

# Thermodynamic and molecular determinants of sterol solubilities in bile salt micelles

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**Abstract** We examined, by reverse-phase high performance liquid chromatography (HPLC), the hydrophilic-hydrophobic balance of cholesterol and 12 non-cholesterol sterols and related this property to their equilibrium micellar solubilities in sodium taurocholate and sodium glycodeoxycholate solutions. Sterols investigated exhibited structural variations in the polar function ( $3\alpha$ -OH,  $3\beta$ -OH,  $3\beta$ -SH), nuclear double bonds (none,  $\Delta^5$ , or  $\Delta^7$ ), side chain length ( $C_{27}$ ,  $C_{28}$ ,  $C_{29}$ ) and side chain double bonds (none,  $\Delta^{22}$ , or  $\Delta^{24}$ ). In general, a sterol's hydrophilic-hydrophobic balance became progressively more hydrophobic (as exemplified by increasing HPLC retention values,  $k'$ ) with additions of side chain methyl ( $C_{28}$ ) and ethyl ( $C_{29}$ ) groups and with  $3\beta$ -SH substitution of the 3-OH polar function. Side chain  $\Delta^{22}$  and especially  $\Delta^{24}$  double bonds rendered the sterols appreciably more hydrophilic, whereas a single nuclear double bond had little influence. Sterol solubilities ( $24^\circ\text{C}$ ,  $0.15\text{ M Na}^+$ ) were uniformly greater in 50 mM solutions of sodium glycodeoxycholate (range 0.15 to 2.5 mM) than in equimolar solutions of the more hydrophilic bile salt, sodium taurocholate (range 0.07 to 0.67 mM). For each bile salt system, a strong inverse correlation existed between micellar solubilities of sterols and their HPLC  $k'$  values, indicating that more hydrophilic sterols had greater micellar solubilities than the more hydrophobic ones. Based upon the aqueous monomeric solubilities of cholesterol ( $C_{27}$ ) and  $\beta$ -sitosterol ( $C_{29}$ ) at  $24^\circ\text{C}$ , we derived free energy changes associated with micellar binding and found that solubilization of both sterols was more energetically favored in glycodeoxycholate solutions. Although cholesterol exhibited a higher binding affinity than  $\beta$ -sitosterol in glycodeoxycholate micelles, solubilization of  $\beta$ -sitosterol in taurocholate micelles was more energetically favored than cholesterol by  $-0.6\text{ kcal/mol}$ . Based upon these results we offer a thermodynamic explanation for the greater micellar solubilities of more hydrophilic sterols and suggest that the high affinity, but low capacity, of a typical phytosterol for binding to trihydroxy bile salt micelles may provide a physical-chemical basis for its inhibition of intestinal cholesterol absorption.—**Armstrong, M. J., and M. C. Carey.** Thermodynamic and molecular determinants of sterol solubilities in bile salt micelles. *J. Lipid Res.* 1987. **28**: 1144–1155.

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Non-cholesterol sterols are important components of invertebrate and plant membranes (1). These sterols are

structurally related to cholesterol but differ in their polar group, nuclear and/or side chain configurations (2, 3). Several of these have been found in small quantities in plasma lipoproteins (4), in human bile (5, 6), and in human gallstones (6, 7), suggesting that they are absorbed from the intestine, transported in plasma and secreted, in part as neutral sterols, into bile.

Plant sterols such as  $\beta$ -sitosterol have been intensively investigated as potential hypocholesterolemic agents (e.g., 8–10). Although mechanisms by which phytosterols lower plasma cholesterol are not understood, it is believed that they may interfere with intestinal absorption of cholesterol (9–12). Studies in human beings have suggested that less than 5% of ingested  $\beta$ -sitosterol, but up to 50% of an equivalent dose of cholesterol, are absorbed from the upper small intestine (9, 12). Intestinal absorption of several other sterols that resemble plant sterols is also much less efficient than that of cholesterol (11, 13–19) whereas certain shellfish sterols that resemble cholesterol are well absorbed (18). Although bile salt solubilization of sterols appears essential for their absorption (20), the question as to whether there is an underlying relationship between their chemical structures and solubilities in bile salt micelles has not been explored.

The present studies were undertaken to quantify systematically the relationship between the molecular structures of sterols and their hydrophilic-hydrophobic balance<sup>3</sup> as determined by reverse-phase HPLC and to determine whether these parameters bore a predictable

Abbreviations: HPLC, high performance liquid chromatography; NaTC, sodium taurocholate; NaGDC, sodium glycodeoxycholate.

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<sup>3</sup>A common expression that refers to the relative contributions of the hydrophilic and hydrophobic parts of an amphiphile to the overall polarity of the molecule. This physical-chemical property can be measured relative to other homologs by a number of chromatographic and phase partitioning methods.

relationship to equilibrium sterol solubilities in micellar bile salt solutions. Since sterol polarity was varied, but polarity of the solubilizing bile salts was kept constant, the present study can be considered the converse of our earlier one (21) wherein the polarity of the sterol (i.e., cholesterol) was constant and the polarities of the bile salt species were varied systematically. In that study (21), we demonstrated an inverse relationship between reverse-phase HPLC mobilities of bile salt monomers and their capacities as micellar solutions to solubilize cholesterol monohydrate. Here we show, in the case of common sterols, that there is a strong positive correlation between the polarity of sterols as inferred by HPLC mobilities and their equilibrium solubilities in bile salt micelles. We provide a thermodynamic basis for the greater micellar solubilities of hydrophilic sterols on the basis of their higher aqueous monomeric solubilities and suggest that the high affinity but low capacity of a typical phytosterol ( $\beta$ -sitosterol) for binding to micellar sodium taurocholate may be a physical-chemical basis for its inhibition of intestinal cholesterol absorption.

## MATERIALS AND METHODS

### Materials

Campesterol, brassicasterol, desmosterol (24-dehydrocholesterol), fucosterol, lathosterol, thiocholesterol, epicholesterol, and the acetates of 22-dehydrocholesterol and 24-methylenecholesterol were obtained from Research Plus (Bayonne, NJ). Cholesterol was obtained from Nu-Chek-Prep, Inc. (Elysian, MN) and cholestanol, stigmaterol, and " $\beta$ -sitosterol" were obtained from Serdary Research (London, Ontario, Canada). All sterols except " $\beta$ -sitosterol" were found to be 98% pure by analytic HPLC (method described below). In the case of the two acetate esters, free (unesterified) sterols were prepared by dissolving the compounds in aqueous MeOH that was saturated with KOH, followed by hydrolysis at 37°C overnight. The precipitates were washed with distilled H<sub>2</sub>O and the recovered sterols were shown to be free of the respective acetates by their differential HPLC mobilities. By HPLC analysis, " $\beta$ -sitosterol", as supplied, was found to contain ~35% impurities, principally stigmaterol, and was purified to ~99% by preparative HPLC (method described below). Cholesterol,  $\beta$ -sitosterol, stigmaterol, and cholestanol were recrystallized thrice from hot absolute EtOH followed by a final crystallization from hot 95% EtOH to produce the monohydrates (21, 22). Radiolabeled [<sup>14</sup>C] $\beta$ -sitosterol (Amersham, Arlington Heights, IL) was shown by TLC (silica gel 60, benzene-diethyl ether-ethanol-acetic acid 50:40:2:0.5 (by vol)) to be ~96% chemically and radiochemically pure, and was further purified by preparative TLC to attain a

purity of greater than 99% by analytic HPLC in the same solvent system.

