than cholesterol (34) following the installation of micellar solution of the two sterols into the duodenum of rat. The conditions of uptake were not identical for the two sterols, however, as cholesterol was administered as a tracer to label endogenous sterol, and the radioactive vitamin was given with 5 nmol of nonradioactive product. In another study, the uptake of the two sterols was comparable in rats with lymph cannulae (35).

Despite the reduced rates of uptake in the calciferol series, the selective recognition of the side chain of cholesterol was retained, although it was not as dramatic as with the 7-dehydrosterols. Studies of calciferol derivatives by nuclear magnetic resonance (36) have shown that these molecules assume an extended 6(7)-transconformation in solution (shown in Fig. 1). Thus, although a contribution of the 6(7)-*cis* conformer cannot be ignored, a planar, rigid ring system is apparently not essential for the influence of the side chain to be expressed during absorption. Selectivity between the analogues of cholesterol and the plant sterols was also retained in the 3-oxo,4-ene series. Unlike the calciferol derivatives, these compounds retain a planar ring structure and X-ray diffraction studies of androst-4-en-3-one and androst-5-en-3β-ol have shown a close conformational similarity between the two structures in the crystalline state (29).

The absorptive influence of the side chain of the plant sterols apparently does require a 3- or 4-ring system for its expression. This was indicated by the loss of selectivity following removal of rings A and B of the steroid nucleus. It is unlikely that this is due to any radical differences in the mode of absorption as the rates of uptake in the des AB,8-one series were similar to those of the 7-dehydro and 3-oxo,4-ene steroids. The possibility of their disruptive influence on the erythrocyte or brush border membranes must also be considered, although no lysis of the red blood cells was detected in the presence of these derivatives. The absence of a hydroxyl group in the des-AB,8-one series is unlikely to be a major influence in view of the results with the keto steroids discussed earlier. Thus, it appears that a ring

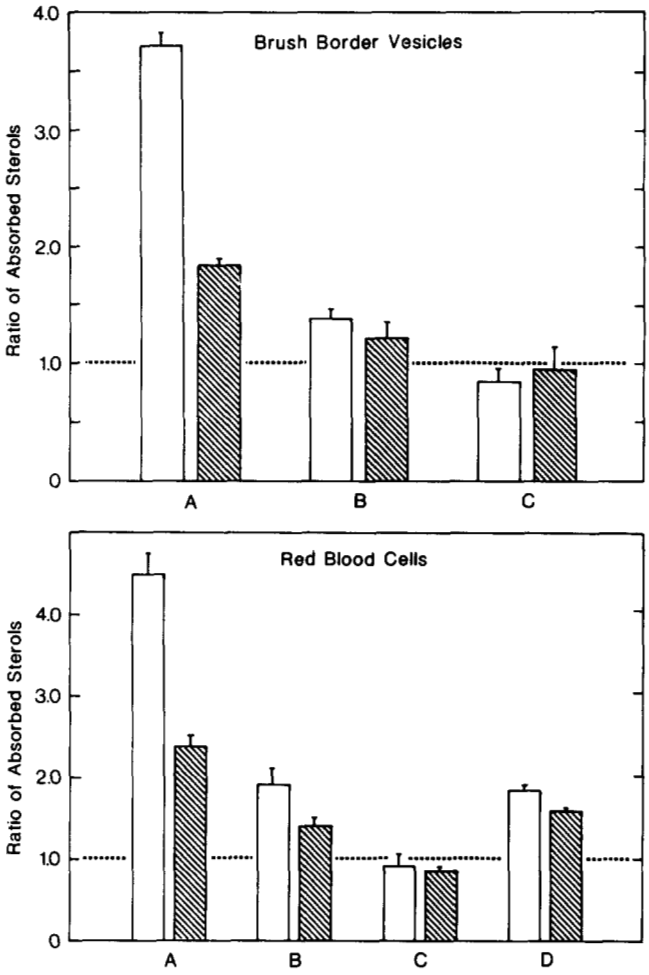

Fig. 10. Comparison of uptake ratios of sterol derivatives in brush border membranes and erythrocytes. Clear bars (▭) refer to the ratio of the cholesterol and sitosterol analogues and the hatched bars (▨) refer to the ratio of the campesterol and sitosterol analogues in the membranes, after 30 min of incubation, in the 7-dehydro (A), calciferol (B), des-AB,8-one (C), and the 3-oxo-4-ene (D) series. The values represent mean ± SD of three or more determinations.

system having the bulk of the cholestane nucleus is required for the differences in the side chain to exert their effect, but a rigid ring B or a 3β-hydroxyl group is not essential. With these structural determinants in mind, it is useful to consider absorptive mechanisms that are consistent with these data.

Mechanism of absorption

The absorption of sterols by the intestine has the characteristics of passive diffusion and is generally considered to involve an initial partition of sterol monomers into the brush border membrane (37, 38). Sylvén (39), Barton and Glover (40), and Barton et al. (41) have suggested that the absorptive discrimination against the plant sterols is the result of an impaired interaction with the acyl chains of phospholipid in this membrane, or others within the intestinal mucosa. It is unlikely that a selective interaction of this type occurs while the sterol is oriented in the bilayer in a fashion similar to that of endogenous cholesterol, since a rigid ring system and a 3β-hydroxyl group are not essential to the selectivity described in this report. These structural features are thought to align cholesterol in the bilayer and to promote the optimal condensing interaction between sterol and phospholipid (31, 32, 42, 43). A selection for cholesterol occurring in the incubation medium, on the other hand, appears to be ruled out by the dependence of the absorptive preference on the nature of the host membrane (4). The pertinent interactions leading to the recognition of the cholesterol molecule must, therefore, occur during partition of the sterols into the membrane, but before they become functionally incorporated into the bilayer.

The passage of the plant sterols through the acyl chains of the outer hemi-leaflet may be restricted, compared to cholesterol, as a result of the additional alkyl groups in their side chains. On steric grounds, the influence of the additional branch points in the side chains of the plant sterols would be expected to be greatest in regions of high acyl chain order. The first 10–12 carbon atoms of the phospholipid acyl chains in a membrane leaflet exhibit the highest degree of order in the membranes examined so far (44) and this region, occupied by the rigid ring system of endogenous cholesterol, is an area through which the side chains of the incoming sterols must pass during their absorption. It is therefore a likely candidate to bring about the recognition of the differences in the bulk and conformation of the cholesterol and plant sterol side chains.

To be consistent with the present findings, the absorptive mechanism must take into account the requirement for a complete ring system in the recognition process. This condition may be met if it is postulated that the 4-membered ring system normally prevents the sitosterol molecule from re-orienting within the membrane to relieve the steric interference caused by the additional ethyl group in the side chain. The truncated ring system of the des-AB,8-one series may allow the bulky side chain to be readily accepted. In fact, the 4-membered ring system may actually cause the sterol to enter the membrane via its side chain. This mode of entry would be consistent with the wedge-like shape of cholesterol (see space-filling model in ref. 42) and would cause the less favorable or disruptive interactions involving the plant sterol side chains to be sensed by the membrane before the bulk of the molecule enters. It would appear unlikely that a branch point in the side chain would have much influence on a molecule already embedded hydroxyl end-first.

We therefore suggest that the differential absorption

of the sterols from micellar solutions occurs as a result of the interaction of the side chains of incoming monomeric sterols with the acyl chains of membrane phospholipids. While this mechanism is highly speculative, it provides a basis for further experimentation to clarify the events underlying the phenomenon of selective sterol uptake and the absorption of cholesterol itself.

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