Uptake of 7-dehydro derivatives of cholesterol, campesterol, and β-sitosterol by rat erythrocytes, jejunal villus cells, and brush border membranes

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Abstract We have determined the effect of the side chain on the uptake of sterols from micellar solutions by isolated rat jejunal villus cells, brush border membranes, and erythrocytes. From an equimolar mixture of 7-dehydrosterols, the uptake decreased with an increasing number of carbon atoms at C_{24} of the sterol side chain in a manner identical to that observed for the parent-Δ^5-sterols. The brush border and erythrocyte membranes were found to absorb 4–5 times more 7-dehydrocholesterol than 7-dehydro-β-sitosterol over a 60-min period of incubation. A somewhat lower specificity of sterol uptake by the villus cells was attributed to the exposure of large areas of the basolateral membrane, which apparently was less able to discriminate between sterols. The higher sterol selectivity was associated with higher membrane organization anticipated from the higher free cholesterol/phospholipid and protein/phospholipid ratios of the brush border and red blood cell membrane. On a mass basis the villus cells and brush borders absorbed 30–60 times more sterol than the erythrocytes. Assuming that the $\Delta^{5,7}$-sterols accurately represent the absorption of the Δ^5 parent sterols, it is suggested that the 3- to 5-fold excess of absorbed cholesterol over β-sitosterol that is typically found in the jejunal wall of the rat following feeding of radioactive sterols arises from an inability of β-sitosterol to enter the brush border membrane as easily as cholesterol.—**Child, P., and A. Kuksis.** Uptake of 7-dehydro derivatives of cholesterol, campesterol, and β-sitosterol by rat erythrocytes, jejunal villus cells, and brush border membranes. *J. Lipid Res.* 1983. **24:** 552–565.

Supplementary key words HPLC • membranes • mixed micelles • phospholipids • bile salts

The plant sterols, campesterol and β-sitosterol, are not absorbed as readily as cholesterol by the mammalian intestine (1–7). The components and interactions responsible for this absorptive discrimination are unknown, but it has been suggested (1,6–8) that it occurs during the initial entry of the sterols into the intestinal wall. To determine whether intestinal villus cells or their brush border membranes are indeed capable of an absorptive recognition of cholesterol, it is necessary to provide a direct demonstration in vitro, where the process may be studied without interference from events occurring in the intestinal lumen, subsequent metabolism, or variations between animals.

Study of the absorptive process in vitro has, until recently, been hampered by the failure of several well-characterized intestinal tissue preparations (7, 9, 10) to distinguish between cholesterol and β-sitosterol during their uptake from solutions containing physiological concentrations of bile salts. We have found that the addition of egg yolk phospholipid to similar micellar mixtures allows a preferential uptake of cholesterol (over β-sitosterol) to occur in isolated jejunal villus cells (11) and erythrocytes (12) from the rat. Phospholipid was shown to reduce the damage to the cell membranes induced by the bile salts (12). These preliminary findings enabled us to perform a direct examination of the ability of purified brush border membranes to recognize differences in the structure of the sterol side chain during absorption from micellar solutions containing bile salt.

This study compares the behavior of the brush border membranes to that of isolated villus cells and rat erythrocytes. We have also taken advantage of the ultraviolet absorbance of the 7-dehydro analogues of cholesterol, campesterol, and β-sitosterol to allow a simultaneous assessment of the uptake of the three sterols by reversed-phase HPLC. The brush border membrane vesicles were markedly superior to the villus cells in selectively absorbing 7-dehydrocholesterol, and displayed a level of selectivity comparable to that found with the parent sterols in the intestinal wall following feeding in vivo.

Abbreviations: 7-dehydrocholesterol, 5,7-cholestadien-3β-ol; 7-dehydrocampesterol, 5,7-cholestadien-24 methyl-3β-ol; 7-dehydro-β-sitosterol, 5,7-cholestadien-24α ethyl-3β-ol; LC–MS, combined liquid chromatography–mass spectrometry; GLC–MS, combined gas–liquid chromatography–mass spectrometry; GLC, gas–liquid chromatography; TMS, trimethylsilyl; HPLC, high pressure liquid chromatography; HEPES, N-2-hydroxyethylpiperazin-N′-2-ethanesulfonic acid; TLC, thin-layer chromatography; HBSS, Hank's balanced salt solution.

MATERIALS AND METHODS

Egg yolk phospholipid was purchased from Sigma Chemical Co. (St. Louis, MO). It was fractionated by TLC to yield separate fractions for phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, and sphingomyelin, which were recombined to give approximately the lipid class ratios found in the host membranes (60% phosphatidylcholine; 40% phosphatidylethanolamine). Reference 7-dehydrocholesterol acetate was obtained from Steraloids Co. (Wilton, NH). All biochemicals and cell incubation supplies were as previously reported (10, 13). The plant sterols were recrystallized from a crude soy sterol mixture supplied by Distillation Products Co. (Rochester, NY) as described (10). [22,23-^{3}H]-β-Sitosterol was purchased from Amersham/Searle Corporation (Don Mills, Ontario) and was purified by TLC before use (10).

Preparation of 7-dehydrocholesterol, 7-dehydrocampesterol, and 7-dehydro-β-sitosterol

The 7-dehydrocampesterol and 7-dehydro-β-sitosterol were prepared simultaneously from a 1:1 mixture of the parent sterols obtained by the acetone recrystallization. Following conversion of the precursor cholesterol and plant sterols to the benzoate esters, the additional double bond was introduced according to the dibromantin/trimethylphosphite method of Blunt and DeLuca (14). The free sterols were regenerated from the benzoates by brief saponification in 0.5 N KOH methanol under a nitrogen atmosphere. The sterols were subsequently stored in ethanol under nitrogen and were purified by TLC before use.

Isolation of mucosal cells

Upper jejunal villus cells were isolated from the small intestine of chow-fed, male Wistar rats (250–350 g) using the hyaluronidase dispersion technique of Hoffman and Kuksis (13). The isolated cells (about 30 mg of cell protein per animal) were suspended in 10 ml Hank's balanced salt solution containing 15 mM HEPES buffer, 5 mM EGTA, and 4% fatty acid-free bovine serum albumin complexed with oleic acid (4 moles per mole of protein). The cells were kept on ice until used. All incubation buffers were well gassed with 95% O_2–5% CO_2 before use and the pH was adjusted to 7.4. Cells were prepared separately from each animal and were not pooled.

Isolation of brush border vesicles

Intestinal brush border vesicles were prepared from fresh rat jejunal scrapings using the method of Kessler et al. (15). For use in the sterol uptake studies, the final brush border pellet was resuspended in a medium identical to the villus cell storage buffer described above.

Isolation of erythrocytes

Fresh blood from male Wistar rats (250–350 g) was treated with 4 mM EDTA. The cells were pelleted immediately at 1200 *g* for 10 min and the supernatant and buffy coat were removed by aspiration. The cells were then washed three times with five volumes of Hank's balanced salt solution containing 15 mM HEPES buffer (phenol red omitted to allow detection of hemolysis) at room temperature. The cells were made up to 30% hematocrit in the same buffer and were used within 2 hr.

Enzyme assays

For the enzyme assays, a sample of the above brush border suspension was pelleted and resuspended in a buffer (pH 7.4) containing 2 mM Tris-HCl and 50 mM mannitol. The total homogenate was suspended in the same buffer. Sucrase activity was determined by the glucose oxidase method of Huebscher, West, and Brindley (16) and alkaline phosphatase by the method of Weiser (17). The assay for succinate dehydrogenase was carried out according to the method of Pennington (18). Monoacylglycerol acyltransferase activity was assayed as described by Manganaro and Kuksis (19).

Electron microscopy

For examination by electron microscopy, the brush border membrane preparation was centrifuged in a small polypropylene tube at 27,000 *g* for 30 min and the pellet was fixed in 2% glutaraldehyde–1% formaldehyde in sodium cacodylate buffer. After fixation for 60 min at room temperature, the pellet was extensively washed and stained with OsO_4. Dehydration was carried out with a graded series of ethanol solutions and the resultant pellets were imbedded in EPON blocks. Slices of the blocks were stained with uranyl acetate prior to microscopy.

