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SOME ASPECTS OF MECHANISM OF INHIBITION OF CHOLESTEROL ABSORPTION BY β-SITOSTEROL

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(1) Mixed bile salt micelle solubilized either cholesterol or β-sitosterol to a comparable extent. When added simultaneously, β-sitosterol restricted the micellar solubility of cholesterol. (2) β-Sitosterol also reduced the cholesterol content in the aqueous (micellar) phase of the intestinal contents of rats, the extent of reduction being comparable with that observed in vitro. The intestinal uptake of cholesterol in vivo was equivalent to the micellar incorporation of cholesterol both in vitro and in vivo. (3) β-Sitosterol had no inhibitory effect on cholesterol absorption from the micellar solution in jejunal loops in situ, whereas the rate of β-sitosterol uptake was only about one-fifth that of cholesterol. (4) The intestinal uptake of β-sitosterol intubated into the stomach of rats was about one-fifth that of cholesterol. The intestinal brush-border membrane discriminated these sterols. These results suggest that the restriction of the micellar solubility of cholesterol, rather than the inhibition of uptake from brush-border membrane, is the major determinant for the interference of β-sitosterol with cholesterol absorption.

Introduction

β-Sitosterol reduces intestinal absorption of cholesterol and hence acts as a hypocholesterolemic agent in humans and experimental animals [1,2]. The mechanism by which β-sitosterol interferes with cholesterol absorption is still unclear, in spite of numerous investigations [1,2]. Some proposed mechanisms involve (i) formation of a non-absorbable complex with cholesterol in the intestinal lumen; (ii) reduction of the micellar solubility of cholesterol; (iii) inhibition of cholesterol uptake in the absorptive site, possibly at the brush-border membrane; and (iv) interference with cholesterol esterification or chylomicron formation in the intestinal mucosal cell.

On the other hand, the absorption in vivo of β-sitosterol itself is estimated to be about 5–10% that of cholesterol [1,2], possibly due to discrimination at the outer surface of the intestinal mucosal cell, because both sterols can distribute in the micellar phase to the same extent in vitro [3]. The discrimination mechanism between cholesterol and β-sitosterol in vivo seems at least to relate closely to the inhibitory effect of β-sitosterol on cholesterol absorption.

In this study, rats were used as a model and several parameters of cholesterol absorption were studied; these included (i) the effect of β-sitosterol on micellar incorporation of cholesterol both in vitro and in vivo; (ii) the influence of the plant sterol on the uptake in situ and in vivo of micellar cholesterol by the intestinal mucosa; (iii) the absorption in vivo of β-[^{14}C]sitosterol and [^{3}H]-cholesterol.

Materials and Methods

Chemicals

[4-^{14}C]Cholesterol (58.4 mCi/mmol), [1α,

2α(n)-^{3}H]cholesterol (60 Ci/mmol) and β-[4-^{14}C]sitosterol (58 mCi/mmol) were purchased from Amersham International and purified by thin-layer chromatography on benzene/ethyl acetate (7 : 3, v/v) prior to use. β-Sitosterol (ICN Pharmaceuticals, Cleveland, OH) was purified by recrystallizing several times from ethyl acetate and methanol to 99% purity as sterol [4]. This preparation contain 7% campesterol and 93% β-sitosterol. Sodium taurodeoxycholate was obtained from Calbiochem, La Jolla, CA. All other chemicals were of reagent grade.

Animals and diets

Male Wistar rats obtained from Kyudo Co., Kumamoto, were used. The animals were fed commercial chow (type NMF, Oriental Yeast Co., Tokyo) or a semipurified diet, the composition of which was, in percent: vitamin-free casein, 20; safflower oil, 10; mineral mixture, 4; vitamin mixture (water soluble), 1; choline chloride, 0.15; cellulose powder, 4 and sucrose to 100. The diet contained 400 IU, retinyl palmitate, 200 IU cholecalciferol and 10 mg α-tocopheryl acetate per 100 g. Mineral and vitamin mixtures were according to Harper [5]. Cholesterol and β-sitosterol were added at the 0.5% level at the expense of sucrose.

