Comparison of synthetic saponin cholesterol absorption inhibitors in rabbits: evidence for a non-stoichiometric, intestinal mechanism of action[1]

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Abstract **The hypocholesterolemic activities of pamaqueside and tiqueside, two structurally similar saponins, were evaluated in cholesterol-fed rabbits. The pharmacological profiles of the saponins were virtually identical: both dose-dependently decreased the intestinal absorption of labeled cholesterol 25–75%, increased fecal neutral sterol excretion up to 2.5-fold, and decreased hepatic cholesterol content 10–55%. High doses of pamaqueside (>5 mg/kg) or tiqueside (>125 mg/kg) completely prevented hypercholesterolemia. Decreases in plasma and hepatic cholesterol levels were strongly correlated with increased neutral sterol excretion. Ratios of neutral sterol excreted to pamaqueside administered were greater than 1:1 at all doses, in opposition to the formation of a stoichiometric complex previously suggested for tiqueside and other saponins. Ratios in tiqueside-treated rabbits were less than unity, a reflection of its lower potency. Pamaqueside-treated rabbits exhibited a more rapid decline in plasma cholesterol concentrations than control animals fed a cholesterol-free diet, indicating that the compound also inhibited the absorption of biliary cholesterol. Intravenous administration of pamaqueside had no effect on plasma cholesterol levels despite plasma levels twice those observed in rabbits given pamaqueside orally. These data indicate that pamaqueside and tiqueside induce hypocholesterolemia by blocking lumenal cholesterol absorption via a mechanism that apparently differs from the stoichiometric complexation of cholesterol hypothesized for other saponins.**—Morehouse, L. A., F-W. Bangerter, M. P. DeNinno, P. B. Inskeep, P. A. McCarthy, J. L. Pettini, Y. E. Savoy, E. D. Sugarman, R. W. Wilkins, T. C. Wilson, H. A. Woody, L. M. Zaccaro, and C. E. Chandler. **Comparison of synthetic saponin cholesterol absorption inhibitors in rabbits: evidence for a non-stoichiometric, intestinal mechanism of action.** ***J. Lipid Res.*** **1999.** 40: **464–474.**

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The effect of dietary cholesterol consumption on total plasma cholesterol (TPC) levels in humans has been known for decades. Lately, there has been renewed interest in cholesterol absorption (1–4) that has led to a variety of pharmacological approaches to inhibit the process (5). Despite this, many of the basic steps in intestinal cholesterol absorption have remained relatively poorly understood at a molecular level. Perhaps not surprisingly then, of numerous endobiotics and xenobiotics that inhibit cholesterol absorption in animals or humans, some act via mechanisms that are at least partially obscure (6–11).

One class of naturally occurring cholesterol absorption inhibitors is the saponins, steroidal glycosides found in a variety of plants including many used as foodstuffs (12). As a result of inhibiting cholesterol and/or bile acid absorption, saponins lower TPC in a variety of species including humans (for summary see ref. 13). By virtue of their purported intestinal action, saponins could provide a non-systemic alternative to the commonly used HMG-CoA reductase inhibitors (statins). Because of their complementary mechanism of action, saponins could be particularly well suited for combination therapy with statins or other systemic hypolipidemic agents.

The molecular mechanism of action of saponins in many experimental studies has been difficult to ascertain for several reasons. As previously mentioned, cholesterol absorption is still not well characterized at the molecular level. Second, naturally occurring saponins are typically of low potency and the high doses that are required to elicit hypocholesterolemia may induce secondary effects that can complicate the interpretation of the data. Finally, in-

Abbreviations: CAI, cholesterol absorption inhibitor; FNS, fecal neutral sterol; GLC, gas–liquid chromatography; HDL, high density lipoprotein; HPβCD, hydroxypropyl-β-cyclodextrin; IV, intravenous; NZW, New Zealand White; TMS, trimethylsilyl; TPC, total plasma cholesterol; WHHL, Watanabe heritable hyperlipidemic.

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vestigators have tended to utilize saponin extracts that are mixtures of individual saponins differing in structure, and therefore potentially also in mechanism of action. Digitonin, perhaps the most widely known saponin, forms an insoluble 1:1 complex with cholesterol in vitro. Despite that supportive in vivo data are lacking, it is generally assumed that an analogous complexation occurs in the intestinal lumen thereby limiting the availability of cholesterol for absorption. Other potential mechanisms for the inhibition of cholesterol absorption by saponins have been summarized (14).

Important contributions to understanding the action of saponins on intestinal sterol absorption and atherosclerosis have been made by Malinow and colleagues (15–19). Through their earlier work with alfalfa saponins, they recognized the importance of isolating pure saponin components in order to better assess their pharmacological and toxicological properties (20). They prepared the synthetic spirostane saponins, diosgenin glucoside and tigogenin cellobioside, and demonstrated their effects on cholesterol absorption (21) and sterol balance in monkeys (22). Tigogenin cellobioside (tiqueside, CP-88,818) was later shown to decrease TPC concentrations in a variety of animals (23) and in humans (24).

More recently, we have identified a series of structurally similar saponins (25) including pamaqueside (11-ketotigogenin cellobioside), a compound that is more potent than tiqueside in the hamster (25, 26). In the present study, we have compared the pharmacological profiles of these two synthetic saponins, pamaqueside and tiqueside, hereafter collectively termed cholesterol absorption inhibitors (CAI), in cholesterol-fed rabbits. Rabbits were fed a fixed amount of cholesterol per kg body weight, and fecal neutral sterols (FNS) were monitored, permitting an accurate assessment of the stoichiometry of cholesterol absorption inhibition in vivo. To address potential systemic mechanisms of action, we also examined the effect of IV administration of pamaqueside on plasma cholesterol levels in rabbits.

MATERIALS AND METHODS

Animals and diets

Dose response study. Male New Zealand white rabbits (NZW) rabbits (~3 kg) were obtained from Millbrook Farms (Amherst, MA). During the initial acclimation period, they were maintained on a chow diet (Harlan Teklad, Inc. Madison, WI). Prior to the experimental period, rabbits hypo- or hyper-responsive to cholesterol feeding were identified by feeding a 0.4% cholesterol (w/w) and 10% peanut oil (w/w) diet for 5 days prior to monitoring TPC levels. Animals were then assigned to treatment groups in such a way as to minimize mean TPC differences between the groups. The following day, the diet was switched to a 0.13% (w/w) cholesterol, 3.3% (w/w) peanut oil diet with or without CAI. Daily rations for each animal were prepared using three stock diets (basal, 0.4% cholesterol/10% peanut oil, and basal + CAI (0.25% pamaqueside or 2.5% tiqueside) that were mixed in several proportions to generate diets with a range of pamaqueside (0.01%–0.1% w/w) or tiqueside (0.1%–1% w/w) contents. Diets were prepared in this manner to ensure a consistent consumption of cholesterol (40 mg/kg), peanut oil (1 gm/kg) and CAI per kg throughout the study and to ensure that there was no physical interaction of CAI with cholesterol during diet preparation or storage. This latter point was potentially important as saponin CAIs could bind cholesterol in a manner analogous to digitonin. Rabbits were treated for either 23 days (tiqueside) or 24 days (pamaqueside) prior to necropsy. Half of the control animals were necropsied each day to control for any duration of treatment-related differences, but as none were observed, the control animals were analyzed as a single group.