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 egg yolk phospholipid, and 30–150 μM sterol in the desired volume. For experiments requiring the presence of radioactive β-sitosterol, [22,23-^{3}H]-β-sitosterol was added to the unlabeled material giving a specific radioactivity of 2.7×10^6 dpm/μmol. Following the evaporation of the solvents under nitrogen, the required volume of HBSS + HEPES was added and the suspension was filtered through a Milex filter disc (0.2 μm pore diameter, Millipore Corp., Mississauga, Ontario) attached to a disposable syringe under light

plunger pressure. All solutions were gassed before use as described for the cell preparations, and the pH was adjusted to 7.4. The mixed micellar solutions were water-clear in appearance. The proportions of the neutral lipids in the final filtered solution were assessed following total lipid extraction by GLC using the TMS derivatives (20). The actual compositions of the solutions are indicated in the legends to the figures.

Measurements of sterol uptake

To compare sterol absorption in different cell and membrane types, jejunal villus cells, erythrocytes, and purified brush border membranes were incubated at 37°C in a micellar medium containing sodium taurocholate, egg yolk phospholipid, and 40 μM each of 7-dehydrocholesterol, 7-dehydrocampesterol, and 7-dehydrositosterol. To compare the uptake of the 7-dehydrosterols and their Δ^5 parent sterols, rat erythrocytes were incubated in media containing taurocholate and phospholipid as above, and either *1*) 30 μM each of campesterol and sitosterol, *2*) 30 μM each of campesterol, sitosterol, and their 7-dehydro analogues, *3*) 30 μM each of 7-dehydrocholesterol, 7-dehydrocampesterol, and 7-dehydrositosterol, or *4*) 30 μM each of the three 7-dehydrosterols plus 50 μM cholesterol.

Incubations were initiated by the addition of 1 ml of the cell or membrane suspension (2–3 mg of protein) to 4 ml of micellar solution prewarmed to 37°C. Following incubation (100 cycles per min in a Dubnoff shaker-bath), samples were withdrawn with an automatic pipette. The samples containing intestinal preparations were released into 5 ml of iced saline (0.9% w/v) containing 7 mM sodium taurocholate. Because of the lysis of erythrocytes under these conditions (12), the latter were released into iced saline. To determine the erythrocyte pellet volumes following the first centrifugation (1200 *g* for 5 min), calibrated 15-ml 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 same solution. Prior to extraction of the resultant pellets, a known amount of 7-dehydrocholesterol acetate was added as an internal standard for HPLC quantitation. Cell pellets were extracted by the Folch method as previously described (10). Protein determinations were carried out on the remaining (Folch) aqueous phase using the biuret reaction as outlined by Hoffman and Kuksis (13). Neutral and phospholipid fractions were isolated from the organic extracts by TLC on silica gel H plates developed with chloroform–methanol–acetic acid–water 100:45:20:7 (10). The neutral lipids running with the solvent front were eluted from the gel quantitatively with a 1:1 mixture of ether–pentane (13).

This fraction, containing UV-absorbing sterols, was dissolved in methanol–acetonitrile 1:1 and chromatographed on a C_{18}-reversed phase HPLC column (5 μm particle size, 25 × 0.4 cm I.D., Supelco #5-8298) installed in a Hewlett-Packard Model 5880 liquid chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a variable wavelength detector. The sterols, monitored at 265 nm, were eluted with isocratic methanol–acetonitrile 1:1 at a flow rate of 1 ml/min and an oven temperature of 30°C. In some cases, total lipid extracts were analyzed by HPLC. Unless otherwise indicated, the profiles depicted in the figures are those of the neutral lipid fraction. In some instances the neutral lipid fraction was also analyzed as the TMS derivative by GLC on a 10-m methyl siloxane capillary column using the conditions described by Kuksis et al. (20).

Analytical methods

Quantitative analyses of phospholipids in the total lipid extracts were carried out following TLC using the solvent system described above. The phospholipid classes were quantitated by determination of phosphorus following digestion with H_2SO_4–$HClO_4$ 2:1 (21). Electron impact capillary GLC/MS of the benzoylated 7-dehydrosterols were carried out as reported for bile acids (22). Chemical ionization LC/MS was performed on the free 7-dehydrosterols using the liquid chromatograph interfaced (LC-MS liquid inlet interface, Hewlett-Packard part No. 05985-60216) with a Hewlett-Packard quadrupole mass spectrometer (Model 5985B). Sterols were eluted from the HPLC column and introduced into the ion source in acetonitrile–water 90:10. Spectra were obtained in the chemical ionization mode at an electron energy of 240 eV and an emission of 300 μamps. Using this system, about 1% of the eluant flow is directed into the ion source with the solvent vapour acting as the ionizing gases. Radioactive sterol fractions collected from the HPLC were evaporated to dryness and 10 ml of Aquasol (New England Nuclear Corporation, Boston, MA) was added. Liquid scintillation counting was carried out using an Isocap 300 counter (Searle Corporation, Oakville, Ontario).

RESULTS

Characterization of 7-dehydrosterols

The synthetic 7-dehydrosterols were characterized by their behavior during TLC, GLC, and HPLC, and by their ultraviolet and mass spectra. The properties of the 7-dehydrocholesterol prepared in the laboratory were indistinguishable from those of the authentic commercial standard. The 7-dehydro derivatives of the

plant sterols also gave the anticipated chromatographic and mass spectral properties and were indistinguishable from 7-dehydrocholesterol in their ultraviolet spectra. Ultraviolet scans taken through the peaks emerging from the HPLC demonstrated the homogeneity of these compounds. The presence of ultraviolet absorption maxima at 262, 271, 282, and 294 nm confirmed (14) the presence of a $\Delta^{5,7}$-conjugated diene. **Fig. 1** shows that the 7-dehydrosterols (detected at 265 nm) are effectively resolved by HPLC from each other and from the cholesterol and plant sterol parent compounds (detected at 210 nm). Partial overlaps between cholesterol and 7-dehydro-β-sitosterol and between β-sitosterol and the internal standard, 7-dehydrocholesterol acetate, did not interfere with the analyses of the 7-dehydrosterols. The absolute retention times for the C_{27}, C_{28}, and C_{29}-$\Delta^{5,7}$-sterols at a flow rate of 1 ml/min were 15.2, 16.9, and 18.8 min, respectively, and those for the corresponding Δ^5-sterols were 18.9, 21.2, and 23.2 min, respectively. **Fig. 2** shows that the detector response at 265 nm was linear over the 1–10 nmol range of 7-dehydrocholesterol acetate tested. From this figure a lower limit of detection was estimated to be 10 pmol (about 4 ng).

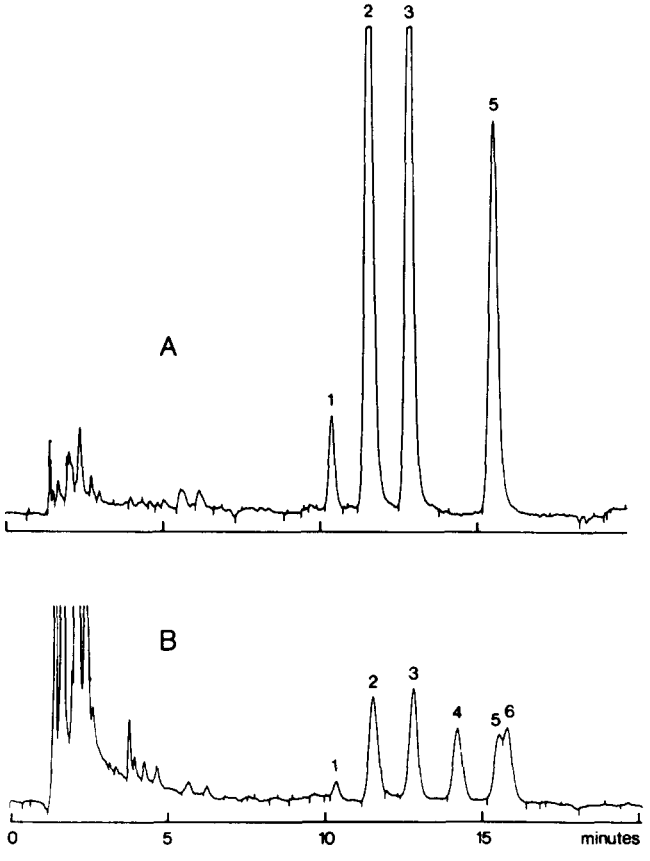

Fig. 1. Resolution of the 7-dehydrosterols by reversed phase HPLC. HPLC profiles of the same sample at two different wavelengths (panel A scanned at 265 nm, panel B at 210 nm) were obtained using acetonitrile–methanol 1:1 as the eluting solvent at a flow rate of 1.5 ml/min. The profiles represent a total lipid extract of a micellar solution containing sterols, egg phospholipid, and sodium taurocholate. An attenuation of 32 was used for both scans. Peak identification is as follows: 1,7-dehydrocholesterol; 2,7-dehydrocampesterol; 3,7-dehydro-β-sitosterol; 4, campesterol; 5,7-dehydrocholesterol acetate; 6,β-sitosterol.