Micellization study in vitro

To the solutions containing either 5.4 mM monoolein, 10.8 mM triolein, 10.8 mM oleic acid and 6 mM sodium taurodeoxycholate in 150 mM phosphate buffer (pH 7.0, system I) or 5 mM monoolein and 5 mM sodium taurodeoxycholate in 50 mM phosphate buffer (pH 6.3, system II) were added various amounts of cholesterol and β-sitosterol and they were sonicated at 110 W for 5 min. The emulsions thus obtained were ultracentrifuged at 100 000 × *g* for 15 h (Hitachi 55P-72, RP-65 rotor, Tokyo). An aliquot of the clear micellar solution was saponified by the ethanolic KOH solution and the unsaponifiable matter was extracted with light petroleum ether. The sterols were determined by gas-liquid chromatography on 3% OV-17 as trimethylsilyl ether with 5α-cholestane (Sigma, St Louis, MO) as an internal standard [4]. All manipulations were carried out at 37°C or at room temperature around 25°C. The particle size of the micelle was less than 150 Å by membrane ultrafiltration (Amicon Corp., Lexington, MA).

Micellization study in vivo

Rats (300–350 g) were meal-fed for 1 h the semipurified diet with 0.5% cholesterol (group C) or with 0.5% cholesterol plus 0.5% β-sitosterol (group C + S) for 1 week. 2 h after withdrawal of the diet, rats were anesthetized with urethane and blood was collected from the abdominal aorta. The small intestine and liver were quickly removed and the intestinal contents were allowed to drain completely into a centrifuge tube by gentle massage. The contents were placed in a water bath at 70°C for 15 min to inactivate lipase and cholesterol esterase [6] and then ultracentrifuged under the same condition as mentioned above. Two phases separated, pellet and clear yellowish supernatant, and were analyzed for the sterol content. Serum and liver cholesterol were determined as described previously [7]. The concentration of bile acids in the micelle was measured enzymatically by using 3-hydroxysteroid dehydrogenase (Sigma) [8].

Cholesterol absorption in vivo

Rats (310–360 g) were meal-fed and killed as in the preceding experiment. The diets contained about 1 μCi of [^{14}C]cholesterol on the day of killing. The intestine was divided into eight equal lengths and the content was washed out with ice-cold saline. The intestinal segment was directly saponified in ethanolic KOH. The intestinal content was dried at 90°C and saponified. The radioactivity in the sterol fraction was measured in an aqueous scintillant (ACS II, Amersham) by liquid scintillation spectrophotometer (Aloka LSC-900, Tokyo). The stomach content was also analysed for the radioactivity. Under the present experimental condition, it seems likely that cholesterol is being actively absorbed while scarcely transported to the lymph [3].

Uptake of cholesterol and β-sitosterol from jejunal loops in situ

Micellar solutions containing trace amounts of [^{3}H]cholesterol and/or β-[^{14}C]sitosterol were prepared by the same procedure using the system I buffer at 37°C.

Overnight-fasted rats (270–320 g) were

anesthetized with urethane and kept in a chamber at 37°C throughout the experiment. The abdomen was opened and two jejunal segments of approx. 7 cm length were ligated without disturbing the intact blood supply. The first segment was just distal to the ligament of Treitz. The second segment was 1 cm distal to the first one. Before tightly closing the segment, 0.5 ml of a micellar solution kept at 37°C was infused to the first segment through one end. The abdomen was closed and rats were kept at body temperature for 30 min. About 2 min before the termination of incubation, the abdomen was opened again and 0.5 ml of the same micellar solution was introduced to the second segment. Immediately after the infusion, two segments were resected and jejunal contents were washed out with ice-cold saline. Washed segments were dried at 110°C for 16 h and weighed. The dried segments were saponified in ethanolic KOH and the radioactivity in the unsaponifiable matter was counted. The second segment served as a zero-time blank.