Plasma cholesterol reversal study. Male and female NZW/Watanabe heritable hyperlipidemic (WHHL) heterozygote rabbits were fed a 0.2% cholesterol, 5% peanut oil diet for 10 mo, assigned to either control or CAI treatment groups and then fed a cholesterol-free rabbit diet with or without pamaqueside (25 mg/kg) given by diet admix. Plasma cholesterol levels were monitored periodically for 6 mo.

Intravenous studies. Male NZW rabbits were fed a cholesterol-containing diet (0.4% cholesterol, 10% peanut oil) for 4 days and they were grouped according to their TPC responses as described previously. A cholesterol-free diet was fed for several weeks to allow TPC concentrations to return to baseline values. Then, rabbits (n = 6) were rechallenged with the same cholesterol-containing diet for 5 days and dosed intravenously with pamaqueside solubilized in hydroxypropyl-β-cyclodextrin (HPβCD) (0.25 mg/kg pamaqueside in 0.14 ml/kg of 20% HPβCD bid) or orally (25 mg/kg) by diet admix. Animals were bled from the marginal ear vein for TPC and pamaqueside mass determinations just prior to their morning IV injections. The TPC concentrations of rabbits treated IV or orally with pamaqueside were compared to control rabbits fed the same cholesterol-containing diet and given the HPβCD vehicle. Based on predictions made from previous pharmacokinetic studies done in rabbits, the IV dose and dosing frequency of pamaqueside were chosen so as to match or slightly exceed the plasma concentrations of pamaqueside observed after oral administration. We chose HPβCD as the vehicle for these studies despite the reported hypocholesterolemic activity of high dose HPβCD (27) because it was well tolerated upon multiple IV infusions. Doses of HPβCD infused in this study were much lower than that reported to be hypocholesterolemic and did not lower TPC concentrations in a group of hypercholesterolemic rabbits (data not shown).

Experimental procedures

Cholesterol absorption. Cholesterol absorption was determined using a method similar to that reported previously (9). Two days before necropsy, each animal received 8 μCi of [^{3}H]cholesterol (Amersham) orally in their meal, and the ^{3}H content of the plasma and liver was quantitated. Data are expressed as the percent of radiolabel recovered in the plasma and liver. Though not suitable for the determination of absolute percentages of cholesterol absorption, this method and variations thereof in our hands have been reliable relative measures of cholesterol absorption in rabbits, hamsters, and mice.

FNS excretion. Feces collected during the last 2–3 days of study were homogenized in distilled water and aliquots were lyophilized, pulverized, weighed, and stored at −20°C until analyzed. Neutral sterols were analyzed by gas–liquid chromatography (GLC) by modification of methods previously described (28, 29). Briefly, 50 mg of lyophilized powdered feces was saponified in 2 ml of 1 N NaOH in 90% ethanol at 80°C for 1 h. After adding 1 ml of water to the saponified mixture, neutral sterols were extracted 3 times with 5 ml of petroleum ether. This extraction procedure routinely resulted in >90% extraction of neutral sterols. The pooled extracts were dried under N_2, and trimethylsilyl

(TMS) ethers were prepared by adding 0.5 ml of pyridine–hexamethyldisilazane–trimethylchlorosilane 9:3:1 and incubating for 30 min at room temperature. GLC was performed using a 60 m × 0.32 mm inner diameter glass capillary SPB-1 column (Supelco, Bellefonte, PA) with a temperature program of 20°C/min from 190°C to 265°C (Varian Vista 6000, Walnut Creek, CA). Sterol analyses were performed in triplicate. An internal recovery standard, 5α-cholestane (Steraloids, Wilton, NH) (50 μg/50 mg sample), was added prior to saponification. The relative retention times and detector responses of TMS derivatives of cholesterol and cholesterol-derived sterols were compared to those of highly purified standards (Steraloids) to identify and quantitate FNS content.

Plasma cholesterol. Whole blood was obtained by venipuncture of the marginal ear vein and collected into tubes containing lithium heparin or EDTA and centrifuged to obtain plasma. At necropsy, blood was drawn via cardiac puncture. Total plasma cholesterol was determined enzymatically using commercial assay kits (Single Vial, Boehringer Mannheim, Indianapolis, IN or Waco, Richmond VA). HDL-cholesterol (HDL-C) was measured after precipitation of apolipoprotein B-containing lipoproteins by the method of Assmann et al. (30), and non-HDL-cholesterol was calculated by difference.

Liver cholesterol determinations. After excision of the liver from each animal, a ~2 g piece was saponified in 5 ml of 2.5 m KOH for 2 h at 70°C. One milliliter of ethanol containing [^{14}C]cholesterol ~0.05 μCi was added to aliquots (200 μl) of the saponified liver. Tubes were mixed and placed at −20°C for 15 min to facilitate precipitation of protein before the addition of 4 ml of hexane. After vigorous mixing, samples were centrifuged at low speed for 5 min to separate phases. Three milliliters of the hexane layer was evaporated to dryness under N_2 before being redissolved in 300 μl of 1% (v/v) Triton X-100 in ethanol. Aliquots were removed for determination of total liver cholesterol using an enzymatic cholesterol assay and for radioactivity by liquid scintillation counting. The cholesterol data were corrected for the recovery of the [^{14}C]cholesterol internal standard in the extraction step.

Plasma CAI analysis. Plasma concentrations of pamaqueside were determined by Oneida Research Services, Inc. (Rome, NY). Plasma was extracted with acetonitrile, derivatized with acetyl chloride, and dissolved in acetonitrile–2-propanol–25 mm ammonium acetate 50:30:20. Pamaqueside was detected using a PE Sciex API III, HPLC/MS/MS triple quadrupole system with atmospheric pressure chemical ionization through a heated nebulizer. Detection was carried out using multiple reaction monitoring. Separation was achieved using a Waters Novapak C18 analytical column at a flow rate of 1.2 ml/min. The run time was 3 min. The internal standard was penta-deuterated pamaqueside that was added to each plasma sample before extraction. The peak area ratio of pamaqueside to internal standard was used to quantify pamaqueside. Upper and lower limits of quantitation for the assay were 15 mg/ml and 100 ng/ml, respectively. Plasma tiqueside concentrations were determined using a reverse phase HPLC system with detection on a Sciex API III mass spectrometer as previously described (31).