Sodium salts of taurocholate (3 $\alpha$ , 7 $\alpha$ , 12 $\alpha$ -trihydroxy-5 $\beta$ -cholanoyl taurine, NaTC) and glycodeoxycholate (3 $\alpha$ , 12 $\alpha$ -dihydroxy-5 $\beta$ -cholanoyl glycine, NaGDC), (Calbiochem-Behring, La Jolla, CA), were recrystallized to achieve a purity of 98% as previously described (21, 22). HPLC grade MeOH and acetonitrile were obtained from Waters, Inc. (Milford, MA). All other chemicals (Fisher Scientific Co., Pittsburgh, PA) were ACS or reagent grade. Filtered H<sub>2</sub>O was deionized and glass-distilled through an automated all-glass Teflon® apparatus (Corning Glass Works, Corning, NY). For measurements of monomeric sterol solubilities, the distilled H<sub>2</sub>O was further purified by distillation over KMnO<sub>4</sub> in an all-glass laboratory assembly and then purged with 99.99% pure N<sub>2</sub> for 1 hr at 24°C. Pyrex glassware was used throughout and cleansed by sequential washing for 24 hr in tanks of EtOH-2 N KOH 50:50 (by vol) and 1 N HNO<sub>3</sub> followed by thorough rinsing in running distilled water.

### Solutions

For HPLC analysis of sterols, gravimetric amounts of each compound were dissolved in MeOH-acetonitrile 60:40 (by vol) to give final concentrations of 1.0  $\pm$  0.1 mg/ml. For sterol solubility studies in micellar bile salt solutions, 50 mM NaTC was prepared on a w/v basis in 0.15 M NaCl, and adjusted to pH 7.0 with a few  $\mu$ l of 2 M NaOH. Equimolar (50 mM) solutions of NaGDC were prepared on a w/v basis with 0.10 M NaCl in 0.05 M Na carbonate-bicarbonate buffer to give a final pH of 10.

### High performance liquid chromatography

Reverse-phase HPLC was performed on a Model 330 Beckman-Altex high performance liquid chromatograph employing two Model 110A pumps and a Model 210 injection valve (all from Beckman Instruments Inc., Fullerton, CA). The reverse-phase columns were Ultrasphere-ODS (Altex Inc., Palo Alto, CA), with dimensions of 250  $\times$  4.6 mm and 250  $\times$  10 mm, for analytical and preparative studies, respectively. Following equilibration of the HPLC column with MeOH-acetonitrile 60:40 (by vol), 1.0 mg/ml of a sterol or sterol mixture dissolved in the same solvent system was injected and eluted with a 15-min linear gradient of MeOH (60  $\rightarrow$  22.5, by vol) and acetonitrile (40  $\rightarrow$  81.5, by vol) at a flow rate of 3.0 ml/min. The sterol elution profiles were monitored continuously at 210 nm or in the case of thiocholesterol, at 240 nm, by means of a Model 100-40 ultraviolet-visible spectrophotometer (Hitachi Ltd., Tokyo, Japan). The normalized HPLC retention factor (*k'*) was calculated from the retention peaks of the solvent front and the sterol of interest as described previously (21, 23).

## Equilibrium sterol solubilities in bile salt solutions

Equilibrium micellar solubilities of sterols were carried out, in triplicate, by the microcrystalline dissolution method described earlier for cholesterol monohydrate (21, 22). We employed the monohydrates of cholestanol, stigmaterol, and  $\beta$ -sitosterol (see Materials), but anhydrous sterols were used in the other dissolution studies. In the latter cases, equilibration times were sufficiently prolonged to ensure that the sterol monohydrates had formed and had equilibrated with the micellar solutions (22). In brief, 50–100 mg samples of crystalline sterols were finely powdered in an agate mortar and then added to test tubes containing 20 ml of 50 mM bile salt solutions. The test tubes were sealed with Teflon®-lined screw caps under a blanket of  $N_2$  and placed in a shaking water bath at 37°C for the duration of each experiment. Small aliquots (0.5 ml) of well mixed solutions with excess crystalline sterol were sampled daily for 7 days and every 7th day thereafter. To remove excess crystalline sterol prior to assay of micellar sterol concentrations, each sample was passed through a prewarmed (37°C) 0.22  $\mu$ m Millipore filter (Millipore Corp., Bedford, MA).

## Quantitation of solubilized sterols

Concentrations of  $3\beta$ -OH sterols were estimated by the cholesterol oxidase method (Cholesterol Reagent Kit, Boehringer-Mannheim Corp., Indianapolis, IN). To validate the enzymatic kit for estimating  $3\beta$ -OH sterols other than cholesterol, solutions containing known amounts (w/v) of cholestanol,  $\beta$ -sitosterol, stigmaterol, and brassicasterol were assayed by the cholesterol oxidase method, employing a standard curve obtained with authentic cholesterol standards (Boehringer-Mannheim, Indianapolis, IN). Quantities of  $3\beta$ -OH sterols estimated in this way agreed within experimental error ( $\pm 5\%$ ) with those measured by gravimetric analyses employing dry-weight determinations. Since thiocholesterol and epicholesterol with  $3\beta$ -SH and  $3\alpha$ -OH groups, respectively, were unreactive in the cholesterol oxidase assay, these sterols were quantified by HPLC, employing standard curves of integrated peak areas as functions of injected sterol mass.

## Aqueous monomeric solubilities of sterols

To deduce the changes in standard free energy ( $\Delta G_s$ ) associated with micellar solubilization, it was necessary to characterize the temperature-dependent sterol binding constant ( $K_1(T)$ ) and for this purpose an accurate knowledge of the sterol's aqueous monomeric solubility was required (24, 25). We chose to carry out these experiments with cholesterol (a  $C_{27}\Delta^5$  sterol) and  $\beta$ -sitosterol (a  $C_{29}\Delta^5$  sterol) whose solubilities differed sixfold and twofold in NaGDC and in NaTC solutions, respectively (cholesterol  $\gg$   $\beta$ -sitosterol, see Results). Aqueous monomeric solubilities of cholesterol monohydrate are known

with precision for several temperatures between 20° and 30°C (26–29), but have not been measured at 24°C, the temperature employed for the present studies. Since a plot of these values increased in a linear fashion with increases in temperature (not displayed), we utilized an interpolated value of  $3.2 \times 10^{-8}$  M for the monomeric solubility of cholesterol monohydrate at 24°C.

To evaluate the aqueous monomeric solubility of  $\beta$ -sitosterol at 24°C, [ $^{14}C$ ] $\beta$ -sitosterol, purified by preparative TLC (see Materials), was dissolved in HPLC grade  $CHCl_3$  and the solution was divided into two portions, each containing approximately 2  $\mu$ Ci. The [ $^{14}C$ ] $\beta$ -sitosterol- $CHCl_3$  solutions were placed in acid-washed 20-ml Pyrex test tubes and the solvent was evaporated to complete dryness in a stream of  $N_2$  and under reduced pressure. Ten ml of  $KMnO_4$ -redistilled  $H_2O$  was then added to each test tube. After replacing air with a blanket of  $N_2$ , the tubes were tightly sealed with Teflon®-lined screw caps and incubated at 24°C for 2 weeks in a shaking water bath (29). The solutions were then transferred to 10.0-ml stainless-steel centrifuge tubes and centrifuged for 1 hr at 105,000  $g$  in a Ti-50 rotor employing a Beckman L-65 ultracentrifuge (Beckman Instruments, Fullerton, CA). Following centrifugation, each solution was filtered through a Whatman GF/F filter (Fisher Scientific Co., Pittsburgh, PA) and a 0.22  $\mu$ m Millipore filter employing stainless-steel filter holders (Millipore Corp., Bedford, MA). Triplicate 100- $\mu$ l portions of the filtrates were dissolved in 4.0 ml of Liquiscint (National Diagnostics, Somerville, NJ) and counted in a liquid scintillation spectrometer (Packard Instrument Co., Sterling, VA). The median value of  $\beta$ -sitosterol monohydrate solubility in two sets of experiments was  $5.02 \times 10^{-9}$  M at 24°C.