Uptake of cholesterol and β-sitosterol in vivo

[^{3}H]Cholesterol and β-[^{14}C]sitosterol dispersed in 0.5 ml of ethanol/saline (6 : 94, v/v) were intubated to the stomach of twelve rats (230–270 g) just after 2-h meal-feeding of rat chow. 2 h after intubation, rats were killed by decapitation and the stomach and intestine were removed and washed with ice-cold saline. The intestines of three rats were divided into eight equal lengths and the incorporation of the labels was measured. From the remaining nine rats were scraped the intestinal mucosa, and the brush-border membrane was fractionated and characterized by marker enzymes [9]. The rat chow contained 0.19% of sterols, the composition of which was: cholesterol, 45%; campesterol, 19%; and β-sitosterol, 36%. Protein was determined by the method of Lowry et al.

Statistical analysis

Data were analyzed by Student's *t*-test [11].

Results

Micellization study in vitro

Fig. 1 shows the effect of β-sitosterol on the micellar solubility of cholesterol in systems I and II. The extent of incorporation of cholesterol into micelles reached plateau at above the certain level of cholesterol added (cholesterol/taurodeoxycholate, 1 : 3 and 1 : 5, molar ratio, for systems I and II, respectively). The contours of micellar incorporation of β-sitosterol depicted apparently the same pattern as that of cholesterol in system I, but it tended to decrease at higher concentrations. When β-sitosterol and cholesterol (1 : 1, molar ratio) were simultaneously added, total sterols incorporated into the micelle were less than those in the micelle containing either one of sterols in both systems and the plateau value for micellar incorporation of cholesterol was less than one-half that observed on cholesterol alone.

When the concentration of cholesterol was kept constant, the micellar incorporation of cholesterol decreased progressively with increasing amounts of β-sitosterol (Fig. 2A). However, at a lower level of cholesterol, the addition of β-sitosterol scarcely affected the micellar solubility of cholesterol (Fig. 2B).

Micellization study in vivo

Though there was no difference in the serum

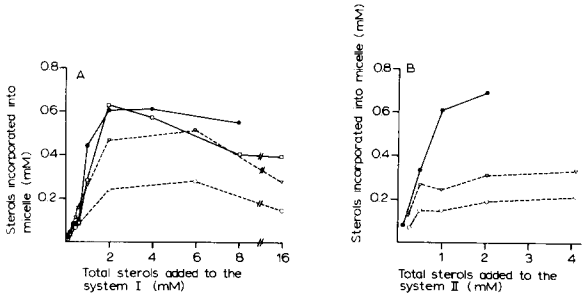

Fig. 1. Micellar incorporation of cholesterol, β-sitosterol and their mixture in system I (Fig. 1A) and system II (Fig. 1B) in vitro. To the solutions containing either 5.4 mM monoolein, 10.8 mM triolein, 10.8 mM oleic acid and 6 mM sodium taurodeoxycholate in 150 mM phosphate buffer (pH 7.0, system I), or 5 mM monoolein and 5 mM sodium taurodeoxycholate in 50 mM phosphate buffer (pH 6.3, system II) were added various amounts of cholesterol and β-sitosterol. After emulsification by sonication, the emulsion was ultracentrifuged and clear micellar solution was harvested. All manipulations were carried out at room temperature (system I) and 37°C (system II). Values were means of two experiments. ●———●, cholesterol; □———□, β-sitosterol; ○- - - - -○, cholesterol in a 1 : 1 mixture of two sterols; ▽- - - - -▽ total sterols in a 1 : 1 mixture of two sterols.

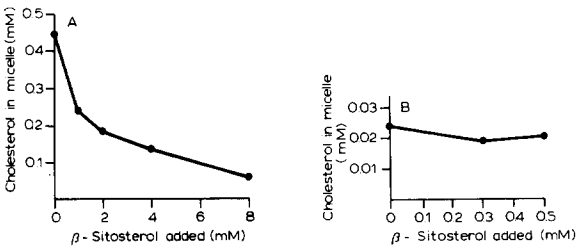

Fig. 2. Effect of varying concentrations of β-sitosterol on micellar incorporation of cholesterol in the system I in vitro. See Fig. 1. Cholesterol concentration was 1 mM (A) and 0.1 mM (B), respectively. Values were means of two experiments.

cholesterol level, the concentration of liver cholesterol was significantly lower in the C + S (cholesterol- + β-sitosterol-fed) group than in the C (cholesterol-fed) group, reflecting the inhibitory effect of β-sitosterol on cholesterol absorption (Table I). Volumes and bile acid concentration of the aqueous phase showed no significant differences between the two groups. The cholesterol content in an aqueous phase of the C group was significantly higher than that of the C + S group, the ratio being 1.75. Cholesterol contents in the stomach and intestinal solid phase, and total sterol (cholesterol plus plant sterols) contents in the aqueous phase were virtually the same in both groups.