Determination of pamaqueside dissociation from HPβCD in vivo. Dissociation of pamaqueside from HPβCD after IV administration was monitored by injecting [^{3}H]pamaqueside:[^{14}C]HPβCD (Cyclodextrin Technologies Dev. Inc., Gainesville, FL) into rabbits and drawing blood from the opposite ear 2 min and 2 h later. Plasma was subjected to FPLC size exclusion chromatography on a Superose 6 column equilibrated with 154 mm NaCl, 1 mm EDTA, 0.02% NaN_3, pH 8.1. Fractions (0.5 ml) were collected and analyzed by liquid scintillation counting.

Statistical analysis. In experiments with multiple treatment groups, a one-way analysis of variance followed by a Student Newman-Keuls test for multiple group comparisons was used to test for significant differences (32). Student's *t* test was used to assess significance in experiments with two treatment groups.

RESULTS

Dose response of CAIs

The dose response effects of pamaqueside and tiqueside on cholesterol absorption, TPC, and hepatic cholesterol content were examined in cholesterol-fed rabbits and are summarized in **Table 1**. Significantly less labeled cholesterol was recovered in plasma and the livers of rabbits treated with either compound; the highest doses of CAI decreased the recovery of labeled cholesterol by 70% to 74% compared to the untreated controls (Table 1). Despite a structure almost identical to tiqueside (**Fig. 1**), pamaqueside was approximately 10-fold more potent than tiqueside in inhibiting the absorption of radiolabeled cholesterol. Inhibition of intestinal cholesterol absorption by the CAIs had profound effects on plasma and hepatic cholesterol levels in these animals. After 23 or 24 days of treatment, hepatic cholesterol levels were significantly lower in CAI-treated rabbits as compared to control animals (Table 1). Consistent with the cholesterol absorption data, pamaqueside was approximately 10-fold more potent than tiqueside. At necropsy, total plasma cholesterol concentrations in CAI-treated animals were up to 80% lower than those of the untreated control group and were not statistically different from the baseline levels established prior to cholesterol feeding (Table 1). There was no adverse effect of either saponin on body weight gain over the course of study (Table 1).

Lipoprotein cholesterol was also monitored throughout the 3.5 wk of CAI treatment. As shown in **Fig. 2**, pamaqueside dose-dependently reduced plasma non-HDL cholesterol concentrations. After 3.5 wk of treatment, rabbits treated with high doses of pamaqueside (12.5 and 25 mg/kg) had non-HDL-C levels that were below the baseline values prior to the initiation of cholesterol feeding. The data in tiqueside-treated animals were qualitatively similar in that non-HDL-C levels in rabbits given high doses of tiqueside (125 and 250 mg/kg) were at the level of chow-fed animals, whereas the levels in rabbits given low doses of tiqueside were not significantly different from that of cholesterol-fed controls (data not shown). There was no effect of pamaqueside (**Fig. 3**) or tiqueside (data not shown) on HDL-C levels at any time during the study.

Neutral sterol balance and stoichiometry

Inhibition of cholesterol absorption by the CAI was also assessed by measuring the fecal excretion of neutral sterols. In agreement with previously published data (33, 34), the vast majority of FNS excreted by control rabbits was cholesterol and coprostanol with a relatively smaller amount of cholestanol detected. Animals treated with either pamaqueside or tiqueside had increased levels of FNS. Overall, total FNS excretion increased approximately 1.5- to 2.5-fold with CAI treatment compared to cholesterol-fed controls (**Table 2** and **Fig. 4**). There was no significant

TABLE 1. Effects of saponin CAIs on plasma and hepatic cholesterol levels and body weight changes in cholesterol-fed rabbits

Group (n)	[^{3}H]Cholesterol Recovery	Hepatic Cholesterol	Plasma Cholesterol	Body Weight Change
	%	*mg/gm liver*	*mg/dl*	*g*
Control (8)	29.3 ± 8.1	7.0 ± 0.6	244 ± 184 *83 ± 37*	227 ± 28
Pamaqueside-treated				
2.5 mg/kg (5)	22.1 ± 5.4[a]	6.3 ± 2.0	159 ± 85 *77 ± 17*	239 ± 58
5 mg/kg (5)	13.1 ± 5.1[a]	5.0 ± 1.1[a]	103 ± 54 *78 ± 21*	284 ± 82
12.5 mg/kg (5)	11.1 ± 4.4[a]	3.8 ± 0.6[a]	55 ± 24[a] *86 ± 21*	295 ± 76
25 mg/kg (5)	8.8 ± 2.8[a]	3.5 ± 0.3[a]	44 ± 13[a] *88 ± 22*	215 ± 70
Tiqueside-treated				
25 mg/kg (5)	26.9 ± 7.0	6.8 ± 0.5	278 ± 140 *102 ± 44*	189 ± 108
50 mg/kg (5)	26.1 ± 6.5	6.6 ± 1.4	231 ± 104 *78 ± 25*	144 ± 70
125 mg/kg (4)	10.8 ± 1.9[a]	4.0 ± 1.0[a]	54 ± 24[a] *78 ± 35*	179 ± 37
250 mg/kg (5)	7.7 ± 3.1[a]	3.8 ± 0.4[a]	52 ± 13[a] *82 ± 21*	221 ± 82

Rabbits were fed a 0.4% cholesterol, 10% peanut oil diet for 5 days and then grouped on the basis of their TPC values. All groups were then switched to a 0.13% cholesterol, 3.3% peanut oil diet with or without added CAI for 23 days (tiqueside) or 24 days (pamaqueside). The number of animals per treatment group is shown in parentheses. A dose of 2.5 mg/kg is approximately equal to 0.01% (w/w). Cholesterol recovery data are the percent of an orally adminstered dose of radiolabeled cholesterol recovered in the plasma and liver of rabbits at necropsy. Plasma cholesterol data are from the terminal blood sample. Data in italics are TPC values for each treatment group prior to the start of cholesterol feeding. Body weight change was calculated from the start of CAI treatment.). Values are means ± SD.

[a] $P < 0.05$ vs. controls.

difference in the magnitude or proportions of the neutral sterols excreted in response to either compound with the exception that pamaqueside induced comparable FNS excretion at one-tenth the dose of tiqueside.

To address whether changes in plasma and hepatic cholesterol levels in CAI-treated rabbits were the result of inhibiting cholesterol absorption, we performed linear regression analysis. Non-HDL-C and hepatic cholesterol levels were inversely correlated with FNS excretion (**Fig. 5A** and **5B**). The regression fits for pamaqueside- and tiqueside-treated animals were virtually identical providing evidence that these compounds may act via the same mechanism(s). FNS excretion in rabbits treated with CAI was also inversely correlated with the recovery of radiolabeled cholesterol from the plasma and liver compartments (Fig. 5C).