## RESULTS

### Chemical structures of sterols

Common and systematic nomenclature as well as empirical formulae,  $M_r$  values, and mean capillary melting points (mp, °C, from commercial suppliers and ref. 2) are listed in Table 1. Fig. 1 displays the chemical structures of the sterols studied. Nuclear, side-chain, and polar group alterations were chosen in order to have systematic variations in each  $C_{27}$ ,  $C_{28}$ , and  $C_{29}$  sterol.

### Reverse-phase HPLC analysis

Fig. 2 depicts representative HPLC elution profiles of nine  $3\beta$ -OH sterols grouped according to the carbon number ( $C_{27}$ ,  $C_{28}$ ,  $C_{29}$ ) and degree of nuclear and side-chain unsaturation ( $\Delta^{5,24}$ ,  $\Delta^{5,22}$ ,  $\Delta^5$ ,  $\Delta^7$ ). As has been noted previously for analysis of shellfish and phytosterols by gas-liquid chromatography on both packed and capillary columns (18), the range of retention times of the  $3\beta$ -OH sterols by HPLC was limited to a  $\approx$ 5-min time



TABLE 1. Nomenclature and properties of sterols

Common Name	Empiric Formula	Systematic Nomenclature <sup>a</sup>	Mean MP	M <sub>r</sub>
<b>Nuclear variations</b>				
Cholesterol (C <sub>27</sub> Δ <sup>5</sup> )	C <sub>27</sub> H <sub>46</sub> O	5α-cholesten-3β-ol	149	386.67
Cholestanol (C <sub>27</sub> Δ <sup>0</sup> )	C <sub>27</sub> H <sub>48</sub> O	5α-cholestan-3β-ol	141	388.69
Lathosterol (C <sub>27</sub> Δ <sup>7</sup> )	C <sub>27</sub> H <sub>46</sub> O	5α-cholest-7-en-3β-ol	122	386.67
<b>Side chain variations</b>				
22-Dehydrocholesterol (C <sub>27</sub> Δ <sup>5,22</sup> )	C <sub>27</sub> H <sub>44</sub> O	cis-cholesta-5,22-dien-3β-ol	135	384.65
24-Dehydrocholesterol (C <sub>27</sub> Δ <sup>5,24</sup> ) (desmosterol)	C <sub>27</sub> H <sub>44</sub> O	cholesta-5,24-dien-3β-ol	117	384.65
Campesterol (C <sub>28</sub> Δ <sup>5</sup> )	C <sub>28</sub> H <sub>48</sub> O	24α-methylcholest-5-en-3β-ol	158	400.69
Brassicasterol (C <sub>28</sub> Δ <sup>5,22</sup> )	C <sub>28</sub> H <sub>46</sub> O	24β-methylcholesta-5,22-dien-3β-ol	148	398.68
24-Methylene-cholesterol (C <sub>28</sub> Δ <sup>5,24</sup> )	C <sub>28</sub> H <sub>46</sub> O	24-methylenecholesta-5,24-dien-3β-ol	144	398.68
β-Sitosterol (C <sub>29</sub> Δ <sup>5</sup> )	C <sub>29</sub> H <sub>50</sub> O	24β-ethylcholesten-3β-ol	140	414.72
Stigmasterol (C <sub>29</sub> Δ <sup>5,22</sup> )	C <sub>29</sub> H <sub>48</sub> O	24α-ethylcholesta-5,22-dien-3β-ol	170	412.71
Fucoesterol (C <sub>29</sub> Δ <sup>5,24</sup> )	C <sub>29</sub> H <sub>48</sub> O	24α-ethylcholesta-5,24-dien-3β-ol	124	412.71
<b>Polar group variations</b>				
Epicholesterol	C <sub>27</sub> H <sub>46</sub> O	5α-cholesten-3α-ol	142	386.67
Thiocholesterol	C <sub>27</sub> H <sub>46</sub> S	5α-cholesten-3β-thiol	95	402.73

<sup>a</sup>This nomenclature has been simplified by the omission of E,Z configurational prefixes and R,S chiral prefixes.

interval (Fig. 2). The HPLC elution times of the C<sub>27</sub> sterols (desmosterol (Δ<sup>5,24</sup>), cholesterol (Δ<sup>5</sup>), and lathosterol (Δ<sup>7</sup>)) showed that they are more mobile than the corresponding C<sub>28</sub> sterols (24-methylene cholesterol

(Δ<sup>5,24</sup>), brassicasterol (Δ<sup>5,22</sup>), campesterol (Δ<sup>5</sup>)), which in turn were more mobile than the C<sub>29</sub> sterols (fucoesterol (Δ<sup>5,24</sup>), stigmasterol (Δ<sup>5,22</sup>), β-sitosterol (Δ<sup>5</sup>)). Within each triad, the HPLC mobility increased, i.e., the sterol

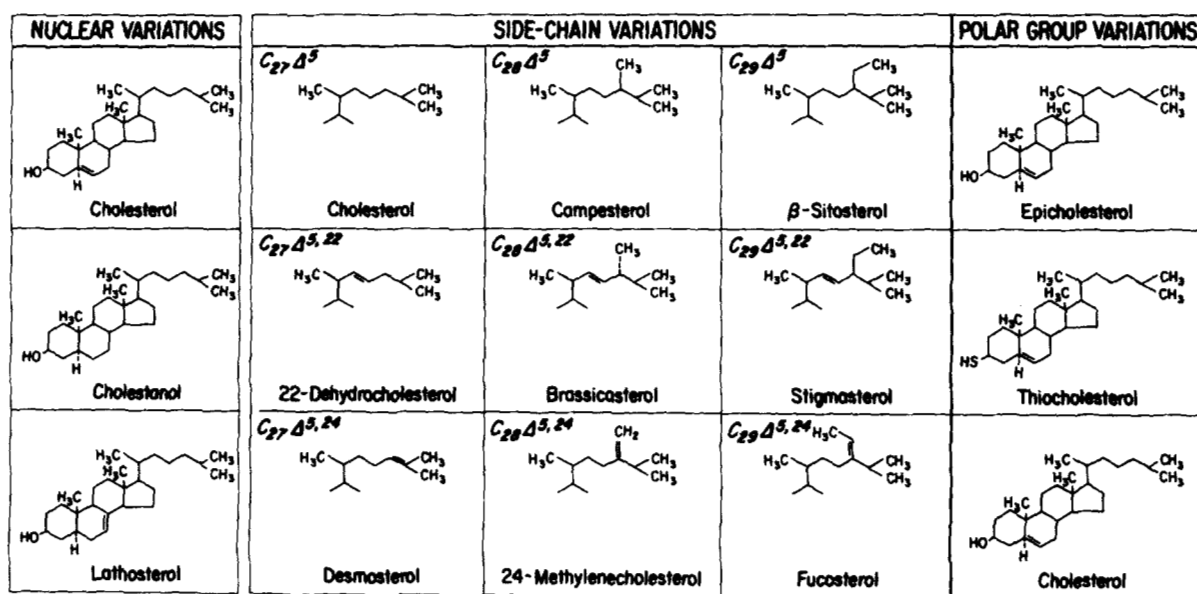
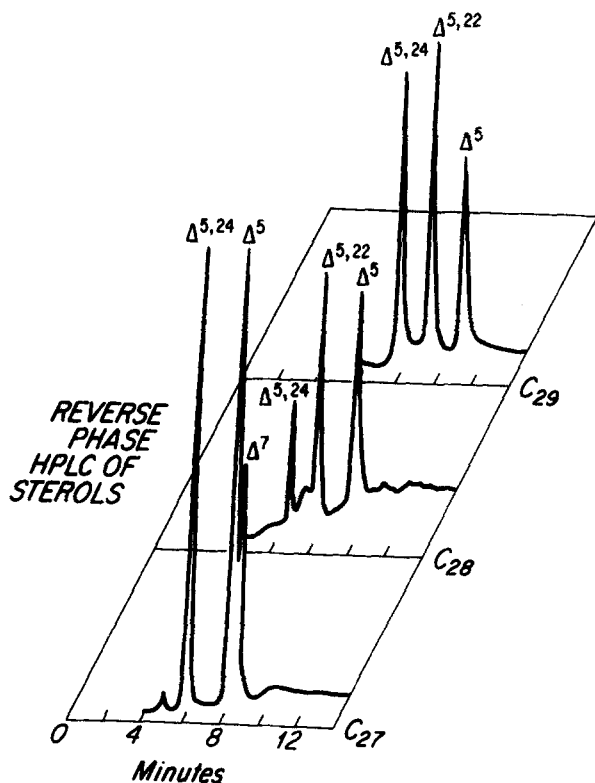