The intestinal absorption of cholesterol in vivo

Serum and liver contained 0.63 ± 0.04% and 4.5 ± 0.3%, respectively, of the radioactivity administered in the C group and 0.23 ± 0.02% and 1.5 ± 0.2% in the C + S group. Total radioactivities in the intestinal mucosa in addition to intestinal and stomach contents corresponded to 84% of the dose in both groups. There were no differences in radioactivity in the stomach contents between two groups. The distribution of [^{14}C]cholesterol in the intestinal mucosa and contents is shown in Fig. 3. Unexpectedly, about one-half of [^{14}C]cholesterol incorporated into the intestine existed in the ileal part. Total intestinal uptake of [^{14}C]cholesterol in the C group was 1.84-fold higher than that in the C + S group. Consequently, more labeled cholesterol existed in the intestinal content in the distal portion of the latter.

The uptake of cholesterol and β-sitosterol from jejunal loops in situ

The jejunal segment incorporated [^{3}H]-cholesterol linearly up to 30 min. However, the uptake at zero time was unreasonably high. In spite of exhaustive washing out by saline, it never reduced to become negligible. Accordingly, the second segment served as a zero-time blank and the value of the first segment after 30 min incubation was corrected for the blank value. The relatively high incorporation into the intestinal mucosa at zero time also occurred when β-[^{14}C]sitosterol was used. The adsorption at zero time was about 3–5% of the labels infused. This is presumably

TABLE I

EFFECT OF β-SITOSTEROL ON THE CONCENTRATION OF SERUM AND LIVER CHOLESTEROL AND ON THE DISTRIBUTION OF CHOLESTEROL IN GASTROINTESTINAL TRACT

Rats were meal-fed for 1 h 0.5% cholesterol (group C) or 0.5% cholesterol plus 0.5% β-sitosterol (group C+S) diets for 1 week. 2 h after withdrawal of the diet, rats were killed and liver, stomach and small intestine were removed. All manipulations were carried out at 37°C. Values were means ± S.E. of seven (group C) or eight (group C+S) rats. Volumes of the aqueous phase were 1.8 ± 0.1 ml and 1.6 ± 0.1 ml for group C and group C+S, respectively. Chol, cholesterol; PS, plant sterols which contained β-sitosterol and campesterol; n.s., no significance.

Groups	Serum chol (mg/100 ml)	Liver chol (mg/g)	Stomach content		Intestinal content				
			chol (mg)	PS (mg)	Aqueous phase		Solid phase		Aqueous phase
					chol (μg)	PS (μg)	chol (mg)	PS (mg)	bile acid (mM)
C	69.0 ± 5.8	10.2 ± 0.9	53.0 ± 5.4	1.6 ± 0.2	710 ± 115	48 ± 7	8.2 ± 0.8	0.5 ± 0.1	40.9 ± 3.3
C+S	63.6 ± 6.8	4.0 ± 0.3	48.7 ± 2.2	54.6 ± 2.4	406 ± 70	385 ± 68	9.8 ± 0.5	13.1 ± 0.6	41.1 ± 4.4
	n.s.	$P < 0.001$	n.s.	–	$P < 0.05$	–	n.s.	–	n.s.