FNS excretion was also expressed on a mg/day basis, permitting an estimate of neutral sterol balance to be made in these animals. Although not a classical sterol balance study, rabbits did consume a measured amount of diet per day with a fixed cholesterol content per kg body weight, corresponding to cholesterol consumptions of

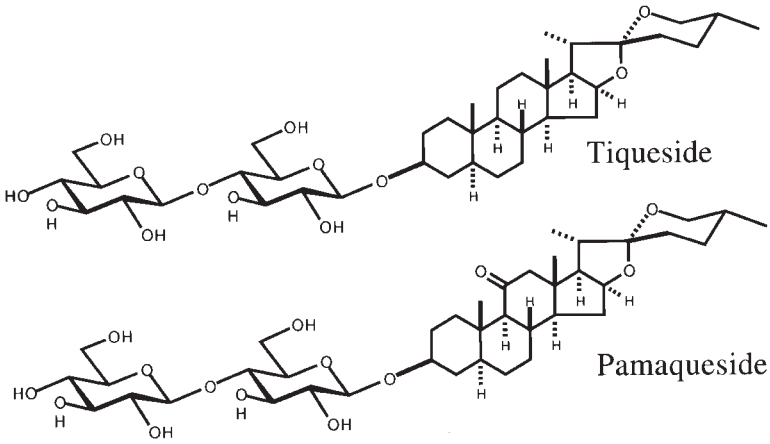

Fig. 1. Structures of synthetic saponin cholesterol absorption inhibitors.

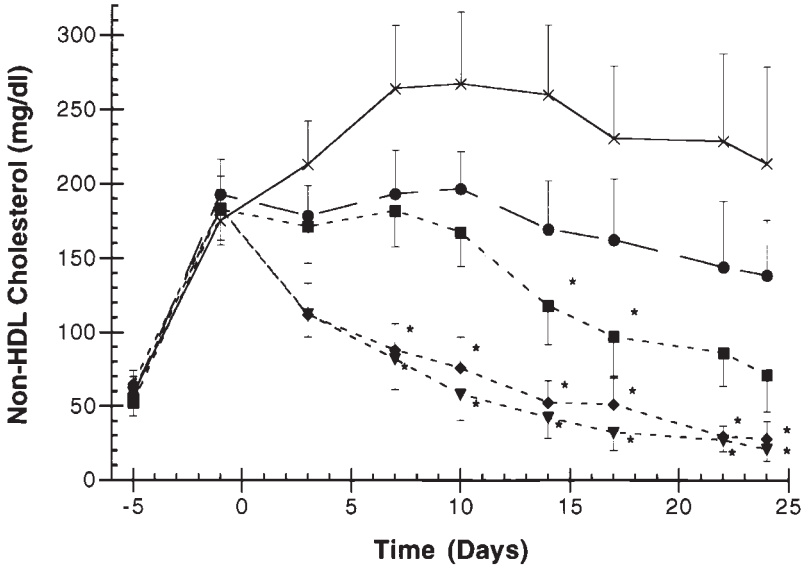

Fig. 2. Non-HDL-C levels in cholesterol-fed rabbits during treatment with pamaqueside. Rabbits were treated as described in the legend to Table 1; (X) untreated controls; (●) 2.5 mg/kg; (■) 5 mg/kg; (◆) 12.5 mg/kg; and (▼) 25 mg/kg pamaqueside. The number of animals per treatment group is the same as shown in Table 1. Values are mean ± SEM. *$P < 0.05$ vs. controls.

118–129 mg/day. As shown in Table 2, treatment with the CAIs increased daily neutral sterol excretion in a dose-dependent fashion from 43 mg/day to 100-110 mg/day. The highest doses of CAIs induced levels of sterol excretion that approached daily dietary cholesterol consumption. The efficiency by which these CAIs inhibit cholesterol absorption was assessed by dividing the sterol excretion due to CAI administration (sterol excretion in the treated groups in excess of that of the control group) by the amount of pamaqueside or tiqueside administered. These data, also shown in Table 2, indicate that the FNS:CAI ratio was as high as 5:1 in pamaqueside-treated rabbits but considerably less than 1:1 in the groups of animals given tiqueside.

Inhibition of biliary cholesterol absorption

To examine whether pamaqueside could also inhibit biliary cholesterol absorption, WHHL-NZW heterozygote rabbits, fed a cholesterol-containing diet for 10 months, were subsequently switched to a cholesterol-free diet with or without 25 mg/kg pamaqueside. Rabbits had TPC values of approximately 950 mg/dl after cholesterol feeding, which decreased upon switching to the cholesterol-free diet, eventually reaching baseline levels in approximately 6 months. The decline in plasma cholesterol levels in both control and CAI-treated rabbits was fairly rapid and reminiscent of that previously reported by Ho and Taylor (35). However, the decrease in TPC was significantly more rapid in the group of rabbits administered pamaqueside (**Fig. 6**).

IV administration

Although both synthetic saponins are poorly absorbed, high doses of pamaqueside and tiqueside yield measurable plasma levels (26, 31). To examine potential systemic actions of these agents, rabbits were administered a choles-

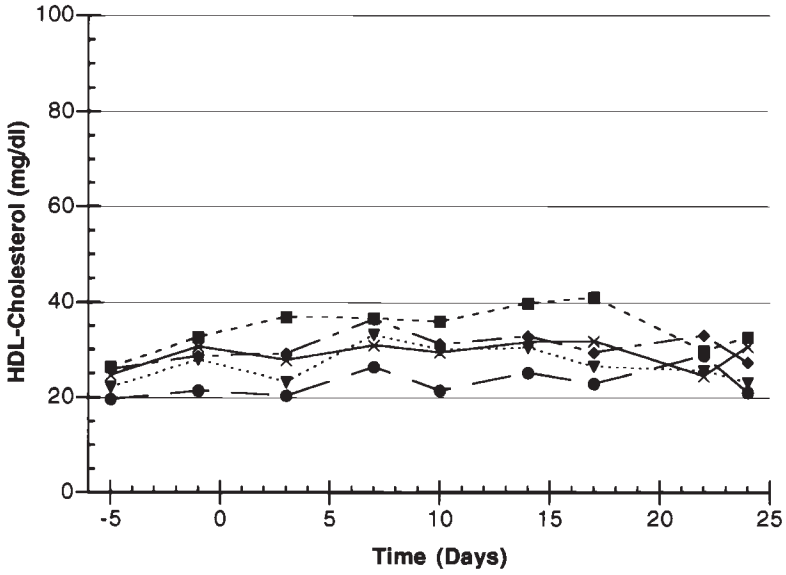

Fig. 3. Time course of HDL-C levels in cholesterol-fed rabbits during treatment with pamaqueside. Rabbits were treated as described in the legend to Table 1; (X) untreated controls; (●) 2.5 mg/kg; (■) 5 mg/kg; (◆) 12.5 mg/kg; and (▼) 25 mg/kg pamaqueside. The number of animals per treatment group is shown in Table 1. Error bars are omitted for clarity.