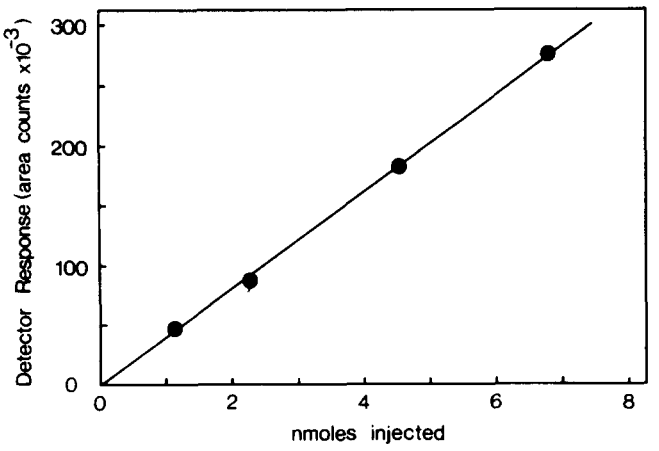

Fig. 2. Variable wavelength detector response curve for 7-dehydrocholesterol acetate. Area counts were obtained using a scan wavelength of 265 nm during HPLC analysis of the samples. For the analysis, solutions of 7-dehydrocholesterol acetate were prepared in acetonitrile–methanol 1:1 following quantitation of the steroid, in ethanol, by ultraviolet absorbance using a molar extinction coefficient of 11,000 at 282 nm (14).

Fig. 3 shows the electron impact and chemical ionization mass spectra of the three 7-dehydrosterols. The presence of the additional double bond in the 7-dehydro derivatives of cholesterol, campesterol, and β-sitosterol is demonstrated by ions at m/z 366, 380, and 394 (M-Bz), respectively, in the electron impact spectra. Similar ions at m/z 367, 381, and 395 [(M + 1) − H_2O] can be seen in the chemical ionization spectra. Localization of both sites of unsaturation in the ring system is further confirmed by the ion at m/z 253 in the electron impact spectrum in each case.

Characterization of brush border preparations

Table 1 gives the marker enzyme distribution of the intestinal brush border preparation. The 25-fold increase in the sucrase activity in the isolated vesicles in relation to the total homogenate compares favorably with enrichments ranging from 18- to 22-fold reported by previous investigators (15, 23). The 3-fold increase in the activity of alkaline phosphatase was less than previous reports (15, 24). The increase in the activity of the monoacylglycerol acyltransferase apparently represented microsomal contamination, which was estimated on the basis of its specific activity to be about 10%. This enzyme is no longer believed to be present

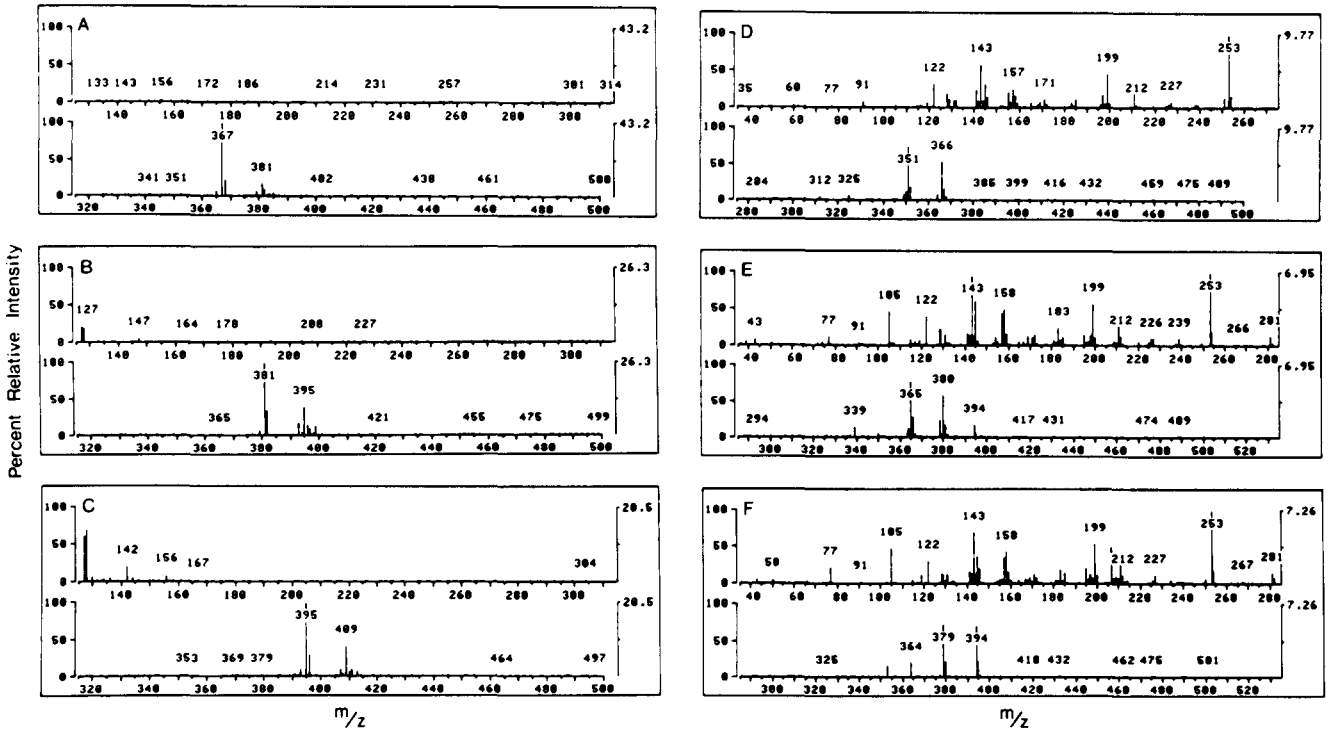

Fig. 3. Chemical ionization and electron impact mass spectra of the 7-dehydrosterols and sterol benzoates. Panels A, B, and C represent the chemical ionization spectra of 7-dehydrocholesterol, 7-dehydrocampesterol, and 7-dehydro-β-sitosterol, respectively, obtained by LC–MS following hydrolysis of the benzoate esters. Panels D, E, and F represent the electron impact spectra of the benzoates of 7-dehydrocholesterol, 7-dehydrocampesterol, and 7-dehydro-β-sitosterol, respectively, obtained by GLC–MS as described previously (22).

in the brush border of the intestinal villus cells (25) despite earlier reports. There was a significant decrease in the activity of succinic acid dehydrogenase in keeping with a relative absence of mitochondria from the brush border vesicle preparation.

Examination of the brush border preparation by transmission electron microscopy confirmed that the membranes were closed vesicles. A representative field is shown in **Fig. 4,** along with a cross-sectional view of intact microvilli at the same magnification. The vesicle diameters (0.05–0.15 μm) are uniform and very similar to those of the microvillar cross sections. The absence of mitochondria is confirmed and structures reminiscent of intracellular or basolateral membranes are not discernable.