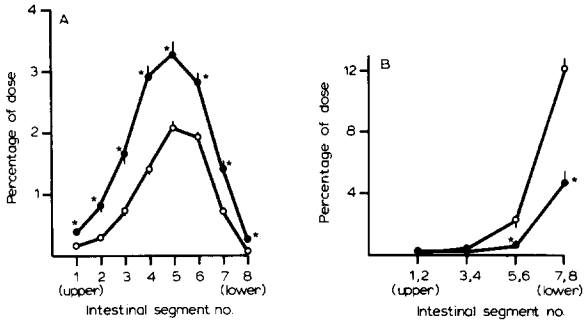

Fig. 3. Effect of β-sitosterol on the incorporation of [^{14}C]cholesterol into intestinal mucosa (Fig. 3A) and the radioactivity remaining in intestinal lumens (Fig. 3B). Rats were meal-fed and killed in the same condition as described in Table I. On the day of killing, the diets containing about 1 μCi of [^{14}C]cholesterol were given. Values were means ± S.E. of seven (cholesterol group) and eight (cholesterol + β-sitosterol group) rats. * Significantly different from cholesterol + β-sitosterol group at $P < 0.01$. ●———●, cholesterol group; ○———○, cholesterol + β-sitosterol group.

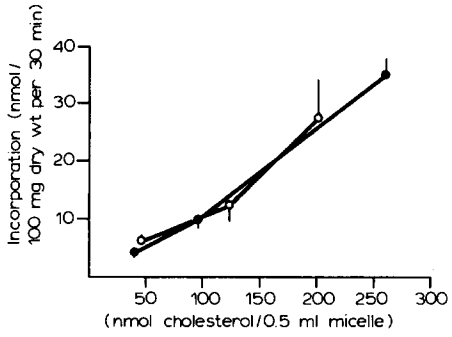

Fig. 4. Effect of β-sitosterol on cholesterol absorption in situ (I). Micellar solution containing a trace amount of [^{3}H]cholesterol which was prepared as the same procedure using the system I buffer at 37°C (see Fig. 1) was infused to the rat jejunal loops in situ. After incubation for 30 min, the segments were resected and the radioactivity incorporated was analyzed. Values were corrected for the zero time blank (see Materials and Methods) and expressed as means ± S.E. of four rats. ●———●, [^{3}H]cholesterol incorporation with mixed micelle containing cholesterol alone; ○———○, [^{3}H]cholesterol incorporation with mixed micelle containing both cholesterol and β-sitosterol (1 : 1).

attributed either to nonphysiological adhesion at the surface of the intestinal mucosa or to incorporation into the unstirred water layer not removed by washing out, and hence it does not represent the real absorption.

The effect of β-sitosterol on cholesterol uptake is shown in Fig. 4. Cholesterol uptake increased in proportion to the micellar concentration of cholesterol increased. β-Sitosterol which was micellized with cholesterol did not affect the cholesterol uptake. In this case, the total sterol content in the micelle containing both cholesterol and β-sitosterol was about 2-fold higher than that containing cholesterol alone. When total amounts of sterols in micelle were kept approximately constant, the rate of cholesterol absorption was also not affected by the increasing amount of β-sitosterol, even in the case when the micelle contained 9-fold β-sitosterol (Fig. 5). Subsequently, even when micelles containing β-sitosterol with a trace amount of [^{3}H]cholesterol were incubated in situ, the rate of [^{3}H]cholesterol uptake was the same as that of micelles containing cholesterol alone (Fig. 6A). Absorption of β-[^{14}C]sitosterol from micelles containing β-sitosterol with a trace amount of [^{3}H]cholesterol was about one-fifth that of [^{3}H]cholesterol (Fig. 6B).

The intestinal uptake of cholesterol and β-sitosterol in vivo

The intestinal tissue and intestinal and stomach contents all together contained 88% of [^{3}H]cholesterol and 96% of β-[^{14}C]sitosterol ingested. The radioactivities remaining in the stomach contents were almost the same between two sterols. The distribution of the labels in in-

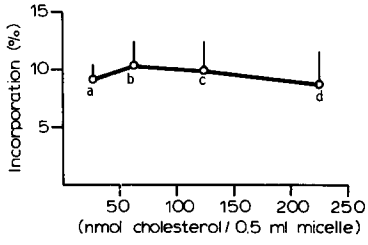

Fig. 5. Effect of β-sitosterol on cholesterol absorption in situ (II). See Fig. 4. The total amount of sterols in the mixed micelle was kept approximately constant. The ratios of β-sitosterol to cholesterol in the mixed micelle were: a, 9/1; b, 3/1; c, 1/1; and d, 0/1 and the concentration of total sterols (cholesterol plus β-sitosterol) (nmol/0.5 ml) were a, 225; b, 211; c, 245 and d, 253. Incorporation (%) was calculated according to the following equation; incorporation (%) = (radioactivity incorporated into 100 mg dry tissue) × 100/(radioactivity infused into jejunal loop). Values were means ± S.E. of five rats.