Fig. 1. Conventional chemical configurations of the sterols investigated. Nuclear variations reflect the absence, presence and position of a single double bond. Side chain variations reflect the presence of a branched methyl or ethyl group and the presence and position of a single double bond. Polar group variations in C<sub>27</sub> sterols are 3α-OH, 3β-SH, and 3β-OH (cholesterol).



**Fig. 2.** Reverse-phase HPLC of sterols employing a 15-min linear gradient of methanol-acetonitrile 60:40 (by vol) to methanol-acetonitrile 22.5:81.5 (by vol) at a flow rate of 3.0 ml/min. HPLC column was Ultrasphere ODS containing 5  $\mu$ m silica C18 particles with external dimensions of 250  $\times$  4.6 mm. The rank ordering of HPLC mobilities was  $C_{27} > C_{28} > C_{29}$  with  $\Delta^{5,24} > \Delta^{5,22} > \Delta^5 > \Delta^7$ .

became more hydrophilic, when a single double bond was introduced into the side chain and a distal double bond ( $\Delta^{24}$ ) produced appreciably greater HPLC mobilities than a proximal double bond ( $\Delta^{22}$ ). In contrast, HPLC mobi-

lities of  $C_{27}$  sterols with nuclear double bonds differed only slightly (Fig. 2).

**Table 2** lists the normalized HPLC retention factors ( $k'$ ) of all sterols studied. These vary from a low value of 5.40 for desmosterol ( $C_{27}\Delta^{5,24}$ ), which was the most mobile and hence most hydrophilic sterol, to 21.58 for thiocholesterol ( $3\beta$ -SH,  $C_{27}\Delta^5$ ), which was the least mobile and therefore most hydrophobic. Whereas alterations in the sterol's side chain produced marked effects on HPLC  $k'$  values, the presence and location of a single double bond in the sterol's nucleus had only small effects on HPLC  $k'$  values (Table 2). Moreover, variations in the single polar function from  $3\alpha$ -OH to  $3\beta$ -OH to  $3\beta$ -SH resulted in appreciable increases in mean HPLC  $k'$  values from 6.3 to 7.4 and 21.6, respectively.

#### Equilibrium micellar solubilities

**Fig. 3** and **Fig. 4** show representative plots of the time course of sterol solubilization in 50 mM NaGDC and 50 mM NaTC solutions. Equilibration times by the dissolution method can be inferred from the constant sterol solubility values that began at approximately 3–7 days. In general, sterols with low micellar solubilities attained equilibrium values somewhat more rapidly than sterols with higher solubilities. **Fig. 3** depicts that the equilibrium solubilities of the more hydrophilic  $C_{29}\Delta^{5,24}$  sterol (fucosterol) in both NaGDC and NaTC solutions was greater than the more hydrophobic  $C_{29}\Delta^5$  sterol ( $\beta$ -sitosterol). **Fig. 4** displays the equilibrium micellar solubilities of cholesterol ( $C_{27}\Delta^5$ ), campesterol ( $C_{28}\Delta^5$ ), and  $\beta$ -sitosterol ( $C_{29}\Delta^5$ ) in both bile salt systems. Whereas in NaGDC solutions, equilibrium micellar solubilities of  $\Delta^5$  sterols with saturated side chains varied in the rank order  $C_{27} > C_{28} > C_{29}$ , the rank ordering was reversed for micel-

**TABLE 2.** HPLC properties and micellar solubilities of sterols<sup>a</sup>

Sterol	$k'$ <sup>b</sup>	Solubility in NaGDC (mM)	Saturation <sup>c</sup> Ratio	Solubility in NaTC (mM)	Saturation <sup>c</sup> Ratio
$C_{27}\Delta^5$	7.38 $\pm$ 0.44	2.20 $\pm$ 0.03	23	0.45 $\pm$ 0.01	111
$C_{27}\Delta^0$	6.58 $\pm$ 0.46	1.88 $\pm$ 0.06	27	0.39 $\pm$ 0.02	128
$C_{27}\Delta^7$	7.69 $\pm$ 0.53	2.43 $\pm$ 0.24	21	0.6 $\pm$ 0.07	83
$C_{27}\Delta^{5,22}$	5.72 <sup>d</sup>	2.50 <sup>d</sup>	20	0.50 <sup>d</sup>	100
$C_{27}\Delta^{5,24}$	5.40 $\pm$ 0.23	1.91 $\pm$ 0.03	26	0.67 $\pm$ 0.03	75
$C_{28}\Delta^5$	9.39 $\pm$ 0.13	0.43 $\pm$ 0.03	116	0.13 $\pm$ 0.0	385
$C_{28}\Delta^{5,22}$	7.43 $\pm$ 0.23	0.15 $\pm$ 0.04	333	0.11 $\pm$ 0.01	455
$C_{28}\Delta^{5,24}$	6.22 $\pm$ 0.31	0.90 <sup>d</sup>	56	0.45 <sup>d</sup>	111
$C_{29}\Delta^5$	10.24 $\pm$ 0.64	0.31 $\pm$ 0.04	161	0.19 $\pm$ 0.08	263
$C_{29}\Delta^{5,22}$	8.51 $\pm$ 0.59	0.94 $\pm$ 0.02	53	0.20 $\pm$ 0.01	250
$C_{29}\Delta^{5,24}$	7.73 $\pm$ 0.22	1.14 $\pm$ 0.02	44	0.47 $\pm$ 0.02	106
Epicholesterol	6.32 $\pm$ 0.43	0.72 <sup>d</sup>	69	0.30 <sup>d</sup>	167
Thiocholesterol	21.58 $\pm$ 0.74	0.54 <sup>d</sup>	93	0.07 <sup>d</sup>	714

<sup>a</sup>Data are presented as means  $\pm$  SD (n = 3).

<sup>b</sup>Normalized HPLC retention value.

<sup>c</sup>Molar ratio of bile salt to solubilized sterol at micellar saturation.

<sup>d</sup>Average of two observations.