Table 2 gives the phospholipid composition of the isolated brush border membrane and of the total homogenate. The brush border contains significantly more phosphatidylethanolamine, phosphatidylserine, and sphingomyelin, and less phosphatidylcholine and lysophosphatidylcholine than the total homogenate. The phospholipid profile of the brush border is in good agreement with that given for rat brush border by Hauser et al. (26). The free sterol/phospholipid ratio of

TABLE 1. Marker enzyme distribution of the total mucosal homogenate and brush border fraction

Marker	Total Homogenate	Brush Border Fraction	Enrichment in Brush Border
Protein (mg)	380	7.9	×0.021
Enzyme (units[a]/mg protein)			
Sucrase	0.15	3.59	×25
Alkaline phosphatase	29.1	94.6	×3.3
Succinate dehydrogenase	7.7	4.5	×0.6
Monoacylglycerol acyltransferase	16.2	41.2	×2.5

[a] The value of 1 enzyme unit is as follows: sucrase, 1 μmol sucrose hydrolyzed per min at 37°C; alkaline phosphatase, 1 μmol p-nitrophenol phosphate hydrolyzed per min at 37°C; succinate dehydrogenase, 1 μmol INT reduced in 15 min at 37°C; monoacylglycerol acyltransferase, 1 nmol diacylglycerol formed from *sn*-2-monoacylglycerol per hr.

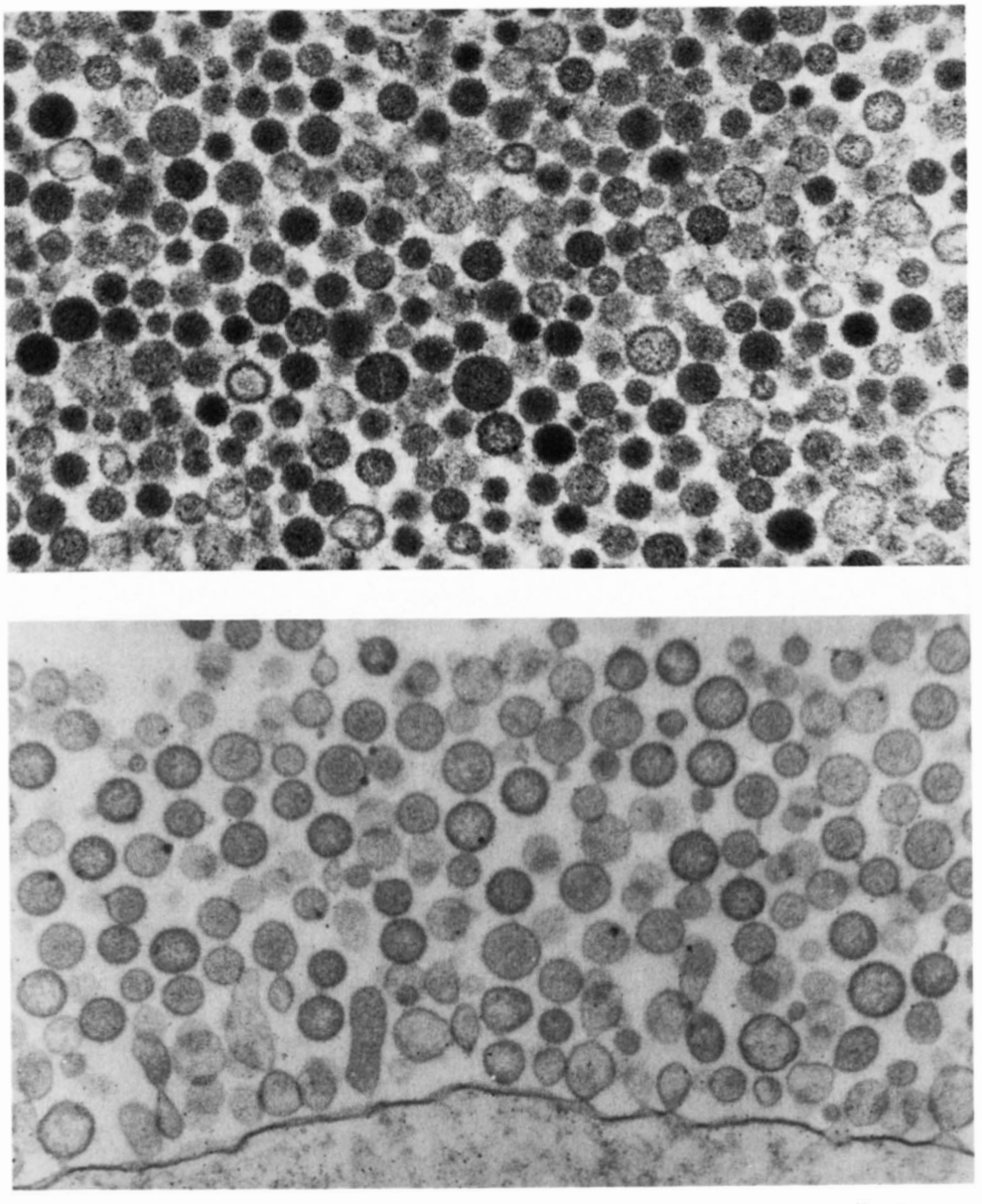

Fig. 4. Electron micrographs of intact microvilli and of a preparation of brush border vesicles. Lower photo, intact microvilli of an isolated villus cell viewed in cross section. Upper photo, purified brush border vesicles. Magnification ×55180 (reduced in reproduction). The cell and vesicle preparations were fixed, stained, and embedded as described in Materials and Methods.

1.14 corresponds closely to values reported by Forstner et al. (24) and by Gray et al. (27), but it is somewhat higher than that recorded by Douglas, Kerley, and Isselbacher (28). The absence of detectable 7-dehydrocholesterol in this fraction was confirmed by analysis of the total lipid extract by HPLC.

Uptake of 7-dehydrosterols by rat erythrocytes

Fig. 5 shows the time course of uptake of the 7-dehydrosterols by rat erythrocytes. The results are expressed as nmol of sterol per ml packed cells, which represents about 1850 nmol of phospholipid under the centrifugation conditions used in these experiments (12). The sterol uptake is linear with time and a preference for the cholesterol analogue is clearly visible following an initial nonspecific accumulation. This trend continues to increase throughout the period of incubation (inset). The ratio of 7-dehydrocholesterol/7-dehydro-β-sitosterol in the cells was 5.5 after a 60-min incubation. The uptake of the C_{28} sterol, 7-dehydrocampesterol, was intermediate between that of the C_{27} and the C_{29} sterols at all time points.

TABLE 2. Phospholipid distribution in the total mucosal homogenate and brush border fraction

Phospholipid Distribution	Total Homogenate	Brush Border Fraction
Total (mg/mg protein)	0.14	0.085
Class composition[a] (mol percentage)		
Phosphatidylethanolamine	16.1	24.4
Phosphatidylserine	2.4	10.4
Phosphatidylinositol	5.0	6.2
Phosphatidylcholine	65.0	45.7
Sphingomyelin	5.2	9.1
Lysophosphatidylcholine	10.2	4.3
Total sterol[b]/total phospholipid (mol/mol)	0.26	1.14

[a] The phospholipid composition was determined by inorganic phosphorus analysis following resolution of the phospholipid classes by TLC and digestion of the fractions with H_2SO_4–$HClO_4$ as described in Methods.

[b] The sterol content was determined by GLC as the TMS ethers following isolation of the neutral lipid fraction from the above TLC plate.

Fig. 6 shows the HPLC profiles of the lipid extract of the erythrocytes prior to incubation (panel A), following 30 min of incubation with the 7-dehydrosterols (panel B) and of the incubation medium (panel C). The 7-dehydrosterols are cleanly resolved from any ultraviolet-absorbing components that are present in the cells. Furthermore, there was no detectable conversion of the 7-dehydrosterols into other ultraviolet absorbing derivatives in either the cell or the medium. A release of erythrocyte cholesterol into the incubation medium was, however, detected by GLC after 45 min of incubation. This release was associated with the onset of hemolysis, hence subsequent incubations with the erythrocytes were limited to 30 min.