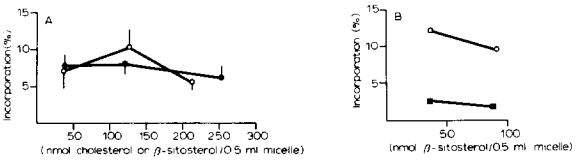

Fig. 6. Effect of β-sitosterol on cholesterol absorption in situ (III). See Figs. 4 and 5. Micellar solution containing β-sitosterol or cholesterol with a trace amount of [^{3}H]cholesterol was infused to the rat jejunal loops in situ (Fig. 6A). In B, micelle also contained β-[^{14}C]sitosterol. Values were means ± S.E. of five (A) and three (B) rats. ●———●, [^{3}H]cholesterol incorporation with the mixed micelle containing cholesterol alone; ○———○, [^{3}H]cholesterol incorporation with the mixed micelle containing β-sitosterol and a trace amount of cholesterol; ■———■, β-[^{14}C]sitosterol incorporation with the mixed micelle containing β-sitosterol and a trace amount of cholesterol.

testinal mucosa and contents is shown in Fig. 7. Approx. 4.5-fold higher radioactivity from [^{3}H]cholesterol was absorbed by the intestine than that from β-[^{14}C]sitosterol. The concentration of luminal cholesterol in the upper quarter of the intestine was roughly 2.5-fold higher than that of β-sitosterol (data not shown). Thus, when specific activities were taken into consideration, quantitatively 10-fold more cholesterol was calculated to be absorbed compared to β-sitosterol.

The ratios of the radioactivities (^{3}H/^{14}C) were apparently identical among the brush border

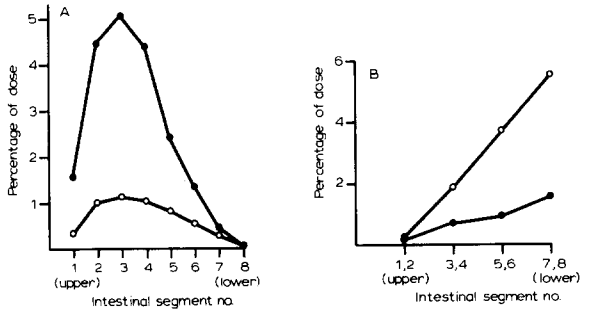

Fig. 7. Incorporation of [^{3}H]cholesterol and β-[^{14}C]sitosterol into intestinal mucosa (Fig. 7A) and the radioactivity remaining in intestinal lumens (Fig. 7B). The labels dispersed in 0.5 ml ethanol/saline (6:94, v/v) were intubated to the stomach of rats just after 2-h meal-feeding of rat chow. 2 h after intubation, rats were killed and the small intestine was removed. Values were means of three rats. ●———●, cholesterol; ○———○, β-sitosterol.

membrane (4.6), supernatant (3.9), pellet (containing organelles) (4.5) and the whole homogenate (4.6).

Discussion

There is a contrasting discrepancy in the rate of absorption of cholesterol and β-sitosterol between in vitro and in vivo [12–15]. Sylven reported that normal blood supply was critical for absorption of cholesterol; when blood supply was disturbed, absorption of cholesterol decreased to the same level as that of β-sitosterol [13]. Administration of metabolic inhibitors such as NaN_2 and KCN or the lowering body temperature of the rat cause the decrease in cholesterol uptake, but not fatty acid uptake, by the rat jejunum in situ [16]. Chow and Hollander suggested the existence of some components to complex with cholesterol in the intestinal mucosal cell [17]. All of these observations suggest that cholesterol absorption proceeds through carrier-mediated and/or energy-dependent processes. Hence, studies on cholesterol absorption, even though in vivo, should be carried out under the conditions exactly reproducible in the intact animals, and almost all the present experiments were carried out with these considerations. The discrimination of absorption by the small intestinal cell between cholesterol and β-sitosterol was evident in our experimental conditions (Figs. 6B and 7A).