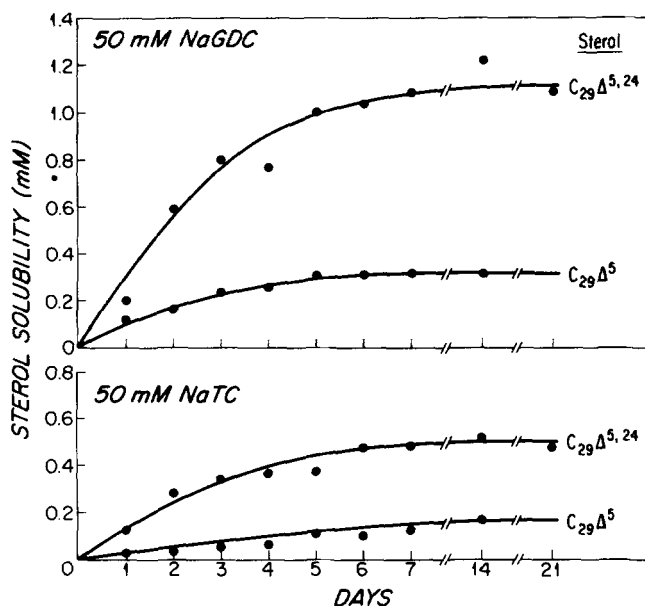


Fig. 3. Solubilities of two  $C_{29}$  sterols by microcrystalline dissolution in 50 mM solutions of NaGDC (pH 10.0) and NaTC (pH 7.0) as functions of time in days. Equilibrium sterol solubilities (plateau in curves) of both fucosterol ( $C_{29}\Delta^{5,24}$ ) and  $\beta$ -sitosterol ( $C_{29}\Delta^5$ ) were greater in NaGDC than in NaTC solutions.

lar solubilities of the  $C_{29}$  and  $C_{28}$  sterols in NaTC solutions (Fig. 4).

Table 2 lists all equilibrium sterol solubilities as well as micellar saturation ratios, that is, the number of bile salt molecules required to carry one sterol molecule in mixed micellar solution at equilibrium. It is clear that micellar solubilities of individual sterols were appreciably higher in NaGDC than in NaTC solutions. In particular, equilibrium micellar solubilities of  $C_{27}$  sterols were markedly higher than  $C_{28}$  and  $C_{29}$  sterols, irrespective of whether the side chains were saturated or unsaturated. However, in comparing the two systems, some anomalies were noted in the solubilities of identical sterols. For example, a  $C_{28}$  sterol that was more soluble than the analogous  $C_{29}$  sterol in NaGDC solutions was less soluble than the  $C_{29}$  sterol in NaTC solutions (Table 2, Fig. 4). Also, side chain unsaturation influenced the rank ordering of solubilities between the two bile salt systems in that the  $C_{27}\Delta^{5,22}$  sterol was the most soluble sterol in NaGDC solutions whereas the  $C_{27}\Delta^{5,24}$  sterol was the most soluble sterol in NaTC solutions. Equilibrium micellar solubilities of the  $C_{27}$  sterols most structurally akin to cholesterol (cholestanol ( $\Delta^0$ ), lathosterol ( $\Delta^7$ )) displayed solubilities in both bile salt systems similar to those of cholesterol, varying in the rank order  $\Delta^7 > \Delta^5 > \Delta^0$ . Thiocholesterol exhibited very low equilibrium micellar solubilities in both NaTC and NaGDC solutions, bracketing the values for  $C_{28}\Delta^5$  and  $C_{29}\Delta^5$  sterols (Table 2). Epicholesterol, the  $3\alpha$  epimer of cholesterol, was also less soluble than cholesterol despite its high HPLC  $k'$  value (Table 2).

Micellar saturation ratios ranged from a low value of 20:1 for the most soluble sterol ( $C_{27}\Delta^{5,22}$ ) in NaGDC solutions to a high value of 714:1 for the least soluble sterol (thiocholesterol) in NaTC solutions.

### Relationships between crystal energies, HPLC parameters and equilibrium micellar solubilities

Relationships between capillary melting points (mp's  $^{\circ}\text{C}$ ), an index of crystal energy, of each 3-OH sterol and their respective micellar solubilities in NaGDC and NaTC solutions are plotted in Fig. 5. The main figure shows that, as the sterol mp decreased, micellar solubilities tended to increase although the data, especially in the case of NaGDC, were highly dispersed. The inset (Fig. 5) displays that there was a weak positive correlation between mp values and HPLC  $k'$  values. Thus, 3-OH sterols with higher mp values tended to be less soluble especially in NaTC solutions and generally less mobile by HPLC (higher  $k'$  values).

Fig. 6 displays equilibrium sterol solubilities in micellar NaGDC and NaTC solutions, as functions of HPLC  $k'$  values. Both relationships are curvilinear and show that

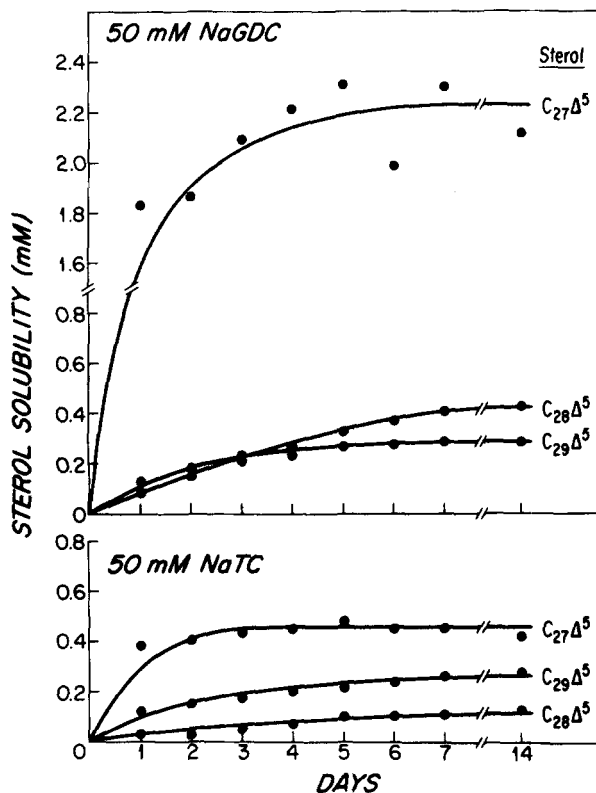


Fig. 4. Solubilities of  $C_{27}$ ,  $C_{28}$ ,  $C_{29}\Delta^5$  sterols by microcrystalline dissolution in 50 mM solutions of NaGDC (pH 10.0) and NaTC (pH 7.0) as functions of time in days. The solubilities of cholesterol ( $C_{27}\Delta^5$ ), campesterol ( $C_{28}\Delta^5$ ), and  $\beta$ -sitosterol ( $C_{29}\Delta^5$ ) in NaGDC are greater than in NaTC solutions. Note that the final rank ordering of solubilities is  $C_{27}\Delta^5 > C_{29}\Delta^5 > C_{28}\Delta^5$  in NaGDC solutions, whereas the rank ordering of solubilities is  $C_{27}\Delta^5 > C_{29}\Delta^5 > C_{28}\Delta^5$  in NaTC solutions.