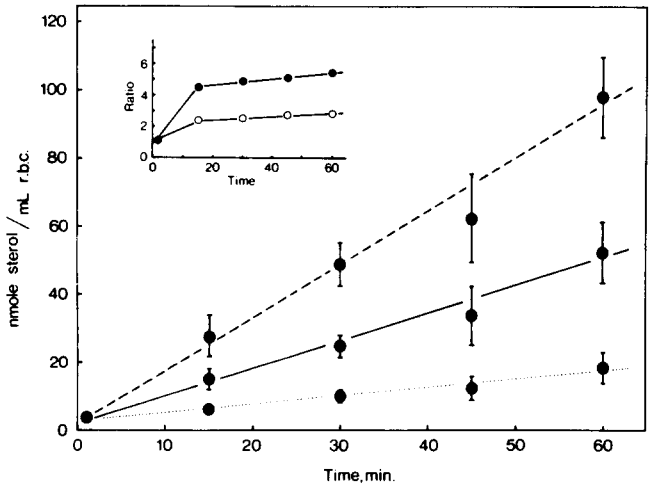

Fig. 5. Time course of 7-dehydrosterol uptake by rat erythrocytes in vitro. Rat erythrocytes were incubated at 37°C in a mixed micellar medium containing 40 μM each of 7-dehydrocholesterol, 7-dehydrocampesterol, and 7-dehydro-β-sitosterol, 6.6 mM sodium taurocholate, and 0.6 mM egg phospholipid. Uptake was determined by HPLC as described in Methods and each point represents the mean ± standard deviation for three erythrocyte preparations. The dashed line indicates 7-dehydrocholesterol; the solid line, 7-dehydrocampesterol; and the dotted line, 7-dehydro-β-sitosterol. Inset: This curve illustrates the change in the cellular ratio of 7-dehydrocholesterol to 7-dehydro-β-sitosterol (closed circles) and 7-dehydrocampesterol to 7-dehydro-β-sitosterol (open circles), with time.

Table 3 demonstrates that the relative rates of uptake of the 7-dehydrosterols parallel those of the parent sterols. For this purpose the erythrocytes were incubated in micellar media containing combinations of campesterol, β-sitosterol, and their 7-dehydro derivatives. At the 30 μM level the uptake of the 7-dehydrosterols, determined by the ultraviolet absorbance during HPLC (Experiment 2), did not differ significantly from that of campesterol and β-sitosterol alone determined by GLC (Experiment 1). Comparable rates of uptake for β-sitosterol and its 7-dehydro derivative were also obtained when the cells were incubated in the presence of campesterol, β-sitosterol (tritium labeled), and their 7-dehydro analogues (Experiment 2). In this experiment the ultraviolet absorbance of 7-dehydro-β-sitosterol was compared to the radioactivity measured in the β-sitosterol fraction recovered by HPLC (there was no radioactivity in the campesterol fraction). In other experiments, the uptake of the 7-dehydrosterols was not significantly influenced by the addition of 50 μM cholesterol to the incubation medium. Thus, each of the sterols appeared to be taken up independently of any other sterol in the 30 to 50 μM range tested, and the ultraviolet-absorbing 7-dehydro derivatives provided a faithful representation of the relative rates of uptake of their parent compounds.

Uptake of 7-dehydrosterols by villus cells and brush border membranes

Under the selected experimental conditions, the uptake of the 7-dehydrosterols by the isolated villus cells and the brush border vesicles was linear with time. As with the erythrocytes, a preference for the cholesterol analogue over the plant sterol analogues was clearly seen, and it continued to increase throughout the experimental period. **Fig. 7** shows the HPLC profiles of the 7-dehydrosterols derived from the villus cells and the brush border membranes following a 30-min incubation. From the HPLC profile of the total lipid extract of the villus cell preparation (panel A), it is evident that there were no interfering substances eluting in the 7-dehydrosterol range in the cells or brush border vesicles prior to incubation. From the concentrated lipid extracts, it was determined that the endogenous level of 7-dehydrocholesterol was less than 0.2 nmol per mg of villus cell protein. There was no evidence of con-

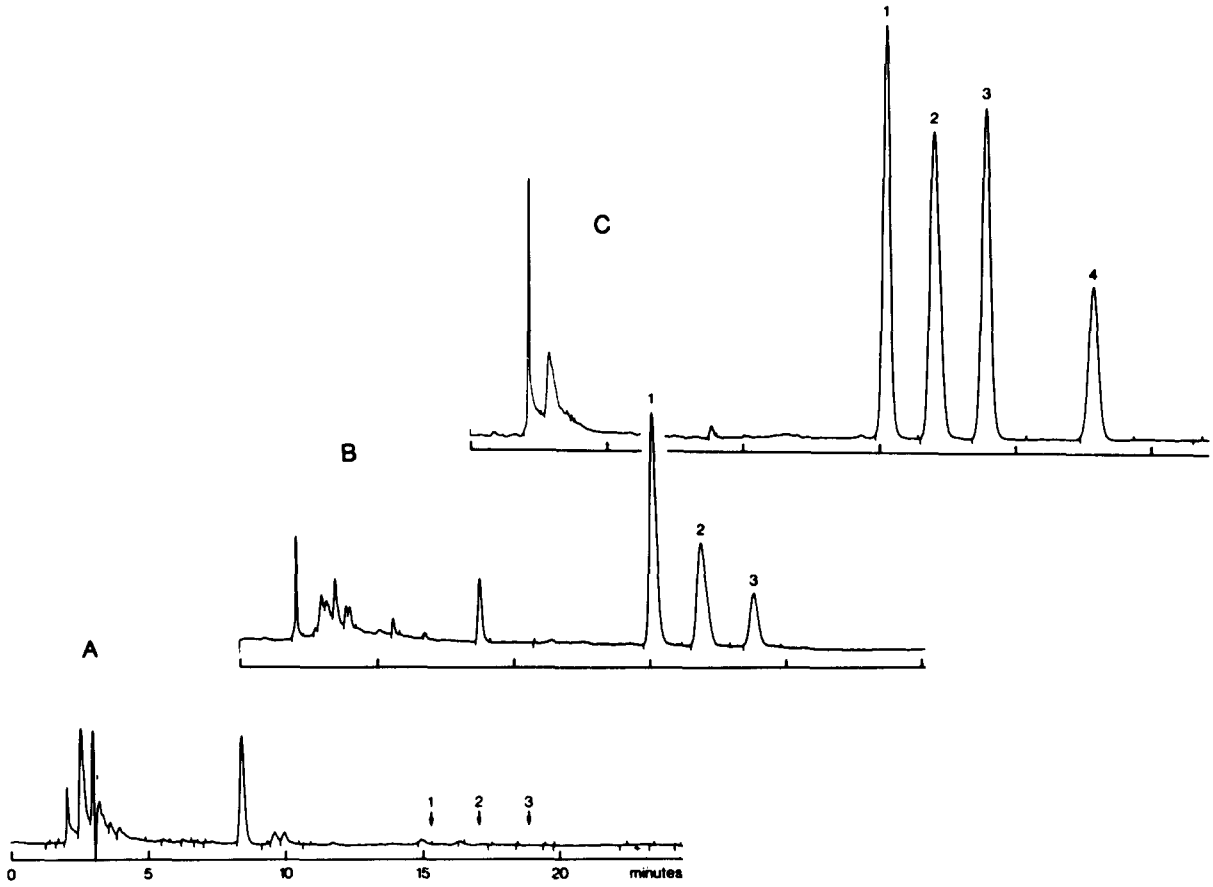

Fig. 6. 7-Dehydrosterol content of rat erythrocytes before and after incubation in a micellar medium. A, total lipid extract of erythrocytes (0.5 ml packed cells) prior to incubation; B, rat erythrocytes after 30-min incubation; C, extract of the incubation medium. The profiles were obtained by HPLC of the neutral lipid fractions as described. The cells were incubated for 30 min in a medium containing 6.6 mM sodium taurocholate, 41 μM 7-dehydrocholesterol (peak 1), 44 μM 7-dehydrocampesterol (peak 2), 42 μM 7-dehydro-β-sitosterol (peak 3), and 0.6 mM egg phospholipid. Conditions for HPLC were as described in Methods. 7-Dehydrocholesterol acetate (peak 4) was added prior to extraction as an internal standard.

version of the sterols into other ultraviolet-absorbing materials by these preparations or by their incubation media. It is seen, from an equimolar sterol mixture in the medium, that the brush border membranes (panel B) are clearly superior to the villus cells (panel C) in displaying a selective absorption of 7-dehydrocholesterol. A quantitative comparison of data obtained from several replicate experiments terminated at the 30-min period is given in **Fig. 8.** The villus cells (panel B) show a 2-fold excess of 7-dehydrocholesterol over 7-dehydro-β-sitosterol, and a 1.4-fold excess over 7-dehydro-campesterol. The brush border preparation shows a 4-fold excess of 7-dehydrocholesterol over 7-dehydro-β-sitosterol and a 2-fold excess over the campesterol analogue. It is evident that the selectivity of the brush border membrane for 7-dehydrocholesterol approaches that seen with the erythrocyte membrane (panel D).