TABLE 2. Effects of saponin CAIs on FNS parameters in cholesterol-fed rabbits

Group	Dietary Cholesterol	FNS Excretion		Sterol CAI
	mg/d	*mg/gm feces*	*mg/d*	*mol:mol*
Control	122 ± 8	1.5 ± 0.6	43 ± 11	—
Pamaqueside-treated				
2.5 mg/kg	128 ± 7	2.3 ± 0.4[a]	65 ± 13	5.2 ± 3.0
5 mg/kg	118 ± 6	2.9 ± 0.4[a]	74 ± 17[a]	4.1 ± 2.2
12.5 mg/kg	120 ± 12	2.8 ± 0.4[a]	74 ± 12[a]	1.6 ± 0.6
25 mg/kg	118 ± 7	3.8 ± 1.1[a]	100 ± 32[a]	1.5 ± 0.8
Tiqueside-treated				
25 mg/kg	129 ± 10	2.1 ± 0.4[a]	59 ± 13	0.4 ± 0.3
50 mg/kg	126 ± 7	2.3 ± 0.4[a]	68 ± 15	0.3 ± 0.2
125 mg/kg	125 ± 6	3.1 ± 0.4[a]	86 ± 18[a]	0.2 ± 0.1
250 mg/kg	127 ± 10	4.2 ± 0.5[a]	114 ± 14[a]	0.2 ± 0.05

Rabbits were treated as described in the legend to Table 1. Feces were collected during the last 2–3 days of CAI treatment and neutral sterols were analyzed by GLC. Sterol/CAI for each animal was calculated by subtracting the mean FNS excretion (in mg/day) of the control group from the FNS excretion of each treated animal and dividing the difference by the mass of CAI administered. The number of animals per treatment group is shown in Table 1. Values are means ± SD.

[a] $P < 0.05$ vs. control rabbits.

terol–peanut oil-containing diet with either oral (25 mg/kg by diet admix) or IV administration of pamaqueside (0.25 mg/kg/day). Previous studies had determined that this IV dose of pamaqueside would result in comparable systemic exposure to a 25 mg/kg oral dose. The TPC of rabbits given HPβCD or HPβCD:pamaqueside IV were indistinguishable, increasing from a mean of approximately 40 mg/dl to 250 mg/dl over the course of 4 days. In contrast, the diet-induced TPC increase in the group of rabbits administered pamaqueside by diet admix (25 mg/kg/day) was significantly blunted, failing to reach 100 mg/dl in the same 4-day period (**Fig. 7A**). Pamaqueside did not completely prevent hypercholesterolemia under these conditions, presumably because a diet with higher cholesterol and peanut oil content (than was used in the dose–response experiment) was fed to enhance the rate of onset of hypercholesterolemia. Plasma pamaqueside levels in the IV group were more than double those found in the orally dosed group (Fig. 7B) thus demonstrating that comparable systemic exposure had been achieved in the IV group of rabbits. Systemic exposure of tiqueside after oral dosing was considerably greater than that of pamaqueside (26). Nonetheless, a similar experiment evaluating the effect of IV tiqueside in hypercholesterolemic rabbits showed that rabbits dosed IV with tiqueside had no change in plasma cholesterol levels whereas animals given tiqueside by diet admix had decreased plasma cholesterol levels (data not shown).

To ensure that complexation with HPβCD did not prevent a potential systemic action of IV administered CAI, plasma from NZW rabbits injected with a [^{3}H]pamaqueside/[^{14}C] HPβCD solution was subjected to FPLC size exclusion chromatography. Plasma prepared from blood collected 2 min after IV pamaqueside administration showed two distinct peaks of radioactivity demonstrating a rapid dissociation of pamaqueside-HPβCD complex (**Fig. 8A**). The retention time of pamaqueside was indicative of its association with plasma proteins of a molecular size comparable to albumin. Plasma from blood collected 2 h after pamaqueside administration had detectable levels of pamaqueside remaining, but contained virtually no HPβCD (Fig. 8B), in keeping with the reported rapid elimination of HPβCD from the bloodstream. The results of the analogous study with [^{3}H]tiqueside showed that it too rapidly dissociated from HPβCD immediately after IV administration (data not shown).

DISCUSSION

The data presented herein are consistent with the hypocholesterolemic activity previously reported for tiqueside and other saponins. Cholesterol-fed rabbits given tiqueside by diet admix had lower plasma and hepatic cholesterol levels than untreated control animals. Plasma cholesterol lowering occurred exclusively in the non-HDL fractions and was dose-dependent. Higher doses of tique-

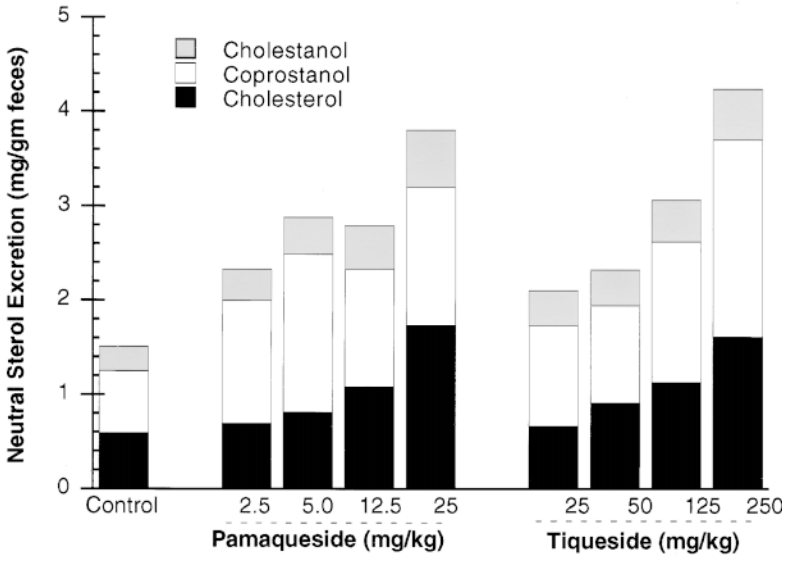

Fig. 4. Fecal excretion of cholesterol-derived, neutral sterols from cholesterol-fed rabbits treated with CAIs. Rabbits were treated as described in the legend to Table 1. Feces were collected during the last 2–3 days of the study and analyzed by GLC. The number of animals per treatment group is shown in Table 1.