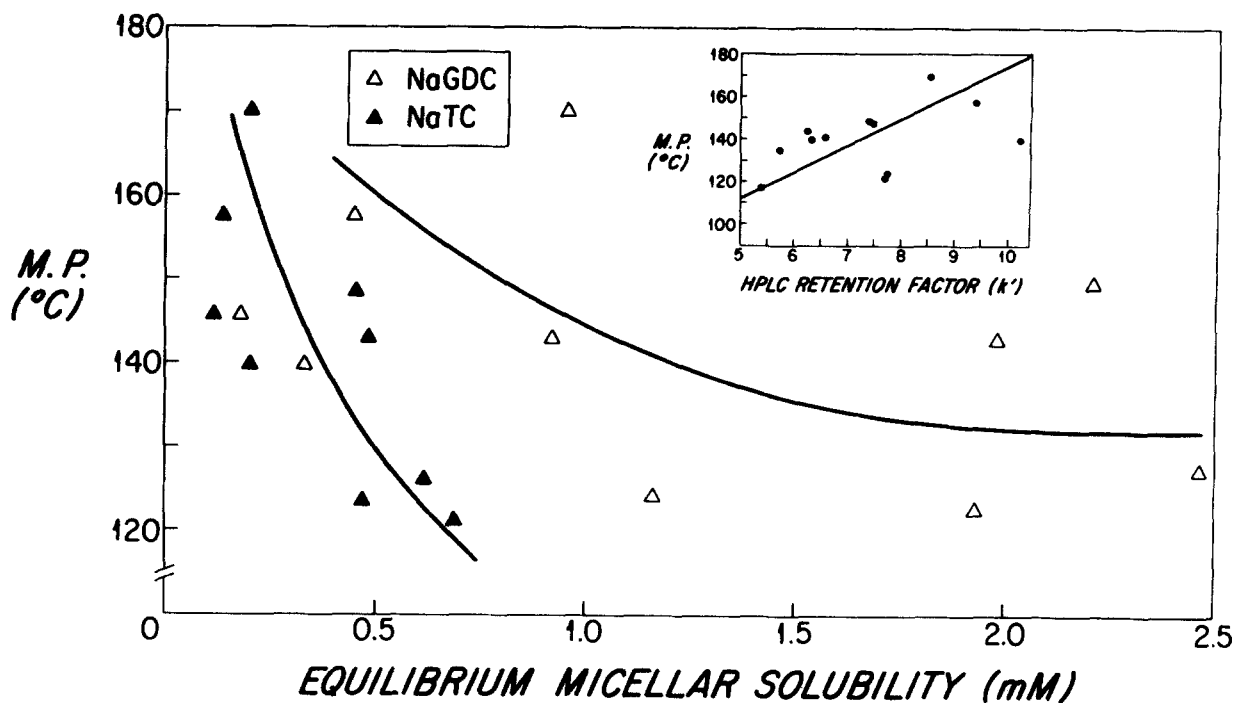


Fig. 5. Relationships of capillary melting points (mp, °C) of all 3-OH crystalline sterols and equilibrium micellar solubilities (mM) in NaGDC and NaTC solutions (main figure). Inset: Relationship between sterol mp (°C) values and HPLC  $k'$  values for all 3-OH sterols.

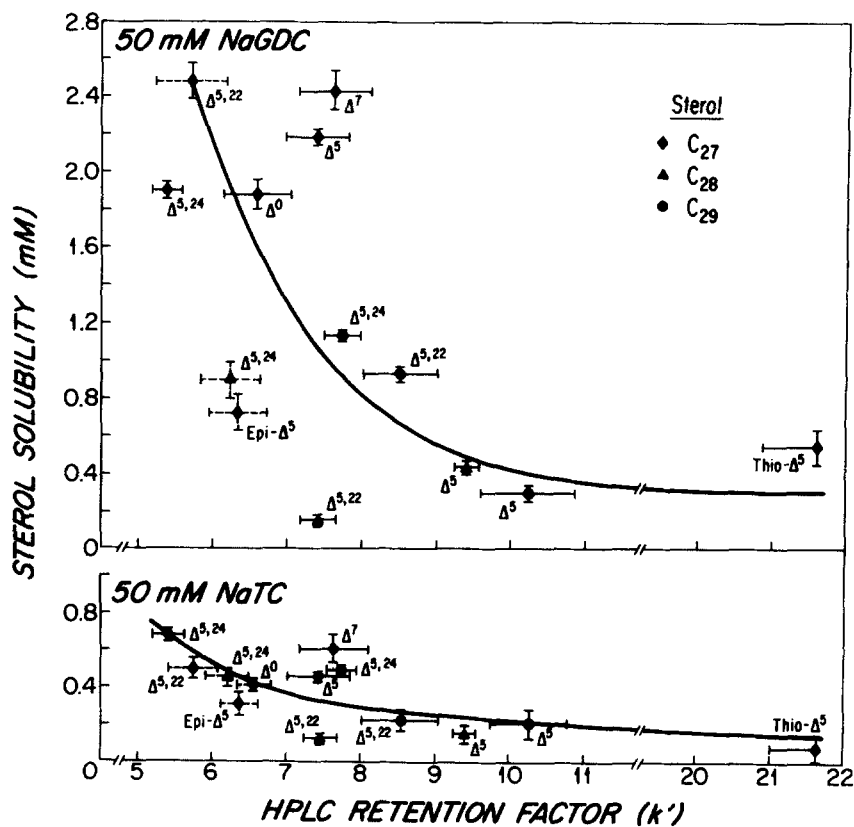


Fig. 6. Relationships of equilibrium sterol solubilities in NaGDC and NaTC micellar solutions as functions of HPLC  $k'$  values (mobility<sup>-1</sup>). Solid bars represent  $\pm 1$  SD; dashed bars are experimental estimates based upon the medians of the data.

as HPLC mobility decreased (to lower HPLC  $k'$  values), the sterol solubilities in each bile salt micellar solution decreased. This decrease was more dramatic in the case of NaGDC than NaTC solutions. Sterol solubilities, as a function of HPLC  $k'$  values in NaTC solution, were statistically fitted by least squares analysis to a curvilinear function ( $y = 7.26 e^{-1.57x}$ ,  $r = 0.775$ ,  $P < 0.01$ ). In contrast, sterol solubilities in NaGDC solutions were too scattered to be statistically correlated with HPLC  $k'$  values. This was principally due to the fact that equilibrium micellar solubilities of epicholesterol (epi- $\Delta^5$ ), brassicasterol ( $C_{28}\Delta^{5,22}$ ), and 24-methylenecholesterol ( $C_{28}\Delta^{5,24}$ ) were much smaller than predicted by their HPLC  $k'$  values (Fig. 6).

### Thermodynamic analysis of micellar cholesterol and $\beta$ -sitosterol solubilities

Because cholesterol solubilization in bile salt micelles does not appreciably alter preexisting micellar size or structure (24, 25), we have quantitatively interpreted the present data by employing an earlier analysis used with common bile salt-cholesterol mixed micellar systems (24, 25). We assume that each bile salt micelle has a single sterol binding site characterized by a temperature-dependent binding constant  $K_1(T)$ . The ratio of bile salt to sterol molecules corresponding to the maximum degree of solubilization (micellar saturation ratio), denoted  $S$  (Table 2) is thus

$$S = \bar{N} \left[ \frac{1 + K_1(T) S_0(T)}{K_1(T) S_0(T)} \right] \quad \text{Eq. 1}$$

where  $\bar{N}$  is the mean aggregation number<sup>4</sup> of the bile salt micelle,  $K_1(T)$  is the sterol binding constant, and  $S_0(T)$  is the temperature-dependent aqueous monomeric solubility of the sterol. To obtain  $K_1(24^\circ\text{C})$  values, we employed mean literature values of  $\bar{N}$  (NaGDC) = 22 and  $\bar{N}$  (NaTC) = 5 in 0.15 M NaCl (30),  $S_0$  (cholesterol) of  $3.2 \times 10^{-8}$  M, and  $S_0$  ( $\beta$ -sitosterol) of  $5.02 \times 10^{-9}$  M (see Results) and appropriate  $S$  values from Table 2<sup>5</sup>. The calculated  $K_1(24^\circ\text{C})$  values were  $6.9 \times 10^8$  l/mol and  $1.5 \times 10^6$  l/mol<sup>6</sup> for cholesterol solubilization and  $3.2 \times 10^7$  l/mol and  $3.9 \times 10^6$  l/mol for  $\beta$ -sitosterol solubilization in NaGDC and NaTC solutions, respectively.

While it is apparent from this analysis that the micellar binding constant for cholesterol solubilization in NaGDC

solution was larger than the value for  $\beta$ -sitosterol solubilization, it was unanticipated that the micellar binding constant for  $\beta$ -sitosterol solubilization would be threefold larger than the corresponding value for cholesterol solubilization in NaTC solutions.