Attempts to relate the uptake of the 7-dehydrosterols by the villus cells or the brush border vesicles with a quantitative release of cholesterol into the medium were unsuccessful. Detectable amounts of cholesterol were released at all times of incubation, but analyses of the cellular or membrane sterol by GLC failed to establish significant changes in the total sterol content following incubation and washing of the cells with bile salts.

Comparative total sterol uptake by various cell preparations

To further investigate the similarities in the uptake of sterols by the different cell membrane preparations it was of interest to normalize the absorption to a common reference point (total cell surface area) to allow a comparison of the absolute values. Estimated surface areas of 1.40 m^2/ml packed red blood cells (29, 30) and 2.15×10^{-3} m^2/mg villus cell protein (10) were used. A value of 6.6×10^{-3} m^2/mg brush border protein was calculated based on the known amount of the brush border enzyme, sucrase, and the estimated surface area of the brush border membrane of the villus cell preparation (see Appendix for details of the calculation). The surface area calculation was based on the assump-

TABLE 3. A comparison of the uptake of $\Delta^{5,7}$- and Δ^5-sterols by rat erythrocytes

Experiment Number	Cellular Uptake[a]					
	$\Delta^{5,7}$-C27[b]	$\Delta^{5,7}$-C28[b]	$\Delta^{5,7}$-C29[b]	Δ^5-C27	Δ^5-C28	Δ^5-C29
1					30.2 ± 3.9[c] (30)	16.7 ± 3.2[c] (30)
2		25.5 ± 5.0 (35)	12.5 ± 2.3 (33)		N.D.[d] (34)	13.8 ± 1.1[e] (33)
3	49.0 ± 6.3 (30)	24.7 ± 3.2 (30)	9.9 ± 1.8 (30)			
4	58.0 ± 12.6 (31)	38.0 ± 7.9 (37)	17.1 ± 3.7 (36)			
5	55.6 ± 9.7 (32)	35.4 ± 6.4 (38)	16.4 ± 3.7 (34)	N.D. (50)		

[a] Rat erythrocytes were incubated for 30 min at 37°C in media containing 6.6 mM taurocholate, 0.6 mM egg yolk phospholipid, and the sterols in the concentrations indicated in the table. Cellular uptake is expressed as nmol sterol/ml packed erythrocytes. Each value represents the mean ± standard deviation of three to six replicates from separate cell preparations. The media concentrations of each sterol (μM) are listed in parentheses immediately below the uptake value.

[b] Sterols assayed by UV absorbance during resolution by HPLC.

[c] Sterols assayed as the TMS ethers by GLC as described in Methods.

[d] N.D., not determined.

[e] Assayed by liquid scintillation counting (using [22,23-^{3}H]β-sitosterol tracer) following resolution of the sterols by HPLC. The uptake of sitosterol was converted from dpm into nmol using the specific radioactivity of the labeled compound in the incubation medium.

tion that the hyaluronidase digestion technique used for the villus cell preparation gave a 100% yield of jejunal enterocytes. **Table 4** shows that the total sterol uptake by the intestinal cell membrane preparations is 40 to 60 times greater than in the erythrocytes. Sterol uptake by the brush border membranes was estimated to be about

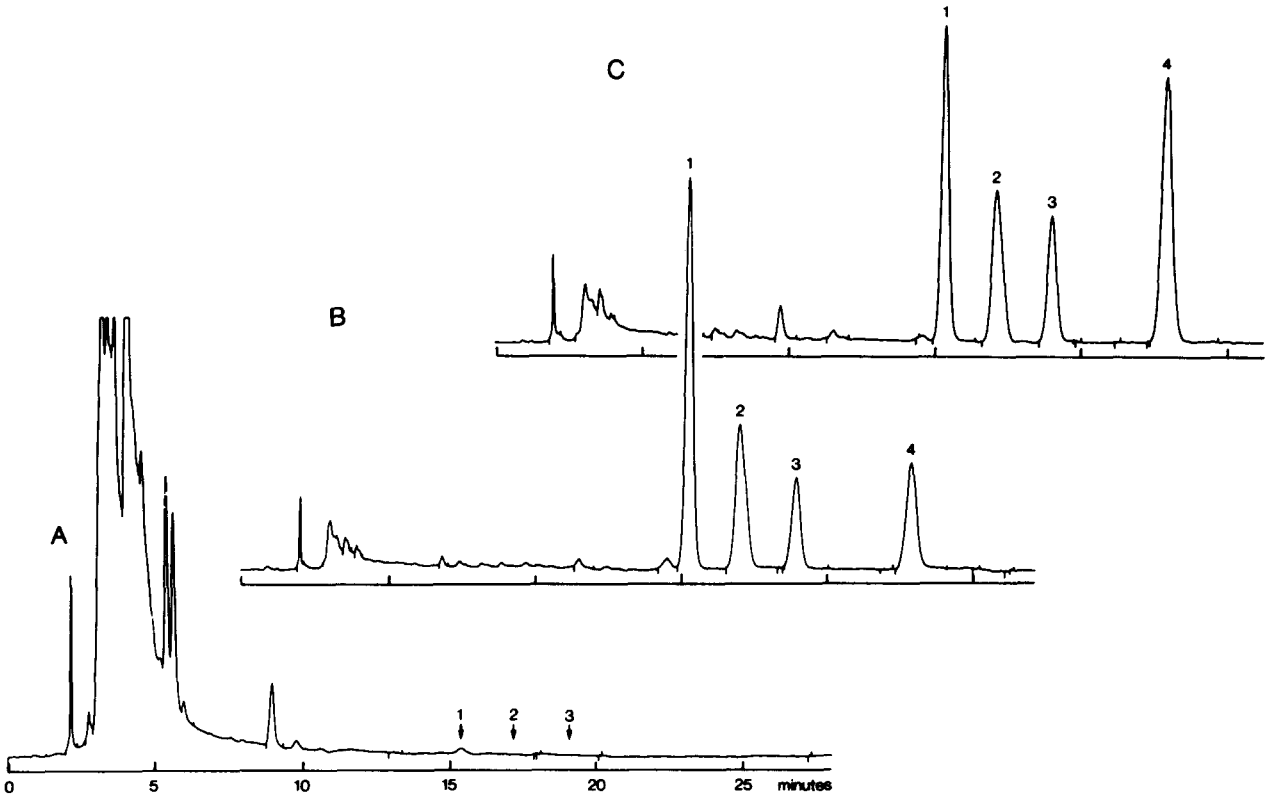

Fig. 7. 7-Dehydrosterol profiles in villus cells and brush border membrane following 30 min of incubation. A, total lipid extract of villus cells (2 mg cell protein) prior to incubation; B, brush border membranes after 30-min incubation; C, villus cells after 30-min incubation. The cells and membranes were incubated in the micellar medium described in Fig. 6. Peak identification as in Fig. 6.

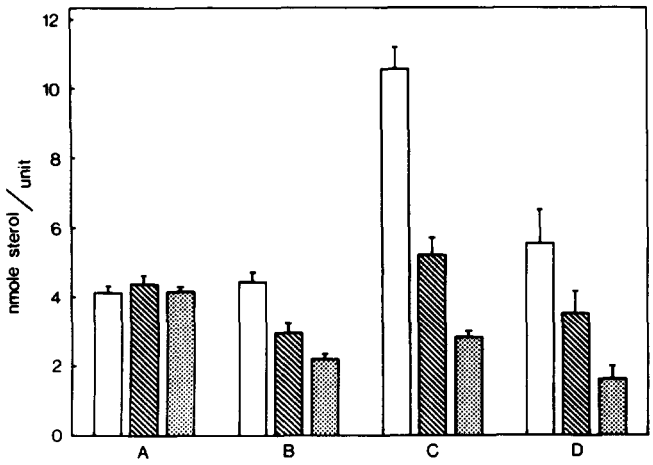

Fig. 8. Comparative uptake of 7-dehydrosterols in isolated villus cells, brush borders, and erythrocytes. Conditions and legends as in Figs. 6 and 7. Sterol content in panel A expressed per 100 μl media. Sterol uptake in panel B expressed per mg villus cell protein, per mg brush border protein in panel C, and per 100 μl packed erythrocytes in panel D. Values are the means ± standard deviations for three determinations with tissue from separate animals.

one half that of the whole villus cells. In view of the uncertainties involved in the estimates of the brush border surface area, the latter difference may not be significant. However, the likelihood that the intestinal preparations internalized the sterol should be considered as a factor contributing to their greater apparent uptake of sterol.