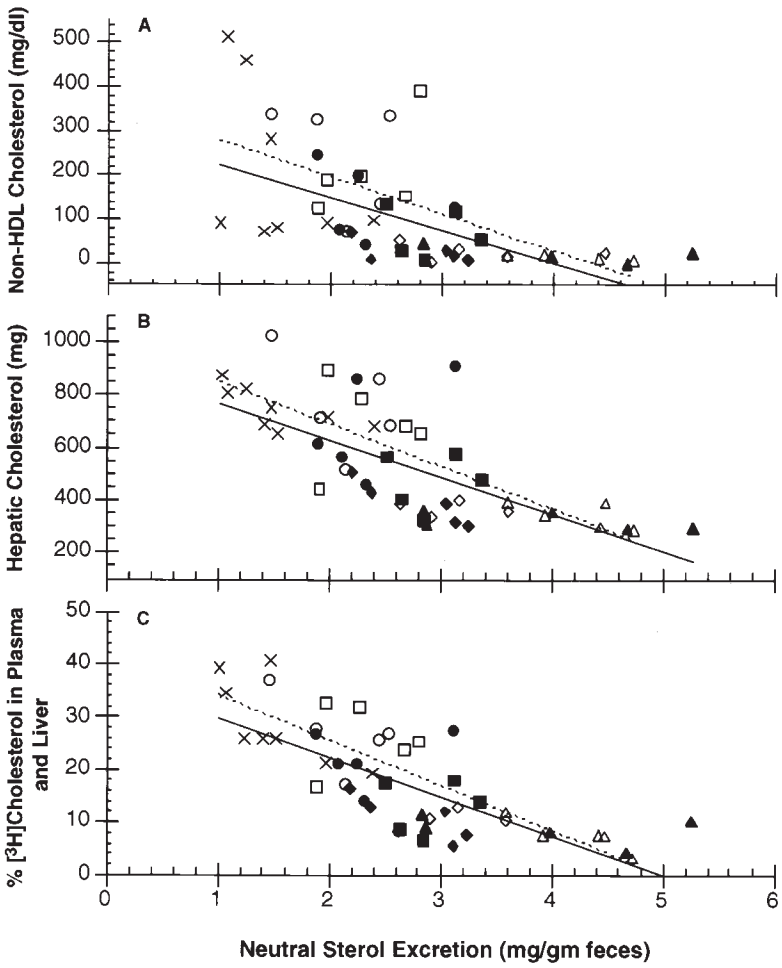

Fig. 5. Relationship betweenbetween fecal neutral sterol excretion and non-HDL cholesterol levels (A), hepatic cholesterol mass (B), and [^{3}H]cholesterol recovery (C) in CAI-treated rabbits. Rabbits were treated as described in the legend to Table 1; (X) controls; (solid symbols) pamaqueside-treated; (open symbols) tiqueside-treated; (●, ○) 2.5, 25 mg/kg CAI; (■, □) 5, 50 mg/kg CAI; (◆, ◇) 12.5, 125 mg/kg CAI; (▼, ▽) 25, 250 mg/kg CAI. The correlation coefficients for pamaqueside (solid line) and tiqueside (dashed line) were: $r = 0.76$, $P < 0.0001$ and $r = 0.84$ and $P < 0.0001$, (A); $r = 0.70$, $P < 0.0001$ and $r = 0.80$ and $P < 0.0001$ (B); and $r = 0.59$, $P < 0.002$ and $r = 0.59$ and $P < 0.002$ (C), respectively.

side completely prevented the rise in plasma non-HDL cholesterol levels induced by cholesterol–peanut oil feeding. The pharmacological profile of tiqueside was virtually indistinguishable from that of a structurally similar saponin, pamaqueside, except that pamaqueside was 10-fold more potent than tiqueside. Considering that the 25 mg/kg dose of each CAI translates to approximately 0.1% (w/w) in the diet, both of these synthetic saponins appear to be more potent than their naturally occurring counterparts. As an example, alfalfa saponins (~1%) only partially prevented hypercholesterolemia in rabbits given 0.1% cholesterol (18).

We sought to extend earlier findings on tiqueside by examining in greater detail the effects of saponin CAIs on intestinal cholesterol absorption. In previous animal studies, tiqueside or pamaqueside treatment decreased absorption of orally administered radiolabeled cholesterol, suggesting that the hypocholesterolemic activity of these saponins was the result of cholesterol absorption inhibition (23, 24, 26). In this paper, we have demonstrated that pamaqueside and tiqueside treatment of rabbits also resulted in increased FNS excretion. Sterol excretion was increased dose-dependently, and was inversely correlated with the fraction of orally administered radiolabeled cholesterol recovered in the plasma and liver compartments at necropsy. The magnitude of FNS excretion was also inversely correlated with plasma non-HDL cholesterol and hepatic cholesterol levels, further supporting the hypothesis that the hypocholesterolemic activity of these synthetic saponins is a consequence of the inhibition of cholesterol absorption.

These data indicate that the CAIs inhibit the absorption of exogenous cholesterol, but a similar effect on endogenous biliary cholesterol cannot be assumed automatically. Dietary cholesterol is initially incorporated into large emulsion particles, and therefore is at least initially distinct from endogenous cholesterol that is secreted into the intestinal lumen as a mixture of bile salt micelles and phospholipid vesicles. As reviewed by Wilson and Rudel (4), some investigators have noted an apparent difference in the absorption of endogenous versus exogenous cholesterol. To address whether the CAIs were likewise effective at inhibiting biliary cholesterol absorption, we examined the ability of pamaqueside to reduce plasma cholesterol levels in hypercholesterolemic animals reverted to a chow diet. The TPC of rabbits fed pamaqueside declined more rapidly than that of control rabbits consuming the chow diet. We have also observed that pamaqueside increases FNS excretion in hamsters fed a cholesterol-free diet (manuscript in preparation). These data indicate that pamaqueside also inhibits the absorption of biliary or endogenous cholesterol as well as dietary cholesterol.

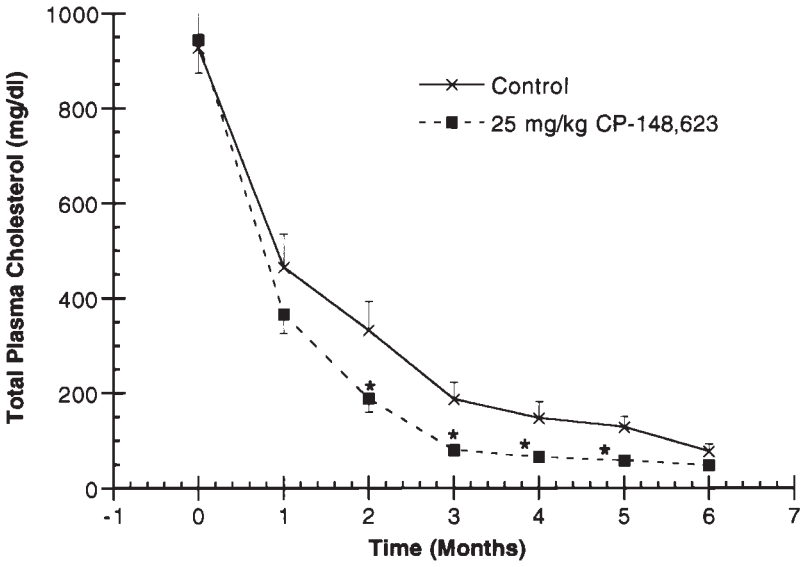

Fig. 6. Time course of TPC concentrations in previously hypercholesterolemic rabbits treated with pamaqueside. Twenty-five NZW–WHHL heterozygote rabbits were fed a 0.2% cholesterol, 5% peanut oil diet for 10 mo. All were then placed on a cholesterol-free diet and half (n = 13) were administered pamaqueside (25 mg/kg/d) by diet admix for 6 mo. Values are means ± SEM. *$P < 0.05$ vs. the corresponding mean of control rabbits. Non-linear regression analysis (data fit to two-phase exponential decay curves) was performed using Prism software, and the curves were significantly different by F test, $P < 0.001$ (48).