The standard free energy change ( $\Delta G_s$ ) associated with sterol solubilization was derived from

$$\Delta G_s = -RT \ln K_{eq}(T) \quad \text{Eq. 2}$$

where  $RT$  has the usual meaning and  $K_{eq}(T)$  is the equilibrium constant which is related to the temperature-dependent sterol binding constant ( $K_1(T)$ ) by:

$$K_{eq}(T) = K_1(T) [H_2O] \quad \text{Eq. 3}$$

where  $[H_2O]$  is the mole fraction of water which, in very dilute solutions, is 55.5. For cholesterol solubilization in NaGDC and NaTC micellar solutions at  $24^\circ\text{C}$ ,  $\Delta G_s$  values corresponded to  $-14.3$  and  $-10.7$  kcal/mol, respectively and, for  $\beta$ -sitosterol,  $\Delta G_s$  values were  $-12.5$  and  $-11.3$  kcal/mol, respectively<sup>7</sup>. Thus, with NaGDC micelles, the binding of cholesterol is the most energetically favored, whereas with NaTC micelles the binding of  $\beta$ -sitosterol is the most energetically favored. In previous work (24) we calculated a  $\Delta G_s$  value of  $-12.0$  kcal/mol by estimating the hydrocarbon area eliminated from water contact on the *external* surface of a primary bile salt micelle when a cholesterol molecule binds. This  $\Delta G_s$  value is bracketed by those experimentally derived for cholesterol solubilization in both NaGDC and NaTC micelles in the present work. The difference of  $-2.4$  kcal/mol between  $\Delta G_s$  values for cholesterol and  $\beta$ -sitosterol solubilization in the two micellar systems is not easily explained. However, the  $-3.6$  kcal/mol larger  $\Delta G_s$  value for cholesterol and the  $-1.2$  kcal/mol larger values for  $\beta$ -sitosterol solubilization in NaGDC micelles compared with NaTC micelles may be related to differences in the nature of the sterol binding sites between primary (NaTC) and secondary (NaGDC) micelles (30) or to subtle alterations in micellar structure upon solubilization of  $C_{27}$  versus  $C_{29}$  sterols. The latter possibility may also explain the dispersity in sterol solubility data in NaGDC solutions (Figs. 5 and 6). Assuming binding of  $\beta$ -sitosterol to the outside of primary NaTC micelles (see ref. 24), the  $-0.6$  kcal/mol larger  $\Delta G_s$  value for  $\beta$ -sitosterol compared with cholesterol would be consistent with the greater hydrocarbon area on the side chain of  $\beta$ -sitosterol that is eliminated from water contact upon micellar solubilization.

<sup>4</sup>Average number of monomers per micelle under specified conditions of temperature, ionic strength, and bile salt concentration.

<sup>5</sup>Differences in  $S$  (saturation ratio) between  $24^\circ\text{C}$  and  $37^\circ\text{C}$  are small and have been ignored in these calculations.

<sup>6</sup>This  $K_1(24^\circ\text{C})$  value is quite similar to the  $K_1(30^\circ\text{C})$  value deduced for NaTC-cholesterol micelles ( $1.34 \times 10^6$  l/mol) in ref. 25.

<sup>7</sup>These  $\Delta G_s$  values are relatively insensitive to large variations in the values of  $\bar{N}$  chosen for NaGDC or NaTC. While the values employed in the calculations may be considered "best" experimental values (see ref. 30), we have shown that, by varying  $\bar{N}$  (NaTC) and  $\bar{N}$  (NaGDC) but holding the ratio constant, the rank ordering of  $\Delta G_s$  values calculated for both sterols is not appreciably altered.



## DISCUSSION

These studies were designed to quantify, through the use of reverse-phase HPLC, the hydrophilic-hydrophobic balance of a series of 13 sterols with differences in polar functions, steroid nucleus and side chain length, configuration, and unsaturation. In turn, the HPLC  $k'$  values of the sterols were shown to bear an inverse relationship to equilibrium sterol solubilities in bile salt micellar solutions and to their own capillary melting points.

Molecular mobility on a reverse-phase HPLC column is a reflection of the partitioning of a monomeric solute between a stationary hydrophobic surface and a hydrophilic mobile phase (31, 32). Micellar solubilization of a sterol molecule reflects similar equilibria, but in this case it is the monomeric solubility of the sterol molecule in water and the binding affinity for the sterol molecule of the bile salt micelle that is important (24, 25). The aqueous monomeric solubility is influenced positively by the hydrophilicity of the sterol (i.e., low  $k'$  by HPLC) and low crystal energy (low capillary mp) (24).

### HPLC properties

The reverse-phase HPLC elution patterns of the free unesterified sterols examined in this study bear an analogous relationship to HPLC analysis of a series of sterol acetates (33). As noted by these authors (33) and ourselves, it is difficult to achieve complete separation with baseline resolution by HPLC of sterols that differ only in the position of a double bond in the sterol's nucleus (e.g., Fig. 2). In contrast, alterations in both the polar function and side chain resulted in marked changes in the HPLC retention behavior (Fig. 2, Table 2). Side chain double bonds increased sterol mobility considerably, which reflected decreased hydrophobicity of the molecule. However, the change in hydrophobicity depended upon the position of the side chain double bond: sterols with double bonds at the C22 position were less mobile (and more hydrophobic) than sterols with double bonds at the C24 position (Table 2). Increases in the side chain length, by the addition of methyl or ethyl groups, resulted in decreased HPLC mobility and increased hydrophobicity. These chromatographic variations are well known and have been observed for phospholipids (34) in which the addition of a methyl group to the acyl chain increased hydrophobicity, whereas the insertion of a double bond in one or both fatty acyl chains decreased hydrophobicity. Similar sequences of relative retention times have been noted for several of the sterols studied here when chromatographed on capillary SE-30 gas-liquid chromatographic columns (18).

### Micellar sterol solubilities

Previous studies from this laboratory have shown that the cholesterol solubilizing capacity of bile salt micelles

increases as the hydrophobicity of the monomeric bile salt increases (21). Similarly, equilibrium solubilities of essentially all sterols in the present study were greater in micellar solutions of the more hydrophobic NaGDC than in NaTC (Table 2, Figs. 3 and 4). However, the differences between the solubilizing capacities of the two bile salts were most marked for the more hydrophilic C<sub>27</sub> sterols, compared with the more hydrophobic C<sub>28</sub> and C<sub>29</sub> sterols (Table 2, Figs. 3 and 4). Thus, increases in the overall hydrophobicity of sterols resulted in a decrease rather than an intuitively expected increase in equilibrium solubilities in bile salt micelles. This lends added support to our earlier hypotheses that sterol monomers associated with the outer (hydrophilic) surfaces of bile salt micelles rather than with the hydrophobic core and that the major driving force for micellar solubilization was the aqueous monomeric solubility of the sterol (21, 24). As suggested by us earlier (24), a possible role of polar interaction with one or more of the OH functions on the exterior surface of bile salt micelles (see also ref. 30) is implied by the low micellar solubilities of epicholesterol (3 $\alpha$ -OH) and especially thiocholesterol (3 $\beta$ -SH) sterols with altered polar groups, despite HPLC  $k'$  values, which demonstrated that these sterols are more hydrophilic and more hydrophobic, respectively, than cholesterol (Table 2).