DISCUSSION

Nature of test sterols

On the basis of the previous work (8, 31–33), it was assumed that the 7-dehydro derivatives of cholesterol and the plant sterols would be handled similarly to the parent sterols. It had been shown that 7-dehydrocholesterol was absorbed by the rabbit intestine as readily as cholesterol (8), and membranes of rat hepatocytes and human erythrocyte ghosts were unable to distinguish between the two sterols during their exchange from phospholipid liposomes (31). In other studies, 7-dehydrocholesterol and cholesterol were equally able to reduce the permeability of liposomes of egg yolk phospholipid to glycerol and Rb^+ (32). Similarly, the electron spin resonance order parameters derived by a study of the influence of various sterols on the structure of liposomes of egg phosphatidylcholine were identical for cholesterol and its 7-dehydro analogue up to 50 mol% sterol (33). The expected similarity was also in evidence in the present experiments. The 7-dehydro plant sterols were absorbed at rates comparable to those of their parent sterols when presented to rat erythrocytes in the form of micellar solutions. This similarity is consistent with the minor effect of the additional double bond on the overall shape of the molecules (34).

The cholesterol used as a precursor of the 7-dehydrosterol in these experiments represents a single molecular species of known structure, but the plant sterols, campesterol and sitosterol, originated from soy bean oil and may contain a mixture of isomers. According to Nes, Krevitz, and Behzadan (35), the C-29 sterol from soybean oil is exclusively the 24-alpha-ethyl isomer, but the C-28 sterol contains both epimers of 24-methyl cholesterol. A partial resolution of these epimers may be responsible for the widening of the 7-dehydrocampesterol peak that is evident in the reversed-phase HPLC profiles. Complete separation has been described using long (110 m) capillary GLC columns (36), but in the present experiment, the resolution was not sufficient to determine whether the various cell or membrane preparations were able to discriminate between the alpha and beta epimers of the side chain substituents in these sterols.

Characteristics of the cell and membrane preparations

The 25-fold enrichment of sucrase activity in the brush border membrane preparation establishes that the bulk of the material is derived from the microvillar membrane. The activity of alkaline phosphatase, however, was low and this was possibly due to the inhibition of the enzyme by inorganic phosphate accumulated by

TABLE 4. Uptake of the 7-dehydrosterols normalized to the surface area of the cellular preparations

	Sterol Uptake (nmol/m^2 · 30 min)			
Preparation	C27	C28	C29	C27 (mol) / C29 (mol)
Villus cells	2065 ± 130	1377 ± 130	1024 ± 65	2.02
Brush border	1603 ± 92	792 ± 74	430 ± 29	3.72
Erythrocytes	37.9 ± 6.6	24.1 ± 4.3	11.1 ± 2.5	3.94

The data used in these calculations were derived from Fig. 8 and Table 3. The values presented are the mean + SD for three replicate cell or membrane preparations. The standard deviations represent the original data only and do not reflect the accuracy of the surface area estimates.

the brush border vesicles during their isolation in buffer containing phosphate. Previous work has shown (15, 24, 28) that the alkaline phosphatase activity parallels that of sucrase during the purification of brush border membranes.

The high purity of the membrane preparation is confirmed by the high ratio of cholesterol/phospholipid and an increased proportion of sphingomyelin, both of which are characteristic of this fraction (24, 26, 27). In addition, there is a striking resemblance of the purified membranes to the intact microvilli in cross section in the electron micrographs. There is no evidence of mitochondria or other intracellular membrane contaminants in the micrographs of the brush border vesicle preparation but the activity of the microsomal marker (25), monoacylglycerol acyltransferase, indicated that some endoplasmic reticulum must have been present. The overall granular appearance and the vesicle diameters in the brush border fraction correspond closely to those reported by others (15, 37) and to those seen in the intact microvilli. Vesicles prepared by this procedure have been shown to retain their right-side-out orientation (37).

The dispersed villus cells prepared by the technique of hyaluronidase digestion display a high level of metabolic activity and a greater than 90% viability when exposed to Trypan Blue and Nigrosin (10, 13, 38). The viability is reduced when the cells are incubated with solutions containing bile salts, free fatty acids, and monoacylglycerols (10, 38) but the inclusion of phospholipid into these micellar solutions has been shown (12) to reduce membrane damage dramatically. Thus, under these conditions, isolated cell and membrane preparations may be incubated in the presence of bile salts for up to 1 hr without disruption of the membranes.

Differential uptake of the 7-dehydrosterols

This study demonstrates that the uptake of 7-dehydrosterols by isolated jejunal villus cells, brush border vesicles, and red blood cells of the rat decreases with increasing number of carbon atoms at C_{24} of the sterol side chain, as has been established for the parent sterols in vivo. This is contrary to previous reports of a comparable uptake of cholesterol and plant sterols by rat intestinal preparations in vitro (7, 9, 10). The unequivocal demonstration of a differential sterol absorption by the present study is due both to the improved measurement of the sterol uptake in the absence of interference from large endogenous pools and to the reduction of damage to the cell membranes by the inclusion of egg yolk phospholipid in the incubation medium. In preliminary experiments (11), increasing the concentration of egg phospholipid above 0.5 mM did not influence the rate of sterol absorption or the selectivity expressed in the uptake.

Sterol concentrations in the range of 30–150 μM were chosen to allow the formation of clear aqueous solutions and to minimize the interaction between sterols during their absorption. This range of sterol concentration also represented conditions under which linear uptake was seen for both erythrocytes and the villus cell preparations. According to McIntyre et al. (39) the concentration of sterol in the intestinal lumen of the rat fed continuously with a sterol-containing diet may be expected to be in the range of 200–1200 μM in the aqueous phase. Therefore, the concentrations of sterols used in the present work would be at the lower end of the physiological range.

The degree of selectivity in the absorption of the 7-dehydro plant sterols in the present work agrees well with that obtained for the parent plant sterols by previous workers. Thus, the mean ratio of 7-dehydrocampesterol/7-dehydro-β-sitosterol (2.2) obtained in the erythrocytes after 30 min of incubation correlates closely with the 2.5-fold excess of campesterol over β-sitosterol determined in similar cells following a 16-hr incubation with liposomes containing the plant sterols and egg phospholipid (40). Under the latter conditions, the accumulation of the plant sterols was at the expense of membrane cholesterol. In the present experiments we were unable to demonstrate a net mass increase in the membrane sterol of the erythrocytes at the low levels of transfer measured. The total 7-dehydrocholesterol uptake of about 50 nmol/ml packed cells in 30 min would increase the total cell sterol by only 3% if net uptake rather than exchange had occurred. Similarly, in the villus cells, the uptake of 5 nmol of 7-dehydrocholesterol per mg cell protein in 30 min would increase the total cellular sterol by only 6–7%. It is evident from previously reported data (10, 12) that these increases are within the bounds of normal experimental error. A mass accumulation of the parent sterol has been demonstrated in rat intestinal villus cells (10, 11) and erythrocytes (12) at higher sterol loads (250 μM). However, the appearance of cholesterol in the medium during the incubation of the erythrocytes indicates that some exchange occurred under the present conditions.

The 2-fold enhancement in the uptake of 7-dehydrocampesterol over 7-dehydro-β-sitosterol in the brush border membranes is comparable to the 1.5/1 ratio of campesterol to β-sitosterol measured in everted rat intestine following incubation with the sterols as albumin dispersions (41), and agrees well with the 14/7.4 ratio reported by the same authors following feeding in an oil carrier in vivo. Similarly, the 4-fold excess of 7-dehydrocholesterol over 7-dehydro-β-sitosterol in the brush borders approximates the 3- to 4-fold excess of cholesterol over β-sitosterol reported for the intestinal wall of the rat after uptake of the two sterols from an oil emulsion meal (6) and from a mixed micellar infusate (4).