Sterol incorporation into the bile salt micelle in vitro depended on the solubilizing capacity of that micelle. Thus, the micellar solubilization of cholesterol was restricted by the addition of β-sitosterol. The results obtained in vitro were ascertained in the in vivo micellization study (Table I). The ratio of cholesterol distributed in the micelle from rats fed cholesterol to that from rats fed cholesterol plus β-sitosterol was very close to that of the radioactivity incorporated into the intestinal mucosa in these two groups of rats (1.74 vs. 1.84) (Table I and Fig. 3A). A jejunal loop technique in situ showed that β-sitosterol had no inhibitory effect on the absorption of micellar cholesterol. The data were consistent with those of a similar type of the in situ study reported by Hollander and Morgan [18]. These observations indicate that the principle determining factor for the inhibitory

effect of β-sitosterol on cholesterol absorption is the reduction of micellar incorporation of cholesterol.

In the in vitro study, the total micellar solubilizing capacity decreased when cholesterol and β-sitosterol existed simultaneously both in system I buffer at 25°C and system II buffer at 37°C. In the in situ experiments with the system I buffer at 37°C, the reduction of solubilizing capacity was also confirmed (data not shown). This phenomenon may be related to the formation of the mixed crystal with animal and plant sterols [19].

According to our results, β-sitosterol did not disturb cholesterol absorption until the micelle was being saturated with sterols in the intestinal lumen. This observation favors available information that for an effective therapeutic use. a reasonable amount of β-sitosterol is needed. As shown in Fig. 2A, β-sitosterol at the cholesterol/β-sitosterol ratio of 1.0 was, in a relative sense, most effective. Since 1–3 g of cholesterol are estimated to pass through the human intestine per day, above 3 g of β-sitosterol seems to be the effective dose. Lees et al. showed that 3 g of β-sitosterol was most efficient for reducing the serum cholesterol level [20].

The mechanism of the inhibitory effect of any other less absorbable sterols on cholesterol absorption, such as cholestanol [21], shell-fish sterols [22], stigmasterol [23] and β-sitostanol [4,24], may similarly be attributed to the restriction of micellar incorporation of cholesterol. The absorbability of these sterols seems to determine the potency of the inhibitory effect; the less they are absorbed, the more they remain in the intestinal lumen and become effective, just as in the relationship between β-sitosterol and β-sitostanol – the latter is known to be a more effective hypocholesterolemic agent than the former [4,7,24,26].

With regard to the discrimination mechanism between cholesterol and β-sitosterol, the classic study of Glover and Green [25] suggested the presence of acceptor lipoprotein with a high affinity for cholesterol in the intestinal mucosa, the inhibition of cholesterol absorption of plant sterols could be explained by assuming that the acceptor lipoprotein becomes partially blocked with the foreign sterol. Our data do not support their hypothesis, because β-sitosterol did not affect the cholesterol absorption from jejunal loops in situ in spite of the discrimination of β-sitosterol from cholesterol.

Sylven and Borgström have suggested that the discrimination for sterol absorption takes place during the process of uptake into the intestinal mucosal cell [3], the assumption being confirmed by our study. The brush-border membrane could primary discriminate cholesterol from β-sitosterol. However, the existence of the active transport system and/or receptor protein remains to be clarified.

In cholesterol absorption study in vivo (Figs. 3A and 3B), cholesterol accumulated considerably in the distal intestine. Intestinal contents were washed out several times with ice-cold saline as in the similar type of experiment on the absorption of cholesterol and β-sitosterol (Fig. 7), where the accumulation of both cholesterol and β-sitosterol in the distal part was indeed low, though the distal intestinal lumen contained large amounts of radioactivity. Thus, it seems likely that the large accumulation of cholesterol in distal mucosa is attributable to the specific experimental conditions employed, i.e., 1-h meal-feeding of high-cholesterol diets rather than the non-specific adsorption. However, because no non-absorbable marker was used to assess non-specific adsorption in the experiment of in vivo cholesterol absorption, more studies are needed to draw any conclusion.

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