### Quantitative aspects of sterol solubilization

We demonstrated earlier (24, 25) that the overall transfer of cholesterol from the crystalline state through the monomer phase to micellar binding sites is unfavorable. We suggested that complete occupancy of bile salt-micellar binding sites does not occur at monomeric saturation of the sterol in the aqueous phase. It was expected, therefore, that as the crystalline energy ( $\sim$  mp) of the sterol increased, the transfer of monomers from the crystalline state to micellar binding sites would become even less favorable, as was observed (Fig. 5). Further, when the HPLC  $k'$  relationships are taken as evidence of the rank ordering of relative hydrophilic-hydrophobic balance and, hence, aqueous monomeric solubilities, then the inverse relationship between micellar sterol solubilities and  $k'$  values (Fig. 6) strongly suggests that the aqueous monomeric sterol solubilities control micellar solubility. However, as inferred from the degree of data dispersion, it is obvious that certain sterols did not fulfill these quantitative criteria (Table 2). These anomalies can be rationalized by considering that the stationary phase of a reverse-phase HPLC column represents a model hydrocarbon matrix bathed in a polar solvent. The HPLC elution system thus models the partitioning of monomeric sterol molecules from polar solutions onto pure hydrocarbon surfaces (31, 32). Our studies of equilibrium micellar solubilities modeled the partitioning of sterols between crystalline, aqueous, and the partly polar exteriors of micelles (25). Thus, the observed equilibrium micellar

sterol solubilities are more complex functions of both aqueous monomeric solubilities and hydrocarbon-polar solvent partitioning coefficients than HPLC analysis provides. These differences were reflected in some of the anomalies observed between micellar solubilities and  $k'$  values (Table 2, Fig. 6) and provide a possible explanation for the lack of clearly defined statistical relationships especially in the case of NaGDC (Fig. 6).

### Relationship of physical-chemical results to degree of intestinal absorption of sterols

Fig. 7 displays intestinal absorption of several non-cholesterol sterols as a ratio of cholesterol absorption in rats and humans (4, 11, 16) plotted against equilibrium sterol solubilities in 50 mM NaGDC solutions. The inset displays absorption ratios against HPLC mobilities ( $k'$  values) obtained in this study. It is apparent that, despite the experimental uncertainties involved in such studies, the absorption efficiency of each sterol increased as the equilibrium micellar solubility increased and, conversely, decreased with increasing HPLC  $k'$  value (Fig. 7, inset). Since more hydrophilic sterols demonstrated greater monomeric aqueous solubilities, e.g., cholesterol, the association of equilibrium micellar solubility and HPLC mobility with increased intestinal absorption suggests that the aqueous monomeric solubility is, per se, an important determinant of sterol absorption. Poorly absorbed sterols such as  $\beta$ -sitosterol and stigmasterol exhibited low equi-

librium micellar solubilities and, as shown for  $\beta$ -sitosterol in this work, also have very low aqueous monomeric solubilities. It must be appreciated that micellar sterol solubilities employed in this analysis reflect equilibrium conditions which are unlikely to occur in the upper small intestine during sterol absorption. However, as shown in the present work (Figs. 3 and 4) and previously (22), initial (30–90 min) dissolution rates of sterols in bile salt systems bear a predictable relationship in terms of rank order to equilibrium sterol solubilities.

### Physiological implications

Whereas the mechanisms responsible for the cholesterol-lowering properties of non-cholesterol sterols are not known, our thermodynamic analysis (see Results) suggests that  $\beta$ -sitosterol would displace cholesterol from binding to NaTC micelles with a favorable free energy difference of  $-0.6$  kcal/mol. Clearly, displacement of cholesterol from NaGDC micelles would not occur, since  $\beta$ -sitosterol binding would be accompanied by an unfavorable free energy difference of  $+1.8$  kcal/mol. These results may be of physiological relevance, since NaTC, in contrast to dihydroxy bile salts, may play a unique, but currently unexplained, promoting role in cholesterol absorption from the intestine (20, 35). A number of experimental observations support our thermodynamic deductions. In *in vitro* solubility studies with bile salt micellar solutions, the addition of equimolar amounts of

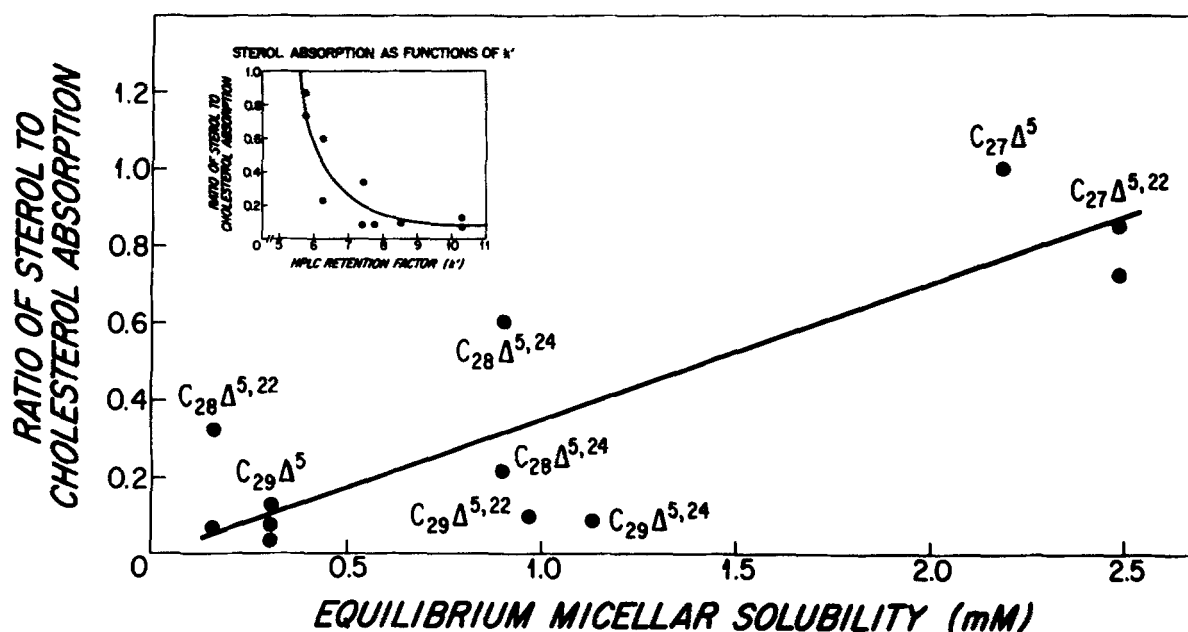


Fig. 7. Ratios of non-cholesterol sterol to that of cholesterol absorbed from the intestinal tract of rats and human beings (refs. 4, 12, 16) as functions of equilibrium sterol solubilities in 50 mM NaGDC solutions (main figure). The inset shows the same ratios for intestinal absorption of non-cholesterol sterols as functions of HPLC  $k'$  values (mobility $^{-1}$ ). Intestinal absorption of non-cholesterol sterols bears a weak positive relationship to equilibrium micellar solubilities and a strong inverse relationship to HPLC  $k'$  values.

$\beta$ -sitosterol and cholesterol decreased the amount of cholesterol solubilized more than would have been expected (19). In an in vivo study in human beings (cited in ref. 36), following a meal containing  $\beta$ -sitosterol, the isolated upper intestinal "micellar phase" was enriched in  $\beta$ -sitosterol and depleted in cholesterol, whereas the insoluble pellet was enriched in precipitated cholesterol.

Our studies carried out with simple bile salt micelles are clearly a great oversimplification of the in vivo situation. During established fat digestion in humans, complex multicomponent mixed micelles containing mixed bile salts, lipid digestion products, and unesterified cholesterol coexist with unilamellar vesicles composed of the same lipids (37). Whether sterol solubilities in these systems bear a predictable relationship to solubilities in the more reductionistic system that we have used is difficult to assess. However, studies with a series of common taurine-conjugated bile salts suggested that low solubility of cholesterol in simple bile salt micelles is also reflected by low solubilities in bile salt-lecithin mixed micelles (38). Whereas our studies provide quantitative relationships between solubilization of sterols by bile salt micelles and their hydrophilic-hydrophobic balance as inferred by reverse-phase HPLC, it is clear that further studies are needed to clarify whether these predictions are borne out physiologically. ■

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