The cellular uptake ratios presented in this report are not, however, expected to represent the maximum possible under these conditions. The apparent selectivity between sterols is expected to be obscured by the presence of nonspecifically bound sterol. The taurocholate washes employed in this study remove adherent sterol from the intestinal preparations but lyse red blood cells (12). Therefore, an estimate of sterol adherent to the outer membrane surface is required with these cells. The small Y-intercept (2–3 nmol 7-dehydrosterol/ml packed red blood cells) in the time course curve indicates that the initial adsorption is limited under these conditions. This observation was confirmed in the intestinal preparations and coincided with estimates of the amount of adherent fluid volume determined previously (10) with the nonabsorbable marker, [^{14}C]inulin. The identity of the sterol composition at the zero time point to that in the incubation medium is also consistent with the initial adsorption of these sterols to the outer surface of the membrane. A nonselective uptake was also obtained throughout incubations of erythrocytes at 0°C in a mixed micellar medium (12). At this temperature the rate of uptake approached 30% of that measured at 37°C. This accumulation may reflect either an inability of the membrane to recognize the differences between the sterol side chains at this temperature, or a buildup of sterol at the cell surface occurring over the 30-min incubation period. A nonselective accumulation would be expected where diffusion of the sterol to the membrane surface is faster than entry into the bilayer. In isolated cell systems, where unstirred water layers are thought to be small (42), this may well be the case. Subtraction of the sterol accumulated at 0°C from that accrued at 37°C in erythrocytes gives rise to a ratio of absorbed C_{27}/C_{29} sterol that approaches 8–10. This is comparable to the level of discrimination that is found in the lymph of a rat after ingestion of radioactive cholesterol and β-sitosterol (1, 3). Ratios of this order were not achieved with the taurocholate-washed intestinal preparations, but other factors, such as contamination with other, less discriminating cell or membrane types, may prevent the expression of maximal selectivity in this case.

Comparison of selectivity of different membrane types

Although the maximal degree of selectivity expressed by a given cell or membrane type during the absorption of sterols may be masked by adherent substrate or contamination with other membrane types, these factors do not offer a rationalization for the marked differences in the ability of villus cells, erythrocytes, and brush border vesicles to recognize 7-dehydrocholesterol. Furthermore, the greater degree of selectivity expressed by the erythrocytes and brush border membranes compared to the villus cells does not appear to correlate with the rate of sterol uptake. The inaccuracy of the estimate of the brush border surface area notwithstanding, the intestinal preparations both exhibit a much greater uptake per unit surface area than the red blood cells. The calculation of the brush border surface area is an underestimate in this case, but even more liberal estimates indicate at least a 15-fold greater uptake of sterol than in the erythrocytes. This may be related to the ability of the intestinal preparations to internalize absorbed sterols as part of their absorptive function. We have previously shown (10) that sterols taken up by isolated villus cells rapidly equilibrate throughout the intracellular membranes, a process that cannot occur in red blood cells.

The wide variation in the preference for 7-dehydrocholesterol that exists between the villus cells, erythrocytes, and brush border vesicles may be the result of compositional differences between the membranes. These dissimilarities suggest that the events leading to the recognition of the side chain of cholesterol occur in the plasma membrane, most likely during or after partition into the outer half of the plasma membrane bilayer. If the selection were occurring in the incubation medium, no difference would be expected between host membrane types. The basis for the preferential absorption of cholesterol has been suggested (43) to arise from a greater tendency of this sterol to cross the hydrophilic region between the carrier micelle and the cell membrane, compared to the plant sterols, but the present experiments clearly demonstrate that the nature of the host membrane is critical. The occurrence of the sterol recognition within the membrane is also indicated by the absence of selectivity during the initial uptake and throughout incubations at 0°C. The loss of the absorptive preference for the cholesterol analogue following removal of egg yolk phosphatidylcholine from the medium (10, 11), a treatment known to lead to disruption of villus cell (11, 38) and erythrocyte (12) membranes, further supports this point of view.

It follows then, that the ability of a membrane to recognize differences between side chains depends upon its structure and composition. Diamond and Wright (44) and Hingson and Diamond (45) have predicted that structural features acting to increase the degree or order within the acyl chains of the membrane phospholipid would enhance the selection against nonelectrolytes with alkyl branch points, compared to nonbranched analogues, during their uptake from aqueous solutions. The basis for their reduced uptake was claimed (45) to be a local disruption of the ordered packing of the fatty acyl chains of membrane phospholipids and a reduction of interhydrocarbon cohesive forces caused by the steric configuration of the branched

solutes. A requirement of this rationale is the predominance of solute-membrane lipid interactions over solute-solute, or solute-solvent interactions. For alkyl substituents, this has been shown to be the case in a variety of membranes (44). Campesterol and β-sitosterol can both be considered branched analogues of cholesterol and similar arguments should apply. The increased molecular weight of the plant sterols is likely not an important factor in the selective absorption, as indicated by the greater uptake of β-sitosterol by the rat intestine compared to its unsaturated counterpart, stigmasterol (5, 46), which is two mass units lighter. Apparently, the bulk and configuration of the side chain is the dominant feature in the recognition. It may be significant therefore that the brush border and rat erythrocyte membranes, which exhibit a similar degree of sterol selectivity, show molar ratios of cholesterol/phospholipid that approach 1:1 (24, 27–29) and a high weight ratio of protein/phospholipid (27, 47, 48), both of which act to increase the internal order of the membrane (27, 47, 49). The isolated villus cells, on the other hand, are less effective in the sterol selection and are largely surrounded by basolateral membranes, which possess a lower cholesterol/phospholipid molar ratio (about 0.5) (27, 28), a lower protein/phospholipid ratio (27, 47), and an increased phospholipid acyl chain unsaturation (27) compared to the brush border membrane. All of these factors contribute to an overall increase in the acyl chain mobility in the basolateral membrane compared to the brush border (27, 47). It is possible, of course, that the integrity of the basolateral membranes was compromised by the procedures used for the isolation of cells. While this would weaken the case for a specific role of lipid composition in the selective absorption, it nevertheless suggests a major role of internal membrane order in the process.

From the results of these experiments in vitro, it can be concluded that the brush border membrane possesses the capability to bring about the absorptive recognition of cholesterol that is found in the intestinal wall in vivo. The inability of β-sitosterol to enter this structure depends upon features that are apparently not unique to the brush border, but may be common to all plasma membranes. Experiments to further establish the role of membrane structure in the absorption of sterols are warranted, and should be facilitated by the availability of in vitro assay systems such as those described herein. Studies with mucosal cell populations having different membrane lipid compositions, as well as with sterols possessing further modifications of the ring system, would be of particular interest.

APPENDIX

The localization of the enzyme, sucrase, to the brush border of the villus cell has been described (15, 26).

We have previously calculated (10) that the surface area of the brush border is 1.88×10^{11} A^2/cell (1.88×10^3 μm^2/cell) in jejunal villus cells. The yield of villus cells by the isolation procedure used in this work is reported (13) to be 5.6×10^7 cells for the upper one-third of the small intestine (75 mg total villus cell protein). Assuming this to represent 100% of the total jejunal villus cell population, the jejunal brush border surface area is 1.04×10^{11} μm^2.

In the homogenate of the scrapings from this segment of the intestine, there were 57 units of sucrase (Table 1). Therefore, the surface area represented by one unit of the enzyme is 1.82×10^9 μm^2. In the brush border preparation, the specific activity of sucrase was 3.59 units/mg brush border protein, giving 6.6×10^9 μm^2 brush border surface/mg brush border protein. This converts to 6.6×10^{-3} m^2 brush border surface/mg protein.

We wish to thank Dr. C. C. Yip for help in obtaining UV scans on the Cary spectrometer, and Messrs. Lajos Marai and Philip Connelly for assistance in obtaining the GLC/MS and LC/MS spectra. This research was supported by grants from the Medical Research Council of Canada and from the Ontario Heart Foundation, Toronto, Ontario. Peter Child was a recipient of a studentship from the Medical Research Council of Canada.

Manuscript received 5 May 1982 and in revised form 23 November 1982.

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