Rabbits had relatively higher plasma levels of CAI after oral administration than did hamsters or dogs (26, 31). Thus, it is conceivable, and perhaps more likely in rabbits than in other species, that systemic mechanisms could contribute to TPC lowering. As an example, diosgenin, a sapogenin very similar in structure to the aglycone of pamaqueside or tiqueside, was shown to substantially increase biliary cholesterol output in an acute bile fistula rat model (36). However, in the current study, TPC lowering was not detected when pharmacologically relevant plasma concentrations of pamaqueside were maintained by IV administration. Thus, we conclude that there is no significant systemic hypocholesterolemic mechanism of these CAIs, at least at pharmacological doses, and the predominant, if not sole, mechanism of plasma cholesterol lowering is via interruption of cholesterol absorption occurring in the intestine.

Though the present study was not intended to rigorously examine sterol balance, the measured consumption of cholesterol diet throughout the study did afford impor-

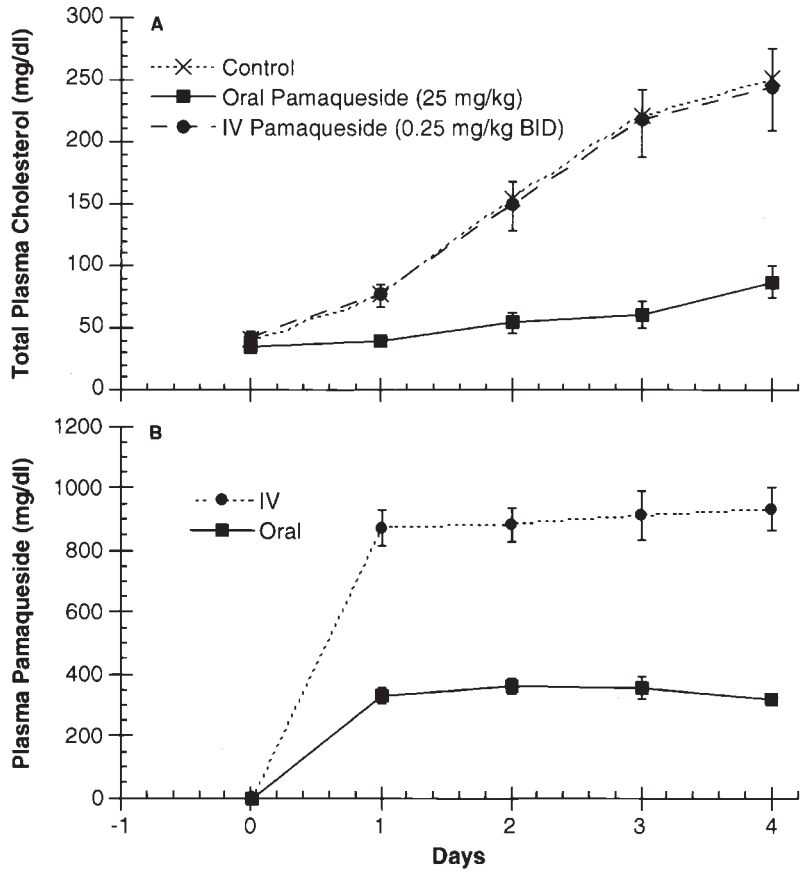

Fig. 7. Comparison of TPC changes in rabbits after oral or IV administration of pamaqueside. Rabbits (n = 6/group) were fed a 0.4% cholesterol, 10% peanut oil diet for 4 days and administered pamaqueside IV or orally via diet admix. All rabbits received IV infusions of either HPβCD vehicle (control and oral pamaqueside groups) or pamaqueside: HPβCD (IV group) BID. Rabbits were bled each morning just prior to their IV infusion and daily feeding for measurement of TPC (panel A) and pamaqueside concentrations (panel B). Values are means ± SEM. *$P < 0.05$ vs. controls.

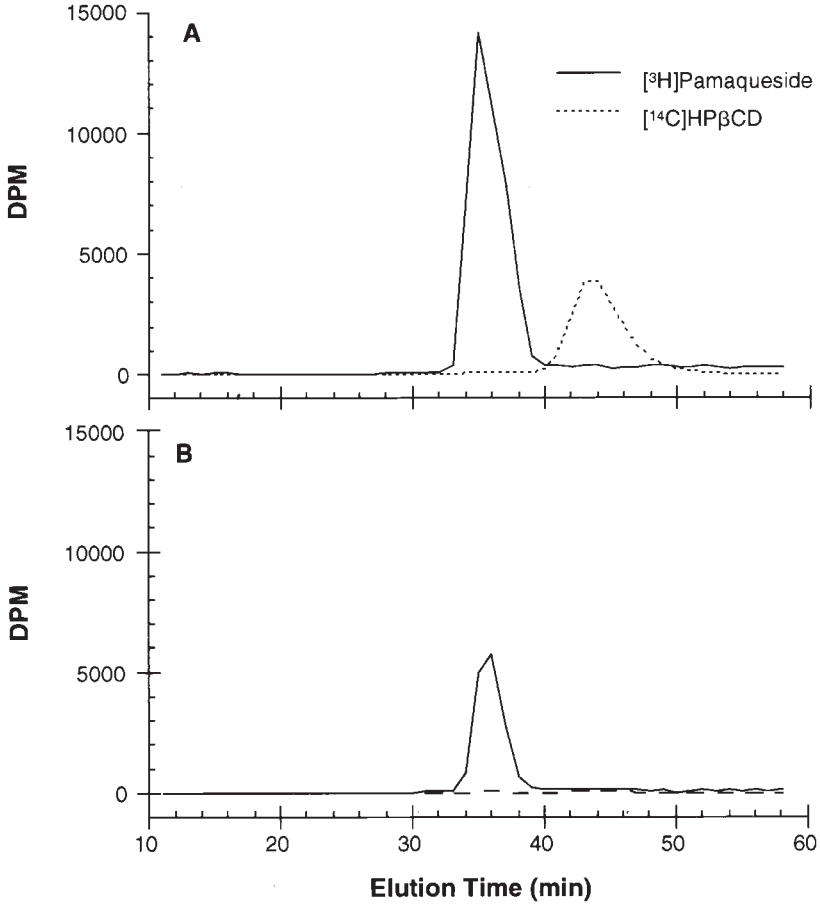

Fig. 8. Dissociation of plasma [^{3}H]pamaqueside:[^{14}C]HPβCD after IV administration in rabbits. Rabbits (n = 2) were injected with an aqueous solution of [^{3}H]pamaqueside:[^{14}C]HPβCD as described in Methods. Blood samples were obtained from the opposite ear at 2 min (panel A) and at 2 h (panel B). Plasma was fractionated on a Superose 6B FPLC column and fractions were analyzed using liquid scintillation counting. Data are means of two animals.

tant information regarding neutral sterol balance and consequently the efficiency by which these saponin CAIs induce FNS excretion. High doses of either compound increased FNS excretion to levels that verged upon daily cholesterol consumption. Fecal acidic sterols were not determined in this study because previous data had indicated that there was no effect of tiqueside on acidic sterol excretion (23). In rabbits, bile acid excretion has been reported to be in the range of 40 mg/day (33, 34). Therefore, assuming a similar level of acidic sterol excretion, high-dose animals in our study had total sterol excretion in excess of dietary sterol intake. This would be consistent with the eventual normalization of TPC levels observed in cholesterol-fed rabbits given the highest doses of pamaqueside or tiqueside. Data from a monkey study testing 1% diosgenin glucoside also indicated a negative sterol balance (22).

While these data indicate that synthetic saponin CAIs effect plasma and hepatic cholesterol lowering by inhibiting cholesterol absorption, the data do not provide much insight into the molecular mechanism by which they act. Probably the most frequently cited mechanism for saponins is the physical interaction with lumenal cholesterol resulting in the precipitation of a 1:1 complex. In contrast to considerable in vitro data, there is little or no definitive evidence for the formation of a saponin:cholesterol complex in the intestinal lumen; complexation is usually inferred as the mechanism by analogy to the in vitro data. To examine the likelihood of such a mechanism occurring with the saponin CAIs, we determined the amount of FNS excreted per day over the last 2–3 days of the study and compared it to the amount of CAI administered. The ratio of FNS excreted to pamaqueside administered was greater than unity in all groups of rabbits and averaged approximately 5:1 in animals given the lowest dose of pamaqueside. This finding stands in opposition to the 1:1 complex formation shown for digitonin and tomatine in vitro and mentioned as a possible mechanism of tiqueside action in vivo (23).

On the other hand, ratios of FNS excreted per saponin in tiqueside-treated animals were substantially less than 1:1, consistent with its lower potency relative to pamaqueside. The formation of a 1:1 complex between cholesterol and tiqueside is conceivable based on the stoichiometry we observed, but because of the close structural similarity of tiqueside and pamaqueside and the virtually superimposable regression lines in pamaqueside- and tiqueside-treated rabbits, we suspect that tiqueside's mechanism of action is similar to that of pamaqueside. Neutral sterol excretion data also support this hypothesis. Insoluble complex formation between either tiqueside or pamaqueside and cholesterol should manifest itself as an increase in the proportion of cholesterol in the FNS extract, as the complexed sterol would in all likelihood escape bacterial transformation. However, there was no significant difference in the proportion of cholesterol relative to total FNS excretion in tiqueside or pamaqueside-treated rabbits at any dose of CAI.

Some evidence does exist in the literature for the formation of adducts containing multiple cholesterol molecules per saponin. Gestetner et al. (37) reported the formation of such complexes between cholesterol and alfalfa saponins with the approximate stoichiometry of 5:1. Though the complexes were formed in vitro, the adducts were not sufficiently stable to isolate. Coupled with the data that tiqueside is more potent at inhibiting cholesterol absorption in rats than are alfalfa saponins (38), it seems unlikely that these labile complexes would be a significant factor in the inhibition of cholesterol absorption in vivo. Saponins also form immunostimulating complexes, ISCOMs, thought to contribute to the adjuvanticity observed with quillaia saponin. Saponins, cholesterol, and phospholipids can form ISCOM-like structures in the absence of antigen (39) but the spirostane saponins digitonin and tomatine, which are structurally similar to pamaqueside and tiqueside, have no adjuvant activity (40, 41). To our knowledge, there are no data indicating that ISCOM formation plays a role in cholesterol absorption inhibition by saponins.

There are other mechanisms besides cholesterol complexation by which saponins are postulated to inhibit cholesterol absorption. Saponins form complexes with membrane cholesterol or extract cholesterol from membranes and this may explain the hemolytic activity of some saponins. *Gypsophylla* or *Saponaria* saponins decrease permeability and active glucose transport in isolated everted jejunal sacs (42), with permeability changes persisting after saponin pretreatment. Saponins isolated from soy, *Saponaria officinalis*, or *Quillaia saponaria* altered the size or shape of micelles (14, 43), and this mechanism is a likely explanation for the inhibition of bile salt absorption by a variety of saponins (13). Plant sterols such as sitosterol and sitostanol, though structurally distinct from saponins, are thought to inhibit cholesterol absorption by competing with cholesterol for micellar solubility. Micellar exclusion of cholesterol was proposed as a potential mechanism for tiqueside (23).

However, none of these potential mechanisms is consistent with the data collected on the synthetic saponin CAIs. Tiqueside did not perturb glucose or cholate uptake in everted sacs and there was no residual inhibition of cholesterol uptake after pretreatment with saponin (44), indicating no significant change in enterocyte transport function. Similarly, there was no change in body weight in this study, suggesting that there was no significant malabsorption of other nutrients in vivo as might be expected if cholesterol complexation or extraction from the brush border membranes resulted in a loss of permeability and/or absorptive function. Sidhu, Upson, and Malinow (44) contrasted the effects of tiqueside with soy saponins that had pronounced inhibitory effects on both cholesterol and bile acid uptake. In our hands, pamaqueside does not affect the absorption of triglycerides (P. Tso, L. Gray, C. E. Chandler, and L. A. Morehouse, unpublished data) or other micellar components in the rat (R. W. Wilkins and L. A. Morehouse, unpublished data). With a structurally related saponin more potent than pamaqueside, inhibition of cholesterol uptake in an in situ ligated hamster intestinal model was observed at concentrations that did not decrease sterol solubility (L. M. Zaccaro and C. E. Chandler, unpublished data). Neither of these synthetic saponins inhibit enzymes (ACAT, pancreatic cholesteryl ester hydrolase, and pancreatic lipase) known to have roles in lipid absorption (data not shown).

The commonly held belief that cholesterol uptake into the enterocyte is a passive process has been challenged recently with reports of a putative apical membrane cholesterol transporter (45) and a potential chaperone function of pancreatic cholesterol esterase (46). While these results are provocative, their contribution to cholesterol uptake in vivo remains to be clarified. Nonetheless, the difference in in vivo potency of pamaqueside relative to tiqueside despite differing only by a keto group and the additional structure–activity relationships reported for these synthetic saponins (25) may suggest the potential interaction with a cholesterol transporter. These saponins are not the only example of high potency cholesterol absorption inhibitors as other researchers have disclosed 2-azetidinones (47) whose molecular mechanism of action is likewise unknown. These two structural series of potent cholesterol absorption inhibitors may facilitate further work on mechanism of intestinal cholesterol absorption.

In summary, the synthetic saponins pamaqueside and tiqueside inhibit intestinal cholesterol absorption in rabbits resulting in decreased plasma and hepatic cholesterol levels. The mechanism of action is probably lumenal but apparently does not involve a stoichiometric complexation with cholesterol. These experimental findings do not appear to be consistent with previously postulated mechanisms of saponin action, leaving open the possibility that these saponin CAIs may inhibit cholesterol absorption by a novel mechanism.

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